

THIRD EDITION

Principles of
TRANSLATIONAL SCIENCE
IN MEDICINE
From Bench to Bedside

Edited by

MARTIN WEHLING



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Martin Wehling

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ACADEMIC PRESS

An imprint of Elsevier

Academic Press is an imprint of Elsevier
125 London Wall, London EC2Y 5AS, United Kingdom
525 B Street, Suite 1650, San Diego, CA 92101, United States
50 Hampshire Street, 5th Floor, Cambridge, MA 02139, United States
The Boulevard, Langford Lane, Kidlington, Oxford OX5 1GB, United Kingdom

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British Library Cataloguing-in-Publication Data

A catalogue record for this book is available from the British Library

Library of Congress Cataloging-in-Publication Data

A catalog record for this book is available from the Library of Congress

ISBN: 978-0-12-820493-1

For Information on all Academic Press publications
visit our website at <https://www.elsevier.com/books-and-journals>

Publisher: Stacy Masucci
Acquisitions Editor: Elizabeth Brown
Editorial Project Manager: Tracy I. Tufaga
Production Project Manager: Omer Mukthar
Cover Designer: Matthew Limbert

Typeset by MPS Limited, Chennai, India



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Chapter 1

Introduction and definitions

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Abstract

Translational medicine (TM) requires a clear definition to enable researchers to describe its remits and goals and to communicate the results of translational processes and their implications. This introductory chapter describes the various stages of translation in biomedical research and the historical background of what is now being described as TM. This chapter provides the framework for the details on TM in the subsequent chapters.

Keywords: Translational medicine; definition; history; drug pipeline

What is translational medicine?

The definition of *translational medicine* given by the European Society for Translational Medicine is as follows: “Translational Medicine (TM) is an interdisciplinary branch of the biomedical field supported by three main pillars: benchside, bedside, and community. The goal of TM is to combine disciplines, resources, expertise, and techniques within these pillars to promote enhancements in prevention, diagnosis, and therapies.” (Cohrs et al., 2014).

On the main website of leading scientific journal *Science*, TM is defined as follows:

Often described as an effort to carry scientific knowledge “from bench to bedside,” translational medicine builds on basic research advances—studies of biological processes using cell cultures, for example, or animal models—and uses them to develop new therapies or medical procedures.

(*Science Translational Science*, 2020)

A 2007 Wikipedia entry included some explanatory sentences that appear to be relevant even today:

In the case of drug discovery and development, translational medicine typically refers to the “translation” of basic research into real therapies for real patients. The emphasis is on the linkage between the laboratory and the patient’s

bedside, without a real disconnect. This is often called the “bench to bedside” definition.

(Wikipedia, 2007)

Translational medicine can also have a much broader definition, referring to the development and application of new technologies in a patient driven environment—where the emphasis is on early patient testing and evaluation. In modern healthcare, we are seeing a move to a more open, patient driven research process, and the embrace of a more research driven clinical practice of medicine.

(Wikipedia, 2007)

patient
driven
Research
Research
driven
clinical
practice

Although these attempts at a definition are probably the most accurate and concise ones at present, a simpler definition may serve the purpose even better: TM describes the transition of in vitro and experimental animal research to human applications (see Fig. 1.1) (Wehling, 2006).

Other names for the same concept are *experimental medicine*, *discovery medicine*, and *clinical discovery*. TM includes major aspects of clinical pharmacology when it relates to drugs, as early clinical trials—a key component in the setting of clinical pharmacology—are major components of translational processes.

The need to develop this discipline reflects the cleft that has been brought about by the separation of medical teaching and pharmaceutical research into preclinical and clinical categories. Another reason for this cleft appears to be the enormous spread and scope of techniques, platforms, models, and clinical approaches that leads to separate “silos” of expertise. Bridging the gaps or clefts by masterminding transgressional processes is crucial to success in curing diseases in humans.

It is obvious that the term *TM* and its claims are born out of a situation in which the transition—the prediction or extrapolation, respectively—from basic findings to human findings has been disappointing in terms of its final success at the patient level. This difficulty often

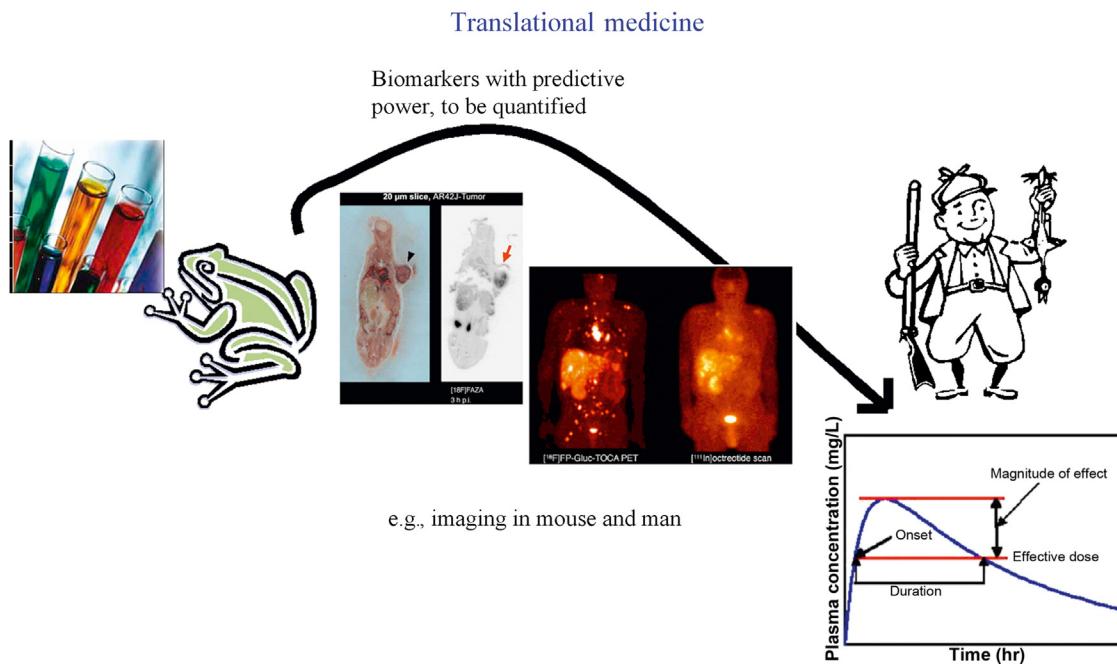


FIGURE 1.1 The main aspects of translational medicine: biomarkers as major tools for the transition from test tube or animal experiments to human trials, with imaging as a major biomarker subset. *From Wehling M., 2006. Translational medicine: can it really facilitate the transition of research “from bench to bedside”? Eur. J. Clin. Pharmacol. 62, 91–95, with kind permission from Springer Nature.*

reflects the differences among in vitro conditions (e.g., cell cultures or test tube experiments), the wide variety of animal species, and, finally, humans. For example, cell cultures of vascular smooth muscle cells are artificial, as they grow only in the presence of serum (e.g., fetal calf serum). In contrast, in vivo, nature does everything to ensure that vascular smooth muscle cells do not encounter serum; endothelium protects them against it. If the cells become damaged and are exposed to serum, all types of vascular pathology commence: hypertrophy, hyperplasia, dedifferentiation, inflammation, and finally atherosclerosis. It is conceivable that results from vascular smooth muscle cells in culture may not reflect even basal physiological in vivo conditions, and projections from such experiments into human pathology may be fruitless or misleading, especially as cells change their phenotypes with increasing culture time or passage numbers (Timraz et al., 2016; Chamley-Campbell et al., 1979).

Cell culture not reflective to organ in vivo
very different

Such artifacts can only raise hypotheses that may or may not be corroborated in animal or, finally, human experiments. The artifactual character of test tube systems is obvious, and differences among species are profound at both the genotype and phenotype levels, so no one is surprised if an intervention works in one species but not another. Although morphine is a strong emetic in dogs, it does not have this effect in rats. It is apparent that this variability applies even more when dealing with human diseases, which may or may not have any correlates in animal models. This is especially

the case with neuropathological diseases for which animal models are either lacking or misleading (e.g., psychiatric diseases such as schizophrenia).

Thus the difficulty of predicting the beneficial or toxic effects of drugs or medicinal devices or the accuracy and value of diagnostic tests is a major problem that prevents innovations from being useful for treating human diseases. From this end, the following is an operational definition of TM:

By optimization of predictive processes from preclinical to clinical stages, translational medicine aims at improving the innovative yield of biomedical research in terms of patient treatment amelioration.

Primary translation versus secondary translation

In the definitions mentioned previously, the focus is clearly on translation in development courses from pre-clinical to clinical stages, in particular as applied to the development of new drugs. These developments would bring innovation to the patients who receive the new drug, test, or device. It seems odd to underscore that some patients may receive the innovation and thus benefit from it, whereas others may not. However, there is yet another gap that prevents innovations from flourishing to their full potential. Even if innovative drugs have changed clinical

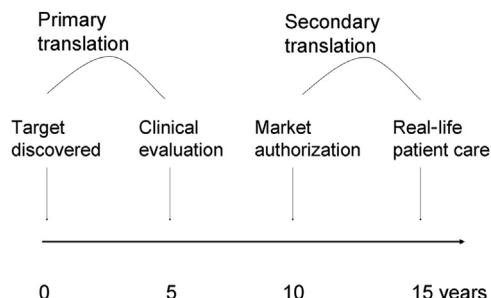


FIGURE 1.2 Scheme depicting the two principal transition phases for translation of a drug project, for instance. The primary translation phase runs from preclinical (target discovery) to clinical development. The secondary translation phase goes from market authorization to real-life patient care.

guidelines and rules and thus been undoubtedly proven to represent beneficial options to suitable patients, they might not be applied in what is commonly termed “real life.”

Undertreatment may result from ignorance, budget restrictions, or patient or doctor noncompliance and often has severe socioeconomic implications: Though potentially correctable in all patients, arterial hypertension is treated to guideline targets only in 20%–50% of patients (Boersma et al., 2003; Tocci et al., 2015); LDL cholesterol in cardiovascular high-risk patients is at target levels in 6%–60% of patients (Böhler et al., 2007; Presta et al., 2019). This means that innovations that have successfully passed all translational hurdles in the developmental process from bench to bedside still might not reach the patients at large, as there is a second barrier between guideline recommendations and real-life medicine (see Fig. 1.2).

This translational aspect of innovation is sometimes called *secondary translation* (in contrast to the developmental primary translation). As problems in secondary translation mainly reflect insufficiency at the level of patient care, socioeconomic structures, education, society, and habits, the scientific challenge is secondary to social and political tasks and obligations. Therefore this book is entirely devoted to the scientific aspects of primary translation and does not deal with secondary translation, although its impact on patient care may sometimes be more crucial than that of primary translation.

Some authors have proposed an even more detailed labeling of translational stages. T1 describes translation from basic genome-based discovery into a candidate health application (e.g., genetic test or intervention); T2 describes translation from application for health practice to the development of evidence-based guidelines; in T3, evidence-based guidelines are moved into health practice; and finally, T4 seeks to evaluate the real-world health outcomes of an application in practice (Khoury et al., 2007).

The history of translational medicine, obstacles, and remits

As described previously, the main feature of TM is the bridging function between preclinical and clinical research. It aims at answering the following simple but tremendously important question: If a drug X works in rats, rabbits, and even monkeys, how likely is it that the drug will be beneficial to humans? Historically, how did this simple and straightforward question, which is naturally inherent to all drug development processes, become of prime relevance in biomedical research?

If all drug, device, or test development components were closely connected within a common structure, the necessity to develop the discipline of TM would probably not have become apparent. As it stands now, however, the current emphasis on TM reflects the wide and strict separation of biomedical research into preclinical and clinical issues, a situation best illustrated by the acronym *R&D*, which is used in pharmaceutical companies to describe their active investments into science as opposed to marketing. *R* stands for research, which largely means pre-clinical drug discovery, and *D* stands for development, which is largely identical to clinical drug development. It is obvious that even the words behind *R&D* arbitrarily divide positions that share many similarities: Clinical development and clinical research are very congruent terms, and compounds are *developed* within the preclinical environment, for example, from the lead identification stage to the lead optimization stage.

In the drug industry the drug discovery and development process follows a linear stage progression. A major organizational transition occurs when a candidate drug is delivered from discovery (*R*) to clinical development (*D*), which is synonymous with trials in humans. When this happens, it is often said that the discovery department has “thrown a compound over the fence.” This ironic or cynical expression exposes the main concern in this context: Clinical issues—that is, the human dimension of a drug project—are not properly and prospectively addressed in the early stages of preclinical discovery or even at the level of target identification or validation. Clinical researchers are then surprised or even upset by what has been sent to be developed in humans. A chemical that had been shaped years earlier with too little or no clinical input or projections may turn out to be impractical for swallowing (e.g., the compound dose may be too large or measured in grams instead of milligrams) or may quickly prove to be too short-lived, requiring multiple dosing schemes that are far out of scope in many therapeutic areas.

Why is this interface problem relevant? Bridging this divide or improving the interface performance is a major prerequisite for success if laboratory or animal data are to

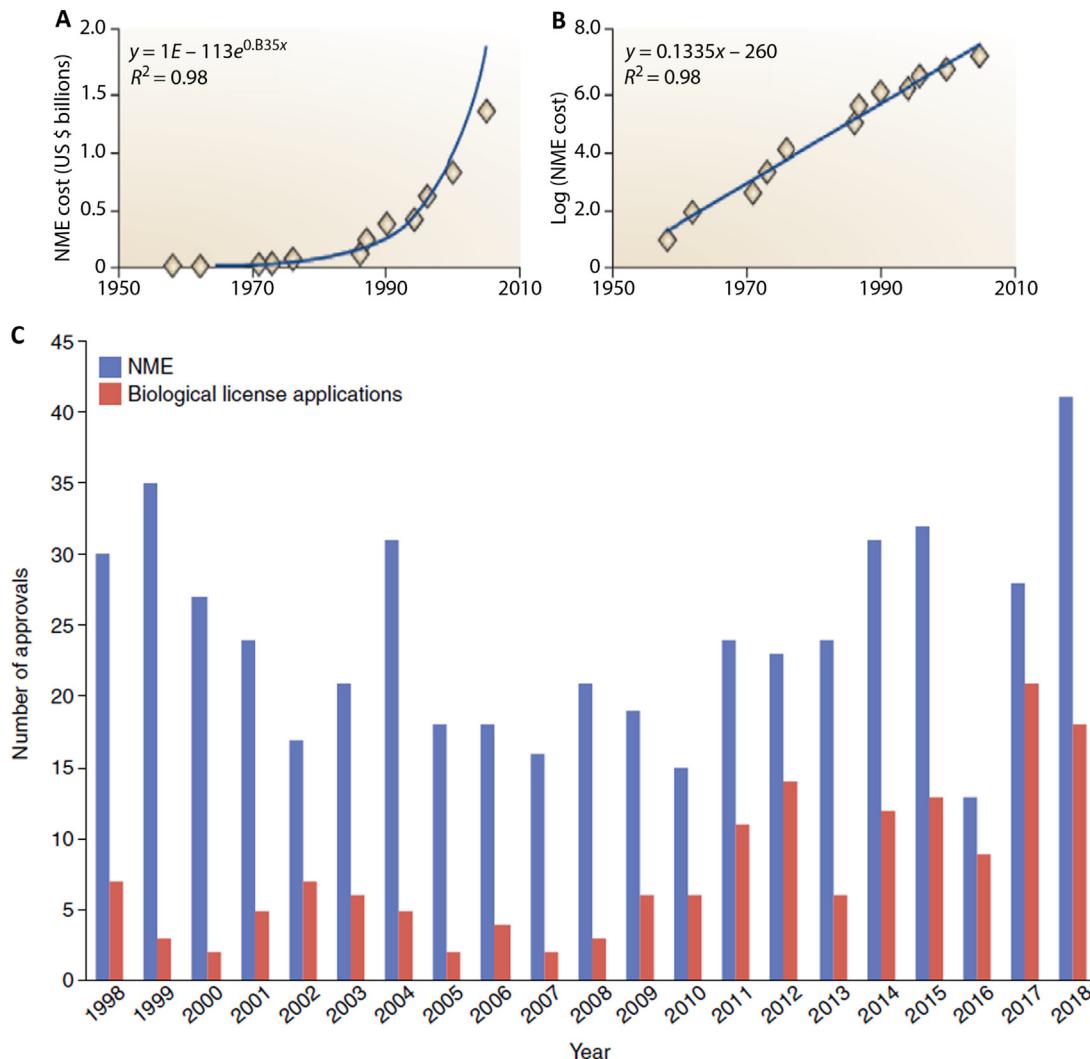


FIGURE 1.3 (A), (B) Increasing R&D costs versus (C) numbers of new drug approvals. NME = new medical entities. From Munos B. (2009) *Lessons from 60 years of pharmaceutical innovation*. *Nat. Rev. Drug Discov.* 8 959–968 ©2009 and Morrison C. (2019) *Fresh from the biotech pipeline—2018* *Nat. Biotechnol.* 37 118–123 ©2009 with kind permission from Springer Nature.

finally lead to treatment of diseases in humans. There is an old dispute over free and basic sciences versus applied sciences, and universities in particular take pride in being independent and free in their choice of research areas and scientific strategies. This *l'art pour l'art* approach is thought to still yield “useful” discoveries—by serendipity or simply as a result of chance findings. Even worse, it is thought that big, applicable discoveries can flourish only in unrestricted, free scientific settings.

Unfortunately, in drug discovery and development the assumption must be that a restricted, structured, and therapy-driven process is the only way to cope with modern standards of drug-approval requirements. Chance findings may trigger the initial steps of drug discovery, but those are rare in clinical stages. (One famous exception is sildenafil, which had been clinically developed as an antianginal and blood pressure lowering agent when its effects on erectile

function were incidentally discovered.) The typical R&D process has to rely on projections across this interface; thus it has to focus its early discovery stages on later applications, that is, the treatment of human disease.

This implies that “throwing a drug over the fence” is not optimal if the final output is to be measured in terms of the number of approved new drugs being sold on the market. Unfortunately, output *is* in fact the major concern: Complaints about this interface problem have been driven largely by the widening gap between surging R&D costs and the steadily and decreasing output of drugs from shrinking pipelines. However, in recent years the numbers of new drug approvals have recovered (Fig. 1.3) (Munos, 2009; Morrison, 2019), mainly owing to the unique successes in oncology and immunology. The exceptional translational paradigm in these therapeutic areas will be discussed in the next chapter.

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Chapter 2

Problems, challenges, and initiatives in translation

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Abstract

The potential but also limitations of the translational approach are discussed, as are the current initiatives and challenges of the still evolving subject. Numbers and reasons for attrition are provided.

Keywords: Initiatives; challenges; obstacles; attrition

Attrition

In the pharmaceutical industry, shrinkage correlates with high late-stage attrition rates, meaning that many drug projects die after billions of dollars and 5–10 years of

investment. This attrition problem particularly applies to expensive clinical phase IIb trials and especially phase III trials. Attrition can be largely attributed to the inability to predict the efficacy and/or safety of a new candidate drug from in vitro, animal, or early human data. From 1991 to 2000, only 11% of all drugs delivered to humans for the first time were successfully registered (Kola and Landis, 2004). In a more recent report on attrition between 2000 and 2010, just 1.2% of compounds nominated for clinical development and 8.8% of compounds entering phase I human trials received marketing approval (Fig. 2.1) (Waring et al., 2015).

It is obvious that there are huge differences among therapeutic areas. For example, success rates in central nervous system (CNS) or oncology drugs are particularly low (7%

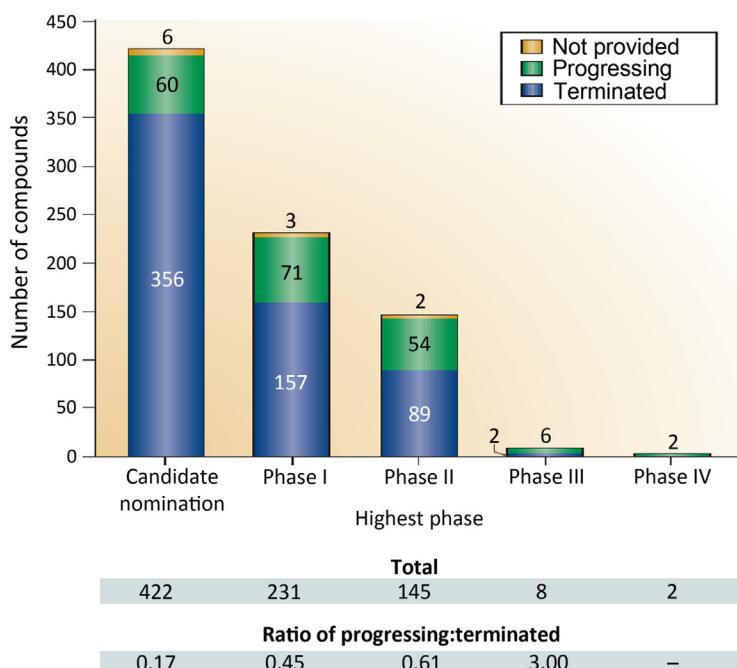


FIGURE 2.1 Success rates from candidate nomination to registration. From Waring M.J., Arrowsmith J., Leach A.R., Leeson P.D., Mandrell S., Owen R.M. et al. (2015). An analysis of the attrition of drug candidates from four major pharmaceutical companies. *Nat. Rev. Drug Discov.* 14, 475–486, reprinted by permission from Springer Nature, ©2015.

and 5%, respectively, compared to a 20% success rate in cardiovascular drugs). This means that in the CNS area, only 1 out of about every 14 compounds that have passed all hurdles to be applied to humans for the first time will ever reach the market and thus the patient. In more than 30% of cases, attrition was related to either clinical safety or toxicology, while just fewer than 30% were efficacy-related, and the remainder was caused by portfolio considerations and other reasons (Kola and Landis, 2004). In the newer data analyzed by Waring et al. (2015), Fig. 2.2, safety was even more prominent as a reason for attrition than in the older data. Attrition caused by portfolio considerations means that the company producing the project has lost interest in it because, for example, a competitor has reached related goals before the project was finished, so the project no longer has a unique selling position.

Late-stage attrition is a problem for all large companies, and lack of innovation is a major reason for the recent stagnation in progress in the treatment of some but not all major diseases (e.g., cardiovascular disease). If the tremendous costs of drug development continue to rise, companies may resort to concentrating on the relatively safe “me-too” approach. This approach aims to find minimally altered compounds that are patentable but resemble their congeners as much as possible in terms of efficacy and safety. These compounds are (sometimes erroneously) thought to be without pharmaceutical risk. Their main disadvantage is the fact that they are not innovative.

Thus tackling the translational challenges in the R&D process may become essential to the struggle for success of the pharmaceutical industry in an increasingly adverse environment. This adverse environment includes reduced remunerations for smaller innovative gains (such as those made by the aforementioned me-too compounds or even innovative drugs that have not proved to be clinically superior to marketed drugs) and ethical issues that continuously undermine the reputation of the drug industry. It now has the lowest reputation among industries, even ranking far below the reputations of the oil and tobacco industries (Gallup’s annual Work and Education poll, 2019). Thus translational medicine, if successfully applied, appears to be an important remedy for improving the ethical (i.e., patient-oriented) and financial success of the R&D process. By improving the treatment of major diseases, it could also help the battered reputation of the drug industry.

It is important to note that translational medicine problems do not pertain only to the drug industry; they are inherent to all developmental biomedical processes and include device and diagnostic tool development as well. They also exist in academia, in which translation is not the primary goal of research or at least is not perceived as such. However, in academia there is also growing awareness of the fact that public funding of expensive biomedical research will not continue forever if this funding is not seen to lead to patient-oriented results. Therefore academic research increasingly utilizes this phraseology as well.

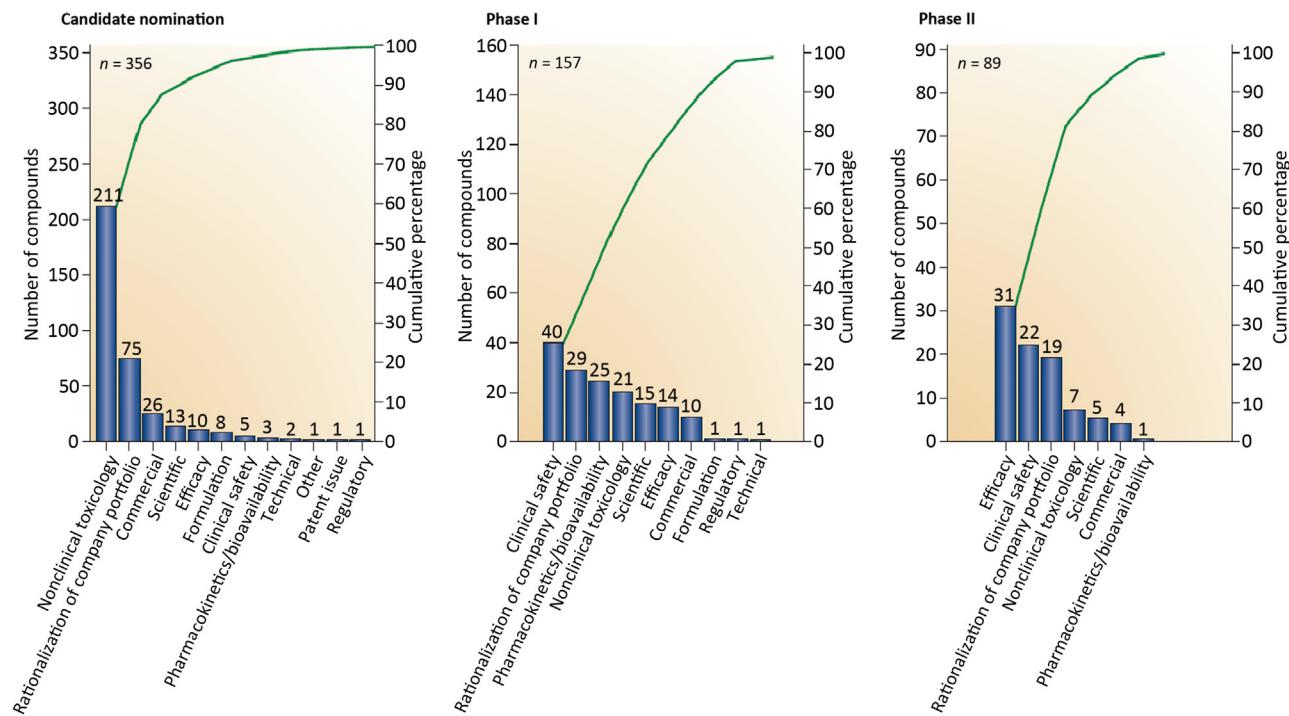


FIGURE 2.2 Main reasons for termination of drug development—for “wasted investment”—from 2000–10. From Waring M.J., Arrowsmith J., Leach A.R., Leeson P.D., Mandrell S., Owen R.M. et al. (2015). An analysis of the attrition of drug candidates from four major pharmaceutical companies. *Nat. Rev. Drug Discov.* 14, 475–486, reprinted by permission from Springer Nature, ©2015.

The persistence of the low-output syndrome in terms of true medical innovations is an obvious threat to the well-being of big pharmaceutical companies (known collectively as “big pharma”). Big pharmaceutical companies are laying off tens of thousands of people. For example, in 2019, Merck announced that it was laying off 500 employees in the United States after chopping 1800 jobs in 2017 ([FiercePharma, 2019](#)). Further cuts in the workforce are being announced almost every week, such as 466 by Sanofi in Europe ([Becker's Hospital Review, 2019](#)).

Academia is also feeling the crunch. Taxpayers will not tolerate expenditures of billions of dollars or billions of euros without measurable treatment improvement. The U.S. Congress has asked researchers what happened to the \$100 billion invested into cancer research from the mid-1980s to mid-1990s in terms of measurable outcome. The inflation-corrected National Institutes of Health (NIH) appropriation fell from its peak value of \$38.1 billion in 2005 to \$29.8 billion in 2014, a drop of 22% ([Levitt and Levitt, 2017](#)).

The effects on society are also a concern. If biomedical research does not improve its utility and create an impressive track record of substantial innovations, biomedical research will be marginalized in the competition for resources as environmental changes, such as climate or energy catastrophes, create tremendous challenges to humankind. In the future, medicine may become static, executed by robots fed by old algorithms, and progress may become a term of the past.

All these negative statements should not overshadow some positive developments that became obvious only recently. If one looks at the number of new medical entities (NMEs) and new biological entities (NBEs) approved by the U.S. Food and Drug Administration (FDA) (see Fig. 1.3C in Chapter 1, Introduction and definitions), some hope seems to loom around the corner. Regarding NMEs and NBEs, only 21 marketing approvals were counted in 2010. This figure was similar or even below average in comparison to the preceding 5 years. In 2018 a record high of 59 NMEs or NBEs were approved. There were 49 in 2017 and 30 in 2013, which are signs of hope. Did translational medicine finally work after more than 15 years of hype? It is obvious that numbers by themselves do not tell much about innovation, as redundant or

minimally divergent drugs may precipitate excessive optimism. Conversely, the surge in biologicals (humanized monoclonal antibodies) as demonstrated in Fig. 1.3 reflects a sound principle with convincing features of translatability, limited toxicity, and targeted efficacy. Biologicals may thus represent one successful approach to tackling the challenge of shrinking (or even vanishing) pipelines. The question remains where this relatively narrow avenue ends or results get saturated; antibodies have no access to intracellular structures, which caps their potential considerably.

What translational medicine can and cannot do

Proponents of translational medicine believe that the high attrition rate can be ameliorated by the main remits of translational medicine, as listed in [Table 2.1](#). The first goal is target identification and validation in humans. Identification has already been achieved by the human genome project, which literally identified all genes in the human body. Validation of known genes is the next task.

Genetics is one of the most powerful tools in this regard, because it tests

1. Disease association genes
2. Normal alleles
3. Mutant genes, especially in oncology
4. Susceptible genes such as BCRabl (imatinib)

To this end, we must ask and attempt to answer the following questions:

1. In general, does the target at least exist in the target cell or tissue, or is expression low or undetectable?
2. Is the target dysregulated in diseased tissues? This relates to functional genomics, for example, Her2neu expression (trastuzumab) or K-Ras ([Parsons and Myers, 2013](#)).

Another approach utilizes test or probe molecules:

1. Can we test the hypothesis with a probe molecule?
 - a. Using a substandard candidate drug or the side effects of a drug used for something else
 - b. Monoclonal antibodies

TABLE 2.1 Main remits of translational medicine.

Target investigation and target validation in humans.
Early evaluation of efficacy and safety using biomarkers in humans.
Use the intact living human as our ultimate screening test system.

- c. Antisense technology
 - d. Fluorescent probes
2. Has someone else tested the hypothesis?
- a. Integrin for VLA4 antagonists in multiple sclerosis

This is just a small fraction of the possible target validation or identification approaches. The basic principle is the early testing of human evidence at a preclinical stage of the drug development process. The reverse could be true as well: Knowledge of the side effects of drugs can be utilized to discover new drugs by exposing this side effect as a major effect. Minoxidil was developed as an antihair loss agent until its ability to lower blood pressure was clinically detected. Although this reverse pharmacology approach has been utilized to find pure blood pressure drugs and pure antihair loss drugs, most attempts have failed so far. However, the principle—human target identification and validation with subsequent feedback into preclinical stages (see Chapter 10, Human studies as a source of target information)—has been proven to be a successful strategy in general.

Another important focus in translational activities is on predicting as early as possible the safety and efficacy of a new compound in humans, mainly by the identification, development, and smart utilization of biomarkers. Several chapters of this book are devoted to biomarkers, which describe physiological, pathophysiological, and biological systems and the impact of interventions in those systems, including those of drugs. This is the most important translational work, and 80% of translational efforts are devoted to finding or developing the right biomarker to predict subsequent success across species, including humans. Biomarker work includes the smart design of the early clinical trials in which those experimental biomarkers are most suitably exploited. This work may also include the validation work necessary to establish the predictive value of novel biomarkers; thus it may

include a developmental program (for the biomarker) that is embedded in the drug development program.

The remit of biomarker work goes far beyond early efficacy and safety prediction: it is increasingly seen as a necessary tool for profiling compounds to better fit the needs of individual patients. The fashionable term in this context is *personalized medicine*, which is a term as old as drugs are. Renal drugs (predominantly excreted by kidneys) have always necessitated tests to assess kidney function and thus require personalized medicine; otherwise, poisoning in renal impairment is inevitable. The novelty in this regard is the use of profiling to achieve better matches between success rates (responder concentration) and thus increase cost-effectiveness. It is thought that this approach will save billions of U.S. dollars in revenue (Fig. 2.3) (Trusheim et al., 2007) when the blockbuster is new. The approach leading from blockbusters to “niche busters” has gained considerable momentum in the development of “orphan drugs,” defined as drugs that are used to treat rare diseases (Kumar Kakkar and Dahiya, 2014).

Another remit of translational medicine is its facilitation of early testing of principles in humans without directly aiming at the market development of the compound being tested. These human trials are called exploratory trials, and they may involve experimental investigational new drugs, which are compounds that are known to have shortcomings (e.g., a compound with a half-life that is too short for the compound to become a useful drug) but could be ideal test compounds. They may prove the basic hypothesis of efficacy in the ultimate test system: the human being. Such tests could validate the importance of, for example, a particular receptor in the human pathophysiology; could substantiate investment decisions; and could speed up developmental processes at early stages. Examples are given in Chapters 18–21.

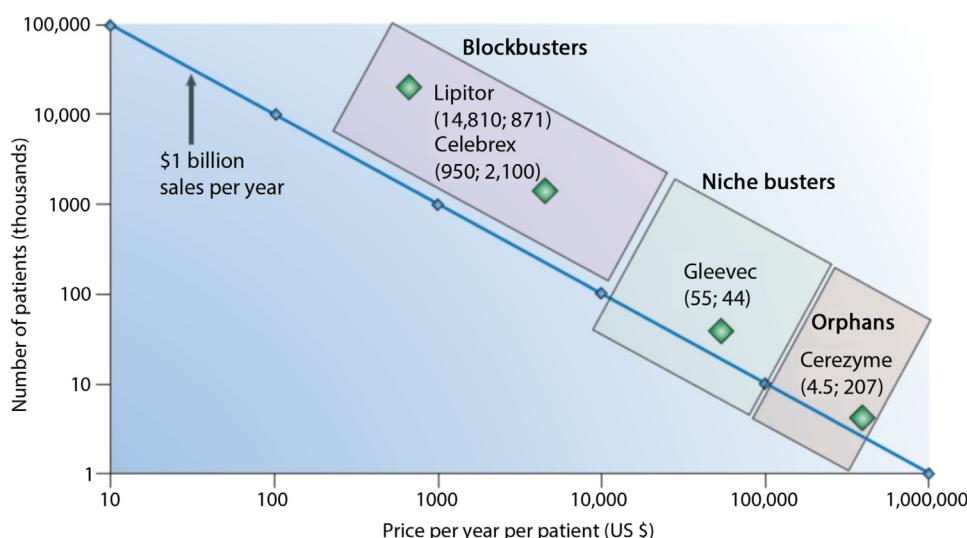


FIGURE 2.3 From blockbuster to “niche buster”, Even the latter can generate billions of dollars in revenues if profiled by personalized medicine approaches that are highly effective and prized. From Trusheim M.R., Berndt E.R., Douglas F.L., 2007. *Stratified medicine: strategic and economic implications of combining drugs and clinical biomarkers*. Nat. Rev. Drug Discov. 6 287–293, reprinted by permission from Springer Nature, ©2007.

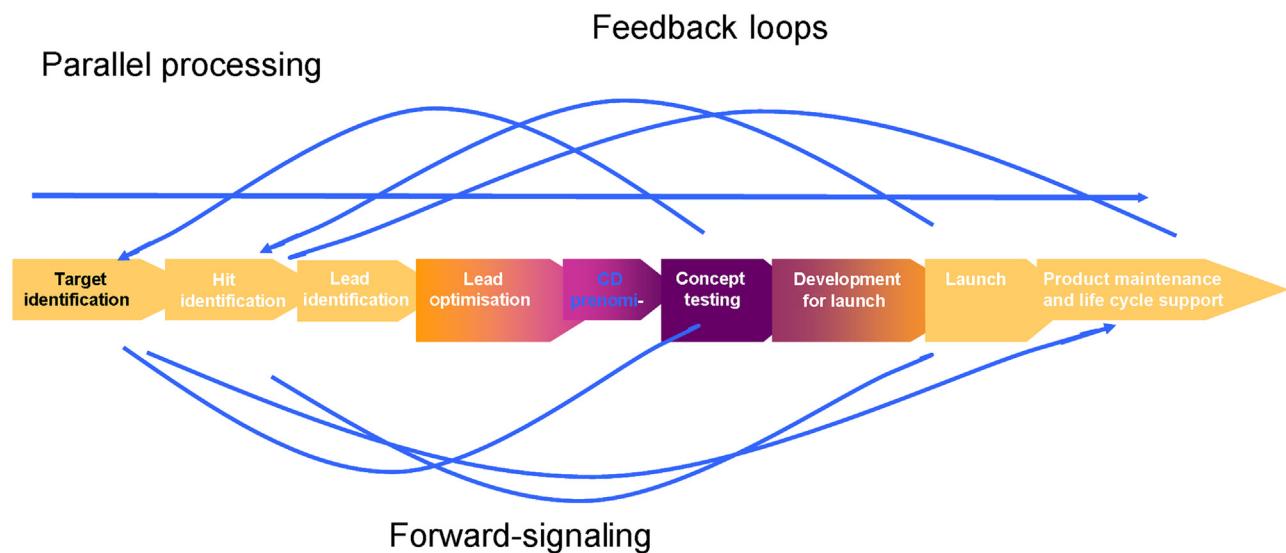


FIGURE 2.4 The pseudo-linear model of drug development, translational medicine creates forward- and reverse-signaling loops and speeds up processes and allows for parallel processing.

This short list of remits is incomplete, but it should demonstrate that the major tool of translational medicine is the early, intensive, and smart involvement of humans as the ultimate test system in discovery and development processes. Its scope reaches from straightforward translation power through reverse pharmacology to personalized medicine.

In an ideal world, translational medicine creates forward-signaling loops and reverse-signaling loops along the artificially linear development line of drugs (Fig. 2.4). It can speed up the process, allow for parallel processing, and generate knowledge for other projects as well (e.g., generic biomarker tools and side effects as target starting points).

Translational medicine cannot replace the most expensive study—the pivotal phase III (safety) trial. However, it can increase the likelihood of success in phase III trials. It cannot invent new targets (all potential targets are gene-related, and all genes have been “invented” and described), but it can significantly help to assess the validity of targets and reduce lapses due to “unimportant” targets at the human level. For these reasons, translational medicine might be the key to helping biomedical research and medicine to thrive in an era in which funding is being transferred to more successful areas of innovation such as energy and climate survival technologies.

The present status of translational medicine (initiatives and deficiencies)

The term *translational medicine* was rarely used in the 1990s. The inflation in its use was caused by increasing efforts to focus on translational issues in all areas of biomedical research. Conversely, its increased use also

caused awareness and attention, including some truly innovative initiatives.

One of the first major initiatives was the NIH Roadmap, announced in September 2003 ([NIH: A Decade of Discovery, 2020](#)). The Roadmap is a series of initiatives intended to “speed the movement of research discoveries from the bench to the bedside” and was introduced by the head of the NIH, Dr. Elias Zerhouni, who took over in 2002. The Roadmap outlined a series of goals that were put into action in 2004 or 2005. The following areas were specified:

New pathways to discovery

The implementation groups in this area are

1. Building blocks, biological pathways, and networks
2. Molecular libraries and molecular imaging
3. Structural biology
4. Bioinformatics and computational biology
5. Nanomedicine

Research teams of the future

The implementation groups in this area are

1. High-risk research
2. Interdisciplinary research
3. Public-private partnerships

Reengineering the clinical research enterprise

The implementation groups in this area are

1. Clinical research networks/NECTAR
2. Clinical research policy analysis and coordination
3. Clinical research workforce training
4. Dynamic assessment of patient-reported chronic disease outcomes
5. Translational research

Part of the strategy of the NIH Roadmap involves the funding of about 60 centers for clinical translation (Clinical and Translational Science Awards, or CTSA) in the United States, an initiative that was started in 2006. The consortium formed from this initiative had five strategic goals:

1. National clinical and translational research capability
2. The training and career development of clinical and translational scientists
3. Consortium-wide collaborations
4. The health of our communities and the nation
5. T1 Translational Research

At present, the goals for translational research are as follows:

1. Train and cultivate the translational science workforce;
2. Engage patients and communities in every phase of the translational process;
3. Promote the integration of special and underserved populations in translational research across the human lifespan;
4. Innovate processes to increase the quality and efficiency of translational research, particularly of multi-site trials; and
5. Advance the use of cutting-edge informatics.

([NIH: About the CTSA Program, 2019](#))

In 2019 the CTSA program funded 60 hubs ([NIH: CTSA Program Hubs, 2020](#)).

Critics are not convinced that the funding will truly be spent translationally, as most of it could be used to finance isolated clinical trials.

The pressure of increasing R&D costs and low output in terms of critically novel drugs forced the FDA to reconsider its own actions and those of major players in biomedical research in terms of timelines, costs, design, and, ultimately, success. The Critical Path Initiative (the official title of which is “Challenge and Opportunity on the Critical Path to New Medical Products”), published in March 2004, represented a milestone in this context ([U.S. Food and Drug Administration. Critical Path Initiative, 2020](#)). It reflects a concerted action initiated by a regulatory authority that has been criticized for its retarding activities, which were claimed to have caused the low-output syndrome described previously. Although certainly not the first public initiative to address translational medicine issues as a major concern,

the Critical Path Initiative was one of the most influential and respected ones.

There is major overlap between the Critical Path Initiative and translational medicine, although the Critical Path Initiative reaches farther into industrialization. However, its first two major goals—safety and medical utility (efficacy)—are entirely dependent on translation.

American universities have also addressed the challenge of translational medicine. Many have established centers for translational medicine, such as those at Duke University and Pennsylvania University. The single most important development regarding translational medicine in the United States was the foundation of the National Center for Advancing Translational Sciences (NCATS) in December 2011; its budget request for the fiscal year 2020 is U.S. \$833 million ([National Center for Advancing Translational Sciences, 2020](#)). CTSA has become part of this institution. The current remits of NCATS are depicted in [Fig. 2.5](#).

The major obstacles for successful translation as seen by NCATS are the following:

1. “Lack of understanding about the science of translation and insufficient rigor in conducting translational research, leading to unpredictability and frequent failure of possible interventions
2. A shortage of qualified translational investigators
3. Organizational structures and incentives that do not encourage the teamwork essential to translational science
4. Inflexible and inefficient clinical trial implementation and low participation in studies
5. A lack of data interoperability
6. Insufficient tools and technologies to predict toxicology and efficacy in safety assessment
7. A shortage of qualified biomarkers for application in disease diagnosis and measurement of treatment response
8. Inadequate development and measurement of appropriate clinical outcome measures or endpoints, including patient reported outcomes” ([National Center for Advancing Translational Sciences, 2017](#))

In addition, there are initiatives in Europe that are worth mentioning, including the European Organization for Research and Treatment of Cancer (EORTC), which is committed to making translational research a part of all cancer clinical trials, and the German Cancer Consortium.

In general, research funding programs in Europe, such as the Horizon 2020 Program of the European Union (EU), use the phrase *translational activities* in most of their topics, but institutionalization is rare in Europe. In the largest EU member state, Germany, there is almost no structural activity (e.g., university departments or independent institutes) covering this most important subject when it comes to prognosis of medical research survival,

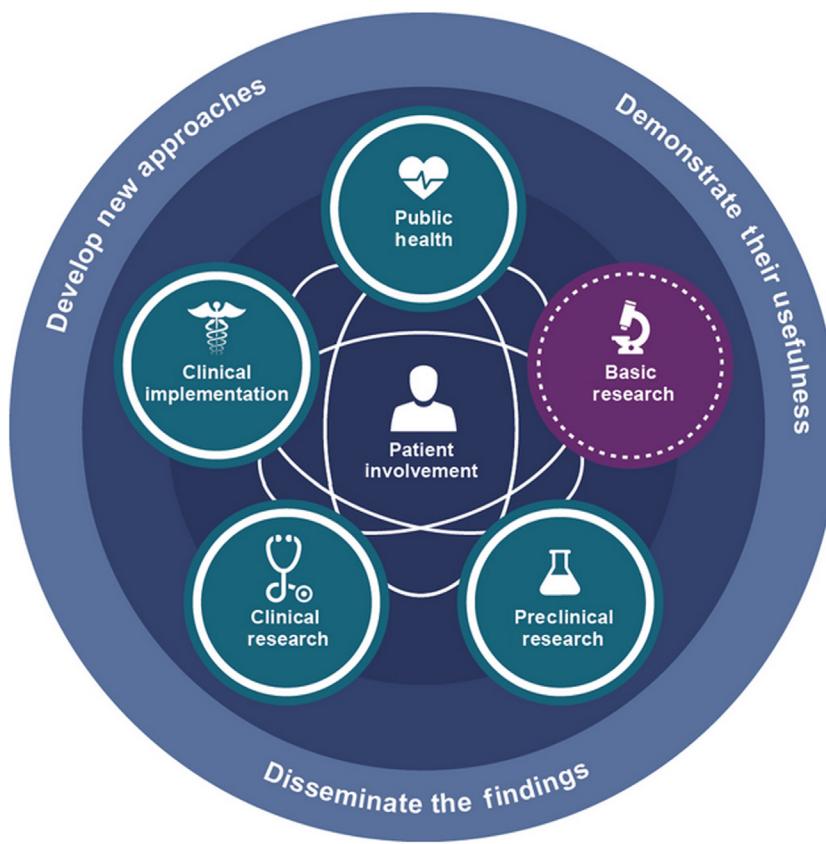


FIGURE 2.5 Remits of translational medicine in the view of the National Center for Advancing Translational Sciences (2018).

although courses in translational medicine exist in some medical faculties. If one screens the scientific programs of disease-specific institutions, such as cancer institutes, the term *translational medicine* will always appear in a prominent position; however, in the absence of dedicated structures, this appears to be phraseology rather than a sound approach. The United Kingdom and maybe the Netherlands seem most advanced in Europe, although distinct structures are still rare.

A major EU investment is the Innovative Medicines Initiative (IMI), fostering public-private partnerships for the development of drugs. Its initial budget of 2 billion euros was the largest in the field worldwide and rose to 3.3 billion euros for 2014–20, with equal contributions from the EU and industry ([Innovative Medicines Initiative, 2020](#)).

Almost all major drug companies have addressed the issue of translational medicine in one way or another. The institutional structures range from independent departments of translational or discovery medicine to entirely embedded dependent structures that are part of the drug discovery or development teams without central facilities. Special interest groups are the least specific modification of the R&D process, although they are not necessarily ineffective. It is obvious that the challenge has been collectively identified by industry and is being met in widely varied ways.

In general, U.S. biomedical players have invested the most (the NIH has announced that it plans to spend a total of up to \$10 billion) and are at the forefront of translational institutionalization, while the individual countries in Europe are lagging behind at different distances. Commercialization of academic inventions has traditionally been much more efficient in the United States than in the EU, and success in translational medicine has a lot to do with expertise accrued during commercialization processes. Later processes can be lucrative only if translational steps have been added to a biomedical invention, and facilitating such early developmental investments has been on the to-do list of successful universities (e.g., the University of California, San Francisco, and Harvard University) and smaller companies for many years. The rapid pace of business development of currently medium-sized or large companies such as Amgen and Genentech may have to do with their early and effective understanding and instrumentalization of translational issues.

Translational medicine in the BRIC countries (Brazil, Russia, India, and China) will be developed as their pharmaceutical and biomedical markets develop. It would come as a surprise if innovation in those countries were to ignore the potential of translational medicine. In fact, China has already developed leadership in segments of this area. The National Research Center for Translational

Medicine in Shanghai and the Suzhou Translational Medicine Research Center by CStone Pharma are examples. There is comparably prominent Chinese participation in, for example, the editorial board of the *Journal of Translational Medicine* (editor-in-chief: Francesco Marincola), and this indicates that awareness of translational medicine has been created in this huge country.

The aforementioned journal, founded in 2003, so far represents one of the only two journals explicitly devoted to translational medicine. On its 2007 home page (no longer available), it stated that it

aims to improve the communication between basic and clinical science so that more therapeutic insights may be derived from new scientific ideas—and vice versa. Translation research goes from bench to bedside, where theories emerging from preclinical experimentation are tested on disease-affected human subjects, and from bedside to bench, where information obtained from preliminary human experimentation can be used to refine our understanding of the biological principles underpinning the heterogeneity of human disease and polymorphism(s).

At present the journal describes its remit as follows:

Journal of Translational Medicine is an open access journal publishing articles focusing on information derived from human experimentation so as to optimise the communication between basic and clinical science. (*Journal of Translational Medicine*, 2020).

In 2009 the American Association for the Advancement of Science established a new journal, *Science Translational Medicine*, which belongs to the *Science* family of high-impact journals. In its fourth year, 2012, the journal already had an impact factor of 10.757, and that rose further in 2018, to 17.1.

In conclusion it appears that translational medicine has been basically understood and substantially funded mainly in the United States, while Europe is lagging behind. In the BRIC countries, China in particular has undertaken serious efforts to catch up with the United States, thus leaving Europe behind if investments remain minor or unstructured in the EU. The IMI of Europe, although it is the most expensive program in the world, still lacks clear and institutional translational structures.

Translational science in medicine: the current challenge

The title of this book (*Principles of Translational Science in Medicine*) contains the main challenge in this context as seen by the authors. It does not use the common phrase *translational medicine* but rather introduces the term *science* as the second, and thus very important, word. Why?

The simpler term *translational medicine* seems to reflect the wishful thinking of people who use it to denote the appropriate direction that should be taken. As with all biomedical science, there is not, nor has there ever been, substantial doubt about the direction to be taken: All efforts should, in a more or less direct sequence, ultimately lead to improvements in patient care. If one accepts this proposition, translational medicine, in a more philosophical sense, is not new. It simply describes the final direction and destination of all biomedical activities (with the exception of forensic or insurance activities in medicine, which certainly serve purposes other than patient care, but this is only a very small segment of the total pie). All modern drugs whose discovery and development followed the classical path from the test tube through animal experiments to human application must have gone through a more or less efficient translational process.

It is obvious that for many—largely ethical—reasons, systematic development of drugs, devices, and medical tests cannot be performed in humans from day one. Thus translation processes have been inherent to all biomedical research ever done under ethical auspices. Therefore it is obvious that translational medicine was “invented” (although not identified as such) by all the biomedical disciplines that have attempted to introduce new treatments for human diseases for thousands of years. There are famous examples of translational processes in many historic documents (e.g., Alexander Fleming’s translation of the in vitro observation of fungi that inhibit bacterial growth into clinically used penicillin). So what is new?

As was noted earlier, the fame of the term *translational medicine* has been brought about by the increasing demand for a successful, reliable, reproducible, and efficient way of translating results from animals or test tubes to humans as the translational paradigms that have successfully worked in the past seem to be failing. Thus neither the claim nor the procedure as such is new, but the quality of the process is not sufficient and needs to be improved if significant innovations in major therapeutic areas are to come back at an appealing pace. The pivotal question is thus how this can be achieved, not how we can convince others of the need for translation. The methods of how translation should take place—rather than the fact that it should take place—should be the true claim of the present translational movement.

However, this claim has generally been neglected in terms of structured approaches and at the level of concept development. The clear formulation of this contemporary challenge is to claim that this aim can be achieved only by the establishment and development of a novel science. The *Encyclopedia Britannica* (2020) defines science as “any system of knowledge that is concerned with the physical world and its phenomena and that entails unbiased observations and systematic experimentation. In general, a science

involves a pursuit of knowledge covering general truths or the operations of fundamental laws.” The science of translational medicine is truly innovative and genuine in that it has not yet been explicitly named, supported, promoted, established, or even recognized. It is thus termed *translational science in medicine*. The specification “in medicine” reflects the fact that translational sciences may be established in other scientific areas as well, such as physics and chemistry.

If it translational science declared a science, what claims and remits should it deal with? The experimental processes and tools or methods used for translational processes in medicine should be clearly defined and used in reproducible, objective, and measurable translational algorithms. Thus toolboxes (as named in the Critical Path Initiative) need to be developed; the strategies described in translational development plans need to be standardized; and decision trees need to be developed, tested, validated, and exercised. The early clinical trial program should be structured according to translational needs (early efficacy and safety testing). Thus in an ideal world the methodology of translational science would comprise a canon of widely applicable, generic procedures that reliably generate quantifiable prediction quality. This includes toolboxes with appropriate biomarkers, their validation, their grading for predictivity, smart early human trial designs, biostatistics methods to cope with multiple readout problems, computational biology, decision tree (go-or-no-go decision) algorithms, and many other activities, which together could give translational science the right to be called a true science.

The basic goals of this book are to help readers start thinking in these terms, to trigger concept development, and to collect available pieces—such as research in the biomarker arena—that can be incorporated into the nascent field of translational science in medicine. We hope that this book will be seminal to trigger the launch of this science and thus contribute to its projected establishment and related benefits.

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Chapter 3

“Omics” translation: a challenge for laboratory medicine

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Abstract

The “omics” revolution of the past 15 years has represented the most compelling stimulus in personalized medicine. Fields with names such as genomics (genetic complement), transcriptomics (gene expression), proteomics (protein synthesis and signaling), metabolomics (concentration and fluxes of cellular metabolites), metabonomics (systemic profiling through the analysis of biological fluids), and cytomics (the study of cell systems—cytomes—at a single-cell level) have been introduced in medicine with increasing emphasis. In this chapter these fields are characterized and profiled for their strengths and weaknesses and their contributions to translational success.

Keywords: Genomics; transcriptomics; proteomics; metabolomics; metabonomics; cytomics

Introduction

The rapid advances in medical research that have occurred over the past several years have allowed us to dissect molecular signatures and functional pathways that underlie disease initiation and progression and to identify molecular profiles related to disease subtypes in order to determine their natural course, prognosis, and responsiveness to therapies (Dammann and Weber, 2012). The “omics” revolution of the past 15 years has represented the most compelling stimulus in personalized medicine, a term that can be simply defined as “getting the right treatment to the right patient at the right dose and schedule at the right time” (Schilsky, 2009). As a matter of fact, among the 20 most-cited papers in molecular biology and genetics that have been published in the past decades, 13 entail omics methods or applications (Ioannidis, 2010). Omics is expected to transform the practice of medicine, including improvements in diagnosis, prognosis, prediction, treatment choice, individualized prevention, and, ultimately, better outcomes (Ioannidis, 2009).

“Omics”: what does it mean?

Omics is an English-language neologism that refers to a field of study in biology focusing on large-scale and holistic data, as derived from its root of Greek origin, which refers to wholeness or to completion. Initially, the suffix *-omics* was used in the word *genome*, a popular term for the complete genetic makeup of an organism, and later, in the term *proteome*. Genomics and proteomics succinctly describe a new way of holistic analysis of complete genomes and proteomes, and the success of these terms led to the trend of using *omics* as a convenient term to describe holistic ways of looking at complex systems, particularly in biology.

Fields with names such as *genomics* (genetic complement), *transcriptomics* (gene expression), *proteomics* (protein synthesis and signaling), *metabolomics* (concentration and fluxes of cellular metabolites), *metabonomics* (systemic profiling through the analysis of biological fluids), and *cytomics* (the study of cell systems—cytomes—at a single-cell level) have increasingly been introduced in medicine (Plebani, 2005). However, beyond these terms, multiple omics fields, with names such as epigenomics, ribonomics, epigenomics, oncopeptidomics, lipidomics, glycomics, spliceomics, and interactomics, have been similarly explored in the study of molecular biomarkers for the diagnosis and prognosis of human diseases.

Each of these emerging disciplines grouped under the umbrella of omics shares the simultaneous characterization of dozens, hundreds, or thousands of genes (genomics), gene transcripts (transcriptomics), or proteins (proteomics) and other molecules that in aggregate and in parallel should be coupled with sophisticated bioinformatics to reveal aspects of biological function that cannot be culled from traditional linear methods of discovery (Finn, 2007). While an increasing body of literature has been produced to prove that omics will irrevocably modify the

practice of medicine, that change has yet to occur, and its precise details are still unclear. The reasonable assumption that the application of omics research will be riddled with difficulties has led to a much better appreciation of concepts of knowledge translation, translational research, and translational medicine.

Proteomics as a paradigm of problems in translational medicine

The paradigm of obstacles in translating new omics insights into clinical practice is a study reporting that a blood test, based on pattern-recognition proteomics analysis of serum, was nearly 100% sensitive and specific for detecting ovarian cancer and was possibly useful for screening (Petricoin et al., 2002a). The approach involved the analysis of a drop of blood using mass spectrometry, resulting in a large number of mass-to-charge ratio peaks (15,000–300,000 peaks, depending on technology) that were then subjected to pattern-recognition analysis to derive an algorithm that discriminates patients with cancer from those without. Which substances cause the peak (e.g., proteins, peptides, or something else) was unknown, as it was unclear whether these substances were released by tumor cells or by their microenvironment.

After this and further supporting work (Adam et al., 2002; Drake et al., 2003; Petricoin et al., 2002b; Vlahou et al., 2001; Zhu et al., 2003), commercial laboratories planned to market a test in late 2003 or early 2004, but plans were delayed by the U.S. Food and Drug Administration (FDA). Questions were raised about whether technological results were reproducible and reliable enough for widespread application in clinical practice.

During the past few years a large number of scientists have been able to identify other candidate protein disease biomarker profiles using patient research study sets and have achieved a high degree of diagnostic sensitivity and specificity in blinded test sets. Nevertheless, translating these research findings to useful and reliable clinical tests has been the most challenging accomplishment. Clinical translation of promising ion fingerprints has been hampered by “sample collection bias, interfering substances, biomarker perishability, laboratory-to-laboratory variability, surface-enhanced laser desorption ionization chip discontinuance and surface lot changes, and the stringent dependence of the ion signature on the subtleties of the reagent composition and incubation protocols” (Liotta and Petricoin, 2008, p. 3). Systematic biases arising from preanalytical variables seem to represent a relevant issue. Examples of nondisease-associated factors include (1) within-class biological variability, which may comprise unknown subphenotypes among study populations; (2) preanalytical variables, such as systematic differences

in study populations and/or sample collection, handling, and preprocessing procedures; (3) analytical variables, such as inconsistency in instrument conditions, resulting in poor reproducibility; and (4) measurement imprecision (Hortin et al., 2006). Biological variability, in particular, may entail potential diurnal variation in protein expression, thus making standardization of sample collection time virtually mandatory. An evaluation of the effects of gender, age, ethnicity, pathophysiological conditions, and benign disorders is also crucial for understanding other possible effects on protein profiling expression. Regarding preanalytical conditions (e.g., collection practices, sample handling, and storage), these may differ from institution to institution, thus influencing the detection of proteins present in biological fluids. Standardization and use of specimens from multiple institutions are hence necessary to reliably demonstrate the efficiency and reproducibility of protein profiling (Lippi et al., 2006; Banks, 2008). Although these preanalytical influences have been recognized for a long time, their impact is likely to be greater in proteomics studies, given the simultaneous analysis of several proteins, resolution of multiple forms of proteins, and detection of peptide fragments arising from active cleavage processes. Moreover, relatively few studies have been performed in such a way that quality control, an essential feature, should be incorporated in proteomic experimental protocols (Hortin, 2005). Reproducibility studies performed with adequate control materials are prerequisites for safe introduction of proteomic techniques in clinical laboratory practice. Table 3.1 summarizes the major problems in translating proteomics insights into clinical practice.

It is now clearly accepted that the lack of standardization in how specimens are collected, handled, and stored represents one of the major hurdles to progress in the hunt for new and effective biomarkers (Poste, 2011). Nevertheless, the significance of assay technical quality has recently been underpinned. Diamandis, for example, has elegantly demonstrated that the assay for a new promising marker for prostate carcinoma was strongly affected by severe methodological drawbacks, including its dependence on the total protein content, namely, the albumin concentration in serum (Diamandis, 2007). The major limitations of this assay are even more important because the apparently spectacular clinical results have been highly publicized to the media whereas potential following failures have not. A review of the literature on translational research in oncology has revealed that most of the 939 publications on prognostic factors for patients with breast cancer that appeared over a 20-year period were based on research assays with poor evidence of robustness or analytical validity (Simon, 2008). This fact should lead journal editors to ask for more robustness of the analytical techniques used for quantification of novel, putative biomarkers (Anderson et al., 2013), since problems such as data manipulation, poor experimental design, reviewer

TABLE 3.1 Obstacles in translation of proteomics research.

Preanalytical factors
a. Patient selection bias
b. Lack of standardization of sample collection, handling, and storage
Analytical factors
a. Technological variability within and across proteomic platforms
b. Different performance of prototype and routine assays
c. Lack of appropriate quality control and external quality assurance data
Statistical/bioinformatics
a. Inappropriate statistical analysis
b. Data overfitting
c. Multiple hypothesis testing
d. Overlapping of training and validation patient cohorts
Clinical validation
a. Poor study design, particularly as regards the verification phase
b. Shortcomings in identifying the true clinical use (e.g., diagnosis vs prognosis or monitoring)
c. Poor evaluation of the effects of the test on clinical outcomes and patient management

bias, and overinterpretation of results are being reported with increasing frequency ([Diamandis, 2006](#)).

Current limitations and open questions regarding clinical proteomics reflect a lack of appreciation of the many steps involved, including evaluation of preanalytical, intraanalytical, and postanalytical issues; interlaboratory performance; standardization; harmonization; and quality control. These are all needed to progress from method discovery to clinical practice ([Plebani and Laposata, 2006](#); [Plebani and Marincola, 2006](#)).

It now seems unquestionable that the problem is more complex, involving not only practical considerations but—more interestingly—a philosophical reasoning. The linear process of hypothesis-driven discovery that characterized the past decades of science has recently been replaced by the hypothesis-generating power of high-throughput, array-based technologies that provide vast and complex datasets that may be mined in various ways using emerging tools of computational biology. David F. Ransohoff has dedicated a series of papers to deepening problems related to this new approach, which has been called *discovery-based research* and in which there is no need to identify targets ahead. Instead, large portions of the genome or proteome may be examined for markers that can be used in diagnosis or prognosis ([Ransohoff, 2004, 2005, 2007](#)).

The evidence that many promising initial results have turned out to be unreliable or scarcely reproducible has resuscitated interest in a more consistent evaluation of result reliability. The term *reliability* refers to whether study results are reproducible and not explained by

chance or bias, so that a conclusion can be used as a solid building block or foundation to ask other questions. Reliability is determined by proper attention to study design and interpretation. No single study can be considered almost perfect, but each study must be fairly interpreted. In particular, results should not be overinterpreted. The primary responsibility for interpretation and specifying study limitations belongs to the investigator(s), although reviewers and editors have some role in the final diffusion of data. According to this concept, compliance with guidelines developed for studies of diagnostic tests (Standards for Reporting of Diagnostic Accuracy, or STARD) is crucial ([Bossuyt et al., 2003](#)). In fact, when compliance with STARD standards is low for traditional tests, it is reportedly even lower for omics-based tests ([Lumbreras et al., 2006](#)). Although Ransohoff has put forward some solutions to improve omics research, including guidelines, recommendations, and use of phases in the development of a diagnostic marker ([Ransohoff, 2007](#)), a fundamental remedy is to facilitate collaboration and communication among diverse audiences, including basic and laboratory researchers, clinical epidemiologists, and clinicians involved in interdisciplinary research on biomarkers. When developing and evaluating new technologies, biologists, technologists, epidemiologists, and physicians need to understand the existence of distinct steps and the limits of each of their fields. Biological and technological reasoning are necessary to develop a promising approach such as pattern-recognition mass spectrometry for analyzing serum proteins. Generally, the first step should give a reliable answer to the question "Might

it work?” After it has been shown that a given technology, in fact, does work, biological and technological reasoning are again necessary to provide insights about the further reasonable question “How does it work?” However, the step in between—which is reflected by the question “Does it work?”—requires multifaceted reasoning that is not specifically biological or technical. That step also requires epidemiological and clinical reasoning, entailing stringent criteria in study design, analysis of data, and—finally—straightforward evaluation of clinical results (Ransohoff, 2005). However, this is not enough, and particularly in the last few years the appropriate evaluation of the clinical validity, clinical utility, and ethical, legal, and social implications (ACCE) is considered a benchmark framework to evaluate emerging technologies that has been updated considering omics (Lal et al., 2014).

Development of biomarkers: from discovery to clinical application

The maturation of high-throughput-omics technologies has set the pace for biomarker discovery to a point at which it has evolved from being an observational by-product of clinical practice into a large-scale systematic process, often referred to as a pipeline (Pavlou et al., 2013). However, despite growing interest and investments, the number of biomarkers receiving FDA clearance has declined substantially to less than one/two protein biomarkers per year (Anderson and Anderson, 2002). Despite a large literature of more than 150,000 papers documenting thousands of putative biomarkers, fewer than 100—a drop in the ocean—have been validated for clinical practice (Poste, 2011). The journey of a new biomarker from the bench to bedside is hence long and challenging, and every step must be meticulously planned and not overlooked in this pipeline.

The Early Detection Research Network (EDRN), established by the Division of Cancer Prevention of the National

Cancer Institute, has identified a widely accepted model, consisting of five separate phases, for development and testing of disease biomarkers (Pepe et al., 2001). This model, however, should be integrated by some particular steps and considerations that are specifically useful for utilization of biomarkers in everyday clinical practice. Fig. 3.1 shows a roadmap for the translational process of biomarkers from discovery to clinical application consisting of six phases (discovery, identification, validation, standardization, clinical association, and clinical application) that better describe both the overall process and the sites involved in the steps from discovery to effective clinical application of putative biomarkers (Plebani et al., 2008).

Discovery

The discovery of new biomarkers should be based on deep knowledge of a given disease, its pathophysiological mechanisms, and clinical needs in terms of both diagnostic and therapeutic goals. The search for biomarkers should be based on possible targets for screening, diagnosis, prognostication, and therapeutic monitoring. Clinical genomics and proteomics research should follow two possible paths. One path (the traditional approach) is based on the “one at a time, lead candidate biomarker approach” (Stanley et al., 2004) through the identification of new candidate markers using omics techniques. The other path (the innovative approach) is based on so-called proteomic/genomic pattern diagnostics or proteomic/genomic profiling (Anderson, 2005). The rationale for this latter approach is that a pattern of multiple biomarkers may contain a higher level of discriminatory information than a single biomarker alone across large heterogeneous patient populations and for complex multistage diseases. Although the search of a unique biomarker has been compared to finding a needle in a haystack, the complex proteomic signature of the disease-host microenvironment may represent a “biomarker amplification cascade” (Semmes, 2005). These two approaches are complementary rather than mutually exclusive.

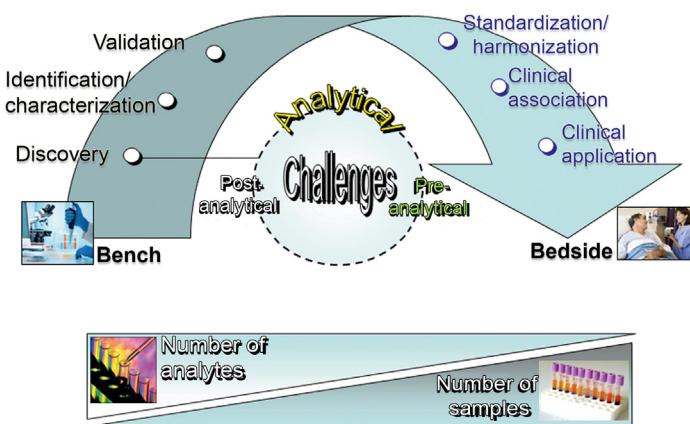


FIGURE 3.1 The six phases of the roadmap for translational research of biomarkers.

Identification/characterization

The molecular/biochemical characterization of a new single biomarker rather than a biomarker pattern is needed to both provide a better understanding of its nature and develop reliable test systems. In the postgenome era the importance of posttranslational modifications, including phosphorylation, glycosylation, acetylation, and oxidation, has been increasingly recognized, since they are linked to disease pathology and are useful targets for therapeutics (Lopez et al., 2012). The recognition of different molecular forms of some biomarkers [e.g., human chorionic gonadotropin (HCG) and prostate-specific antigen (PSA)] has allowed us to better elucidate their nature and to develop different and more specific immunoassays (Morris, 2009). Therefore the accurate characterization of the nature of each novel biomarker is an essential prerequisite for further steps in the development pipeline.

Validation

Validation of biomarkers is a challenging process that requires multisite clinical studies. The initial validation process is intended to perform a preliminary evaluation of clinical performances (in particular, sensitivity, and specificity) in selected groups of patients and a reference population (i.e., controls) using a research method (technique). The term *test research* refers to the type of study that follows a single-test or univariable approach, which means studies focusing on a particular test to quantify its sensitivity, specificity, likelihood ratio, or area under the receiver operating characteristic (ROC) curve (Moons et al., 2004). The most widely acknowledged limitation of test research is that some studies are based on an improper patient recruitment and study design (Ransohoff and Feinstein, 1978). A typical example is the use of cardiospecific troponins for diagnosing myocardial infarction in the emergency department (ED). In this setting, the definition of the upper limit of the reference range of a given troponin immunoassay should not be based on a population of healthy individuals because a differential diagnosis of myocardial infarction in the ED is made on patients who are inherently unhealthy (Lippi et al., 2013). In the case of clinical proteomics an additional and severe bias is represented by the lack of studies aiming to demonstrate the transferability of data obtained at one site to other sites.

Standardization/harmonization

The pipeline for translation of new biomarkers into routine laboratory tests seems to have a bottleneck at the early stage of translation of research markers into clinical tests (Hortin et al., 2006). Research groups performing

discovery and initial validation rarely have enough resources and skills to develop prototype analyzers or reagent sets, to manufacture them, or to proceed with other steps in commercialization. This is also due to the increasingly complex system of evaluation and regulation required for any diagnostic test by regulatory agencies such as the FDA and similar European authorities. These steps usually rely on the in vitro diagnostic industry, which has reduced funding for development of new biomarkers as a result of increased competition on cost and decreased revenue of laboratory products/services.

With specific emphasis on clinical proteomic profiling (although this has provided breakthroughs in the discovery of new disease markers), initial discovery methods have been typically poorly suited for clinical application. Glen Hortin, in an interesting editorial, has emphasized the "lack of appreciation of the many steps, such as evaluation of interlaboratory performances, from method discovery to clinical practice" (Hortin, 2005).

The need to achieve standards of practice is not limited to the analytical step; it extends to preanalytical and postanalytical issues. In particular, optimal materials and methods for sample collection and processing should be determined, including the selection of appropriate sample matrix, protease inhibitors, and collection tubes (Ayache et al., 2006).

Studies should address patient preparation, evaluating the effects of diurnal variation, and fasting versus nonfasting along with additional aspects. For any individual, many analytes present remarkable fluctuations according to the time of day, fasting state, or age. Although these changes may not be clinically relevant, they do add an additional level of complexity in elucidating disease-induced protein changes from modification due to biorhythm.

In addition, the effects of temperature, sample type (plasma vs serum) anticoagulants, and centrifugation methods used have to be carefully evaluated, as these variables strongly affect the accuracy and reliability of many biomarkers, including miRNA expression profiles, as shown in Fig. 3.2 (Basso et al., 2017).

Similarly, analytical and postanalytical issues such as quality control, external quality assurance, reference intervals, and decisional levels should be carefully considered (Plebani, 2013). The definition of standards of practice and quality indicators for preanalytical, intraanalytical, and postanalytical phases is a prerequisite for evaluating a new biomarker in the clinical setting (Plebani et al., 2013).

A reliable evaluation of the clinical utility of newly developed assays, particularly concerning the omics, must take into account the careful examination of analytical performances. Ideally, for established analytes the performance characteristics limits are determined by several factors, including outcome studies, clinical requirements, published professional recommendations, and goals set by regulatory agencies. For a novel analyte, no such

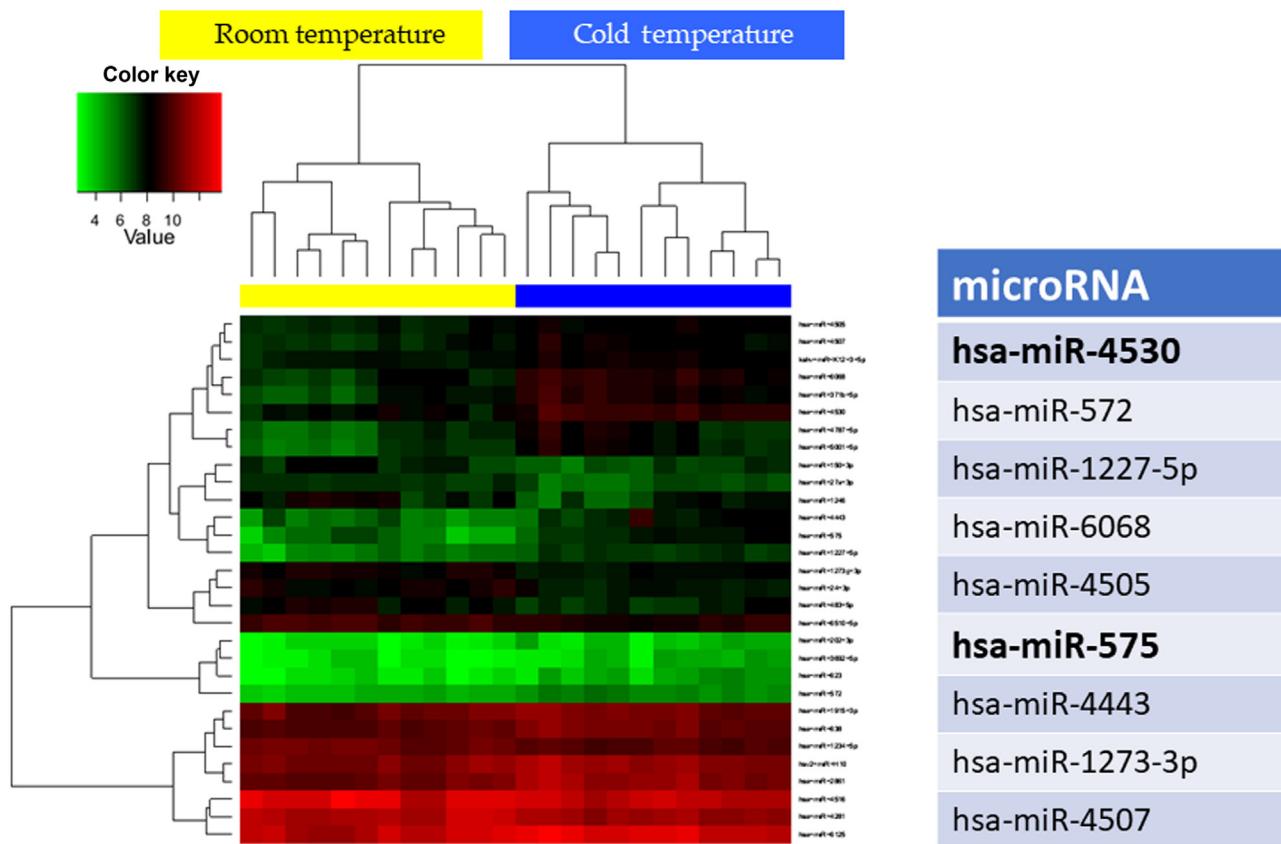


FIGURE 3.2 Effects of different temperatures on miRNA expression profiles. Modified from Basso D., Padoan A., Laufer T., Aneloni V., Moz S., Schroers H., et al., 2017. Relevance of pre-analytical blood management on the emerging cardiovascular protein biomarkers TWEAK and HMGB1 and on miRNA serum and plasma profiling. *Clin. Biochem.* 50, 186–193.

performance specification may be available, but analytical performance comparable to an established, similar assay is expected. Briefly, fundamental analytical performances are grouped as indicators of accuracy, indicators of precision, and indicators of analytical measurement range.

- 1. Indicators of accuracy.** Trueness and accuracy are similar but not identical concepts to describe this aspect of assay performance. *Trueness* is the closeness of agreement between the average analyte value of different samples and the true concentration value, whereas *accuracy* is the closeness of the agreement between the value of a measurement and the true concentration of the analyte in that sample (Dybkaer, 1997; International Standards Organization, 1994). Trueness reflects bias, a measure of systematic error, whereas accuracy reflects uncertainty, which comprises both random and systematic errors. For a novel assay, the evaluation of bias is challenging because reference methods and materials do not usually exist, so an alternative approach should be applied.
- 2. Indicators of precision.** Repeatability and reproducibility are measures of precision and are used to quantitatively

express the closeness of agreement among results of measurements performed under stipulated conditions (Dybkaer, 1997; NCCLS, 1999). Repeatability refers to measurements that are performed under the same conditions, whereas reproducibility is used to describe measurements performed under different test conditions. Imprecision strongly affects the interpretation and reliability of a single result. For example, it is now accepted that the analytical imprecision goal for cardiac troponins is to have a coefficient of variation (CV%) that is less than 10% at the decisional level (Thygesen et al., 2012). This represents a challenge for both manufacturers and clinical laboratories. The former are required to commercialize a reliable diagnostic system; the latter have to select the most appropriate method or diagnostic system with regular basis monitoring in order to guarantee both the achievement of satisfactory performance and the appropriate utilization of the marker in clinical practice. Precision and accuracy are intimately related, and they strongly affect the clinical reliability of a test result as expressed by the concept of total error that is the sum of bias and imprecision.

- 3. Indicators of analytical measurement range.** Linearity and limits of detection and quantification must be assessed to determine the range of analyte values over which measurements can be performed with acceptable precision and accuracy. In particular, the limit of detection is defined as the lowest value that significantly exceeds the measured value obtained with a sample that does not contain the protein or analyte of interest (International Standards Organization, 2000).

These simple concepts and the more complex rules for ensuring a good analytical performance are typically neglected and poorly recognized by experienced and trained physicians and sometimes by basic scientists. However, all laboratory results should ensure consistency and comparability. The magic word *standardization* underlines the importance of ensuring reliability, reproducibility, and accuracy of data released in a laboratory report, particularly for tests that strongly affect clinical reasoning and patient management.

Clinical association and clinical benefit

When a reliable method or technique is available in a format that should allow interlaboratory agreement and comparability of results, retrospective and prospective studies are needed in order to evaluate the effective clinical performances in the real world and not simply in selected groups of patients. The predictive values of a given test vary across patient populations, and a particular test may have different sensitivities and specificities within different patient subgroups, underscoring the need to move from test research to diagnostic research. The term *diagnostic research* refers to studies that aim to quantify a test's added contribution beyond test results already available to the physician for determining presence or absence of a particular disease (Reid et al., 1995; Rutjes et al., 2005). The focus is on the value of the test in combination with other previous tests, patient history, and physical examination. As any test result in real life is always considered in light of other patient characteristics and data, diagnostic accuracy studies that address only a particular test have limited relevance to clinical practice. Therefore well-designed studies are needed that should take into account possible spectrum effects and should therefore be based on well-defined and evidence-based inclusion and exclusion criteria, reference, and gold standards as well as reliable statistical analyses. The traditional statistical methods that are used by epidemiologists to assess etiologic associations are inappropriate for assessing the potential performance of a marker for classifying or predicting risk (Kattan, 1989). While this aspect is not widely appreciated, some authors have recently emphasized the limitations of some traditional methods, including the odds ratio, in gauging the performance of a marker (Baker et al., 2002; Pepe et al., 2004). Techniques that

directly address classification accuracy (e.g., ROC curves) rather than traditional logistic regression techniques should be used. The need to evaluate the test performance in the setting of the specific clinical application has been highlighted, particularly when the gold standard is not appropriately defined (Pepe and Longton, 2005; Pfeiffer and Castle, 2005). Therefore as we proceed to develop technologically sophisticated tools for individual-level prediction and classification, we must be careful to use appropriate statistical techniques when evaluating research studies aimed at assessing their performance. Rigorous criteria should also be identified and used in assessing the economic and clinical outcomes when these new biomarkers are translated into clinical practice and used alone or in combination in public health practice. The evidence on the importance of translating a new biomarker into clinical practice can hence be considered in a hierarchy, all elements of which are important in making the decision.

The technical performance is the foundation of any evidence and has an important bearing on diagnostic performance. In laboratory medicine, there are well-defined and accepted guidelines for evaluating technical performances of a diagnostic system or method, including precision, accuracy, analytical range, and interferences (Linnet and Boyd, 2006). Other recommendations exist for evaluating preanalytical factors including the sample matrix of choice (plasma or serum), potential type and concentration of anticoagulants, and storage conditions (Young et al., 2006). Diagnostic performance represents the second level of the hierarchy and provides an assessment of the test in terms of objective use, namely, sensitivity and specificity. The evaluation of the clinical impact or benefit of the test and its contribution to clinical decision making, which represents the third level of the hierarchy, is rarely accomplished, and this is the main reason for the poor appropriateness of test request and interpretation in clinical practice. The evaluation of organizational impact and cost-effectiveness, including both clinical and economic outcomes (Fig. 3.3), lies at the top of the hierarchy of the framework known as ACCE (analytical validity, clinical validity, clinical usefulness, and ethical implications) (Lumbreras et al., 2006; Plebani, 2010).

Translating omics into clinical practice

Omics techniques generate multiparametric datasets in which the number of dimensions or variables usually greatly exceeds the number of samples. Consequently, differences between the datasets (potential biomarkers) that enable the discrimination between any arbitrary combinations of datasets can easily be defined. This sample heterogeneity makes it virtually impossible to thoroughly validate any biomarker or combination of biomarkers based only on the training set (the initial group of selected samples used to identify biomarkers), even with cross-validation. Accordingly, each

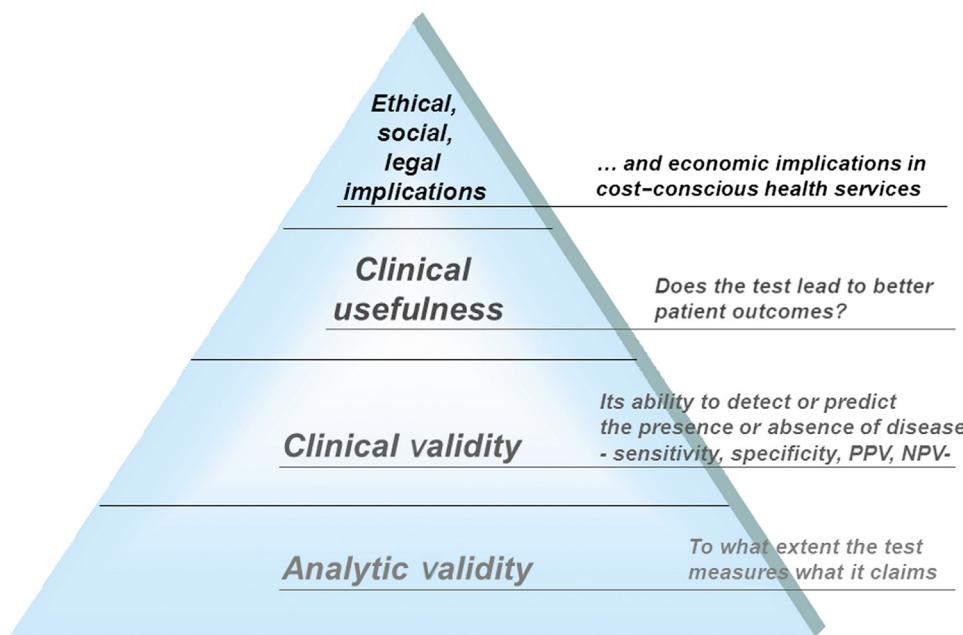


FIGURE 3.3 The ACCE framework for laboratory tests evaluation.

TABLE 3.2 Recommended steps for clinical proteomics.

1.	Define a clear clinical question and how the eventual biomarker or panel of biomarkers would improve the diagnosis and/or treatment of the disease.
2.	Define patient and control populations, clinical data to be collected, and protocols for sampling and sample preparation/storage.
3.	Define types of samples needed for discovery and validation phases.
4.	Define and validate analytical platform(s) for discovery.
5.	Perform a pilot study on validated discovery platform(s).
6.	Evaluate data from the pilot study with appropriate statistical treatments, and calculate the number of cases and controls for the training set.
7.	Perform a study of the training set, and evaluate findings with appropriate statistical treatments.
8.	Transfer the assay to the diagnostic platform, and evaluate technical and clinical performances.
9.	Standardize preanalytical and postanalytical procedures, and define reference ranges and interpretation criteria.
10.	Transfer into clinical use to demonstrate whether the findings may improve clinical outcomes.

Source: Modified from Mischak H., Apweiler R., Banks R.E., Conaway M., Coon J., Dominiczak A., et al., 2007. Clinical proteomics: a need to define the field and to begin to set adequate standards. *Proteom. Clin. Appl.* 1, 148–156.

clinical omic study must include, as a mandatory step, the validation of findings using an appropriate set of blinded samples analyzed independently. **Table 3.2** summarizes some suggested steps in clinical proteomics that might be used, in general, for clinical application of omics (Mischak et al., 2007).

Table 3.2 does not include ethical considerations such as the need to obtain informed consent from participants and/or approval by ethical committees or other issues such as the time to deposit datasets in a public database or patenting and licensing.

It should be emphasized that proper integration of multiomics approach allows the achievement of deeper insights into disease etiology, such as unveiling the myriad ways in which the microbiome may play a part in mitigating or enhancing disease risk (Graw et al., 2020). In fact, no “ome” exists in isolation. Context is critical and represents the net result of nonlinear interactions between the multiple levels of phenotypic control. The value of studying the “interactome” has finally been appreciated. It is now broadly recognized that multiple dimensions of omics data must be interrogated simultaneously to fully

understand biological systems. The field of systems biology was largely born out of the omics era and aims to model complex biological networks by integrating multiple layers of omic data using a holistic rather than traditionally reductionist approach (Kalita-de Croft et al., 2016).

Continuum of translation research and omics

In a study of the "natural history" of promising diagnostic or therapeutic interventions over a 15-year period, Contopolous-Ioannidis et al. showed that only 5% of basic science findings that were considered highly promising were eventually licensed for clinical use, and only 1% were actually used for the licensed indication (Contopolous-Ioannidis et al., 2003). In a cited editorial, Lenfant lamented that basic sciences and clinical research findings are usually "lost in translation" and questioned the evidence that if old insights related to traditional laboratory tests and/or well-known drugs are not used in clinical practice, the gap could be even larger with new insights related to more complex issues such as omics (Lenfant, 2003). In addition, "it takes an average of 17 years for only 14% of new scientific discoveries to enter the day-to-day clinical practice" (Westfall et al., 2007, p. 403). A review of the literature on translational research in oncology has revealed that most of the 939 publications over a 20-year period on prognostic factors for patients with breast cancer were based on research assays without sufficient demonstration of robustness or analytical validity (Simon, 2008).

The magnitude and nature of the work that is required to translate findings from basic research into valid and effective clinical practice have been underestimated for a long time, and the meaning of the term *translational research* seems to be controversial despite the increasing interest in the issue, established translational research programs, and no fewer than 40 journals listed in Medline with the term *Translational* somewhere in their title. This is partly due to the fact that different stakeholders look at distinct aspects of this issue (Woolf, 2008). For academia, translational research represents a general desire to test novel ideas generated from basic investigation in the hope of turning them into useful clinical applications. For academic purposes, translational research also responds to the need to identify novel scientific hypotheses that are relevant to human pathology through direct observation of humans and their diseases. For the commercial sector, translational research refers more to a process aimed at expediting the development of known entities particularly in early phases and/or identifying ways to make early "go" or "no-go" decisions when the cost of product development is still relatively contained. For people more directly involved in clinical practice (physicians, clinical laboratory professionals, and patients), translational

research responds to the need of accelerating the capture of benefits of research, closing the gap between "what we know and what we practice" (Littman et al., 2007).

For many, the term *translational research* refers to the "bench-to-bedside" enterprise of harnessing knowledge from basic sciences to produce new drugs, devices, and treatment options for patients. For others, especially healthcare scientists, laboratory professionals, and physicians, *translational research* refers to translating research into practice and ensuring that new diagnostic tools, new treatments, and research knowledge actually reach the patients or populations for whom they are intended and are correctly implemented. The distinction between these two definitions was articulated by the Institute of Medicine's Clinical Research Roundtable, which described two "translational blocks" in the clinical research enterprise, which have been labeled as T1 and T2. The first roadblock (T1) was described as "the transfer of new understandings of disease mechanisms gained in the laboratory into the development of new methods for diagnosis, therapy, and prevention and their first testing in humans," and the second block (T2) has been described as "the translation of results from clinical studies into the everyday clinical practice and health decision making" (Zerhouni, 2003, p. 64). They include the translation of basic science laboratory work in animals into an understanding of basic human medical biochemistry and physiology and the translation of basic human biochemistry and physiology into improved diagnostic tests, which, in turn, should improve patient management. The final crucial step is the delivery of recommended care to the right patient at the right time, resulting in improvement in individual patient and community health. The research roadmap is a continuum, with an overlap between sites of research and translational steps. Regarding the omics, in the T2 roadblock, some relevant aspects include assay validation, development of appropriate diagnostic platforms, standardization, quality control, and quality assurance protocols.

In particular, diagnostic platforms to be used in clinical practice are seldom the same as the ones used in the discovery and validation steps. In addition to diagnostic accuracy, clinical laboratories should ensure standardization, reproducibility, robustness, appropriate turnaround time, efficiency, and consistency in everyday practice. The construction of a coherent biomarker pipeline with a higher likelihood of success than that of past approaches calls for a stronger involvement of laboratory professionals and for a collaborative rather than competitive relationship between clinicians and scientists (Anderson et al., 2013; Lippi et al., 2007; Pavlou et al., 2013; Rifai et al., 2006).

If the pipeline and roadmap seem to be straightforward, the expansion of practice-based research, which is grounded in, informed by, and intended to improve practice, is mandatory. Practice-based research should help to (1) identify the problems that arise in daily practice and

that may create the gap between recommended and actual care, (2) provide an interdisciplinary context in which the development and validation of new biomarkers advance in a linear way, (3) demonstrate whether and how the new tests improve clinical and economical outcome, and (4) evaluate the need to change diagnostic and therapeutic clinical pathways.

Conclusions

The reality about omics is still uncertain, but expectations are not commensurate with the current data. After many thousands of studies using microarrays, only a handful of tests for breast cancer prediction have moved into clinical practice, and even these tests lack definitive evidence of clinical effectiveness (Marchionni et al., 2008). An even larger literature on pharmacogenetic and /pharmacogenomics has left very few applications with proven improvement in clinical outcomes (Rodén et al., 2006). Careful roadmaps should be implemented to include all steps that must be followed before successful translation, to demonstrate that new diagnostic tests are able to change clinical practice and reduce costs, by either improving people's health or eliminating ineffective and expensive treatments. This holds particularly true in the increasing cost-conscious healthcare environment (Meckley and Neumann, 2010). The complexity of translating basic discoveries into clinical trials and, finally, into clinical practice requires, in addition to well-designed pipelines and roadmaps, some changes in training programs. In fact, evidence has been gathered to demonstrate the existence of gaps in the knowledge of how to use and interpret omics technologies in everyday clinical practice (Ioannidis, 2006). Omics studies rely on large numbers of data, appropriate statistical analyses, and a considerable investment of time, skilled personnel, and money. Current tools utilize clustering, networking, data reduction, and Bayesian analysis. Because of the ever-increasing acquisition of data, the so-called big data, resulting in large datasets and increasing numbers of them, artificial intelligence and machine learning will become more and more necessary for effective analysis and data mining (Graw et al., 2020). In an era in which big data are rapidly surpassing the development of computational methods to extract biologically and clinically relevant knowledge, this may seem to be a daunting challenge, but we are beginning to see exciting progress in this area (Kalita-de Croft et al., 2016).

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Chapter 4

The power of genomics, metabolomics, and other omics for target identification and validation

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Abstract

The application of omics ranges from drug discovery and development to diagnosis of diseases, by following their progression, improving efficacy and safety of treatments, optimizing patient selection and identification of responders/nonresponders, adapting dose regimens of drugs, and helping to decide which therapy is most appropriate. For obvious reasons an integrated approach should be followed as a strategy to avoid misinterpretations of data. For example, changes found at the gene level may not necessarily translate into altered levels of proteins. Here, we address the basis of genomics and metabolomics, highlighting the most used techniques in each case and its current and future applications in human health and disease.

Keywords: Omics technologies; transcriptomics; proteomics; metabolomics; biomarkers

Introduction

Omics technologies play a key role in several dimensions of human health nowadays. The application of omics ranges from drug discovery and development to diagnosis of diseases, by following their progression, improving efficacy and safety of treatments, optimizing patient selection and identification of responders/nonresponders, adapting dose regimens of drugs, and helping to decide which therapy is most appropriate.

In systems biology it is widely accepted that no single omics approach suffices in studying complex organisms and biological processes. As so, the study can focus on several levels of information, namely the genome (genomics) (Robinson et al., 2017), RNA (transcriptomics)

(Sager et al., 2015), proteins (proteomics) (Greco et al., 2018, Messner et al., 2020), and metabolites (metabolomics) (Kohler et al., 2017, Sussolini, 2017).

For obvious reasons an integrated approach should be followed as a strategy to avoid misinterpretations of data. For example, changes found at the gene level may not necessarily translate into altered levels of proteins. In considering the omics cascade, many authors contend that the metabolome is most predictive aspect of the phenotype, as it is located downstream in this cascade; however, it is modulated by events located upstream.

In this chapter we address the basis of genomics and metabolomics, highlighting the most used techniques in each case and its current and future applications in human health and disease.

Genomics

Cells are the fundamental building blocks of every living system, and all the instructions required to control cellular activities are encoded within the DNA molecules, the total amount of DNA in a single cell being called the genome. The human genome includes approximately 3 billion nucleotide pairs of DNA, while bacteria possess the smallest known genome for a free-living organism (Pray, 2008; see also Table 4.1). The study and use of genomic information and technologies, associated with biological methodologies and computational analyses in order to assess genes function, may be considered the basis of modern genomics. The main purpose is to understand how the instructions coded in DNA lead to a functioning human being, deriving meaningful knowledge from the DNA sequence.

TABLE 4.1 Comparative genome sizes of humans and other model organisms.

Organism	Genome size (base pairs)	Estimated number of genes
Human (<i>Homo sapiens</i>)	3.2 billion	25,000
Mouse (<i>Mus musculus</i>)	2.6 billion	25,000
Fruit fly (<i>Drosophila melanogaster</i>)	137 million	13,000
Roundworm (<i>Caenorhabditis elegans</i>)	97 million	19,000
Yeast (<i>Saccharomyces cerevisiae</i>)	12.1 million	6000
Bacterium (<i>Escherichia coli</i>)	4.6 million	3200
Human immunodeficiency virus	9700	9

Specialists in genomics strive to determine complete DNA sequences and perform genetic mapping to evaluate the purpose of each gene and the associated regulatory elements. Modern research also aims to find disparities in DNA sequences between people and assess their relevance at a biological level. The findings from these investigations are being applied in the development of genome-based strategies for the early detection, diagnosis, and treatment of disease. Furthermore, this research will enable researchers to build up novel technologies to analyze genes and DNA on a large scale and to store genomic data efficiently (Mandl et al., 2020). To achieve such goals, genomic research has a number of solutions available that integrate sequencing, arrays, and polymerase chain reaction (PCR)-based systems.

Genomic tools

Sequencing

Sequencing is the process of determining the order of nucleotide bases within a stretch of DNA or RNA. Sequencing the entire genome of many animals, plants, and microbial species is indispensable for basic biological, forensics, and medical research. Since the early 1990s, DNA sequencing has been carried out almost exclusively with capillary-based, semiautomated implementations of the Sanger biochemistry (Chen, 2014). This method, developed by Sanger and colleagues and also known as dideoxy sequencing or chain termination methods, relies on denaturing DNA into single strands using high temperatures and further annealing to an oligonucleotide primer as originally described (Sanger et al., 1977). Afterwards, the oligonucleotide is extended by means of a DNA polymerase, using a mixture of normal deoxynucleotide triphosphates (dNTPs) and modified dideoxynucleotide triphosphates (ddNTPs), which terminate the DNA strand elongation process. These modified ddNTPs lack the 3' OH group to which the next dNTP of the emergent DNA sequence would be added. Without the

3' OH the process is terminated. The resultant newly synthesized DNA chains are a blend of lengths, depending on how long the chain was when a ddNTP was randomly added (Fig. 4.1). This technology reached its peak in the development of single-tube chemistry with fluorescently marked termination bases, heat-stable polymerases, and automated capillary electrophoresis, after which a plateau in technical development was reached (Nelson et al., 2008). The main problems of the Sanger sequencing dealing with larger sequence output were the use of gels or polymers as separation media, the limited number of samples that could be handled in parallel, and the difficulties with complete automation of the sample preparation.

Since completion of the first human genome sequence in April 2003, the demand for cheaper and faster sequencing methods has increased greatly. This demand has driven the development of second-generation sequencing methods, or next-generation sequencing (NGS). NGS platforms perform massively parallel sequencing, during which millions or even billions of DNA fragments (50–400 bases each) from a single sample are sequenced in just one run (Aziz et al., 2015). This technology enables high-throughput sequencing, which in turn allows a whole small genome to be sequenced in just a few hours (Levy and Myers, 2016). In the past decade, several NGS platforms have been developed that provide low-cost, high-throughput sequencing. Furthermore, NGS allows the identification of disease-causing mutations with application in diagnosis by targeted sequencing and offers a robust alternative to microarrays in gene expression studies (Di Resta et al., 2018).

Among the most successful DNA sequencing methodologies used by different NGS platforms, two should be highlighted: sequencing by ligation and sequencing by synthesis.

Sequencing by ligation

Sequencing by ligation is a DNA sequencing method that harnesses the mismatch sensitivity of DNA ligase to

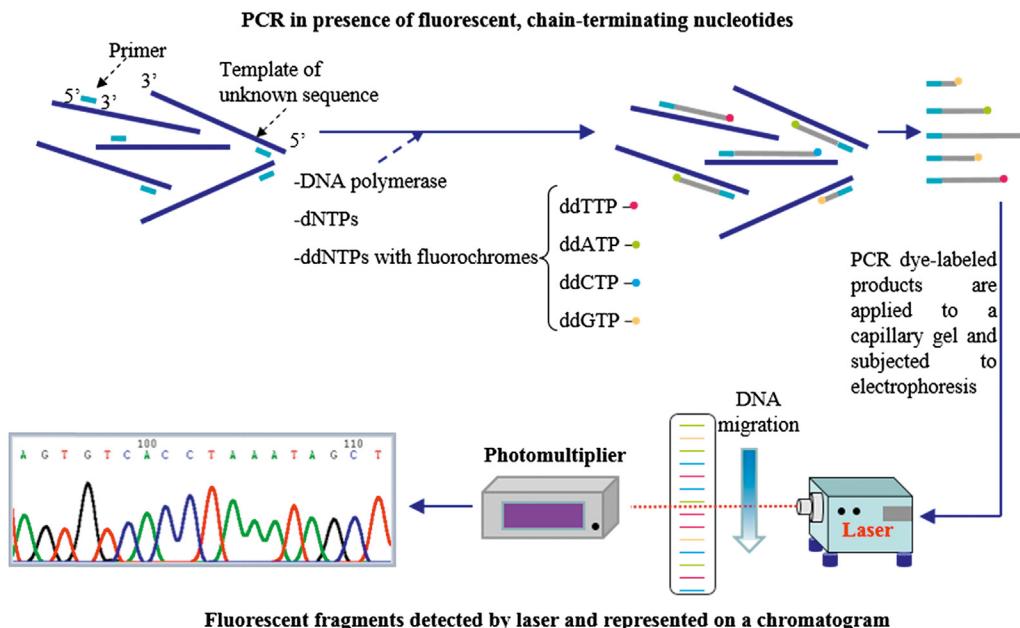


FIGURE 4.1 Strategy for automating the Sanger DNA sequencing system. Adapted from Nelson, D.L., Lehninger, A.L. Cox, M.M. 2008. *Lehninger Principles of Biochemistry*, Macmillan.

determine the underlying sequence of nucleotides in a given DNA sequence (Ho et al., 2011; Kchouk et al., 2017). Platforms based on this method use a pool of oligonucleotide probes of varying lengths, which are labeled with fluorescent tags, depending on the nucleotide to be determined. The fragmented DNA templates are primed with a short known anchor sequence, which allows the probes to hybridize. DNA ligase is added to the flow cell and joins the fluorescently tagged probe to the primer and template; thus the preferential ligation by DNA ligase for matching sequences results in a signal informative of the nucleotide at that position. After a single position has been sequenced, the query primer and anchor primer are stripped from the DNA template, effectively resetting the sequencing (Ho et al., 2011; Kchouk et al., 2017). The process begins again, sequencing a different position by using a different query primer, and is repeated until the entire sequence of the tag has been determined. The support oligonucleotide ligation detection (SOLiD) platform from Life Tech is the main representative of sequencing by ligation. In such a platform, fragmented or mate-paired, primed libraries are enriched by means of emulsion PCR on microbeads, which are afterward adhered onto a glass slide. A set of four 1,2-probes (each tagged with a different fluorophore) composed of eight bases is added to the flow cell, competing for ligation to the sequencing primer (Fig. 4.2) (Metzker, 2010). The first two positions of the probe encompass a known dibase pair specific to the fluorophore; these two bases query every first and second base in each ligation reaction. Bases 3–5 are degenerate bases separated from bases 6–8 by a

phosphorothiolate linkage. A matching 1,2-probe is linked to the primer by DNA ligase. After fluorescence imaging to assess which 1,2-probes were connected, silver ions break the phosphorothiolate link, thus regenerating the 5' phosphate group for subsequent ligation. This procedure—primer hybridization, selective ligation of the probes, four-color imaging, and probe cleavage—is repeated continuously, the number of cycles determining the eventual read length (Levy and Myers, 2016). After a satisfactory length has been reached, the extended product is separated, the procedure is begun anew, and the template is reset with a primer complementary to the $n - 1$ position of the previous round of primers. The template is elongated through successive ligations, and then reset four more times (five rounds of primer reset are completed for each sequence tag). This primer reset procedure results in each base being queried in two independent ligation reactions by two different primers, a check-and-balance system that is determined through the creation and alignment of a series of four-color images analyzed through space and time to assess the DNA sequence.

Sequencing by synthesis

Sequencing by synthesis includes a group of methodologies that make use of a DNA polymerase enzyme to incorporate a single nucleotide or short oligonucleotides (provided either one at a time or fluorescently labeled) containing a reversible terminator. This allows identification of the base type of the incorporated nucleotide without interrupting the extension process (i.e., the sequencing

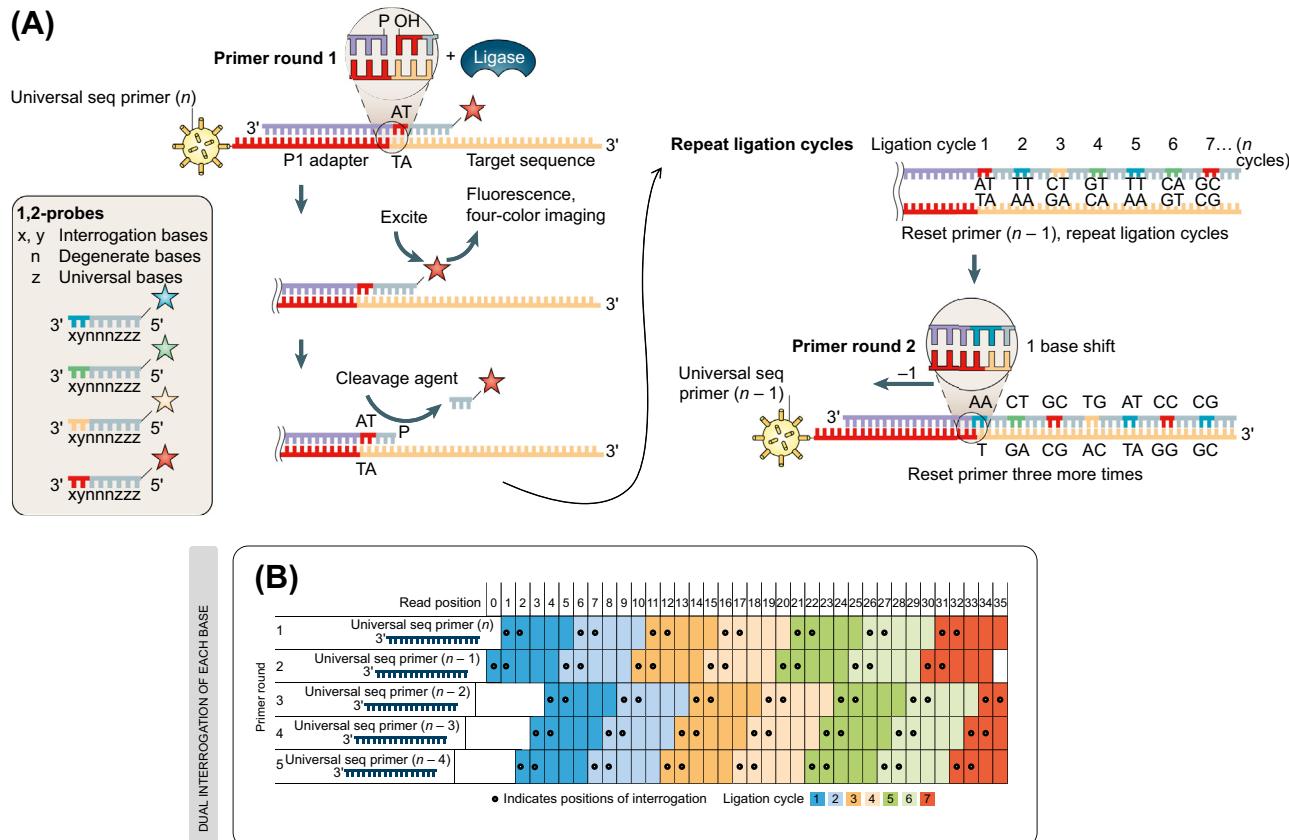


FIGURE 4.2 Illustration of the sequencing by ligation method using the SOLiD platform. (A) Scheme of the different steps followed by the four-color ligation SOLiD method: primer hybridization, selective ligation of the probes, four-color imaging, and probe cleavage. The SOLiD cycle is repeated nine more times. The extension product is removed, and the template is reset with a primer complementary to the $n - 1$ position for a second round of ligation cycles. (B) Five rounds of primer reset are accomplished for each sequence tag. Through the primer reset procedure, practically every base is queried in two independent ligation reactions by two different primers. Adapted with permission from Springer Nature: Metzker, M.L. 2010. Sequencing technologies—the next generation. *Nat. Rev. Genet.*, 11, 31–46. ©2010 and Applied Biosystems website.

process). Sequencing by synthesis may follow a single-molecule- or ensemble-based approach, the former involving the sequencing of various identical copies of a DNA molecule. In addition, it may follow real-time sequencing or synchronous-controlled strategies. The synchronous-controlled strategy involves a priori temporal knowledge to assist in the identification procedure in a stop-and-go iterative fashion (Mardis, 2017). One of the most employed techniques is denominated pyrosequencing, which is a simple, synchronous-controlled robust method that quantitatively monitors the real-time nucleotide incorporation through the enzymatic conversion of released pyrophosphate into a proportional light signal (Mardis, 2017). The light signal that is detected is then used to establish the sequence of the template strand. The wide application of this methodology stems from its accuracy, flexibility, parallel processing capacity, and easy automation as well as from avoiding the use of labeled primers, labeled nucleotides, and gel electrophoresis. The Illumina HiSeq systems represent another widely used sequencing by synthesis platform, based on a reversible

terminator approach (Fig. 4.3). This technology relies on the attachment of randomly fragmented genomic DNA to a flat, transparent surface on a flow cell. These fragments are sequenced by a four-color DNA sequencing-by-synthesis technology that makes use of reversible terminators with removable fluorescence. Tagged nucleotides are incorporated at each cycle, and high-sensitivity fluorescence detection is achieved using laser excitation and total internal reflection optics. Images are compiled and processed to produce base sequences for each DNA template (Raffan and Semple, 2011).

The most commonly used platforms in research and clinical labs besides those already mentioned include the GS FLX Titanium XL + (Roche Applied Science) and PacBio RS (Pacific Biosciences). A comparison of the features, chemistry, and performance of each mentioned platform is presented in Table 4.2. The current limitations of NGS platforms include the high cost of the platforms, inaccurate sequencing of homopolymer regions on various platforms, the time required, and the special know-how needed to analyze the data.

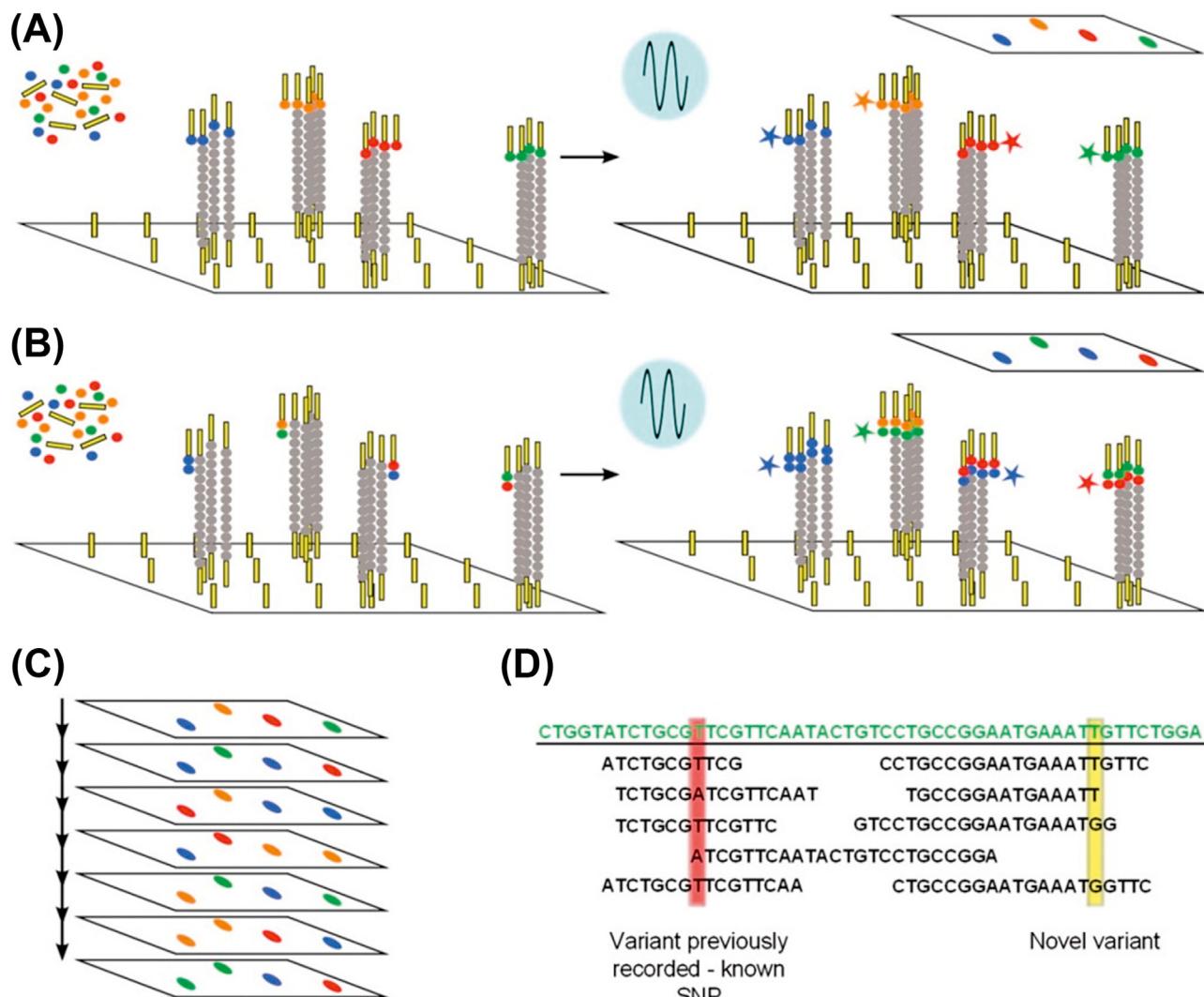


FIGURE 4.3 Illustration of the Illumina sequencing process. (A) The first sequencing cycle starts by adding a solution with four labeled reversible terminators, primers, and DNA polymerase. Temperature cycling allows just one base extension per cycle. Bases are integrated into the sequence, complementary to the amplified template. After laser excitation the emitted fluorescence from each cluster is detected, and the first base is identified. (B) During the next cycles, new labeled reversible terminators, primers, and DNA polymerase are added to generate a fluorescence pattern that will become a fingerprint of the sequences of bases in the fragment (C). (D) Data are aligned and compared to a reference sequence, and sequencing differences are identified. When a difference is detected, its frequency is used to assess whether the variant is heterozygous (as represented in this illustration) or homozygous. Reference databases are searched to assess whether variants are novel or previously recognized as single nucleotide polymorphisms. Reprinted with permission from Oxford University Press; Raffan, E. Semple, R.K. 2011. Next generation sequencing—implications for clinical practice. *Brit. Med. Bull.*, 99, 53–71.

Arrays

The fundamental principle of all microarray procedures is that tagged nucleic acid molecules in solution hybridize, with high sensitivity and specificity, to complementary sequences immobilized on a solid substrate, thus easing parallel quantitative measurement of numerous sequences in a complex mixture (Bumgarner, 2013). DNA microarrays are a well-established technology for measuring gene expression levels (potential to measure the expression level of thousands of genes within a particular mRNA sample) or to genotype multiple regions of a genome. Microarrays

designed for this purpose use relatively few probes for each gene and are biased toward known and predicted gene structures. Although several methods for building microarrays have been developed, two high-density microarrays have prevailed: oligonucleotide microarrays and cDNA microarrays (Fig. 4.4) (Schulze and Downward, 2001).

Oligonucleotide microarrays

Oligonucleotide microarrays have emerged as a preferred platform for genomic analysis beyond simple gene expression profiling. These microarrays have several relatively

TABLE 4.2 Commercial next-generation sequencing platforms for human whole genome sequencing.

	Platforms			
Main features	Titanium XL +	HiSeq 2500/2000	5500xl W	PacBio RS
Method	Polymerase (Pyrosequencing)	Polymerase (reversible terminator)	Ligase (octomer)	Polymerase single molecule
Read length (base pairs)	700	50–250	75 + 35	3000
Reads per run	1 billion	Up to 3 billion	1.2–1.4 billion	35,000–75,000
Gb per run	0.7	600	320	3
Template prep	Emulsion polymerase chain reaction (PCR)	Bridge PCR	Emulsion PCR	None
Advantages	Read length	High throughput	Low error rate	No artifacts Read length
Disadvantages	High error rate	Short reads	Short reads	High error rate
		Time-consuming	Time-consuming	

small probes (typically 25 m) for evaluating the transcript abundance of each gene. Probes are directly synthesized onto the surface of the array using *in situ* chemical synthesis technology (photolithography and combinatorial chemistry). Each chip may contain over 6 million features, each feature comprising millions of copies of a distinct probe sequence (Nesterov-Mueller et al., 2014)

cDNA microarrays

cDNA microarrays are made by mechanically printing/attaching probes, generally amplified PCR products, oligonucleotides, or cloned DNA fragments onto a solid substrate, typically glass with desired physicochemical characteristics (e.g., excellent mechanical stability and chemical resistance). This type of array generally possesses a lower feature density than the *in situ* synthesized oligonucleotide arrays, typically of about 10,000–40,000 elements per microscope slide (Jaksik et al., 2015; Jayapal and Melendez, 2006; Nishimura et al., 2015).

Although the latter method is relatively affordable and offers higher flexibility without requiring primary sequence information to print a DNA element, it is laborious in terms of synthesizing, purifying, and storing DNA solutions. In addition, cross-hybridization phenomena may be regarded as a major disadvantage. Nowadays, oligonucleotide arrays are the preferred platform for whole-genome analysis. This technology offers higher specificity, speed, and reproducibility. It requires only the DNA sequence under study to be known, thus eliminating the need to collect and handle cloned DNA or PCR products. Furthermore, the relatively small probe length, together

with the flexibility of using several overlapping probes representing the same genomic area, makes oligonucleotide arrays the best option to detect the wide range of genomic features, such as small polymorphisms or splice variants. The disadvantages related to oligonucleotide arrays include the associated costs and low sensitivity due to short sequences used in this technology.

Quantitative reverse transcription polymerase chain reaction and low-density arrays

The real-time reverse transcription PCR is a cost-effective, robust, highly sensitive, easy-to-use, and reproducible technology that is suited for validation of microarray-generated data, especially for low-expressed genes (Bustin et al., 2009; Taylor et al., 2019). This technique uses fluorescent indicator molecules to screen the amount of amplification products during each cycle of the PCR reaction, thus eliminating the requirement for post-PCR processing. This enables nucleic acid amplification and detection steps to be combined into one homogeneous assay, which allows the quantitative evaluation of the expression of single genes in multiple sample, while eliminating the necessity of performing gel electrophoresis to detect the amplification products. The high sensitivity of this technique in terms of mRNA detection and quantification makes it a viable option for samples with a limited number of RNA copies. Some of the newest systems based on this technology, low-density arrays, allow the simultaneous quantification of large numbers of target genes in single samples (multiplexing of PCR assays) or measurement of single markers in

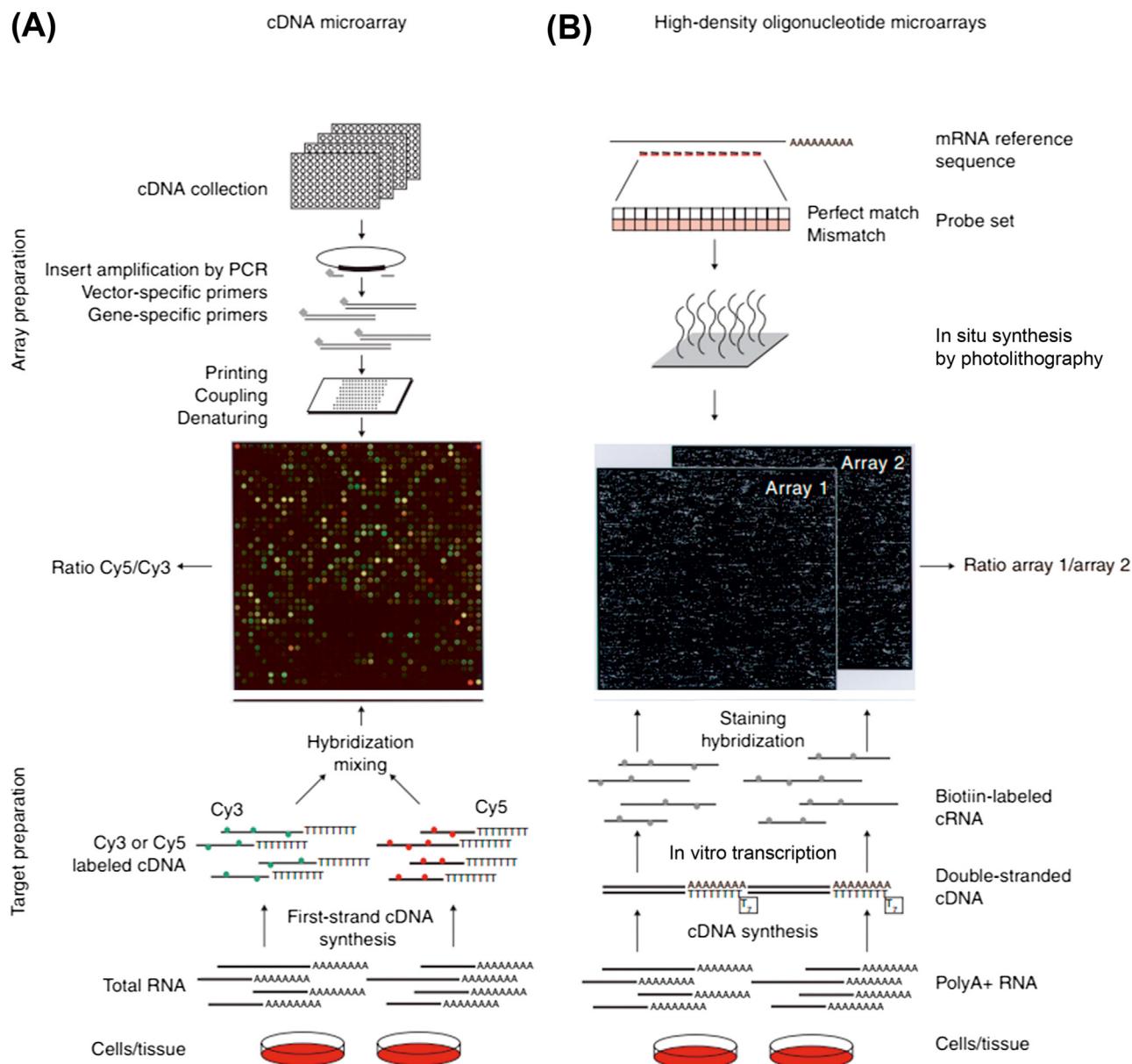


FIGURE 4.4 Illustration of arrays and target preparation. (A) cDNA microarrays: amplification of cDNA inserts by vector-specific or gene-specific primers. Polymerase chain reaction (PCR) products are mechanically attached onto a glass slide. Target preparation is performed by extracting RNA from two different tissues (sample and control), which will then be converted into cDNA in the presence of nucleotides labeled with different tracking molecules (e.g., Cy3 and Cy5). After competitive hybridization of the mixed labeled cDNA against the PCR products spotted on the array surface, a ratio of RNA abundance for the genes represented on the array is determined by high-resolution confocal fluorescence scanning. Array software often uses a green color to symbolize upregulated genes in the sample, red for downregulated genes, and yellow to represent genes that are of equal abundance in experimental and control samples. (B) Oligonucleotide arrays: sequences of 16–20 short oligonucleotides are chosen from the mRNA reference sequence of each gene and are synthesized in situ by photolithography to create high-density arrays. Target preparation is performed by extracting polyadenylated RNA from two different tissues (control and sample), which will be used to generate the corresponding cDNA. During in vitro transcription, biotinylated nucleotides are incorporated into the synthesized cRNA molecules. After hybridization, biotinylated cRNA bounded to the array is stained with a fluorophore conjugated to streptavidin and detected by laser scanning. Fluorescence intensities of probe array element sets on different arrays are used to determine relative mRNA abundance for the genes represented on the array. Reprinted with permission from Springer Nature; Schulze, A. Downward, J. 2001. Navigating gene expression using microarrays—a technology review. *Nat. Cell Biol.*, 3, E190–E195. ©2001.

parallel on microfluidics cards, while retaining the sensitivity of qPCR (Raitoharju et al., 2013).

Applications of genomics in molecular medicine

Through the use of conventional genetic methodologies (e.g., Southern blot or loss of heterozygosity techniques), almost 2000 genes for single-gene disorders had been identified by the year 2000. These discoveries enabled the accurate diagnosis of single-gene disorders, such as Huntington's disease, Marfan syndrome, hemophilia, cystic fibrosis, hereditary spherocytosis, and sickle cell anemia. Many of these discoveries have been used in the development of essential newborn blood spot screening programs, which allows testing of all newborns for early signs of a number of treatable congenital disorders (e.g., cystic fibrosis and sickle cell anemia) (Ulph et al., 2015). However, the most recent advances in genomic science, as well as the falling costs, have given scientists and clinicians the tools to assess the role of genetic and environmental factors in disorders such as diabetes, coronary heart disease, asthma, obesity, and several types of cancer. This information has been quickly integrated into clinical practice, enabling earlier and more precise diagnostics, even prevention in some cases, improved therapeutic strategies, evidence-based approaches for demonstrating clinical efficacy, side effect prediction and better decision-making tools for patients and providers. The current accessibility of over 1000 genetic assessments, targeted therapies, and pharmacogenomic data for drug and dosage selection shows that genomics is already integrated into health care and that it will be a game changer.

Molecular diagnostics

Methodologies such as NGS platforms or microarrays are currently used to assess genomic expression patterns. The study of such patterns allows researchers to distinguish between normal and abnormal genetic processes, thus enabling the discovery of mutations and targeting of genes with known heterogeneous distribution of mutations. Furthermore, it is possible to target larger segments of the genome to identify known or novel variations, thus making these new methodologies invaluable additions to laboratory testing and clinical evaluation, yielding diagnostic, therapeutic, and prognostic information. These methodologies are currently being applied in prenatal and postnatal, cancer, or neurological diagnoses, among other disorders. The evolution of prenatal diagnosis of disorders such as trisomies represents one example of the use of genomics as a tool for diagnosis. Until the 2000s, such a diagnosis would be time-consuming and require a chorionic villus sampling or second-trimester amniocentesis, with its associated risks, to assess the chromosomal

abnormalities (Newberger, 2000). Nowadays, there are several noninvasive clinical diagnostic tests on the market that use maternal DNA and require just a blood sample to run in an NGS platform (e.g., Verify by Illumina). These tests allow detection of trisomy 21, 18, and 13 chromosome abnormalities (along with other genetic disorders) without any risk for the pregnant woman or her unborn child while providing results with a sensitivity similar to that of the invasive tests (Jiang, 2013).

The evolution of cancer diagnosis represents another example of the use of genomics as a diagnostic tool. The extensive diversity of cancer types that have been sequenced and studied using genomic tools has revealed many novel genetic mechanisms governing cancer initiation, progression, and maintenance. For example, about 90% of breast cancers are due to genetic abnormalities in high-penetrance genes, such as *p53*, *BRCA1*, *BRCA2*, *NBS1*, *PTEN*, *ATM*, and *LKB1*. Microarray technology has been used to characterize a number of gene variations associated with breast cancer, leading to a new molecular taxonomy, which is currently being used to identify and classify breast cancer (Hackmann et al., 2016; Ng et al., 2015). Another example of how genomics may contribute to breast cancer diagnosis established a 70-gene tumor expression profile as a powerful predictor of disease outcome in young breast cancer patients (Cardoso et al., 2016); This microarray is currently available in the market as the MammaPrint (by Agendia) diagnostic kit; it claims to determine the probability of early-stage breast cancer distant recurrence in the first 5 years following diagnosis. The resulting profiles are scored to determine the risk of recurrence and, with it, the need for adjuvant therapy.

Pharmacogenomics

The occurrence of large population differences at the genetic level is responsible for different responses to pharmacotherapy. In fact, it is projected that genetics may be responsible for 20%–95% of variability in drug disposition and response (Chenoweth et al., 2020; Relling and Evans, 2015; Weinshilboum and Wang, 2017).

Pharmacogenomics addresses the question of how an individual's genetic variation for specific drug-metabolizing factors, such as enzymes, may affect the body's response to drugs. This is attained by correlating gene expression or single-nucleotide polymorphisms with drug absorption, distribution, metabolism, and elimination as well as drug receptor target effects (Chenoweth et al., 2020; Relling and Evans, 2015; Weinshilboum and Wang, 2017). Knowledge of whether a patient carries any genetic variation that may influence his or her response to drugs can help prescribers to individualize drug therapy, thus decreasing the chance for adverse drug events and increasing the effectiveness of drugs. Pharmacogenomics has been

applied in disorders such as HIV (Limou et al., 2019; Yu et al., 2020), diabetes (Lam et al., 2019; Pearson, 2019), cancer (Jeibouei et al., 2019; Kyrochristos et al., 2020; López-Cortés et al., 2020), and depression (Jha and Trivedi, 2018) by successfully guiding drug selection, which results in therapeutic benefits and minimizes the potential for toxicity. The rate at which the field has been evolving is such that even COVID-19, a disease that was only months old at the time this chapter was being written, has already been the subject of published information regarding the relevance of pharmacogenomics in its onset and progression (Das et al., 2020; Takahashi et al., 2020).

The positive impact of pharmacogenomics in the evolution of treatment of cardiovascular disorders may be regarded as an accurate illustration of this technology's impact on current medicine.

A high number of enzymes are directly linked with the pharmacokinetics and pharmacodynamics of cardiovascular drugs. For instance, in the case of antihypertensive drugs, the difference in blood pressure response is markedly different between patients. The key proteins involved in patients' response to a drug are cytochrome P450 (CYP) 2D6, CYP2C19, CYP3A4, and the ABCB1 transporter (Rysz et al., 2020). These enzymes/transports are responsible, to a great extent, for the variability in cardiovascular drug response. Nowadays, genotyping of the most important CYP is easily performed by microarrays or NGS platforms. The use of these technologies to analyze, for example, the highly polymorphic CYP2D6 gene provides information regarding whether the patient is an extensive metabolizer (possessing a fully functional enzyme), a poor metabolizer (low or no CYP2D6 enzyme activity), or an ultrarapid metabolizer (excessive enzyme activity). This information is particularly important if we consider that poor metabolizers display an exaggerated drug response and may be at greater risk for toxicity if a drug is metabolized mainly by CYP2D6 or presents a narrow therapeutic index such as propafenone, carvedilol, or metoprolol (Dávila-Fajardo et al., 2019; Johnson and Cavallari, 2013).

Recent research on novel cancer therapies has focused on developing agents that have the capacity to interfere with critical molecular reactions responsible for tumor phenotypes. This novel approach has been transforming the treatment of various cancers, improving the translational impact of genetic information on clinical practice. Molecular targeted agents hold great potential to higher treatment efficacy compared with conventional cytotoxic and genotoxic therapies. The success of this approach is centered on distinguishing responders from nonresponders in clinical practice so that the drug is targeted to patients who have a particular molecular abnormality. In the past two decades, several studies have demonstrated that dihydropyrimidine dehydrogenase (DPD, also known as

dihydrouracil dehydrogenase and uracil reductase) is an important regulatory enzyme in the metabolism of one of the most prescribed chemotherapy agents, 5-fluorouracil (5-FU). This enzyme is responsible by the pyrimidine catabolic pathway, as is the rate-limiting step in 5-FU catabolism (Sharma et al., 2019). Variability in this enzyme activity widely influences systemic exposure to fluorodeoxyuridine monophosphate and, consequently, the incidence of adverse effects to 5-FU, such as death. DPD activity is entirely or partly abnormal in 0.1% and 3%–5% of individuals in the general population, respectively. This deficiency has been correlated with multiple polymorphisms in the DPYD gene, which results in a decreased activity by this enzyme (Amstutz et al., 2018; Boisdrorn-Celle et al., 2017). Quantification of DPD mRNA by reverse transcription PCR as a means of determining intratumor DPD levels and analysis by microarrays of the variation in the DPYD gene are currently being proposed as promising approaches to identify high-risk patients for severe 5-FU toxicity.

In targeted anticancer treatment directed to nonsmall cell lung cancer, a great portion of the oncologic patients do not respond to gefitinib, a tyrosine kinase inhibitor that targets oncogenic epidermal growth factor receptor and is used as monotherapy when patients do not respond to standard chemotherapy (Singh et al., 2016). Nonetheless, about 10% of the patients have a rapid and often dramatic response to such therapy, owing to somatic mutations clustered around the ATP-binding pocket of the tyrosine kinase domain of epidermal growth factor receptor (Gridelli et al., 2011). These are responsible by causing enhanced tyrosine kinase activity in response to epidermal growth factor and increased sensitivity to inhibition by gefitinib. One solution provided by genomics may be to assess patients who will have a response to gefitinib by using a screening for such mutations among lung cancer patients.

Metabolomics

Metabolomics or metabonomics?

Following the earlier introduction of the terms genome, transcriptome, and proteome the word metabolome was introduced by Oliver in 1998 (Oliver et al., 1998). In the following year, Nicholson introduced metabonomics (Nicholson et al., 1999), and in 2000, Fiehn coined the term metabolomics (Fiehn et al., 2000). A case can be made regarding what is considered a metabolite. Following a literal definition, all molecules that result from metabolism are metabolites. However, this definition would include several types of compounds, from peptides to other macromolecules. This is not the case, as it is generally accepted that metabolomics addresses small molecules, thus leaving out peptides, proteins, and other biological entities.

In the field of omics there is a fair amount of discussion regarding the nomenclature used, with different authors adopting different definitions. In this chapter the authors chose to use the following interpretation: *Metabolomics* addresses the metabolome, that is, the complete set of molecules that constitute an organism. Thus metabolomics can be seen as a snapshot of the chemical composition of a tissue, organ, or organism in a given moment. By contrast, *metabonomics* addresses the temporal and spatial changes in the chemical composition of the sample. In the field of biomedical science and molecular medicine, metabonomics is frequently used to profile endogenous metabolites in biological fluids (e.g., urine, blood, tissue homogenates, cerebrospinal fluid, bronchoalveolar lavage) in order to characterize the metabolic phenotype and its response to stimulation or disease (Johnson et al., 2016; Liu and Locasale, 2017; Newgard, 2017; Wishart, 2016).

For obvious reasons, it still is not possible to study the complete set of all metabolites within an organism. This arises as a consequence of the high number of different small molecules present in organisms and the low concentration at which they may occur. This objective is further hindered by the fact that no single analytical technique or combination of several techniques allows the identification of all metabolites, a consequence of their diversified physical and chemical properties and the wide concentration range in which they occur.

Nevertheless, from a biological point of view, the metabolome is the biological endpoint that links genotype to function. In addition to genotypes, environmental factors, such as nutrition, circadian rhythm, aging, stress, and hormonal changes, are known to affect phenotype through the metabolome, thus being key players in the assessment of health and disease and the impact of pharmacotherapy.

Several experimental approaches can be used in the field of metabolomics. In the following sections, we highlight a few.

Metabolite profiling

In metabolite profiling, the emphasis is placed on compounds that share chemical similarity (e.g., fatty acids, sugars, eicosanoids) or that are related through a common metabolic pathway. In this design, selective extraction procedures are commonly used to remove other metabolites that are regarded as interferences, thus improving the analysis of the target molecules. From an analytical point of view, liquid chromatography–mass spectrometry (LC-MS) (Engskog et al., 2016), gas chromatography–mass spectrometry (GC-MS) (Zarate et al., 2017), and nuclear magnetic resonance (NMR) (Larive et al., 2015) are the most used techniques.

Metabolite fingerprinting

Fingerprinting analysis involves collecting spectra of unpurified and chemically complex matrices and initially ignoring the problem of making individual assignments of peaks, which frequently overlap. Multivariate statistical methods are then used to identify clusters of similarity or difference (De Livera et al., 2018; Pereira et al., 2010; Zacharias et al., 2018). In subsequent studies, only peaks that are different between groups are analyzed, thus avoiding the time-consuming identification of all peaks. In this metabolomic strategy, sample preparation is reduced because the objective is a global, rapid, and high-throughput analysis of crude samples.

The most frequently used technique for metabolic fingerprinting is NMR, and several types of applications can be found in the literature. Multivariate analysis of unassigned ¹H NMR spectra is among the most used approaches.

In addition to NMR, MS-based investigations are also possible. Here, the metabolite fingerprints are represented by *m/z* values and corresponding intensities of the detected ions. When a separation step takes place prior to the MS analysis, retention times may also be used to index metabolites.

Metabolic footprinting

The widespread and increasingly robust use of cell cultures in several research areas has created a new approach, metabolic footprinting, which addresses the exometabolome. In this case, the analysis is performed on the culture media or other extracellular environments, thus allowing the study of compounds excreted during cellular metabolism without the need for cell disruption or lysis.

This approach may also present some advantages in regard to data complexity and analysis time. For instance, the intracellular metabolism is more dynamic, and so the turnover of most metabolites is extremely fast, requiring an efficient quenching of cell metabolism, followed by an effective separation of intracellular and extracellular metabolites and subsequent extraction of intracellular compounds (Cappelletti et al., 2017; Pimentel et al., 2018).

Analytical techniques in metabolomics/metabonomics

Nuclear magnetic resonance

NMR spectroscopy explores the magnetic properties of the nuclei of certain atoms. From an instrumental point of view, it relies on the phenomenon of NMR, which can provide a wide range of information, including structure, reaction state, and chemical environment. Molecules containing at least one atom with a nonzero magnetic moment are potentially detectable by NMR, such isotopes including ¹H, ¹³C, ¹⁴N, ¹⁵N, and ³¹P. These signals are

characterized by their frequency (chemical shift), intensity, fine structure, and magnetic relaxation properties, all of which reflect the environment of the detected nucleus. NMR is the analytical method that provides the most comprehensive structural information, including stereochemical details.

Most applications of NMR are simple one-dimensional NMR experiments of the ^1H nuclei; other techniques are able to provide more information, such as ^{13}C or 3–4 dimensional techniques for the study of complex molecules. Studies of 1D NMR are very useful in classifying similar groups of samples; however, when there are several overlapping peaks, identification of metabolites can be hindered. In this situation, two-dimensional NMR studies can prove to be very useful because they provide much more information (Emwas et al., 2019; Markley et al., 2017).

The high amount of structural information provided by NMR is countered by its relatively low sensitivity, samples in the range of 1–50 mg usually being required, in contrast to MS, which can use samples in the picogram-to-nanogram range. For this reason, extensive research has been conducted to improve NMR sensitivity, one of the most promising findings being the use of cryoprobes. In this approach, the detection system is cooled while the sample remains at room temperature (Pereira et al., 2012), which limits the noise voltage associated with signal detection and, when compared with regular probes, the signal-to-noise ratio is improved by a factor of 3–4 (Krishnan et al., 2005).

Despite the issues related with sensitivity, NMR presents a number of advantages, including the fast and simple sample preparation required, quick measurements, and quantification of analytes without the need for standards. NMR coupled with LC is an even more powerful analytical tool, but its prohibitive price has prevented its widespread use.

One important advantage separates NMR from MS: It is not a destructive technique, so further analysis of the same sample is possible.

Mass spectrometry

MS relies on the fragmentation of molecules following exposure to a high energy input. Several degrees of information can be drawn from this action, from molecular weight to the presence of a certain functional group, sometimes even differentiation of certain types of isomers. The fragmentation products are impelled and focused through a magnetic mass analyzer to be further collected and each selected ion measured in a detector. The choice of the ionization method to be employed depends on the physicochemical properties of the analyte (s) of interest, namely, volatility, molecular weight, and thermolability, and on the complexity of the matrix in which the analyte is contained, among others.

MS can also detect molecules that comprise NMR-invisible moieties, such as sulfates. For some chemical classes the fragmentation pattern (both peak mass and abundance) can allow the characterization of the unknown compound's structure. However, with a few exceptions (Ferreres et al., 2010), MS does not allow differentiation of isomers, which is particularly important in a biological context, in which the activity of molecules is highly influenced by the isomer and its conformation.

In a general way, MS is used in combination with a prior separation step of analytes, either LC or GC. In LC, several variables can be controlled, such as column length, particle size, flow, and solvent system. In GC, ideal candidates are molecules with high to medium volatility, although nonvolatiles can sometimes be studied if a derivatization step is introduced. Still, GC-MS offers excellent reproducibility and low detection limits. Given the fact that several databases of compound identification exist, a tentative identification in the absence of standards is possible.

In terms of ion sources, widely used options include electron ionization (EI), chemical ionization (CI), electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and matrix-assisted laser desorption/ionization (MALDI). The bases of each ion source have been addressed elsewhere (Badu-Tawiah et al., 2013; Dreisewerd, 2014; Wang et al., 2016) and are beyond the scope of this chapter.

Among the several mass analyzers available, we can point to the widespread use of single-quadrupoles, triple quadrupoles, time-of-flight (TOF), ion traps (IT), and Fourier transform ion cyclotron resonance (FT-ICR).

When MS is used, two levels of compound identification can be achieved: provisional and positive. Positive identification can be achieved when reference compounds are available, thus allowing comparison of both retention time and mass spectra. However, in the case of new or rare compounds, no standards are available, in which case provisional identification takes place.

In recent years, ultra performance liquid chromatography (UPLC), especially in tandem with MS, has been increasingly used in pharmaceutical analysis and biomarkers discovery. This technique retains many of the principles of HPLC while requiring lower analysis time and solvent consumptions. Specially designed columns, which incorporate particles of lower sizes, typically less than 2 μm , are used, and the system operates at higher pressures than HPLC, with results that show marked improvements in resolution and sensitivity (Nassar et al., 2017).

MS-based metabonomics offers a few advantages when compared to NMR, namely, higher sensitivity ($\text{pM}-\mu\text{M}$), rapid profiling, and, in some cases, databases for structure assignments. There are also disadvantages, such as extensive sample preparation in some cases, challenging quantifications with ion suppression when internal

standards are not available, and lack of standardized data structures.

Several approaches have been developed to combine the information provided by MS and NMR analysis. One such approach is statistical heterospectroscopy, which allows the combination of the information provided by MS and NMR, thus increasing the number of detectable significantly changed metabolites. This approach allows the identification of relevant molecules that could not have been identified by either method separately (Bertrand, Azzolini et al., 2016; Li et al., 2019).

Omics and biomarkers

A biomarker is a measurable indicator that informs on the biological state of a subject. The use of biomarkers in health and disease relies on the changes in their concentrations or flux that corresponds to a particular phenotype when compared to the control phenotype. It is important to highlight that the use of biomarkers goes beyond disease; they can be used across several fields, such as toxicology (Gupta, 2019), nutrition (Picó et al., 2019; Stoppe et al., 2020), and pharmacokinetics and pharmacodynamics (Kohler et al., 2017; Rodón et al., 2015).

After discovery, a potential new biomarker has to undergo validation to ensure that the molecule can be associated with the biological state under study in a specific way (Goossens et al., 2015; Mordente et al., 2015). Contrary to what happens in the discovery step, in which 100 samples are often enough, for validation it is advisable that at least 1000 samples are used. The purpose of this approach is to ensure that the sensitivity and specificity of the biomarker are robust enough to withstand the marked variation found in biological samples of a given population. Between discovery and validation it is common to address the verification phase, in which a few hundred samples are used, the difference residing in the few (tens of) compounds under study, rather than the discovery phase, which requires more molecules.

In the cohorts used, it is important to use not only healthy individuals but also patients suffering from pathologies similar to the one under study. In this way, it may be possible to discharge the lack of specificity that could arise, for example, from a shared pathway, such as the cascades of the inflammatory pathway, which are common to several different pathological conditions.

The typical samples used are serum or plasma, owing to the relative ease with which they can be obtained and the general rule that they represent the current state, either physiological or pathological, of the human body as a whole. Owing to the high number of proteins in these matrices, immune depletion of highly abundant proteins is frequent, the reproducibility and efficiency of this technique having already been evaluated and demonstrated. Further fractionation

techniques, such as cation exchange chromatography, are also common (Lee et al., 2019; Vanmeter et al., 2012).

Taking into account the several steps involved in the acceptance of new biomarkers, the verification phase is currently regarded as one of the major bottlenecks.

Metabolomics and metabonomics in clinical use

Several works are available in the literature showing the application of metabolomics as a tool for diagnosis in several pathologies. Cancer is one of the most widely studied diseases, owing to the pivotal role of an early diagnosis. MS and NMR-based techniques have been successfully used in several types of cancer. Here, we briefly present some examples of the application of metabolomics in diagnosis; we also present our apologies in advance to the authors whose work we are unable to quote.

Hirata and colleagues used GC-MS/MS to analyze 15 candidate metabolites in 55 early pancreatic cancer patients, several molecules being changed when compared with control group (Hirata et al., 2017). When these were combined with classical biomarkers such as carbohydrate antigen 19-9 (CAS19-9), the authors report sensitivity up to 90.7% and specificity around 90%. Several studies are available on this topic, many of which were reviewed in a recent meta-analysis study (Mehta et al., 2017).

UPLC-MS has been successfully used to characterize serum profiles from hepatocellular carcinoma (HCC), liver cirrhosis (LvC), and healthy subjects. The analysis of the UPLC-MS profiles of the subjects yielded 13 potential biomarkers involved in metabolic pathways involving organic acids, phospholipids, fatty acids, and bile acids (Wang et al., 2012). The use of the metabolomic profile was compared with the classical marker alpha-fetoprotein (AFP), the former being able to discriminate patients from controls and also to distinguish HCC from LvC with a sensitivity and specificity of 100%. Canavaninosuccinate was decreased in LvC and increased in HCC, while glycochenodeoxycholic acid was pointed as an indicator for HCC diagnosis and disease prognosis (Wang et al., 2012). In a different study, a good separation between healthy subjects and HCC patients has been described by using an UPLC-TOF-MS system, using 65 plasma samples from the former and 70 plasma samples from the latter (Li et al., 2018).

In another study, using a nontargeted method, three metabolites from rat HCC cancer models yielded taurocholic acid, lysophosphoethanolamine 16:0, and lysophosphatidylcholine 22:5 as potential metabolites with application in grading the different stages of hepatocarcinogenesis. They also represent the abnormal metabolism during the progress of HCC in patients (Tan et al., 2012). For recent reviews on this topic, see Ayoub et al. (2019) and De Stefano et al. (2018).

In the case of kidney cancer, urine is the sample of choice because it displays metabolic signatures of many biochemical pathways. In fact, urine is increasingly studied for its use in cancer diagnosis, even for other types of cancer (Dinges et al., 2019). Nizioł and colleagues described a pilot study using NMR and surface-assisted laser desorption/ionization MS-based serum metabolomics of urine as a strategy to discriminate between patients with kidney cancer and healthy subjects (Nizioł et al., 2020). Overall, eight biomarkers were identified as possible candidates for sorting the renal carcinoma patients from the healthy patients.

In a study by Wen et al. (2010) aiming to use NMR-based metabolomics in the diagnosis of biliary tract cancer, bile was collected from patients with cancer and benign biliary tract diseases. The metabolomic two-dimensional score plot revealed good separation between cancer and benign groups, and the signals contributing to these differences were further studied using a statistical TOCSY approach. The diagnostic performance assessed by leave-one-out analysis exhibited 88% sensitivity and 81% specificity, better than the conventional markers carcinoembryonic antigen, carbohydrate antigen 19-9, and bile cytology.

Zhang and colleagues used LC-MS for studying biomarkers useful in distinguishing between the profile of esophageal cancer (40 samples) and the corresponding normal mucosa (40 samples) (Zhang et al., 2017a). Oleic acid LysoPC(15:0), uracil, inosine, and choline were associated with lymphatic metastasis, while glutamine, serine, uracil, and kynurene were associated with survival time. Over 20 molecules had a statistically significant score with $p < 0.05$, most of them amino acids and fatty acids. Similar studies are available regarding serum biomarkers of chemoradiosensitivity in this cancer (Fujigaki et al., 2018) as well as a systematic review on metabolomics profiling of gastric and esophageal cancer (Huang et al., 2020).

While many of the biomarkers that result from the aforementioned studies are still under evaluation and not widely used in clinics, significant advances have already been made. For example, in the case of prostate cancer the most frequent tests used for diagnosis rely on prostate-specific antigen (PSA), which suffers from poor accuracy. Lokhov et al. (2010) used an MS-based metabolite fingerprinting approach to analyze blood plasma of patients. This new technique displayed sensitivity, specificity, and accuracy of 95%, 96.7%, and 95.7%, respectively, in contrast to the enzyme-linked immunosorbent assay (ELISA) PSA test, which exhibited sensitivity, specificity, and accuracy of 35%, 83%, and 52%, respectively.

The range of application of metabolomics platforms is not confined to cancer, being increasingly used in neurological disorders such as Parkinson's (Hatano et al., 2016; Shao and Le, 2019), metabolic diseases such as diabetes

(Pallares-Mendez et al., 2016), and some types of obesity (Bakar et al., 2015; Zhang et al., 2017b).

For detailed reviews of the application of metabolomics in biomarker discovery for fields such as cancer, neurological disorders, and others, see Burton and Ma (2019), Guo et al. (2018), Hurtado et al. (2018), and Kohler et al. (2017).

Conclusion

There is no doubt that omics technologies, in their multiple dimensions, are now regarded as a powerful tool for diagnosis, disease monitoring, and personalized medicine. While many gaps are still present, the remarkable rate at which several technological solutions are evolving will result in omics approaches becoming an unavoidable reality in the near future.

In terms of instrumental apparatus, improved MS and NMR hardware will yield faster analysis with higher sensitivity. The challenge is believed to be the integration of the information generated by the different omics approaches. Thus when information from genomics, transcriptomics, proteomics, and metabolomics is combined, a true integrated view of organisms in a systems biology context can be attained. Statistical tools are likely to play an important role in this area, as only robust statistics can shed a light on the relevant information to be extracted from the enormous datasets generated by the aforementioned techniques.

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Chapter 5

Potency analysis of cellular therapies: the role of molecular assays

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Abstract

All cellular therapy products must be demonstrated to be safe, pure, potent, stable, and effective for human use. Potency is a measure of the critical biological properties of a product, and measuring the potency of cellular therapies is very challenging. The potency of most cellular therapies is dependent on multiple functions, some of which may not be known. Biological variability in the starting material and variability in complex manufacturing processes also increase the difficulty in measuring cell therapy potency. Despite these difficulties, measuring potency is one of the most important aspects of assessing cellular therapy products. All products should meet established minimal levels or ranges of potency, and potency levels should be consistent across manufacturing lots. Potency measures are also used to ensure that product effectiveness is maintained as manufacturing methods are scaled up and out.

Keywords: Cellular therapy; immune therapy; gene therapy; mesenchymal stem cells; bone marrow stromal cells; hematopoietic stem cells; potency; gene expression analysis; RNA sequencing

Introduction

Cellular therapies are making a major contribution to the emerging field of biological therapy. The possibilities for the clinical application of new cellular therapy products are expanding rapidly, as is their clinical promise. The diversity and effectiveness of cellular therapies that are now available have encouraged the development of new clinical applications and improved the quality of life of patients. These therapies include adoptive immune therapy utilizing enriched or in vitro manipulated autologous or allogeneic

immune cells to treat cancer and viral infections (Powell et al., 2006; Leen et al., 2006; Kochenderfer et al., 2010, 2012, 2013; Porter et al., 2011; Kalos et al., 2011; Grupp et al., 2013; Brentjens et al., 2011, 2013), β islet cell transplantation (Shapiro et al., 2000), hematopoietic stem cells (HSCs) for transplantation, HSC therapy for cardiac ischemia (Kinnaird et al., 2004; Nanjundappa et al., 2007), gene therapy (Ariga, 2006; Corsten and Shah, 2008), mesenchymal stem cell (MSC) therapy (Caplan, 2007; Le Blanc and Ringden, 2006), and cancer immunotherapies (Wall and Krueger, 2020). The ability to produce large quantities of biological products with predictable quality and quantifiable potency is critical to this field.

The complexity of cellular therapies is increasing. For example, the initial immune therapy protocols that were adopted to treat cancer involved only the administration of autologous tumor-infiltrating leukocytes (TIL) (Rosenberg et al., 1986) or leukocyte-activated killer cells (Rosenberg et al., 1987; Rosenberg et al., 1986). Now adoptive immune therapy protocols are combination therapies that include high-dose chemotherapy, the administration of in vitro activated and primed TIL, and autologous HSCs (Wrzesinski et al., 2007). Immunosuppressive chemotherapy depletes the patient's naturally occurring repertoire of lymphocytes, including T regulatory cells and myeloid-derived suppressor cells (MDSCs). The lack of T regulatory cells and MDSCs and increased levels of cytokines, including IL-7, that are associated with leukopenia allow for the rapid and marked in vivo expansion of TIL administered with HSCs (Wrzesinski et al., 2007).

Adoptive cellular therapy with genetically engineered T cells is becoming an important cancer therapy. This

therapy involves the genetic engineering of autologous T cells to express high-affinity T cell receptors (TCRs) or chimeric antigen receptors (CARs) that are reactive with tumor antigens or cancer testis antigens. T cells engineered to express TCRs that are reactive with melanoma antigens MAGE1 and gp100 are being used to treat metastatic melanoma (Morgan et al., 2006), and those expressing TCRs that are reactive with the cancer testis antigen NY-ESO-1 have been used to treat melanoma and sarcoma (Robbins et al., 2011). In addition, TCRs reactive to human papillomavirus (HPV)16 E6 oncoprotein are being used to treat patients with HPV-associated squamous cell carcinomas and adenocarcinomas of the cervix, anus, and vagina (Doran et al., 2019). Autologous T cells that have been engineered to express CAR that consist of the single chain fragment variable (scFv) of an antibody directed to a tumor antigen, the TCR CD3 zeta chain, and a costimulator molecule such as CD28 or 4-1BB ligand are being used to treat patients with cancer, leukemia, and lymphoma. CAR T cells that are specific for CD19 antigen have shown good clinical results in treating patients with acute lymphocytic leukemia, chronic lymphocytic leukemia (CLL), and B cell lymphoma (Kochenderfer et al., 2010, 2012, 2013; Porter et al., 2011; Kalos et al., 2011; Grupp et al., 2013; Brentjens et al., 2011, 2013; Turtle et al., 2017; Maude et al., 2018; Neelapu et al., 2017). Two CD19-CAR T cell products have been approved by the U.S. Food and Drug Administration and are being manufactured commercially (Wall and Krueger, 2020). CAR T cells directed to B cell maturation antigen (BCMA) are being used in clinical trials to treat multiple myeloma, and the results have been promising (Brudno et al., 2018; Raje et al., 2019). The methods used to produce T cells expressing CARs and high-affinity TCRs are similar. They require T cell collection by apheresis from the blood of the patient to be treated, T cell stimulation, transduction with a retroviral or lentiviral vector, and expansion (Tumaini et al., 2013). The effectiveness of these adoptive T cell therapies is dependent on in vivo proliferation and persistence of the genetically engineered T cells (Fry and Mackall, 2013).

Similarly, HSC transplants have become more complex. While HSC transplants have been used successfully for more than 25 years to treat leukemia, this therapy has been constantly evolving. These changes involve tailoring and optimizing its efficacy by using HSC transplantation in combination with immune therapy to treat leukemia. Either manipulated or unmanipulated lymphocytes from HSC transplant donors are often administered to recipients following transplantation to prevent or treat disease relapse and enhance immune recovery (Montero et al., 2006; Fowler et al., 2006). T cells that are specific for viruses such as cytomegalovirus (CMV), Epstein-Barr virus (EBV), and parvovirus are being expanded and used to prevent or

treat infections following HSC transplantation (Leen et al., 2013; Gerdemann et al., 2013; Tzannou et al., 2017).

Regenerative medicine is becoming an important new part of cellular therapy. Bone marrow stromal cells (BMSCs), which are also known as mesenchymal stromal cells or MSCs, are now being widely used in tissue engineering and regenerative medicine applications. BMSCs are found in the marrow, where they support hematopoiesis. They can also differentiate into osteoblasts, chondrocytes, and adipocytes. BMSCs can be collected from the marrow but must be expanded ex vivo in order to obtain sufficient quantities for effective therapy. When BMSCs are administered with an appropriate scaffold, they form bone, and they are being used to repair bone (Marcacci et al., 2007; Bruder et al., 1998; Quarto et al., 2001). BMSCs also secrete a number of cytokines, chemokines, and growth factors that confer angiogenic, antiinflammatory, and immune modulatory properties on BMSCs (Stagg and Galipeau, 2013; Nuschke, 2014; Elman et al., 2014). These characteristics have led to clinical trials of BMSCs for the treatment of left ventricular failure due to ischemic heart disease (Zhou et al., 2009; Chan et al., 2020), inflammatory bowel disease (Forbes et al., 2014), acute respiratory distress syndrome (Matthay et al., 2019), and acute graft versus host disease (GVHD) following HSC transplantation (Le Blanc et al., 2008; Ringden et al., 2006). The quality of BMSC products are dependent on a number of factors, including the degree of expansion, composition of the culture media and media supplements, and age of the donor. There is also considerable interdonor variability in BMSCs (Ren et al., 2013).

Pluripotent embryonic and induced pluripotent stem cells (iPSCs) hold even more potential for regenerative medicine. Since iPSCs can be produced from easily obtained material such as skin fibroblasts or CD34⁺ cells isolated from the blood (Merling et al., 2013), intense focus has been placed on using iPSCs for regenerative therapies. However, the production of iPSCs is complex, requiring reprogramming with four to five factors (Takahashi and Yamanaka, 2006) and initial expansion on feeder cells followed by expansion on extracellular matrix and prolonged culture. The characteristics of the iPSCs can be affected by the starting cellular material, the reprogramming methods, and the culture conditions. For most clinical applications, iPSCs will likely be differentiated before they are administered. While methods for the differentiation of iPSCs into many cell types have been described, the differentiation is often not complete and is dependent on the starting material used for the production of the iPSCs. Retinal pigmented epithelial cells derived from iPSCs are in clinical trials for the treatment of age-related macular degeneration (Vitillo et al., 2020).

Many of these clinical cellular therapy products require cell mobilization, collection, subset isolation,

in vitro or in vivo stimulation, and culture of cells over a period of several days. The production of some cellular therapies involves serial isolation steps and multiple stimulation and/or culturing steps and gene transfer. Cellular therapy product manufacture is further complicated by donor or patient genetic and physiological heterogeneity. The final product is often markedly different from the starting material. Because of the complex nature of producing cellular therapy products and the clinical importance of the final products, most institutes conducting cellular therapy have developed specialized good manufacturing product (GMP) laboratories devoted to the production of these therapeutic agents. The goal of these cell-processing laboratories is to produce cellular therapy products that provide the desired clinical effect without resulting in adverse effects. These specialized laboratories ensure that an adequate dose of cells is provided to each patient, each product meets release specifications, and lot-to-lot variation is minimized. To produce consistently high-quality products, quality assurance has become a critical part of cellular therapy laboratories.

All cellular therapy products must be demonstrated to be safe, pure, potent, stable, and effective for human use. Objective standards based on clinical trial and manufacturing data should be established to evaluate safety and quality characteristics of clinical products during production and at the time of lot release. Also known as product specifications, these standards are intended to ensure that cellular products consistently meet regulatory and industry requirements for sterility, safety, purity, identity, and potency. Tests to measure and evaluate these parameters are performed at critical steps in the manufacturing process (in-process testing) and at the end of production prior to the release of the product for clinical use (lot release testing). The results of in-process and lot release assays should fall within specified ranges and meet predetermined acceptance criteria before the product can be released for human use. In-process testing and lot release testing are important for ensuring individual product quality as well as lot-to-lot consistency. For cellular therapies these assays include tests of sterility, including mycoplasma, viability and identity, and assessment of product potency. One of the most important aspects of assessing the quality of cellular therapy products is to ensure that all products meet established minimal levels or ranges of potency and that potency levels are consistent across manufacturing lots.

Potency testing

Potency testing involves the quantitative measure of biological activity of a product. The biological activity describes the ability of a product to achieve a defined biological effect. Potency testing is the quantitative measure of a biological activity which is linked to relevant

biological properties of a product. The biological activity that is measured should be closely related to the product's intended biological effect, and ideally, it should be related to the product's clinical response (Stroncek et al., 2007).

Potency assessments are meant to measure a cellular therapy product's critical biological activity within a complex mixture by quantifying the product's activity in a biological system. Measurement of the potency of a product is not the same as measuring clinical efficacy but rather is a means to control product consistency. Generally, potency testing is performed at the time of product lot release and across all production lots.

Since potency assays for cellular products usually take a considerable amount of time to develop, the development of potency assays is generally progressive. The development of potency assays usually begins during pre-clinical and early clinical development. Development starts with identifying the critical biological activity of the product and formulation of an approach to potency determination. A potency assay should be validated prior to phase III clinical trials.

Complexities associated with potency testing of cellular therapies

Potency testing of cellular therapies is particularly challenging for several reasons (Table 5.1). First since most cellular therapies are patient-specific, there is usually a limited quantity of suitable source material and therefore a limited amount of final, ready-to-administer biological material to use for lot release and potency testing. The starting materials for most cellular therapies are cells collected from human subjects. The subjects may be the person being treated (autologous products) or a living donor (allogeneic products). For both situations the quantity of starting material that can be collected is limited; consequently, the amount of material produced is limited. As a result an entire production lot of a cellular therapy is usually administered to a single patient, and the use of large

TABLE 5.1 Challenges associated with potency testing of cellular therapies.

- Limited quantity of final product to test
- Usually limited time to perform lot release testing
- Limited stability of most cellular therapy products
- Limited availability of reference standards
- Generally very high variability among lots

quantities of the product for lot release testing may adversely affect the dose and clinical effectiveness of the product. This limitation on the quantity of material available prevents the use of some assays and/or limits the number of analytes that can be tested.

Second, the time to test the product is limited, since cellular therapy products must be tested at the time production is complete but before being released for clinical use. This is particularly problematic for cellular therapies, since the potency of most living cells is affected by prolonged storage at physiological temperatures. In fact, some products must be administered within hours upon production completion. In addition, handling affects the potency of some products.

Most potency assays require reference preparations with an established potency, which are used as assay standards. The limited availability of reference standards complicates potency testing for cellular therapies. Often in-house reference standards must be developed. When reference standard are commercially available, they are may be expensive.

Finally, cellular therapy products typically show a large degree of lot-to-lot variability. Product variability is due in part to inherent variability in the starting cells or tissues. Donor genetic factors likely contribute to differences in potency of the final cellular therapy product. Genetic polymorphisms in cytokines, growth factors and their receptors affect the cellular immune response (Howell and Rose-Zerilli, 2006; Howell et al., 2001; McCarron et al., 2002; Hollegaard and Bidwell, 2006). It is likely that these polymorphisms affect the response of cells to cytokine and growth factor stimulation *in vitro* and the behavior of cells during culture. Epigenetic changes may also be important. The same type of cells obtained from different donors at different time points and under different physiological conditions could vary significantly, owing to genetic heterogeneities, epigenetic differences, or transcription regulation diversities.

Factors affecting the potency of cellular therapies

Despite the difficulties associated with potency testing of cellular therapies, potency is particularly important for these products, since the complexities associated with their production can result in considerable differences in potency among different lots of the same product (Table 5.2). These differences are related to the multiple steps required to produce most cellular therapies, variations in starting materials, limited stability of the final product, complex mechanisms of action of the product, and genetic differences among individuals donating the starting cells.

TABLE 5.2 Factors contributing to the complex nature of cellular therapies.

- There may be variations in the starting cellular material.
- Multiple biological products may be used in the manufacturing process.
- Multiple steps can be involved in the manufacturing process.
- Clinical effectiveness may depend on multiple cellular functions.

Advanced cellular therapies may incorporate multiple components. For example, cellular products used for cancer vaccines may require more than one peptide to educate immune cells *in vitro*, followed by cytokine stimulation. Genetically engineered T cells require gene transfer followed by ex vivo cytokine-stimulated expansion. A manipulated lymphocyte component prepared from a HSC transplant donor may involve isolating and recombining multiple different types of cells. Regenerative therapies require the reprogramming of somatic cells to produce iPSCs followed by ex vivo expansion and differentiation. The multiple cell types that are present in many cellular therapies have the potential to interfere with one another or to act synergistically.

Many cellular therapies are subject to extensive manipulation, including manufacturing processes such as cytokine, growth factor, or antigen stimulation; culture; expansion; and treatment with vectors or toxins. For these products, slight variations in the starting cellular material, reagents, processing methods, or culture conditions may result in significant variation in the final product, leading to heterogeneous clinical outcomes of the same therapies. Variability in starting materials is particularly problematic, since many cellular therapies make use of autologous cells as starting material and these cells are often affected by the patient's disease or treatments.

Finally, the *in vivo* function of most cellular therapies is dependent on multiple factors in the host environment. HSCs must traffic to specific sites, expand, and differentiate into several mature cell types. Immune therapies must migrate from the site of administration, interact with tumor or other immune cells, and respond to stimuli and/or stimulate other cells. iPSCs and iPSC-derived cells must differentiate into the desired cell type but not create unwanted or abnormal cells *in vivo*.

Measuring potency of cellular therapies

Potency can be tested in a number of ways including *in vivo* and *in vitro* systems (Table 5.3). Testing potency

TABLE 5.3 Assays used for potency testing.**In vivo assays**

- Animal models

In vitro assays

- Cell-based assay systems
- ELISA
- Flow cytometry
- ELISPOT
- Proteomics
- Quantitative real-time PCR

using in vivo animal models is generally preferred over in vitro test systems, since animal models assays have the ability to directly measure a product's functional activity. However, existing animal models may not be relevant, and new animal models may be difficult to develop. In addition, the results of in vivo tests are often variable and difficult to reproduce. Furthermore, these assays usually take a considerable amount of time to complete, making it difficult to use these assays for routine lot release testing. Many in vivo assays are best suited for use in product development, as an in-process control, or to evaluate the potential effect of changes in the manufacturing process or materials.

In vitro assays involve the measurement of biochemical or physiological responses at the cellular level. The in vitro measurement of cell surface markers, activation markers, secretion of factors, or protein expression do not directly measure the function of a cellular product; however, they have been used as surrogates for potency. When an in vitro assay is used as a surrogate for potency, a correlation should be demonstrated between the assay results and the intended biological activity. Typical in vitro assays used as surrogates for potency testing include ELISA, ELISPOT, flow cytometry, proteomic analysis, and cytotoxicity assays.

When the mechanism of action of a cellular therapy can be attributed to the expression of specific cell surface antigens, the measurement of antigens by flow cytometry can be used as an in vitro potency assay. In fact, the measurement of biomarkers by flow cytometry is often used as a surrogate measure of cell potency. Flow cytometry is useful because of the large number of reagents and assays that are available as well as the relatively quick turnaround time. It can be used to measure the expression of cell surface markers, viability, and the production of cytokines. Extensive analysis of cell surface markers using flow cytometry has been used to assess cellular therapies,

but the maximum number of markers that can be analyzed is limited by the availability of specific antibodies, instrumental detection limits, and final product quantity. In addition, the markers that may be most useful might not be known.

In vitro cell function assays have also been used to measure cell potency. Cytotoxicity assays are sometimes used to reflect the function of adoptive immune therapies. Cytokine release by stimulated cells can also be used to measure cell function. However, cell function assays have many limitations. While they may be able to detect differences in relevant biological activity, these assays are typically highly specialized for each cell type, are labor intensive, and require highly skilled staff. Different types of cells and cell subsets require completely different types of cell function technologies. Many cells require the measurement of multiple functions to adequately assess potency. Furthermore, the function(s) that best predict cell potency may not be known. In fact, for many cellular therapies, all aspects that contribute to in vivo activity are not completely understood. In addition to these limitations, many cell-processing laboratories working with cellular therapies in phase I and phase II clinical trials prepare several different types of cellular therapies. It is possible but may not always be feasible for a centralized laboratory to perform several different types of cell function assays.

Cell counts and viability measurements are often performed on cellular therapies. However, since these assays do not measure a relevant biological activity, they are not potency measures.

Gene expression arrays for potency testing

Measurements of the expression of genes related to a specific cellular activity or function could be used as an in vitro biomarker of potency. Quantitative real-time PCR assays are useful tools for assessing the expression of individual genes in order to assess the activity of immune cells. The measurement of changes in interferon gamma transcription by quantitative real-time PCR has been used to as a marker for T cell activation following stimulation with a recall antigen (Provenzano et al., 2002, 2003; Kammula et al., 2000; Panelli et al., 2002). Quantitative real-time PCR has recently been used to measure the production of mRNA encoding interferon- γ , interleukin-2 (IL-2), IL-4, and IL-10 by stimulated T cells (Provenzano et al., 2003). Quantitative real-time PCR arrays are also available to assess angiogenesis, apoptosis, cell cycle, insulin-signaling pathways, cytokines and receptors, nitric oxide-signaling pathways, and JAK/STAT-signaling pathways.

While using quantitative real-time PCR to measure the expression of single genes or groups of genes is helpful in

assessing cell function, the complete assessment of the function of cellular therapies requires the measurement of a broad range of gene transcripts, especially when the mechanisms that are responsible for effective therapy are not thoroughly understood. The analysis of cells using gene expression microarrays allows the simultaneous assessment of the expression of thousands of genes. One practical advantage of gene expression microarray assays over other analytical assays is that very few cells are needed. Enough RNA can be isolated from 1×10^4 to 1×10^6 cells for analysis with a 17,500-gene cDNA expression microarray (Wang et al., 2000).

Microarrays with 40,000 genes or oligonucleotide probes have been used clinically to characterize lymphomas (Dave et al., 2006), prostate cancer (Halvorsen et al., 2005), ovarian cancer (Wang et al., 2005), small cell lung cancer (Taniwaki et al., 2006), melanoma (Basil et al., 2006) and many other cancers. We have used cDNA gene expression microarrays with 17,500–40,000 genes to investigate the immunological changes associated with high-dose IL-2 therapy for renal cell carcinoma (Panelli et al., 2004) and imiquimod, a TLR-7 ligand, therapy for basal cell carcinoma (Panelli et al., 2007). We have also used cDNA microarrays to assess the effects of IL-10 on NK cells (Mocellin et al., 2004; Nagorsen et al., 2005; Stroncek et al., 2005) and several different types of interferon on lipopolysaccharide (LPS)-stimulated mononuclear cells, the in vitro response of mononuclear cells to IL-2 (Jin et al., 2006), and the molecular basis of cutaneous wound healing (Deonarine et al., 2007). More recently, we have used RNA sequencing to measure gene expression.

While gene expression analysis has been widely used to assess changes in cells in response to stimuli, or to classify different types of cancers, it has been used to only a limited extent to assess cell potency. However, since gene expression analysis simultaneously measures the expression of thousands of genes, it captures a snapshot of all possible gene expression signatures that are associated with cellular function and hence could be a very important tool for assessing the potency of cellular therapies. The comprehensive nature of gene expression analysis makes it ideal for measuring both expected and unexpected cell functions. This is particularly important for the analysis of cells with complex and multiple critical functions, such as dendritic cells (DCs), cytotoxic T cells, embryonic stem cells, HSCs, and BMSCs.

In addition to assessing potency, gene expression can assess other important aspects of cellular therapy products such as stability, purity, comparability, maturation, and differentiation status. Since gene expression analysis can detect the activation of apoptosis pathways that signal the onset of cell death, it has the potential to provide useful information about the effects of storage or manipulation on cell viability. The assessment of the expression of

apoptosis genes is likely to be much more sensitive in assessing cell viability than dye exclusion assays or the flow cytometric measurement of Fas or annexin. Gene expression profiles can also detect subpopulation of cells and therefore provide information concerning cell purity.

There are some limitations in the use of gene expression microarrays for potency testing. Gene expression microarray analysis involves multiple steps including RNA isolation, amplification, fluorescent labeling, hybridization, and data analysis. It is impossible at the current technology stage to complete the whole procedure within a few hours, so these global expression microarrays cannot yet be used for lot release testing. However, if global microarrays can identify specific sets of gene whose expression is associated with potency, tailored chips or quantitative real-time PCR kits that assess only specific “potency genes” could be developed and used for lot release testing. One limitation of gene expression analysis is the time required to perform these assays. Recently, a novel, easy-to-use expression platform has become available: the nanostring. The technology involves digital color-coded probes that are attached to a single target-specific probe that is directed to a specific gene. This technology allows for the quantitation of the expression of specific genes without amplification (Tsang et al., 2017; Beard et al., 2013). Kits are available for the evaluation up to 800 immune response genes. This platform has been used along with flow cytometry to evaluate the characteristics of a CD19-CAR T cell product that contributes to its potency (Rossi et al., 2018).

Next-generation DNA/RNA sequencing platforms allow for RNA sequencing (RNA-seq) and the accurate measurement of the expression of the entire transcriptome. This technology is being used in place of microarrays for the assessment of gene expression for many applications. RNA-seq uses the capabilities of high-throughput sequencing methods to provide insight into the transcriptome of cell products. Compared with the quantitative real-time PCR and microarrays, RNA-seq has a deeper coverage and a higher resolution. In addition to gene expression analysis, RNA-seq can identify further analysis, including alternative splicing, genetic variants and fusion genes, which also could be used for potency testing (Lynn et al., 2019; Singh et al., 2020; Guedan et al., 2020; Tong et al., 2020).

Potential applications of gene expression profiling for potency testing

Predicting the confluence of human embryonic kidney 293 cells

Gene expression microarrays have been demonstrated to be useful for some cell therapy applications. They can be used to predict the quality of cells that are used to

manufacture biological products. Human embryonic kidney (HEK) 293 cells are often used to manufacture products such as adenoviral gene therapy vectors and vaccines (Han et al., 2006). These cells can be grown in bioreactors, tissue culture flasks, and roller bottles. However, when HEK 293 cells grow to form a confluent monolayer, their phenotype changes, as does the quality of the vector or vaccine produced by these cells. Cell confluence can be readily assessed by visual inspection of cells grown in flasks and roller bottles, but for cells grown in bioreactors, the assessment of confluence by visual inspection is not always possible. Gene expression profiling has been used to identify genes whose expression predicts cell confluence (Han et al., 2006). HEK 293 cells that have been grown to 90% confluence have a unique gene expression signature compared to those grown to 40% confluence. A set of 37 of these signature genes is able to predict the quality and confluence of HEK 293 cells. While this use of gene expression profiling does not represent a potency assay, it demonstrates the potential of the use of gene expression profile assays.

Cell differentiation status analysis of embryonic stem cells

Human embryonic stem cells (hESC) have the potential to be useful for a number of clinical applications. Since cultured hESC may undergo spontaneous differentiation, it is important to determine whether cultured hESC have maintained their stem cell qualities or have begun to acquire properties of more differentiated cells. Gene expression profiling may be useful for assessing cultured hESC. Gene expression profiling has been used to identify genes that are uniquely expressed by hESC (Player et al., 2006). Player and colleagues have found that 1715 genes were differentially expressed between hESC and differentiated embryonic cells (Player et al., 2006). The analysis of the expression of genes that are expressed by hESC but not by differentiated cells is likely to be useful in determining whether cells in culture have maintained their embryonic stem cell characteristics.

Embryonic stem cells must be differentiated before they can be used clinically. One of the first steps in the differentiation of hESC into mature cells and tissues for clinical use is the production of embryoid bodies (hEB). The production of hEB involves the aggregation of embryonic cells but the prevention of separation of cells into germ lines by plating them onto a nonpermissive substrate. After these hEB are isolated, they can be induced to generate several different types of cells, including hematopoietic cells, neuronal, myogenic, and cardiac muscle cells. A comparison of genes expression profiles of hESC and hEB has found that the expression of several genes were

downregulated and several were upregulated, including 194 whose expression was more than threefold greater in hEB (Bhattacharya et al., 2005). This unique set of genes should also be useful in assessing hESC differentiation.

Potency testing of hematopoietic stem cells

HSCs are widely used for several clinical applications, and better potency assays for these therapies are needed. Potency assays for HSC products used for transplantation should measure the ability of the product to reconstitute bone marrow hematopoietic cells and peripheral blood cells in the transplant recipient. The potency assay should reflect the period of time when neutrophil, platelet, and red blood cells counts return to and remain above specified levels independent of transfusion therapy. In other words, if the potency assay indicates that a product meets minimum criteria, the therapy should result in at least minimum acceptable neutrophil, platelet, and RBC counts in the recipient for a minimum specified duration of time.

Liquid culture of LTC-IC and the repopulation of marrow in nonobese diabetic (NOD)/severe combined immunodeficiency mice assays are considered to be the best measure of the quantity and quality of hematopoietic stem cells. However, these assays require several weeks to complete, highly specialized reagents, and highly trained staff. As a result, these assays have seldom if ever been used as potency assays.

The measurement of myeloid, erythroid, and mixed colony formation in methylcellulose culture systems has been the standard method for assessing bone marrow and peripheral blood stem cell (PBSC) concentrates, but they have been used mainly as in-process controls. The measurement of colony formation in methyl cellulose is an effective biological assay that directly measures a relevant function of HSCs. However, these assays take approximately 14 days to complete; consequently, they cannot be used as a potency assay.

Traditionally, total nucleated cells counts were used to assess the potency of bone marrow and are still used as a measure of potency of umbilical cord blood (UCB) components prepared for transplantation. Regulations suggest that UCB components contain 90×10^7 or more total nucleated cells including nucleated RBC and that 85% or more of nucleated cells are viable. However, the measurement of CD34⁺ cells by flow cytometry has become the universal assay for measuring the potency of HSC products collected by apheresis from subjects who have been treated with hematopoietic growth factors. The number of CD34⁺ cells in a HSC product can be measured within a few hours, using anti-CD34 and flow cytometry, and this assay is well suited for lot release testing. Generally, a dose of 1×10^6 CD34⁺ G-CSF-mobilized PBSCs is considered adequate for an autologous transplant and

$3-5 \times 10^6$ for an allogeneic transplant. UCB components must contain 1.25×10^6 or more viable CD34⁺ cells.

While CD34 antigen expression is widely used as a measure of potency of HPCs collected from the peripheral blood, HSCs expressing CD34 antigen do not represent a homogenous population. Several distinct subpopulations or phenotypes of CD34⁺ cells have been described (Tjonnfjord et al., 1994). Some subpopulations are more primitive, while others are more likely to differentiate into myeloid cells, erythroid cells, or megakaryocytes.

Despite the heterogeneity of CD34⁺ cells, the measurement of CD34⁺ cells has been an effective measurement of potency of PBSC concentrates collected by apheresis. This is likely because PBSC components are relatively similar in that almost all PBSC components are collected from subjects given granulocyte colony-stimulating factor (G-CSF) alone or in combination with chemotherapy. However, the sources of HSCs and types of mobilizing agents used for transplantation are changing. UCB components are being used in place of PBSC concentrates and marrow for unrelated donor HSC transplantation. A new stem cell-mobilizing agent, plerixafor, is being used with G-CSF to mobilize stem cells for autologous transplants (Flomenberg et al., 2005) and will likely soon be used for allogeneic donor transplants (Burroughs et al., 2005; Laroche et al., 2006). CD34⁺ cells from both UCB and plerixafor-mobilized PBSC concentrates differ from those found in G-CSF-mobilized PBSC concentrates and the quantity of CD34⁺ required for a successful transplant from some of these types of products will likely differ from the quantity required for a successful G-CSF-mobilized PBSC transplant.

Plerixafor mobilizes stem cells by a different mechanism than G-CSF. Plerixafor is a CXCR4 antagonist, and it mobilizes HSCs within 6 hours by disrupting the binding of stem cell CXCR4 with SDF-1, CXCL12, on marrow osteoblasts (Nervi et al., 2006). In contrast, G-CSF mobilizes stem cells indirectly by downregulating the expression of SDF-1 on marrow osteoblasts and by releasing neutrophil and monocyte proteolytic enzymes, including neutrophil elastase, cathepsin G, and matrix metalloproteinase-9 that degrade important HPC trafficking and adhesion molecules c-kit, VCAM-1, CXCR4, and SDF-1 (Nervi et al., 2006). Because of the differences in mechanisms of mobilization between plerixafor and G-CSF, plerixafor mobilizes a CD34⁺ cell population with a greater long-term marrow-repopulating capacity and with a different phenotype than G-CSF (Laroche et al., 2006).

The potency of UCB CD34⁺ cells also differs from that of G-CSF-mobilized peripheral blood CD34⁺ cells. The potency of CD34⁺ cells from UCB as measured by the ability to repopulate NOD SICD mice is greater than the potency of CD34⁺ cells from bone marrow or G-CSF mobilized PBSCs (Bhatia et al., 1997; Broxmeyer, 2005;

Broxmeyer et al., 2006). In addition, UCB CD34⁺ cells show increased proliferative capacity compared to bone marrow and G-CSF-mobilized PBSC CD34⁺ cells in methylcellulose culture (Broxmeyer et al., 2006; Broxmeyer et al., 1992; Broxmeyer et al., 1989).

Since the potency of CD34⁺ cells is dependent on the number of and subtypes of CD34⁺ cells and since the sources of HSCs used in transplantation is increasing, new potency assays are needed. A preliminary comparison of CD34⁺ cells mobilized by G-CSF and G-CSF plus plerixafor has found that these two types of HSCs can be differentiated by gene expression profiling (Fig. 5.1) (Jin et al., 2008; Fruehauf et al., 2006). These two types of HSCs were not only mobilized with two different protocols, but two different monoclonal antibodies were used to isolate the HSCs: CD34 and CD133 (Jin et al., 2008). It is not certain whether the differences noted in the HSCs were due to the mobilization protocols or the antibody used for HSC isolation. In additional studies, CD34⁺ cells isolated from rhesus macaques that had been given G-CSF or plerixafor were analyzed by global gene expression analysis. When compared to G-CSF-mobilized CD34⁺ cells, plerixafor-mobilized CD34⁺ cells were enriched for B cell, T cell, and mast cell genes, and G-CSF-mobilized CD34⁺ cells were enriched for neutrophil and monocyte genes (Donahue et al., 2009).

Potency testing of dendritic cells

DCs are potent professional antigen-presenting cells that are capable of capturing and processing antigens in order to present peptides to prime T cells (Bhattacharya et al., 2005). They express both HLA class I and class II molecules and present peptides to CD4⁺ and CD8⁺ T cells. They also express costimulatory molecules such as CD80, CD86, CD40, ICAM-1, and LFA-3. For immune therapy, DCs can be generated from peripheral blood mononuclear cells (PBMCs) after GM-CSF and IL-4 stimulation in vitro, or they can be generated by coculturing in vitro with irradiated tumor cells or virus infected cells, proteins, or peptides. Mature DCs are administered to patients to stimulate cytotoxic T cells in vivo. Immunotherapies with DCs are being used to treat melanoma, renal cell carcinoma, prostate cancer, HER2-expressing cancers, and leukemia (Schmitt et al., 2007; Berzofsky et al., 2018).

Since few DCs are present in the blood, they must be produced from other types of cells. DCs for clinical therapies produced from CD34⁺ cells are known as plasmacytoid DCs, and those produced from circulating mononuclear cells are known as myeloid-derived DCs. Either mature or immature DCs can be produced. Immature DCs express lower levels of HLA class II antigens and lower levels of costimulatory molecules but higher levels of Fc and mannose receptors. The ability of immature DCs to phagocytose and process antigens is

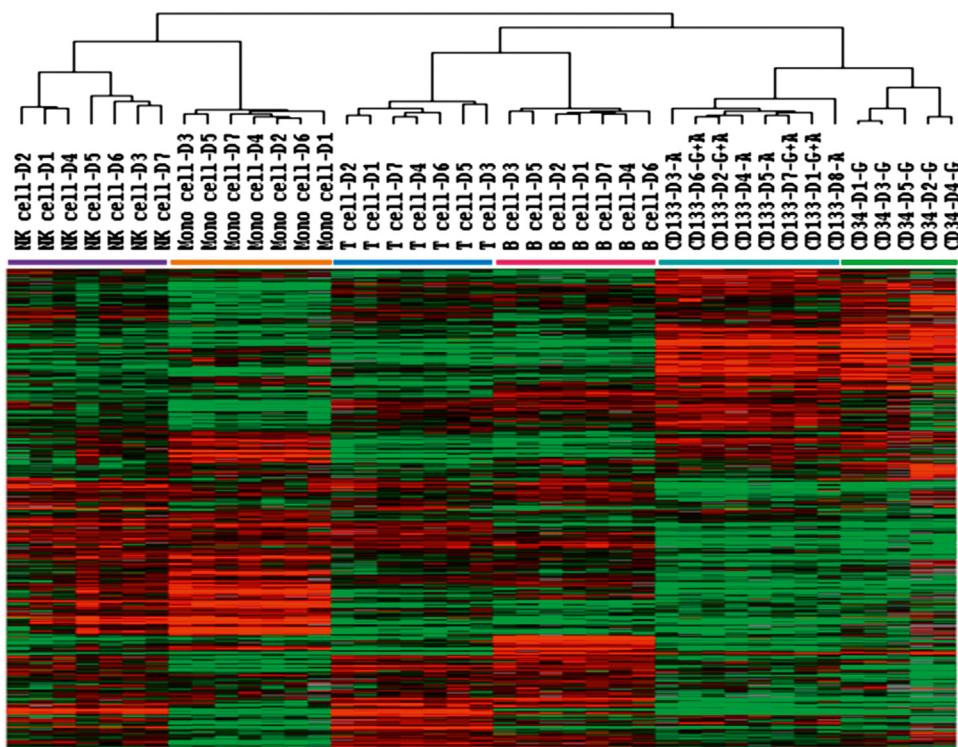


FIGURE 5.1 Gene expression profiles of hematopoietic stem cells (HSCs) and peripheral blood leukocytes (PBLs). cDNA was isolated from T cells, B cells, monocytes, and NK cells from seven subjects; G-CSF (G)-mobilized CD34⁺ cells from five subjects; plerixafor or AMD3100 (A)-mobilized CD133⁺ cells from four subjects; and plerixafor plus G-CSF (A + G)-mobilized CD133⁺ cells from four subjects. cDNA expression was analyzed using an expression microarray with 17,500 cDNA. Unsupervised hierarchical clustering of Eisen was used to analyze the 11,023 genes that remained after filtering (cDNA expressed in ≥ 80% of samples). Adapted from Jin, P., Wang, E., Ren, J., Childs, R., Shin, J.W., Khuu, H., et al., 2008. Differentiation of two types of mobilized peripheral blood stem cells by microRNA and cDNA expression analysis. *J. Transl. Med.* 6, 39. *Epub 2008/07/24. doi: 10.1186/1479-5876-6-39. PubMed PMID: 18647411; PubMed Central PMCID: PMC2503968.*

better than that of mature DCs, but mature DCs present antigens better than immature DCs. While the function of mature and immature DCs differ, it is not possible with standard analytic assays to precisely distinguish the degree of maturation of DCs.

The potency of DCs can be tested by assessing the ability of DCs loaded with antigen to stimulate autologous T cells (Hinz et al., 2006). However, this is difficult because of the low percentage of T cells in most patients who are responsive to tumor antigens. One alternative to overcome the low number of autologous T cells is to generate and expand T cell clones that respond to specific antigens. Even so, only T cells with the same HLA restriction elements and antigen specificity could be used in a DC potency assay. For example, HLA-A*0201 T cell clones specific to a melanoma antigen such as MART-1 would not be useful for testing DCs prepared from subjects with other HLA types such as HLA-A*03 or other antigens such as CMV pp65. Consequently, separate clones must be developed for each antigen and HLA restriction being studied.

The potency of DCs can be assessed by using test peptides from recall antigens that are able to stimulate memory

T cell responses (Hinz et al., 2006). These antigens include HLA-restricted tetanus toxin, influenza virus, and EBV antigens, since most people have been immunized against these antigens. However, assays using recall antigens do not directly test DCs' ability to present tumor-associated antigens and efficacy to stimulate tumor-specific T cells. So these assays cannot be used as a lot release test for DCs used for cancer therapy, although testing the ability of DCs to present recall antigens and stimulate T cells is useful as an in-process control.

The measurement of DC costimulatory activity has been used to measure the potency of DCs. Costimulation plays a critical role in the induction of antigen-specific immunity. One method to measure costimulation is the mixed lymphocyte culture reaction that is based on the stimulation of responder cells with replication competent allogeneic DC stimulator cells. However, it is not known to what degree alloreactivity and costimulation contribute to T cell stimulation.

Alternatively, gene expression profiling is likely to be useful in assessing the potency of DCs used for clinical therapies. It has been used to characterize the differentiation of monocytes into macrophages and their polarization

to macrophages with a type 1 or type 2 phenotype (Martinez et al., 2006) and has also been used to characterize the response of monocytes to LPS and cytokine stimulation (Nagorsen et al., 2005; Stroncek et al., 2005). Preliminary data in our laboratory have also shown that gene expression profiling can distinguish monocytes from immature DCs and immature DCs from mature DCs. The ability of gene expression analysis to assess cells globally may allow them to determine the potency of DCs by evaluating unstimulated cells or cells that have been stimulated with a recall antigen. However, genes whose expression reflects DC maturation as well as specific DC functions must be identified before gene expression profiling can be used a potency assay for DCs.

One important use of a potency assay is comparability testing. Comparability testing is an essential part of cellular therapies. While it is desirable to maintain the same manufacturing methods and to use the same materials throughout a clinical trial or as a product moves from early-phase to late-phase clinical trials and licensure, change is inevitable. There are many reasons that the manufacturing process or reagents and instruments used in manufacturing may change. The supplier of reagents used in the manufacturing process may change, the methods used to produce a reagent or they may stop manufacturing the reagent entirely and a new reagent supplier must be used. Devices used to collect the starting material and containers and bioreactors used for cell culture may also change. In addition, as cellular therapies move from early phase to late phase clinical trials and to licensure the manufacturing process must be scaled up and possibly out and this almost always requires some changes to the manufacturing process. It is essential to show that the products manufactured using a new reagent, container or instrument are comparable to the existing products. Potency assays can be used to show that products are comparable. We have shown that even if the potency of a product is not entirely understood, global gene expression analysis can be used to show that the products are comparable.

We have used gene expressing profiling to show that immature DCs produced from two different types of starting materials were equivalent. The production of clinical cellular therapies often involves multiple centers. For example, cells such as PBMCs are collected from a donor or patient at one center and then shipped to a specialized cellular therapy laboratory where they are processed to produce cells such as DCs. When the processing is complete, the product is shipped to the site where it is administered to a specific patient. The centralized cell processing laboratory may be hundreds or thousands of miles from the collection center and it may take up 48-hours after the collection is complete for the starting cellular material to reach the cell processing laboratory.

We wished to determine if immature DCs produced from PBMCs that were stored at 4°C for 48 hours differed from those produced from fresh PBMCs (Shin et al., 2008). Immature DCs were produced from fresh PBMCs and PBMCs from the same donors that were stored for 48 hours. Global gene expression analysis was used to analyze the fresh and stored PBMCs, monocytes isolated from the PBMCs, and immature DCs manufactured from the PBMCs. Hierarchical clustering analysis separated the fresh and stored PBMCs and monocytes into separate groups, but immature DCs prepared from fresh and stored PBMCs were in the same cluster (Fig. 5.2). Comparison of genes differentially expressed by fresh compared stored products (paired *t*-tests, $P < .005$) found 273 genes that differed between fresh and stored PBMCs, 711 that differed between elutriated monocytes prepared from fresh and stored PBMCs, but only 3 that differed between immature DCs prepared from elutriated monocytes isolated from fresh PBMCs and immature DCs prepared from elutriated monocytes isolated from stored PBMCs (Shin et al., 2008). These results showed that immature DCs made from 48-hour stored PBMCs did not differ from those prepared from fresh PBMCs and demonstrated that gene expression profiling can be used to show equivalence of DC products manufactured using different methods.

We have also taken a novel approach to identify candidate markers for potency testing of DCs. We assessed the variability of genes expressed by DCs and identified the least and most variable genes in order to determine if they might make good candidates as genes for potency testing (Castiello et al., 2013b). In these studies we manufactured DCs from peripheral blood monocytes using methods and reagents used to manufacture DCs in our clinical GMP manufacturing facility. The monocytes were cultured for 3 days with IL-4 and GM-CSF to produce immature DCs and they were then cultured for 1 day with LPS and IFN- γ to produce mature DCs. We first assessed the variability of DCs and sources of variability to determine if lot-to-lot differences in DCs might be significant enough to contribute to differences in clinical outcome. To assess intra-donor variability mature DCs were manufactured from monocytes collected from the same donor on 5 different occasions. For the assessment of manufacturing variability DCs were made five different times using the monocytes obtained from a single apheresis collections. To assess interdonor variability DCs were manufactured from nine different donors. The interclass correlation coefficient of genes from each sample was calculated to assess the degree that each of these sources of variability effected mature DCs. We found that manufacturing variability was less than intra-donor variability and interdonor variability was the greatest. These results show variability in the starting material; both

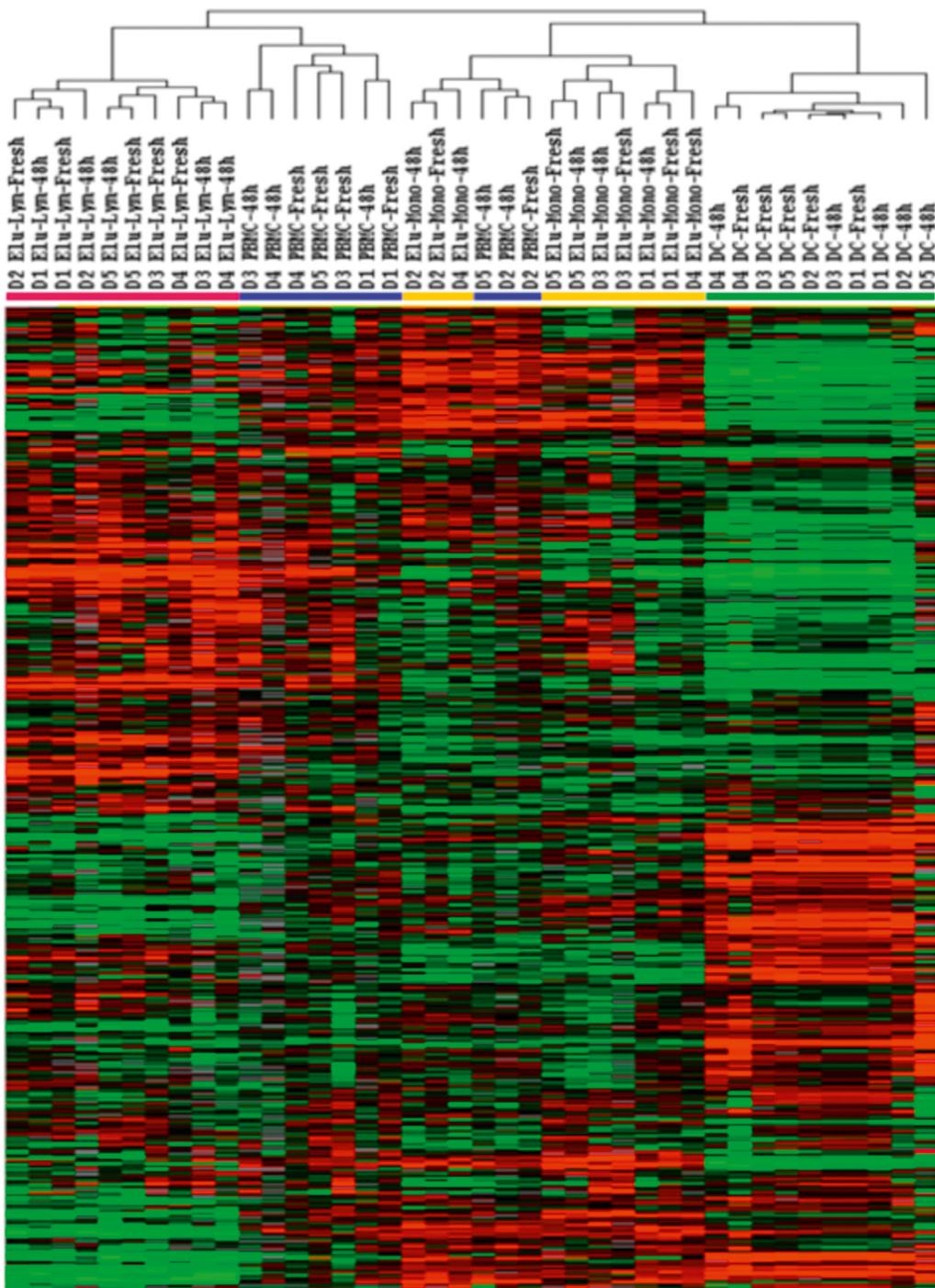


FIGURE 5.2 Gene expression profiling of fresh and 48-hour stored PBMC products and elutriated lymphocytes, elutriated monocytes, and immature dendritic cells (DCs) prepared from fresh and stored PBMCs. Products from five healthy donors were analyzed. The 8661 genes that remained after filtering (expressed in 80% of samples) were analyzed by unsupervised hierarchical clustering of Eisen. PBMC products are indicated by a blue bar, elutriated lymphocytes by a red bar (Elu-Lym), elutriated monocytes by a yellow bar (Elu-Mono), and immature DCs by a green bar (DC). Adapted from Shin, J.W., Jin, P., Fan, Y., Slezak, S., David-Ocampo, V., Khuu, H.M., et al., 2008. Evaluation of gene expression profiles of immature dendritic cells prepared from peripheral blood mononuclear cells. *Transfusion* 48 (4), 647–657. Epub 2008/02/20. doi: 10.1111/j.1537-2995.2007.01615.x. PubMed PMID: 18282241; PubMed Central PMCID: PMC2710576, by permission from John Wiley and Sons.

intradonor and interdonor variability effect the final DC product and that these factors were more important than manufacturing variability.

We next looked for quality markers for mature DCs (Castiello et al., 2013b). We hypothesized that good markers for assessing the quality of manufactured DCs would

be genes whose expression significantly differed by a large degree among monocytes, immature DCs and mature DCs. Using the global gene expression data we identified all genes whose average expression differed among mature DCs and monocytes by more than fivefold and whose difference in expression was highly significant, with a false discovery rate (FDR) less than 0.005 and $P < .001$. Similarly, we identified all genes whose average expression differed among mature DCs and immature DCs by more than 5, FDR $< .005$ and $P < .001$. Using these criteria, we identified 1186 genes whose expression differed among mature DCs and monocytes and 647 whose expression differed among mature DCs and immature DCs. Among these two sets of genes, 323 were common to both sets. Among these genes we selected 291 with good assay reproducibility. We then assessed the variability of each of these 291 genes among mature DCs to identify those with the greatest index of variability that would likely make good markers for identity and potency testing and those with the least index of variability, which may be good potency markers. Among the 29 least variable genes were AIM2, FEM1C, APOL1, NUB1, IMAZ, DRAM1, AK4, IFI27, WARS, PSME2, and ICAM1 (CD54), all of which are functionally important immune-related genes (Table 5.4). Among the 29 most variable genes were the functionally important DC genes CD80, CCL1, CCRL1, and CD70 (Table 5.4). Many groups manufacturing DCs are using the expression of CD80 as measured by flow cytometry as a lot release criterion for mDCs. We are currently working to validate these genes by comparing their expression in mDCs manufactured for clinical therapy and correlating their expression with clinical outcomes.

Cultured CD4⁺ cells

One critical aspect of HSC transplants involving HLA-matched siblings is donor T cell responses. Donor T cell responses directed toward recipient cells promote HSC engraftment but can cause GVHD, while donor T cell responses directed toward leukemia or lymphoma cells can prevent posttransplant disease relapse. Considerable effort has been spent on manipulating HSC grafts in order to minimize donor T cell-mediated GVHD and maximizing T cell-mediated antitumor effects. Fowler and colleagues have found that after allogeneic HSC transplantation, the infusion of CD4⁺ donor T cells that had been cultured with anti-CD3/anti-CD28-coated beads and IL-4 in the presence of the mTor pathway inhibitor rapamycin results in a low incidence of both GVDH and disease relapse in patients with B cell lymphoma (Fowler et al., 2013). While these so-called T Rapa cells have shown promising clinical results, their mechanism of action is not certain, and consequently, potency markers

have yet to be identified. These cells have been difficult to analyze, since rapamycin inhibits many aspects of cell metabolism, which limits the effectiveness of the ELISA and flow cytometry assays that are standard assays for assessing T cell function. We used global gene expression analysis to search for the critical biological functions of T Rapa cells manufactured over 6 (T-Rapa₆) and 12 days (T-Rapa₁₂), both of which have been tested in clinical trials. Using conventional analytic methods, cytokine release and surface marker expression, we found that T-Rapa₆ and T-Rapa₁₂ cells had similar phenotypes. To further compare the potency of these two types of cells, we analyzed both types of cells and input CD4⁺ cells using global gene expression analysis. When compared to CD4⁺ cells, 6641 genes were differentially expressed in T-Rapa₆ cells, and 6147 genes were differentially expressed in T-Rapa₁₂ cells (Castiello et al., 2013a). The genes that were differentially expressed in the T-Rapa₆ and T-Rapa₁₂ cells were similar in terms of gene families that were upregulated (cell cycle, stress response, glucose metabolism, and DNA metabolism) and downregulated (inflammatory response, apoptosis, and transcriptional regulation). However, when T-Rapa₆ and T-Rapa₁₂ cells were compared, 1994 genes were differentially expressed. These results show that the T-Rapa₆ and T-Rapa₁₂ cells have similar yet distinct properties, and they may have slightly different clinical effects. Conventional analyses failed to reveal these differences. The clinical trial of T-Rapa₁₂ cells has been completed (Fowler et al., 2013), but the trial of T-Rapa₆ cells is ongoing, so it is not yet certain whether these molecular differences result in differences in their in vivo function (Castiello et al., 2013a).

Bone marrow stromal cell

Potency testing is challenging for BMSCs because they are used for many diverse purposes, and the critical properties of BMSCs that contribute most to their clinical effectiveness likely differ among applications. For example, when BMSCs are used for treating peripheral vascular disease or left ventricular failure due to coronary artery disease the angiogenic and antiinflammatory properties of BMSCs are likely to be important, but when BMSCs are used to treat steroid-resistant acute GVHD, their immune modulatory functions are likely to be most important. However, it would be desirable to have potency markers that could be used for all applications of BMSCs.

We hypothesized that markers of BMSC senescence would be good for potency testing. When BMSCs undergo prolonged culture, their proliferation slows and finally stops; the cells become senescent. Senescence is also associated with changes in morphology, phenotype, and function. In addition to becoming less proliferative,

TABLE 5.4 Dendritic Cell genes with the least and greatest index of variability.

Least index of variability		Greatest index of variability	
Brain and acute leukemia, cytoplasmic	BAALC	Basic helix-loop-helix family, member e22	BHLHE22
Apolipoprotein L, 1	APOL1	Dynein, axonemal, heavy chain 10	DNAH10
Tumor necrosis factor, alpha-induced protein 6	TNFAIP6	Neuregulin 2	NRG2
Phospholipase A1 member A	PLA1A	Annexin A3	ANXA3
PREDICTED: hypothetical LOC100131733	LOC100131733	Endothelin 1	EDN1
Hyaluronan and proteoglycan link protein 3	HAPLN3	Ladinin 1	LAD1
Absent in melanoma 2	AIM2	S100 calcium binding protein A14	S100A14
Tryptophanyl-tRNA synthetase	WARS	Cingulin-like 1 (CGNL1), mRNA.	CGNL1
MYC-associated zinc finger protein	MAZ	Dystrophin	DMD
Sorcin	SRI	Chemokine (C-C motif) ligand 1	CCL1
Myosin, heavy chain 11, smooth muscle	MYH11	Serine/threonine-protein kinase NIM1	MGC42105
Fem-1 homolog c (<i>Caenorhabditis elegans</i>)	FEM1C	CD70 molecule	CD70
Insulin induced gene 2	INSIG2	Secreted frizzled-related protein 4	SFRP4
Mucolipin 2.	MCOLN2	Immunoglobulin superfamily, member 3	IGSF3
DNA-damage regulated autophagy modulator 1	DRAM1	Neuregulin 2	NRG2
Adenylate kinase 4	AK4	Guanylate cyclase 2 F, retinal	GUCY2F
Serpin peptidase inhibitor, clade G	SERPING1	Chemokine (C-C motif) receptor-like 1	CCRL1
AT rich interactive domain 5B (MRF1-like)	ARID5B	CD80 molecule	CD80
Negative regulator of ubiquitin-like proteins 1	NUB1	LIM and calponin homology domains 1	LIMCH1
Neuralized homolog 3 (<i>Drosophila</i>) pseudogene	NEURL3	Lymphotoxin alpha (TNF superfamily, member 1)	LTA
Interferon induced with helicase C domain 1	IFIH1	Erythrocyte membrane protein band 4.1 like 4 A	EPB41L4A
BTG family, member 3	BTG3	Regulating synaptic membrane exocytosis 2	RIMS2
Intercellular adhesion molecule 1	ICAM1	Tissue factor pathway inhibitor 2	TFPI2
Interferon, alpha-inducible protein 27	IFI27	Synaptopodin 2	SYNPO2
Interleukin 7	IL7	Cytochrome P450, family 3, subfamily A, polypeptide 4	CYP3A4

senescent BMSCs lose their ability to form bone and cartilage, and their immune modulatory properties change (Ren et al., 2013). We believed that gene expression changes associated with senescence could be used as a measure of the quality of BMSCs.

BMSCs from marrow aspirates of seven healthy subjects were isolated and cultured until they became senescent (Ren et al., 2013). The expression of the senescence-associated marker beta galactosidase, stromal cell surface

markers, and global gene expression was assessed on BMSCs from serial passages measured on cells from passages 2 through senescence. A gene expression signature that was associated with senescence was identified, and changes in these senescence-associated genes begin before changes in proliferation, colony formation, beta galactosidase expression, and cell surface marker expression. Changes in the BMSC genes expression transcriptome occurred with every passage, but a change from an

early passage transcriptional profile to a later passage transcription profile occurred between passages 4 and 8, while changes in cell proliferation, surface marker expression, and senescence associated beta galactosidase staining occurred at later passages.

We also used a least-angle regression algorithm to identify the minimum-sized set of genes whose expression could be used to calculate BMSC replicative age as expressed as a percentage of maximum time in culture or time to senescence. We found that a set of 24 genes could predict the replicative age of BMSCs (Table 5.5). We have used these genes to assess clinical BMSCs from 12 production lots manufactured by our clinical cellular and gene therapy laboratory. Unsupervised hierarchical clustering analysis separated the 12 lots into two groups, one cluster with 8 lots that has the lowest predicted replicative ages and one cluster with 4 samples with the greatest predicted replicative age. Interestingly, the 8 samples in the low replicative age cluster all met lot release criteria, while only 1 of 4 samples in the high replicative age cluster met lot release criteria. Two lots failed owing to slow proliferation of the primary culture, and one lot failed owing to high expression of CD34 antigen. These results show that the predicted replicative age of BMSCs as measured using the expression of a 24-gene set is useful for assessing BMSC quality.

CAR T cells

An approximate measure of CAR T cell potency is the expression of the transgene. In fact, most CAR T cell products are dosed as a total quantity of CAR T cells or quantity of CAR T cells per kilogram of recipient weight or per square meter of body surface area. However, patients who are given the same doses of CAR T cells have variable clinical outcomes and variable toxicities. While disease, treatment, and other patient factors likely account for some of this variability, it is known that some CAR T cell characteristics can affect their potency and toxicity.

The clinical effectiveness of CAR T cells used to treat B cell malignancies is associated with in vivo cell expansion. Patients who achieved complete responses (CR) had greater CAR T cell expansion than those who responded poorly (Brudno et al., 2018; Lee et al., 2015). Patients who responded better to CAR T cell therapy were also more likely to experience greater toxicity or higher grades of cytokine release syndrome (CRS) (Brudno et al., 2018), and greater T cell expansion has been associated with higher levels of CRS (Brudno et al., 2018; Lee et al., 2015; Brudno and Kochenderfer, 2019).

The phenotype of the preinfusion CAR T cells likely affects their potency. Animal models have shown that tumor antigen-specific T cells expressing more naïve T cell phenotypes are more potent (Gattinoni et al., 2011).

TABLE 5.5 Twenty-four genes and coefficients used to calculate a predicted replicative age of expanded BMSCs. The expression of the genes was measured by using a microarray platform, and the predicted replicative age was calculated by using the formula: $\sum i c_i x_i + 0.143$ where c_i and x_i are the coefficient and gene expression for the i th gene, respectively.

UG cluster	Symbol	Coefficient
Hs.559718	AK5	0.007
Hs.439145	SCN9A	0.007
Hs.535845	RUNX2	-0.003
Hs.124638	TMEM90B	-0.011
Hs.656071	ADAMTS9	-0.001
Hs.269764	BACH2	-0.014
Hs.646614	KLF8	-0.001
Hs.244940	RDH10	0.038
Hs.483238	ARHGAP29	-0.026
Hs.1407	EDN2	0.008
Hs.131933	PLBD1	-0.068
Hs.369265	IRAK3	-0.009
Hs.148741	RNF144B	0.005
Hs.525093	NDFIP2	0.003
Hs.124299	FAM167A	0.009
Hs.183114	ARHGAP28	0.002
Hs.596680	EYA4	-0.005
Hs.652230	TM7SF4	-0.001
Hs.89640	TEK	0.032
Hs.377894	GCA	-0.017
Hs.439145	SCN9A	0.02
Hs.681802	FLJ00254 protein	0.019
Hs.492974	WISP1	-0.001
Hs.122055	C7orf31	-0.006

Source: Adapted from Ren, J., Stroncek, D.F., Zhao, Y., Jin, P., Castiello, L., Civini, S., et al., 2013. Intra-subject variability in human bone marrow stromal cell (BMSC) replicative senescence: molecular changes associated with BMSC senescence. *Stem Cell Res.* 11 (3), 1060–1073. Epub 2013/08/21. doi: 10.1016/j.scr.2013.07.005. PubMed PMID: 23959330; PubMed Central PMCID: PMC3818332.

In patients with B cell lymphoma treated with CD19 CAR T cells (axicabtagene ciloleucel) the production of interferon gamma by preinfusion cells following coculture with CD19 expressing cells was not associated with clinical response of CRS (Locke et al., 2020); however, the

presence of polyfunctional T cells in the final CAR T cell product was associated with efficacy and toxicity (Rossi et al., 2018). CAR T cell products from patients with non-Hodgkin lymphoma contain polyfunctional T cell subsets capable of producing multiple types of cytokines and chemokines, such as interferon gamma, IL-17A, IL-8, and macrophage inflammatory protein 1 alpha. The presence of greater quantities of polyfunctional CAR T cells was associated with better clinical response and higher grades of CRS (Rossi et al., 2018).

One study of patients with chronic lymphocytic leukemia treated with CD19 CAR T cells found that antitumor activity is, in part, dictated by intrinsic potency of the CAR T cells. Gene expression analysis of the CD19 CAR T cells revealed that those from patients who had better clinical outcomes, CR or partial responses (PR) with transformed disease (PR_{TD}), had different transcript profiles than those from patients with less favorable clinical responses, partial response (PR) with lower levels of cell persistence or no response (NR) (Fraietta et al., 2018). The gene expression profile of CAR T cells from patients with CR and PR_{TD} were enriched in genes involved with early memory differentiation and those from patients with PR or NR were enriched in the expression of key regulators of late memory and effector T cell differentiation, apoptosis, and aerobic glycolysis (Fraietta et al., 2018).

MicroRNAs as potency assays

MicroRNAs (miRNA) are likely to be important indicators of HSC, MSC, embryonic cells, and immune cell potency. MicroRNAs are an abundant class of endogenous non-protein-coding small RNAs of 19–23 nucleotides that are derived from pre-miRNA of 60–120 nucleotides. Mature miRNAs negatively regulate gene expression at the posttranscriptional level. They reduce the levels of target transcripts as well as the amount of protein encoded; 541 human miRNAs have been identified so far. In general, miRNAs are phylogenetically conserved and therefore have conserved and defined posttranscription inhibition function. Some miRNAs are expressed throughout an organism, but most are developmentally expressed or are tissue-specific.

MicroRNAs play an important role in many cellular development and metabolic processes, including developmental timing, signal transduction, tissue differentiation, and cell maintenance. Most miRNAs are tissue specific. For example, the expression of miR-1 is restricted to the heart (Lagos-Quintana et al., 2002) and miR-223 to granulocytes and macrophages (Chen et al., 2004). Recently, miRNA have been found to have a role in stem cell self-renewal and differentiation. Several different miRNAs are

involved with the differentiation of hematopoietic progenitor cells. MiR-155 is important in preventing the differentiation of CD34⁺ cells toward myeloid and erythroid cells (Georgantas et al., 2007). In addition, miR-221 and miR-222 prevent the differentiation of HSCs into erythroid progenitors (Felli et al., 2005). MiR-181 is involved in the control of lymphopoiesis (Chen et al., 2004).

MicroRNA seems ideally suited for distinguishing primitive from committed hematopoietic, embryonic, mesenchymal, and other stem cells as well as different types of lymphocytes and mononuclear phagocytes. However, they have not been evaluated to determine whether they would be useful in this capacity. MicroRNA profiles of mononuclear phagocytes and DCs have not been studied extensively, but if miRNA profiles differ between immature and mature DCs, they may be useful in assessing the potency of DCs produced in vitro.

The high-throughput analysis of miRNAs requires at least 10 times greater quantities of cells than gene expression profiling, since miRNAs contribute only about 1% of a cell's total mRNA. MiRNA amplification methods have not yet been fully validated and hence are not considered reliable. However, targeted miRNA analysis requires a relatively small number of cells, 1×10^6 .

An advantage of miRNA expression profiling compared to gene expression profiling is that miRNA expression profiling requires smaller arrays and chips, making it possible to analyze multiple samples on the same slides containing subarrays. While gene expression cDNA microarrays contain 10,000–35,000 probes, the number of miRNA that have currently been identified is only in the hundreds.

We have shown that miRNA expression can be used to differentiate HSCs from PBMCs and different types of HSCs (Jin et al., 2008). We used miRNA and gene expression profiling to compare plerixafor-mobilized CD133⁺ cells, G-CSF-mobilized CD34⁺ cells, and peripheral blood leukocytes (PBLs). Hierarchical clustering of miRNAs separated HSCs from PBLs (Fig. 5.3). miRNAs upregulated in all HSCs included hematopoiesis-associated miRNA: miR-126, miR-10a, miR-221, and miR-17–92 cluster. MicroRNAs upregulated in PBLs included miR-142-3p, -218, -21, and -379. Hierarchical clustering analysis of miRNA expression separated the plerixafor-mobilized CD133⁺ cells from G-CSF-mobilized CD34⁺ cells. Gene expression analysis of the HSCs and PBLs also naturally segregated samples according to mobilization and isolation protocol and cell differentiation status (Jin et al., 2008).

Conclusions

As more and more new cellular therapies are being developed and used to treat an increasing variety of diseases

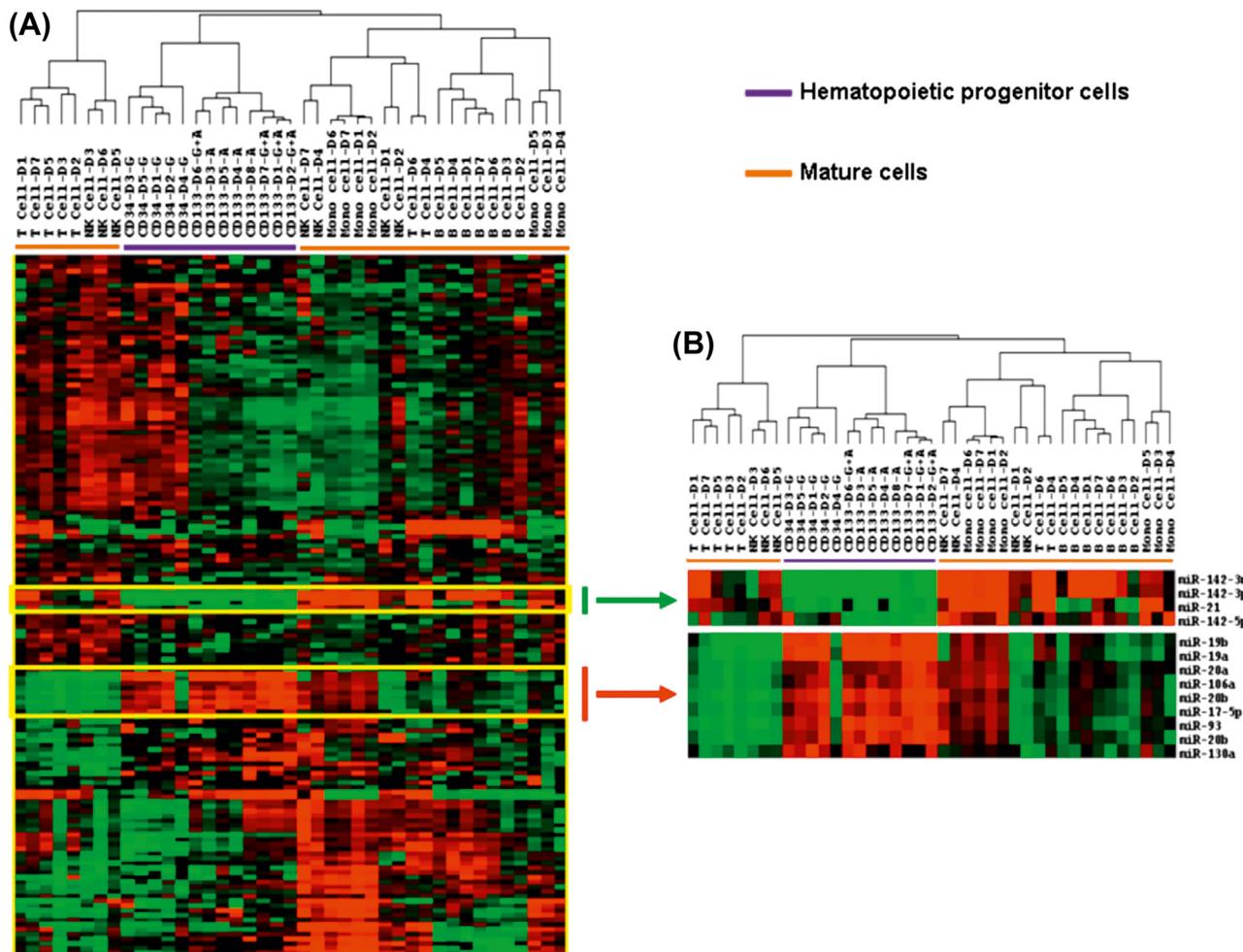


FIGURE 5.3 MicroRNA (miRNA) expression profiles of hematopoietic stem cells (HSCs) and peripheral blood leukocytes (PBLs). RNA was isolated from T cells, B cells, monocytes, and NK cells from seven subjects; G-CSF-mobilized CD34⁺ cells from five subjects; AMD3100 (A)-mobilized CD133⁺ cells from four subjects; and AMD3100 plus G-CSF (A + G)-mobilized CD133⁺ cells from four subjects. The miRNA expression was analyzed by using an expression array with 457 human miRNAs. (A) Unsupervised hierarchical clustering of Eisen was used to analyze the 148 miRNAs that remained after filtering (miRNA expressed in ≥ 80% of samples). (B) Signature miRNAs whose expression was markedly upregulated in HSCs or PBLs. Adapted from Jin, P., Wang, E., Ren, J., Childs, R., Shin, J.W., Khuu, H., et al., 2008. Differentiation of two types of mobilized peripheral blood stem cells by microRNA and cDNA expression analysis. *J. Transl. Med.* 6, 39. *Epub 2008/07/24. doi: 10.1186/1479-5876-6-39. PubMed PMID: 18647411; PubMed Central PMCID: PMC2503968.*

and patients, potency testing is becoming a critical and required part of the production of cellular therapies. Existing assays, such as function, flow cytometry, and ELISA, are important but limited by the number of factors analyzed. Gene and miRNA expression assays have the potential to become important in potency testing. They are well suited for the assessment of the potency of cellular therapies in phase I and phase II clinical trials. As data are collected during clinical trials, the results of analysis with the gene and miRNA expression microarrays should be compared with the results of traditional function assays and genes whose expression is associated with critical biological function identified and used to develop assays to rapidly measure the expression of genes associated with cell potency.

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Chapter 6

Translational pharmacogenetics: pharmacogenetically driven clinical decision making

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Abstract

In patient care, diagnostics of genetic variants may lead to improved drug safety and efficacy. Both clinicians and drug regulatory agencies are making efforts to disseminate knowledge of pharmacogenetic diagnostics and validate the benefit for patients. More than 150 drug labels have been extended with information on pharmacogenetics or other biomarkers that should be determined during the course of drug therapy. In this chapter, pharmacogenetics as a tool for improving individual drug therapy is discussed for some major diseases and drugs used to treat them.

Keywords: Pharmacogenetics; companion diagnostics; personalized therapy; antidepressants; antipsychotics; NSAIDs; PPIs; cancer drugs; cardiovascular drugs; regulation

Introduction

Despite more than 50 years of pharmacogenetic research showing many examples of the impact of inherited variability in the reaction to drugs, this knowledge is not being used in a broad way for improving individual drug treatment. In patient care, diagnostics of genetic variants may lead to improved drug safety and efficacy, both clinicians and drug regulatory agencies are making efforts to disseminate knowledge of pharmacogenetic diagnostics and validate the benefit for patients.

However, the current development of companion diagnostics in the treatment of diseases provides tremendous opportunities for personalizing therapy as, for example, in the field of cancer with more and more available tests that lead to stratified treatment with substances that act on molecular substructures. More than 150 drug labels have been extended with information on pharmacogenetics or

other biomarkers that should be determined during the course of drug therapy. Some variants are to be identified even before the onset of therapy, others to exclude contraindications, and others for safety issues as identified by the U.S. Food and Drug Administration (FDA) and by the European Medicines Agency (EMA) at this time (see PharmGKB website). The EMA and FDA have provided regulatory guidance for the inclusion of pharmacogenetic diagnostics in clinical drug development and in pharmacovigilance in a proactive strategy incorporating human diversity into clinical drug trials and further developing the clinical use of pharmacogenetic diagnostics.

Pharmacogenetics as a tool for improving individual drug therapy

Drug safety and efficacy vary considerably among patients, and few molecular or clinical factors exist that might help to better predict an individual's response to a given drug. Many patients will experience the desired drug effect, some may suffer from well-known adverse drug reactions (ADRs), others may experience no effects, and very rarely a patient will die from severe side effects. It is currently difficult for physicians to prescribe the optimal drug in the optimal dose for each patient because prediction of a patient's response to any one specific drug is rarely possible. The absolute number of deaths per year related to adverse drug effects or ADRs is estimated to be around 100,000 in the United States, and estimations from population-based studies show that ADRs account for approximately 3% of all deaths in the general population ([Shepherd et al., 2012](#); [Wester et al., 2008](#)). For instance, according to several studies, the incidence of adverse

drug effects in hospitalized patients is about 6%–10%, and 0.4%–4% of these cases end with death (Schurig et al., 2018; Just et al., 2020; Pirmohamed et al., 2004). Adverse drug effects are the fifth leading cause of death in the United States, directly after coronary heart disease, cancer, stroke, and lung diseases and before accidents, diabetes mellitus, and pneumonia (Spear et al., 2001). ADR-related costs are estimated to be between \$1.4 and \$4 billion per year in the United States (Lazarou et al., 1998). For Europe, similar data have been published (Schneeweiss et al., 2002). One study investigated the burden of ADR through a prospective analysis of 18,820 patients aged more than 16 years admitted during a 6-month period to two large general hospitals in Merseyside, United Kingdom (Pirmohamed et al., 2004). The study found that ADR was prevalent in 6.5% of cases and that the ADR directly led to admission in 80% of cases. The overall fatality rate was 0.15%.

To understand pharmacogenetic mechanisms and why individual patients respond differently to drugs, it is helpful to envisage the progress of a particular drug from its administration to its observed effect. The effect of a drug will depend first on its systemic concentration and its concentration at the drug target. The systemic concentration of a drug depends on several pharmacokinetic factors, commonly referred to by the acronym ADME (drug absorption, distribution, metabolism, and elimination). It has long been known that pharmacogenetic factors may influence the pharmacokinetics of a drug; therefore a doctor might take this into account when determining dosage for a given patient. In many cases, drug concentration at its target will represent a mere function of the systemic concentration. Active transport processes, however, may influence local target concentrations; for example, the blood-brain barrier is an important determinant of drug concentration in the cerebrospinal fluid. It has become increasingly clear that hereditary variances in drug-metabolizing enzymes (DME) and drug transporters can exert considerable influence on drug concentrations and drug exposure.

The second crucial determinant of an observed drug effect is the response of the drug target to a given drug concentration. Differences in activity or gene expression sometimes caused by hereditary variants have been discovered and characterized in receptors, drug transporters, ion channels, lipoproteins, coagulation factors, and many other factors involved in immune response, cell development, cell cycle control, and other functions; these differences significantly influence the manifestation and course of diseases. At the same time many of these polymorphic structures are targets of specific drugs and can thus potentially influence the effect that a specific drug concentration will exert at the drug target site.

Types of drug therapies that might profit from pharmacogenetic diagnostics

Not all drug therapies are well suited for genotype-based optimization concepts. One problem is that results from genetic testing are typically generated during the first or second day after blood sampling. Thus drug therapies in which quick decisions are demanded probably will not profit from genotype-based dose adjustments. Further, drug therapies with a large therapeutic range do not justify the effort and costs required for pretherapeutic genotyping. In contrast, drug therapies for which the dosage has to be carefully chosen and slowly increased are suited for individual optimization by means such as genotyping. For example, treatment of heart failure with beta blockers demands careful dosing at low dosages; thus more in-depth knowledge of genetically caused differences in drug metabolism would be of great interest. The use of beta blockers for treatment of hypertension, in contrast, can be monitored by measuring the blood pressure and therefore probably will not further profit from genetic diagnostics.

Indications for genotyping in patient care are emerging and being used more and more, starting in the field of oncology but also spreading out to other conditions in general medicine. For some drug therapies, when certain genotypes lead to an altered risk of a severe ADR or when indication for a drug depends on a certain genotype, a direct improvement of efficacy can be expected, or pharmacogenetic diagnostics has to be performed for individualizing dosage before the start of drug therapy. For example, this is the case for fluoropyrimidine drugs such as 5-fluorouracil and capecitabine, for which the degrading enzyme dihydropyrimidine dehydrogenase (DPD) has to be genotyped before onset of therapy to avoid severe side effects according to the EMA, 2020 (<http://www.ema.europa.eu/>). Dose adjustments based on pharmacogenetic testing are also recommended for antidepressant drug therapy with tricyclic antidepressants (which is largely affected by the CYP2D6 polymorphism), anticoagulant drug therapy, and polymorphism of CYP2C9, as well as codeine treatment and CYP2D6.

In anticoagulant drug treatment, bleeding complications occur mostly at the beginning of treatment, so genotyping for CYP2C9 polymorphisms will make the most sense before or during the start of therapy. In other drug therapies, genotyping might be used as a further diagnostic tool for explaining therapeutic failure or adverse drug effects or for explaining abnormally high or low plasma levels during therapeutic drug monitoring. This is the case, for example, in general psychiatric drug treatment. Because many antidepressant and antipsychotic drugs are metabolized by the polymorphic enzyme CYP2D6, genotyping of this enzyme is a common diagnostic tool after adverse drug effects have occurred or after therapeutic failure.

Thus pharmacogenetic implementation guidelines also comprise tools for deciding at which time point of which drug therapies genetic tests might be useful.

The status of translational pharmacogenetics in various drug therapy fields

Depression

The pharmacotherapy of depression, a major psychiatric disorder, is characterized by long-running drug therapy, a relatively narrow therapeutic index, and poor predictability of individual response. About 30% of all depression patients do not respond sufficiently to the first antidepressant drug that is given to them (Bauer et al., 2002). Failure to respond to antidepressant drug therapy and intolerable side effects not only result in personal suffering for individuals and their families but also impose considerable costs on society. At present, it is not possible to reliably predict an individual's response probability before onset of a drug treatment.

Many drugs used in psychiatry, such as antidepressants, antipsychotics, and mood stabilizers, are extensively metabolized by polymorphic DME, with CYP2D6 being involved in the metabolism of approximately half of the commonly prescribed psychotropic drugs (Mulder et al., 2007). Since differences in plasma concentration due to individual variability in drug clearance often vary by 10-fold and even higher, the possibility of rationally justified pharmacogenetic dose adjustments is given in reflection of pharmacokinetic data such as oral drug clearance (Hicks et al., 2013; Swen et al., 2011; Stingl et al., 2013).

Inadequate drug exposure may constitute a risk due to nonresponse or toxicity depending on the therapeutic range of the drug. In some cases, imprecise dosing may have little or no clinical consequences because of a wide therapeutic range. In antidepressant drug treatment, poor metabolizers (PM) for CYP2D6 have been associated with longer time requirements to find the appropriate drug and with more frequent drug switches (Bijl et al., 2008). There are also hints that PM suffer more frequently from ADRs than extensive metabolizers (EM) and register longer hospital stays, whereas ultrarapid metabolizers (UM) have a higher risk of therapeutic failure (Bijl et al., 2008; Chou et al., 2000; Ruano et al., 2013).

The pharmacokinetic-based dose adjustment approach is now being followed by issuing clinical recommendations for specific drug-genotype pairs (Clinical Pharmacogenetics Implementation Consortium, CPIC) (Hicks et al., 2013; Relling and Klein, 2011). Such evidence-based dosing guidelines are now available for 27 drugs and made accessible in the PharmGKB database (Caudle et al., 2014). Of all genes involved in these evaluations, CYP2C19 and

CYP2D6 have been shown to be the most important DME in dose adjustments of psychotropic drugs.

Individuals to whom tricyclic antidepressants are prescribed could benefit from CYP2D6 genotyping if the dose is adjusted for the group of PM and UM of CYP2D6. Within the group of selective serotonin reuptake inhibitors (SSRI), cytochrome (CYP) inhibition poses a problem for drug interaction, and there is growing evidence that the cytochrome P450 2C19 polymorphisms influences exposure and safety of SSRI therapy (Bråten et al., 2020; Jukic et al., 2018).

For mirtazapine it was shown that the CYP2D6 genotype had a significant influence on the variability in the plasma concentration; however, when UM was compared to EM, the magnitude of concentration differences was only moderate (Kirchheimer et al., 2004b).

CYP2D6 is responsible for the transformation of venlafaxine into the equipotent O-desmethyl-venlafaxine (Fukuda et al., 2000, 1999; Otton et al., 1996; Veefkind et al., 2000). However, a higher risk for cardiotoxic events might exist in PM, as cases of severe arrhythmia have been reported in four patients treated with venlafaxine who all were PM according to CYP2D6 (Garcia et al., 2017; Castanares-Zapatero et al., 2016).

As has been shown in several studies, differences in pharmacokinetic parameters caused by genetic polymorphisms affect the outcome and the risk of ADRs of antidepressants and antipsychotics. The effect of the CYP2D6 genotype on adverse drug effects and nonresponse during treatment with CYP2D6-dependent antidepressants, has been shown in several studies (Bråten et al., 2020; Jukic et al., 2018, 2019; Milosavljevic et al., 2020). Recent studies on TDM in patients with different genotypes indicate the influence of CYP2D6 and CYP2C19 variants on adherence and therapy failure related to drug exposure (Bråten et al., 2020; Jukic et al., 2018, 2019; Milosavljevic et al., 2020). Fig. 6.1 illustrates the differences in mean oral clearance among carriers of none, one, two, and more active CYP2D6 genes as percentages of the dose adjustments for antidepressants.

In conclusion, for treatment with antidepressants there is considerable evidence that CYP2D6 and, to a lesser extent, CYP2C19 polymorphisms affect the pharmacokinetics of several antidepressants and antipsychotics and possibly the therapeutic outcome and adverse drug effects. However, the usefulness of genotyping procedures in depressed patients has not been confirmed in prospective clinical trials; therefore translational pharmacogenetic diagnostics is limited to a few hospitals and to patients who experienced adverse drug effects or did not respond to standard treatment regimens.

Cardiovascular disease

Cardiovascular diseases are the most common cause of morbidity and mortality in developed countries. The following discussion addresses the clinical implications

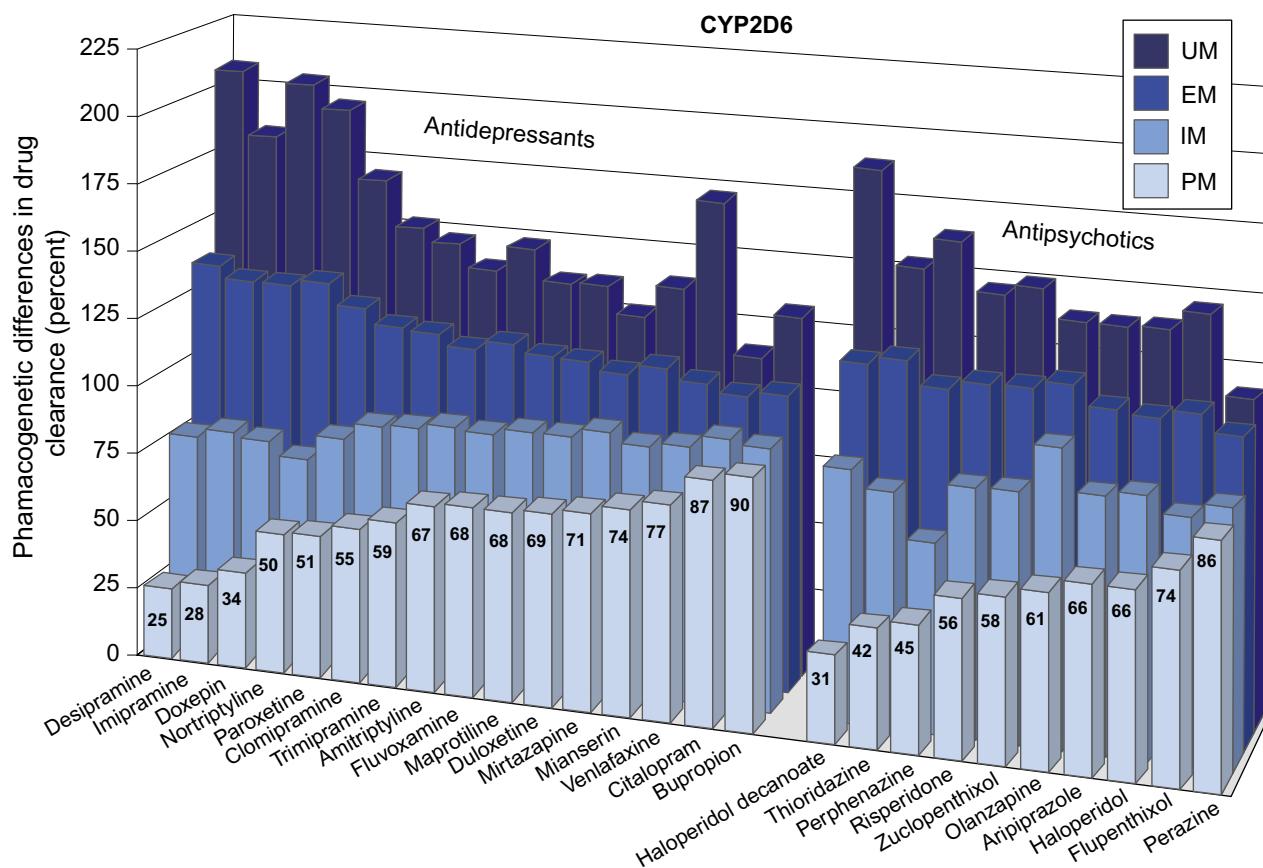


FIGURE 6.1 CYP2D6 PM (light blue), IM (medium blue), extensive metabolizers (blue), and ultrarapid metabolizers (dark blue). Dose adaptations were calculated as described by [Stingl et al. \(2013\)](#). Dose adaptations are based on an average dose of 100% and are relevant to the Caucasian population. Data from studies in Asiatic, African, or other populations were not used in this graph. IM, intermediate metabolizers; PM, poor metabolizers. With permission taken from [Stingl J.C., Brockmoller J., Viviani R., 2013. Genetic variability of drug-metabolizing enzymes: the dual impact on psychiatric therapy and regulation of brain function. Mol. Psychiatry 18 273–287. CYP2D6 genotype-dependent quantitative changes in pharmacokinetics of antidepressant and antipsychotic drugs expressed as percent dose adaptations.](#)

resulting from pharmacogenetic research for some of the drugs used in the therapy of cardiovascular diseases, such as statins, oral anticoagulants, beta blockers, and sartans.

Beta blockers

For metoprolol, one of the most often-prescribed beta blockers, the role of CYP2D6 genetic polymorphisms in its pharmacokinetics seems to be well established. CYP2D6 catalyzes O-demethylation and, even more specifically, α -hydroxylation of the drug ([Lennard et al., 1982a](#)). Not only metoprolol plasma concentrations but also effects on heart rate correlated significantly with the CYP2D6 metabolic phenotype ([Lennard et al., 1982b](#)). In a study with 281 individuals, heart rate response differed significantly by CYP2D6 phenotype with PM and intermediate metabolizers (IM) showing greater reduction ([Hamadeh et al., 2014](#)). In UM carrying the CYP2D6 gene duplication, total clearance of metoprolol was about 100% higher compared with the EM as the reference group (367 L/h vs 168 L/h)

([Kirchheimer et al., 2004a](#)). The reduction of exercise-induced heart rate by metoprolol in the UM group was only about half of that observed in the EM.

On the basis of the considerable impact of the CYP2D6 polymorphism on the disposition of CYP2D6 substrates, it has often been suggested that CYP2D6 PM are more susceptible to adverse effects than EM at standard doses of metoprolol ([Rau et al., 2002; Wuttk et al., 2002; Zineh et al., 2004](#)), which is the same finding as for antidepressive and antipsychotic drugs. For instance, the CYP2D6 PM genotype was overrepresented among 24 patients with severe metoprolol-associated adverse effects in a retrospective study ([Wuttk et al., 2002](#)). However, metoprolol is well tolerated in the majority of patients with cardiovascular diseases irrespective of CYP2D6 genotype ([Hamadeh et al., 2014](#)). The effect of beta blockers on the quality of life of hypertensive patients has not been extensively studied. The wide therapeutic range of metoprolol may explain why it is well tolerated in the majority of CYP2D6 PM and IM, despite several-fold higher plasma concentrations.

Another beta blocker, the racemate carvedilol, which has been approved as an adjunctive therapy in the treatment of heart failure, is known to be stereoselectively metabolized by cytochrome P450 enzymes (Neugebauer and Neubert, 1991). CYP2D6 polymorphism has been shown to alter the stereoselective disposition of carvedilol with PM demonstrating an impaired clearance of the R-enantiomer; thus the PM were affected by a more pronounced α 1-blockade, which might outweigh the beneficial β 1-blocking effects (Zhou and Wood, 1995). Hence CYP2D6 genotypes were related to drug exposure and therapeutic outcomes in patients treated with carvedilol (Luzum et al., 2017; Ingram and Valente, 2020; Jung et al., 2018). Inhibition of CYP2D6 metabolism by administration of fluoxetine in EM led to significant changes in plasma pharmacokinetics in favor of the R-enantiomer, without any effect on blood pressure and heart rate, which casts doubt on the clinical significance of the CYP2D6 genotype for treatment with carvedilol in antihypertensive therapy (Graff et al., 2001).

In summary, CYP2D6 genotyping might be beneficial, if at all, for long-term treatment with metoprolol in indications such as heart failure or in postmyocardial infarction patients when no surrogate parameter such as blood pressure is available to predict long-term efficacy. However, pharmacogenetic diagnostics is not generally done in patients using beta blockers, and at present it does not seem likely to become a general tool for therapeutic improvement.

Vitamin K antagonists

The S-enantiomers of all three vitamin K antagonists—acenocoumarol, phenprocoumon, and warfarin—are substrates of CYP2C9. Warfarin is mainly prescribed in North America and Asia, whereas acenocoumarol and phenprocoumon are more commonly used in Europe. Both CYP2C9 alleles—CYP2C9*2 and *3—have a substantial effect on the intrinsic clearance of (S)-warfarin (Loebstein et al., 2001; Scordo et al., 2002; Takahashi et al., 1998). For acenocoumarol, heterozygous carriers of CYP2C9*3 had only 40% of the clearance measured in CYP2C9*1/*1 carriers, whereas differences in the international normalized ratio (INR) between these two groups resulted in a mean daily dose that was 70% that of wild-type patients in *1/*3 carriers (Tassies et al., 2002; Thijssen et al., 2001; Thijssen and Ritzen, 2003; Verstuyft et al., 2001; Visser et al., 2004a). Many clinical studies reported a higher risk of adverse drug effects, especially bleeding complications in patients at the beginning of treatment with oral anticoagulants (Visser et al., 2004b).

The vitamin K antagonistic effects of the coumarin derivatives are mediated by inhibition of the vitamin K epoxide reductase (Rost et al., 2004). The dosage of

vitamin K antagonists required to achieve the target-level anticoagulation depends largely on the activity of the vitamin K epoxide reductase (VKOR). About 25% of variability in warfarin dose was shown to be explained by the VKORC1 haplotypes, with one single functional single nucleotide polymorphism (SNP) being responsible for the differences in gene expression (Geisen et al., 2005; Rieder et al., 2005). The mean daily dose requirement of phenprocoumon differs largely in the dependence of the G-1639A polymorphism of VKORC1 with GG carriers, requiring more than twice the mean dose compared to AA carriers (Reitsma et al., 2005). In correspondence with the lower dose requirement in AA carriers, those individuals had a higher risk of bleeding even when the INR values were stable. For acenocoumarol it has been shown that the 1639AA genotype is the greatest risk factor for over-anticoagulation, with an odds ratio of 10.5 (3.3–34.1), and that it predisposes to larger INR variations (Osman et al., 2006; Quteineh et al., 2005).

A lot of discussion worldwide on the implementation of pharmacogenetic diagnostics to guide anticoagulant drug treatment is ongoing, and guidelines exist both recommending and not recommending the incorporation of genotype information into therapy, mostly for warfarin (Johnson et al., 2011). Conflicting results of recent large clinical trials keep the discussion on the true clinical utility of genotyping ongoing. The European Pharmacogenetics of Anticoagulant Therapy (EU-PACT) study reported that the time to receive therapeutic range during the initial weeks of anticoagulation was better in the genotype dose-adjusted study arm than in the comparison group during warfarin initiation (Pirmohamed et al., 2013). The Clarification of Optimal Anticoagulation through Genetics trial (COAG), which also was a multicenter randomized trial evaluating the effect of genotype-guided dosing on time within therapeutic range, did not detect a difference between the pharmacogenetic arm and the clinical algorithm guided arm (Kimmel et al., 2013).

The FDA has recommended genotyping for CYP2C9 and VKORC1 prior to onset of the anticoagulant therapy in addition to dosing according to INR, in the drug labeling of warfarin, rendering the vitamin K antagonists one of the first drugs in which pharmacogenetic factors have been completely translated into drug therapy.

Statins and proton pump inhibitors

Statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) are generally regarded to be safe and have become the most commonly prescribed agents for the treatment of dyslipidemia to reduce cholesterol and cardiovascular events. The most common side effect (occurring in 1%–5% of exposed subjects) is skeletal myopathy (Buettner et al., 2012). Statin-related myopathy includes weak symptoms of myalgia up to severe muscle damage and kidney

injury (rhabdomyolysis). Patient risk factors for statin-induced myopathy include high statin exposure, drug-drug interactions, high statin dose, female sex and low body mass index, intense physical exercise, endocrine abnormalities such as hypothyroidism, and ethnicity.

The transporter OATP1B1 (encoded by the SLCO1B1 gene) mediates the hepatic uptake of statins; therefore the activity of the transporter influences bioavailability of statins to a large degree. Interaction studies with cyclosporine, a strong inhibitor of CYP3A4 and OATP1B1, have shown threefold to eightfold increases of the area under the curve (AUC) for simvastatin acid, thereby markedly increasing the risk for statin-related muscle damage (Niemi et al., 2011).

SLCO1B1 polymorphisms clearly affect the pharmacokinetics of simvastatin and, to a lesser degree, the pharmacokinetics of other statins (Niemi, 2010). For simvastatin the evidence linking myopathy to rs4149056 in SLCO1B1 is strongest with data from randomized trials and clinical practice-based cohorts. Because the association of rs4149056 with myopathy is less compelling for other statins, the CPIC guideline focuses on recommendations for simvastatin (Wilke et al., 2012). At lower simvastatin doses (e.g., 40 mg daily), a modest risk for myopathy in carriers of the C allele of rs4149056 is expected, with a relative risk of myopathy of 2.6 per copy of the C allele at rs4149056. Thus the utility of routine CK surveillance may be recommended to clinicians. If lower doses of simvastatin are not sufficient, physicians may consider an alternative statin based on (1) potency differences, (2) kinetic differences, (3) comedications, (4) hepatic function, (5) renal function, and (6) relevant comorbidities (Wilke et al., 2012). However, a recent study on screening for pharmacogenetic risk variants in SLCO1B1 in patients showed that fear for myopathy in carriers of variants may lead to undertreatment, resulting in higher rate of cardiovascular events in the genotype group. Thus genotyping before treatment is not recommended as a conclusion of that study (Vassy et al., 2020).

The purpose of the CPIC guideline for simvastatin was to provide interpretive guidance when the SLCO1B1 genotype was already available. Thus it is not argued directly in this guideline that SLCO1B1 genotyping is absolutely necessary. However, guidance is provided for clinicians treating patients with simvastatin in the event that SLCO1B1 genotype is available during routine care (Wilke et al., 2012).

Proton pump inhibitors (PPI), such as omeprazole, esomeprazole, lansoprazole, pantoprazole, or rabeprazole, are commonly prescribed in combination with antibiotics for *Helicobacter pylori* eradication and for patients with peptic ulcer as well as gastroduodenal reflux disease. These drugs undergo extensive presystemic biotransformation in the liver with the involvement of genetically polymorphic CYP2C19 (Klotz et al., 2004; Schwab et al., 2004). CYP2C19*2 and CYP2C19*3 are the most common

nonfunctional alleles; they are responsible for the majority of PM phenotypes of CYP2C19 (de Morais et al., 1994).

Andersson and colleagues observed that in PM of mephenytoin (a model substrate of CYP2C19), the AUC for omeprazole, lansoprazole, and pantoprazole at a steady state was fivefold higher compared with EM, indicating that approximately 80% of the dose for all three PPI is metabolized by CYP2C19 (Andersson et al., 1998). For different PPI, drug exposures, which were defined with AUC values, were 3–13 times higher in PM and about 2–4 times higher in heterozygous EM as compared to homozygous EM of CYP2C19 (Klotz et al., 2004). At the same time, the elevation of intragastric pH as a pharmacodynamic response to PPI can be shown to be directly related to the respective AUC, and a much higher pH can be monitored for 24 hours following the administration of PPIs in PM than in EM (Klotz et al., 2004).

In a systematic review of pharmacogenetic studies with PPI, Chong and Ensom evaluated the effects of CYP2C19 genetic polymorphism on the clinical outcomes, that is, the effects of the *H. pylori* eradication rates on therapy with these drugs (Chong et al., 2003). The results of most studies supported the hypothesis that eradication rates vary with CYP2C19 genotype and PMs have a significantly better PPI efficacy.

Furuta and colleagues showed that the eradication rates of *H. pylori* on the triple therapy comprising PPI, clarithromycin, and amoxicillin was 72.7%, 92.1%, and 97.8% in CYP2C19 EM, IM, and PM, respectively. The authors concluded that the CYP2C19 genotype seems to be one of the important factors associated with the cure rates of *H. pylori* infections on the PPI-based therapy (Furuta et al., 2001). The same authors conducted a prospective clinical study showing in a randomized prospective way that CYP2C19 genotyping provides a cost-effective benefit in *H. pylori* eradication treatment (Furuta et al., 2007).

The role of CYP2C19 polymorphism in the eradication of *H. pylori* was also studied in the Caucasian population. However, in contrast to the Asian population, the prevalence of PM of CYP2C19 in Caucasians is much lower: about 2.8% in Caucasians compared to 21.3% in Japanese (Wedlund, 2000). We can show significant differences in the efficacy of lansoprazole-based quadruple therapy among Caucasian patients carrying wild-type alleles, one CYP2C19 defect allele, and two CYP2C19 defect alleles, with cure rates of 80.2%, 97.8%, and 100%, respectively (Schwab et al., 2004).

Treatment of gastroesophageal reflux disease (GERD) demands long-term application of PPI. Two retrospective analyses in Japanese patients showed that the healing rate of GERD using lansoprazole was 20%–40% higher in PM or IM compared to EM of CYP2C19 and that PM even benefit in the prevention of relapse of GERD (Furuta et al., 2002; Kawamura et al., 2007; Kawamura et al., 2003).

In summary, according to the results of numerous clinical studies, CYP2C19 polymorphisms are an important factor affecting the pharmacokinetics of most PPI, the value of intragastric pH, and the eradication rates of *H. pylori*. Therefore genotyping for CYP2C19 polymorphisms, especially in Asian populations, characterized by a high prevalence of defect CYP2C19 alleles, is recommended in the recent guideline on CYP2C19 and PPI dosing (Lima et al., 2020). Higher dosages for EM and/or adjusted treatment regimens are recommended in this guideline evaluating all data from clinical trials.

Pain treatment

One large field in which the CYP2C9 polymorphism plays a role is the treatment of pain and inflammation with nonsteroidal antiinflammatory drugs (NSAIDs). At least 16 different registered NSAIDs are currently known to be at least partially metabolized via CYP2C9. They include aceclofenac, acetylsalicylic acid, azapropazone, celecoxib, diclofenac, flurbiprofen, ibuprofen, indomethacin, lornoxicam, mefenamic acid, meloxicam, naproxen, phenylbutazone, piroxicam, suprofen, and tenoxicam (Rendic, 2002). There were significant intergenotypic differences in the pharmacokinetics of celecoxib, flurbiprofen, ibuprofen, and tenoxicam that could be translated into dose recommendations based on CYP2C9 genotype. Celecoxib was one of the first drugs for which the manufacturers' drug information recommends caution in administering celecoxib to "poor metabolizers of CYP2C9 substrates," as they might have abnormally high plasma levels (Celebrex drug information, Searle Pfizer, Chicago, United States). In contrast, the relative contribution of CYP2C9 to the pharmacokinetics of diclofenac was found to be independent of CYP2C9 polymorphisms in several clinical studies (Brenner et al., 2003; Dorado et al., 2003; Kirchheimer et al., 2003; Yasar et al., 2001). Recently, a systematic metaanalysis by CPIC resulted in a dosing guideline for NSAID dependent on the CYP2C9 genotype (Theken et al., 2020) recommending 25%–50% reduced dosages for poor metabolizer compared to normal.

Codeine is converted to morphine via CYP2D6. After several cases of severe or fatal intoxication with codeine in children, the FDA and the EMA restricted the use of codeine and issued a warning about the issue that variable drug metabolism capacity may lead to dangerous intoxication with morphine. According to the EMA, codeine is now contraindicated in all pediatric patients (0–18 years of age) who undergo tonsillectomy or adenoidectomy for obstructive sleep apnea syndrome and in patients of any age who are known to be CYP2D6 UM (up to approximately 10% of Caucasians are CYP2D6 UM, but prevalence differs according to racial and ethnic group), owing to an increased risk of developing serious and life-threatening adverse reactions,

and in women who are breastfeeding (Tobias et al., 2016; Crews et al., 2014) (<http://www.ema.europa.eu/>).

Malignant diseases

Most anticancer drugs are characterized by a very narrow therapeutic index and severe consequences of overdosing or underdosing in the form of life-threatening ADRs or treatment failure, respectively.

Tamoxifen

The clinical benefit of the antiestrogen tamoxifen for the treatment of estrogen receptor (ER) positive breast cancer has been evident for more than three decades, but 30%–50% of patients undergoing adjuvant tamoxifen therapy relapse or die. There are several lines of evidence that suggest that the metabolites 4-hydroxy tamoxifen (4-OH-TAM) and 4-hydroxy-N-desmethyl tamoxifen (endoxifen) are the active therapeutic moieties because, compared to the parent drug, these two metabolites have up to 100-fold higher potency in terms of binding to ER (Destra et al., 2004). CYP2D6 is one of the key enzymes for the formation of 4-OH-TAM and endoxifen (Coller et al., 2002), and CYP2D6 PM have been shown to have very low endoxifen plasma levels (Borges et al., 2006) and nonfavorable clinical outcomes (Goetz et al., 2007). It is of note that between 15% and 20% of European patient populations carry genetic CYP2D6 variants associated with a pronounced impairment in the formation of antiestrogenic tamoxifen metabolites. Tamoxifen-treated patients carrying the CYP2D6 alleles *4, *5, *10, and *41 associated with impaired formation of antiestrogenic metabolites had significantly more recurrences, shorter relapse-free periods (HR = 2.24; 95% CI, 1.16–4.33; $p = 0.02$), and lower rates of event-free survival (HR = 1.89; 95% CI, 1.10–3.25; $p = 0.02$) compared to carriers of functional alleles. Moreover, in 2006 Sim and colleagues reported the first genetic evidence for the CYP2C19*17 polymorphism as a putative supplementary biomarker for the classification of patients with favorable treatment outcome (Sim et al., 2006). Patients with the CYP2C19 high enzyme activity promoter variant *17 had a more favorable clinical outcome (HR = 0.45; 95% CI, 0.21–0.92; $p = 0.03$) than carriers of *1, *2, and *3 alleles. CYP2C19 contributes to tamoxifen metabolism toward the antiestrogenic metabolite 4-OH-TAM with in vitro activities similar to CYP2D6 (Coller et al., 2002; Destra et al., 2004). These studies support the feasibility of treatment outcome prediction for tamoxifen based on the patients' genetic constitution.

Altogether these findings are particularly important in light of the current debate on the effectiveness of tamoxifen for postmenopausal women with hormone receptor positive breast cancer. Because the definition of patient groups with nonfavorable and favorable tamoxifen treatment appears to be feasible

through a priori genetic assessment and given the long-term use of tamoxifen as a safe and effective antihormonal treatment, it is possible to use the results of the pharmacogenetic evidence to refine the choice (Brauch et al., 2013). The Royal Dutch Pharmacists Association—Pharmacogenetics Working Group has issued therapeutic recommendations for tamoxifen based on CYP2D6 genotypes (Swen et al., 2011). For PM and IM genotypes they recommend considering using aromatase inhibitors for postmenopausal women, owing to increased risk for relapse of breast cancer with tamoxifen. For IM genotypes they recommend avoiding concomitant CYP2D6 inhibitor use and/or sequencing of hormonal therapy in prospective clinical trials (Swen et al., 2011).

Thiopurines

Thiopurines, such as 6-mercaptopurine and thioguanine, are used largely in the treatment of acute leukemias, whereas autoimmune diseases and chronic inflammatory bowel diseases are common fields of application for azathioprine (Teml et al., 2007). The cytosolic enzymes thiopurine S-methyltransferase (TPMT) and xanthin oxidase (XO) are the predominant catabolic enzymes in the metabolism of thiopurines. TPMT-dependent methylation is critical in white blood cells, leading to an enhanced cytotoxic effect in patients with low TPMT activity. In 1980, Weinshilboum and Sladek first reported on a trimodal frequency distribution of TPMT activity with about 10% IM and 1 out of 300 homozygous for the trait with very low or undetectable TPMT activity (Weinshilboum and Sladek, 1980). To date, 24 mutant alleles responsible for variation in TPMT enzyme activity have been described, and most of these alleles are characterized by one or more nonsynonymous SNP, resulting in decrease or loss of enzyme activity (Schaeffeler et al., 2006). In several independent studies, the TPMT genotype showed excellent concordance with the TPMT phenotype; the most comprehensive analysis, conducted by Schaeffeler and colleagues, included 1214 healthy Caucasian blood donors (Schaeffeler et al., 2004). The overall concordance rate between the TPMT phenotype and genotype was 98.4%, and specificity, sensitivity, and the positive and negative predictive power of the genotyping test were estimated to be higher than 90%. In addition, patients with TPMT deficiency who are treated with standard doses of AZA or 6-MP are at high risk of developing severe myelosuppression, independent of the underlying disease (e.g., childhood leukemia or inflammatory bowel disease), within a few weeks after commencing drug therapy (Evans et al., 1991; Sebbag et al., 2000). This can be explained by an 8- to 15-fold increase in 6-thioguanine nucleotide (6-TGN) levels in red blood cells compared to wild-type patients, subsequently leading to an exaggerated cytotoxic effect. Moreover, an increased risk of

hematotoxicity in TPMT heterozygous subjects receiving standard thiopurine therapy is also well known from patients with childhood acute lymphocytic leukemia (ALL) and rheumatologic diseases (Black et al., 1998; Relling et al., 1999). Thus dose adjustment, at least in TPMT-deficient patients, is required, with an initial dose reduction to 10%–15% of the standard dose as exemplarily shown for patients with ALL and Crohn's disease (Evans et al., 1991; Kaskas et al., 2003). Moreover, it was also demonstrated that the TPMT phenotype or genotype influences the effectiveness of therapy. Low TPMT activity has been associated with higher 6-TGN levels and improved survival, while high TPMT activity has been associated with lower 6-TGN concentrations and an increased relapse risk (Lennard et al., 1990). The German ALL BFM Study Group recently reported on the association of the TPMT genotype and minimal residual disease (MRD) in 810 children with childhood ALL (Stanulla et al., 2005), indicating a significant impact of the TPMT genotype on MRD after administration of 6-MP in the early course of childhood ALL. This may offer a rationale for genotype-based adaptation of 6-MP dosing in the early course of childhood ALL, provided that the described observations translate into improved long-term outcomes.

In the CPIC guidelines for Thiopurine Methyltransferase Genotype and Thiopurine Dosing: 2013 Update, the literature published between June 2010 and November 2012 was reviewed, and no new evidence that would change the original guidelines was identified (Relling et al., 2013). Therefore the previously issued dosing recommendations remain clinically relevant. For patients with one nonfunctional TPMT allele, it is recommended to start with reduced doses of thioguanine; for patients with malignancy and two nonfunctional alleles, it is recommended to adjust the dose on the basis of the degree of myelosuppression and disease-specific guidelines. In addition, it is recommended to consider alternative nonthiopurine immunosuppressant therapy for patients with nonmalignant conditions and two nonfunctional alleles (Relling et al., 2011).

For 6-MP the FDA has included in the product label respective pharmacogenetic data considering the impact of the polymorphic TPMT on severe toxicity as well as the availability of genotypic and phenotypic testing (Maitland et al., 2006).

5-Fluorouracil

Following administration, 5-FU undergoes complex anabolic and catabolic biotransformations that play an eminent role for both antitumor activity and toxicity. Although the effectiveness of 5-FU depends on its bioactivation, resulting in 5-fluoronucleotides that interfere with normal DNA and RNA function preferentially in cancer cells, more than 80% of a given dose is rapidly metabolized by DPD. This rate-limiting

enzyme of pyrimidine catabolism converts 5-FU to the inactive 5-fluoro-5,6-dihydrouracil (Heggie et al., 1987). Diasio and colleagues were the first to suggest the potential role of DPD as a factor in determining 5-FU toxicity; they showed a complete deficiency of DPD enzyme activity in a patient with the rare familial metabolic disorder pyrimidinemia who had developed severe neurotoxicity during 5-FU treatment (Diasio et al., 1988). Later studies also showed a correlation between lymphocyte DPD activity and 5-FU clearance (Etienne et al., 1994; Soong and Diasio, 2005).

Other polymorphic candidate genes considered as potential factors for 5-FU toxicity include thymidylate synthase (TYMS), which is strongly inhibited by 5-FU, as well as methylenetetrahydrofolate reductase (MTHFR), which forms the reduced folate cofactor essential for TYMS inhibition (Robien et al., 2005).

The CPIC Dosing Guideline for fluoropyrimidines (5-fluorouracil, capecitabine, or tegafur) recommends an alternative drug for patients who are homozygous for DPYD nonfunctional variants—*2A (rs3918290), *13 (rs55886062), and rs67376798A (on the positive chromosomal strand)—as these patients are typically DPD deficient (Caudle et al., 2013). Recently, genotyping for DPD deficiency was introduced into drug labels as mandatory by the EMA prior to drug therapy with fluoropyrimidines (<http://www.ema.europa.eu/>). Patients with a decrease or deficiency in DPD activity screened either by genotyping or by phenotyping of uracil in plasma should be either not put on drug therapy with 5-FU or dosed individually to prevent fluoropyrimidine-related toxicities (Knikman et al., 2020).

Irinotecan

Recently, much attention has been paid to the importance of pharmacogenetic polymorphisms in uridine diphosphate glucuronosyltransferase (UGT) and its role in toxicity and therapeutic response in cancer patients undergoing treatment with the potent antitumor agent irinotecan. SN-38, the active metabolite of irinotecan acting as topoisomerase I inhibitor, is glucuronidated to its inactive form by UGT1A1, the UGT isoform, which is also responsible for the glucuronidation of bilirubin. Several variant alleles leading to reduced enzymatic UGT1A1 activity have been identified in the UGT1A1 gene. These polymorphisms become manifest as unconjugated hyperbilirubinemias in the form of the Crigler-Najjar or Gilbert's syndromes; in addition, in patients treated with irinotecan, they result in reduced SN-38 glucuronidation rates (Van Kuilenburg et al., 2000). For UGT1A1 genetic variations, major interethnic differences have been shown, whereas the UGT1A1*28 promoter polymorphism (TA repeat in the promoter) is quite common in the Caucasian population, UGT1A1*6 and UGT1A1*27 situated in the coding region of UGT1A1 are found mainly in Asians (Sai et al., 2004).

The impact of UGT1A1 polymorphisms on irinotecan toxicity, which is characterized by severe diarrhea and neutropenia, has been studied in several retrospective as well as prospective studies. It can be shown that the presence of common UGT1A1 mutant alleles, even in heterozygous carriers, significantly changed the disposition of irinotecan, causing severe toxicity in patients (Araki et al., 2006; Innocenti et al., 2001; Sai et al., 2004). Finally, given the cumulative evidence, the FDA has approved a new labeling for irinotecan in favor of UGT1A1*28 genotyping, which reflects the prevalence of the UGT1A1*28 allele in Caucasian Americans. A reduced initial dose of irinotecan has been recommended for homozygous carriers of this variant allele, as they are at increased risk of neutropenia (see the FDA's website at <http://www.fda.gov>). The Royal Dutch Pharmacists Association—Pharmacogenetics Working Group has evaluated therapeutic dose recommendations for irinotecan based on UGT1A1 genotype (Swen et al., 2011). They recommend reducing the dose for *28 homozygous patients receiving more than 250 mg/m² (Swen et al., 2011).

Translational pharmacogenetics and the need for clinical studies to support pharmacogenetically driven prescribing

The use of pharmacogenetic markers as a diagnostic tool to improve and individualize drug therapy depends on the existing evidence of the clinical impact of the respective variant. In addition to functional characterization of a polymorphism on the molecular level, further clinical studies that use prospective methods to characterize the clinical consequences in different situations in drug therapy are necessary. The lack of clinical data often hampers the application of pharmacogenetic analyses as a diagnostic tool and therefore impairs the translational process of pharmacogenetic research.

A variety of parameters are of interest in validating the usefulness of a pharmacogenetic variant as a biomarker for therapeutic outcome prediction. Data should be available characterizing the impact of a variant on pharmacokinetics or the direct pharmacological effects of drugs that are affected by the pharmacogenetic variant. In addition, surrogate markers for drug response or so-called intermediate phenotypes should be studied with regard to the genetic variant. Finally, data on complex phenotypes such as drug response or toxicity are of interest. Beyond this, data on long-term parameters such as hospitalization time, quality of life, disability, or survival are warranted in order to estimate the cost–benefit ratio of a pharmacogenetic test.

In conclusion, to achieve translation of pharmacogenetic data into clinical practice, data from a variety of clinical studies in different fields of drug therapy are required, and large cooperative approaches are necessary

to obtain the critical mass of evidence needed to convince clinicians and healthcare providers to use and pay for pharmacogenetic diagnostics as a tool to improve and individualize patient treatment.

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Chapter 7

Tissue biobanks

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Abstract

Biobanks represent complex systems of diverse human specimen archives and cover a multidisciplinary approach to the human health. Biobanks play an important role in the whole process of disease prevention and prediction, patient treatment, follow-up, and therapy monitoring. They are an important compound of precision medicine and strongly support the scientific progress in stratification of population and biomarker as well as drug discovery.

Keywords: Biobanking; personalized medicine; molecular profiling; biospecimen; biomedical research

Introduction

The concept of biobanking has gone through a tremendous transformation over the past decades, and there is no real consensus as to the definition of the term *biobank* (Loft and Poulsen, 1996; Coppola et al., 2019). The field has progressed extensively from simple repositories of biological specimens to modern and complex networks that encompass state-of-the-art infrastructure to conduct high-quality clinical and translational research (Hewitt, 2011; Harati et al., 2019). A 2009 article in *Time* magazine included biobanks in the list of “10 Ideas Changing the World Right Now” and described them as organic bank accounts in which one puts biomaterial in and earns medical interest in the form of knowledge and therapies that grow out of that deposit (Park, 2009). The latest developments followed the advent of so-called omics technologies (genomics, transcriptomics, epigenomics, metabolomics, and proteomics) and required advances in storage and computing capacity to process such big data in association with medical data (Zisis, 2016; Savatt et al., 2019; Ritchie et al., 2019). The recent technological progress in molecular biology and genetics have allowed the discovery of molecular patterns of disease associated with clinical data. This has proven particularly relevant in

oncology, in which next-generation sequencing technologies have enabled the identification of individual-specific genomic alterations that can be correlated with individual-specific drug susceptibilities. This has provided the rationale for the development of novel targeted therapies, which have progressively contributed to major advances in cancer management and have driven the development of precision medicine (Aboulkheyr et al., 2018; Bolck et al., 2019). Precision medicine leverages tumor genomic characterization data to tailor effective therapeutic strategies for each patient. Biobanking is the cornerstone for the development of personalized medicine by providing patient-derived material and associated clinical data for prediction of the risk for a particular disease, disease prevention, personalization of treatments based on genotypic and phenotypic alterations, and increasing participation and awareness of the potential associated benefits (Paskal et al., 2018).

Biobanking evolved in a decentralized manner with local repositories supporting specific research questions. Different biobanking facilities have been applying different technical requirements for biospecimen collection, processing, and storage conditions. This decentralized evolution has generated a high degree of sample heterogeneity among biobanking institutions and poses a barrier for sample sharing and international collaboration (Coppola et al., 2019; Savatt et al., 2019). For this reason, harmonization of biobanking procedures needs to be done at a large scale. Key aspects in standardizing the workflow comprising biospecimen collection, annotation, and biobanking involve hospital pathologists. Pathologists have a critical role in determining the nature and origin of biospecimens that should be biobanked, in assessing biospecimen quality, and in evaluating biospecimen suitability for clinical or research purposes (Paskal et al., 2018; Bevilacqua et al., 2010). International infrastructures dedicated to biobanking, such as the Biobanking and Biomolecular Resources Research Infrastructure (BBMRI), were created to provide the necessary framework and support the development of

standard operating procedures for harmonization of sample collection, handling, storage, retrieval, and distribution (Campbell et al., 2018; van Ommen et al., 2015).

The concept of biobanking

Biobanks provide a long-term repository of biospecimens, which can be distributed together with their associated medical and often epidemiological data in support of current or future research. Biospecimens can be collected for a wide range of scientific purposes, including research or clinical use (Coppola et al., 2019; Hewitt, 2011; Campbell et al., 2018). Biobanks allow patients access to molecular-based diagnosis and a greater analysis of prognostic and predictive biomarkers. They also provide data representing large numbers of individuals for basic and translational science. For example, genome-wide association studies can identify disease-associated single-nucleotide polymorphisms from thousands of patients as potential disease biomarkers (O'Donnell, 2020). The Cancer Genome Atlas is another example of how biobanks representing large patient cohorts were leveraged to create a comprehensive atlas of cancer genomic profiles (Weinstein et al., 2013). Linking biospecimens and associated clinical data to large-scale whole-genome sequencing analysis permitted characterization and classification of tumor subtypes based on distinct genomic alterations. Biobanks have become a key resource for the development of personalized medicine, for translational research applications, and for promoting drug discovery and drug development (Hewitt, 2011; Savatt et al., 2019; O'Donnell, 2020).

Biobanks usually incorporate cryogenic storage facilities for biospecimens. Laboratories that are accredited for biobanking must meet required standards for ensuring reproducible biospecimen collection, processing, and cryostorage and for cataloging and accessing biospecimens. Moreover, appropriate donors' consent granting use for research must be acquired in a timely manner (Bevilacqua et al., 2010; Campbell et al., 2018). Biobanks are commonly maintained by hospitals, universities, nonprofit organizations, and pharmaceutical companies. Institutions harboring a biobank are responsible for its maintenance, sustainability, and accessibility while guaranteeing protection of donors' identities and rights (Nicol et al., 2016; Hämäläinen et al., 2018). Biobanks can be classified by purpose or design into population-oriented biobanks and disease-oriented biobanks. In addition, biobanks can be physical or virtual (Coppola et al., 2019; Hewitt, 2011; Harati et al., 2019; Abdaljaleel et al., 2019).

Population-oriented biobanks

Population-oriented biobanks store biospecimens from large cohorts without specific inclusion or exclusion

criteria. Collection of germline DNA from individuals of a general population and umbilical cord blood are typical examples of biospecimens that are stored in population-oriented biobanks. The aims are to mirror the status of the general population and to determine possible development of common and complex diseases (Harati et al., 2019). Population-oriented biobanks examine the role of individual genetic susceptibility and exposure to external factors in the development of specific diseases by combining molecular data with clinical, lifestyle, genetic, epidemiologic, socioeconomic, and environmental data. As biospecimens are collected prior to disease onset, they are an invaluable source for longitudinal studies and enable identification and validation of early biomarkers of disease. Donors' biospecimens can also be prospectively linked to updated electronic clinical data, thus facilitating prescription of drugs and preventive medicine (Coppola et al., 2019; Hewitt, 2011; Harati et al., 2019; Gurwitz et al., 2009).

Disease-oriented biobanks

Setting inclusion or exclusion criteria during collection of samples constitutes the basis of disease-oriented biobanks, which store biospecimens from well-defined donors with specific diseases, primarily cancer (Harati et al., 2019; Paskal et al., 2018). The aim is to study disease pathogenesis and identify possible therapeutic strategies. With appropriate consent, both normal tissue and tissue with specific pathologies can be made available for research (Coppola et al., 2019; Hewitt, 2011). Such repositories offer endless possibilities to apply a wide range of new omics technologies to discover and validate new molecular targets, model disease progression, and promote development of novel targeted drug treatments (Bevilacqua et al., 2010).

Virtual biobanks

Virtual biobanks are virtual representation of biospecimens, such as digital pathology images and molecular data. They serve as catalogs of information about physical biospecimens, including annotation and location tagging, and associated data (Harati et al., 2019; Zisis, 2016). Virtual biobanking gives researchers an easy way to identify resources that are currently available without having to initiate new collection efforts. This allows easy information sharing without the need to process and physically use the biospecimens and facilitates the development of national and international collaborations (Coppola et al., 2019; O'Donnell, 2020; Abdaljaleel et al., 2019). The utilization of virtual biobanks to address new scientific questions must be based on patient consent and the related research protocol.

Biobanking for research

Biobanking efforts are the cornerstone for procuring high-quality biospecimens for translational research aimed at disease modeling, biomarker discovery and validation, pharmacogenomics-based response prediction, and identification of new therapeutic targets. Research on biospecimens usually incorporates ex vivo analyses and high-throughput omics techniques, including, whenever possible, both diseased samples and donor-matched healthy tissues (Bevilacqua et al., 2010; Morente et al., 2008).

Biobanking for clinical use

Biobanking is essential for the establishment and implementation of novel biomarkers into clinical use. Clinical trials are the common scenarios in which new potential biomarkers are validated. This promotes the discovery of new molecular targets for therapy, advances drug discovery, and identifies new therapeutic susceptibilities. Patients can thereby benefit from newly developed biomarkers, have access to molecular-based diagnosis and treatment, and benefit from advances in personalized medicine (Bevilacqua et al., 2010; O'Donnell, 2020; Morente et al., 2008).

Types of biospecimens

Along with diagnostic biospecimens, dedicated biospecimens should be collected exclusively for biobanking and subsequent research activities. Biospecimens should be kept separately and processed through distinct standardized workflows by trained personnel (Williams et al., 2019). The method that is chosen to process a certain biospecimen will depend on the intended use. Since it is difficult to anticipate all possible uses, biospecimens should be either cryostored as tissue pieces or processed by using the most stringent requirements and aliquoted in small amounts to maximize their use in several applications (Coppola et al., 2019; Paskal et al., 2018; Mandy et al., 2018). The types of biospecimens that are collected during clinical practice include tissues and body fluids. From both types of biospecimens, viable two-dimensional (2D) and three-dimensional (3D) cell models can be generated and subcellular components (e.g. DNA, RNA, and proteins) can be extracted (Coppola et al., 2019; Campbell et al., 2018; Mandy et al., 2018; Caixeiro et al., 2016). Such biospecimens add value to the biobank and extend the potential applications for biomedical research and clinical practice.

All human biospecimens, regardless of their origin or known disease, must be considered potential biohazards. The reason behind this is to ensure that laboratory personnel wear the appropriate personal protective equipment in

order to protect themselves from being exposed to any potentially contagious disease that may be unknown to the donor (Mandy et al., 2018; Hojat et al., 2019).

Tissue

The collection of tissue samples represents the major source of biospecimens for cancer research. Most tumors are heterogeneous at the cellular and molecular level, differing from one entity to another as well as within different areas of the same tumor (e.g. intratumor heterogeneity). It is therefore advisable to sample different regions of the tumor tissue. In addition, stromal cells associated with the tumor are commonly present and constitute a critical biospecimen with high value for research (Hosein et al., 2020; Piersma et al., 2020).

Human tissues are usually obtained from biopsy interventions, surgeries, and autopsies (Tashjian et al., 2019). Tissue sampling for biobanking should occur only after sufficient high-quality tissue has been collected for diagnostic procedures. The time a surgical sample is still within the body but with a compromised blood supply is called *warm ischemia* and is a critical parameter that influences tissue degradation before fixation (Mandy et al., 2018; Betsou et al., 2010; Shabikhani et al., 2014). Immediately after surgery, pathologists perform a gross morphological and histopathological examination of tissues, including photographic records and weight and size measurements. Biospecimens are then transported to the processing laboratory, where microscopic examination of the surgical material (such as tumor cell content, stromal components, necrosis, or fibrosis) will be performed (Hojat et al., 2019). The time between resection to stabilization (e.g., fixation) is called *cold ischemia* and has been shown to influence gene expression in surgical tissues (Shabikhani et al., 2014; Grizzle et al., 2016). Recording all preclinical parameters is critical for decreasing variability in downstream applications (Bevilacqua et al., 2010; Campbell et al., 2018).

Before the advent of omics technologies, tissues were typically fixed in formalin and embedded by using paraffin (FFPE). This method provides optimal tissue structure for microscopic morphology analysis. However, FFPE tissue has disadvantages for molecular applications and thus limits its potential use for research (Bolck et al., 2019). Type and duration of fixation as well as long-term storage duration and conditions are essential information for guaranteeing high reproducibility of the intended research with tissue biospecimens (Mandy et al., 2018; Hojat et al., 2019; Betsou et al., 2010). Snap-freezing of tissues in Optimal Cutting Temperature (OCT) compound allows a wide variety of immunostainings on cryosections and microscopic examination with subcellular resolution. OCT-embedded tissue can additionally be used for

comprehensive genomic, transcriptomic, and proteomic studies (Coppola et al., 2019; Bolck et al., 2019; Paskal et al., 2018; Mendi et al., 2018).

Body fluids

Blood is one of the most common biospecimens collected for research (Coppola et al., 2019; Perry et al., 2019). Blood can be collected in tubes containing preservatives and additives that are specific for the blood fraction needed and the intended research application. Tubes and kits from commercial suppliers that have been extensively validated should be preferentially used for blood collection and processing. Plasma is the liquid fraction of anticoagulated blood, and serum is the liquid fraction of clotted blood. Plasma samples are usually collected in tubes with anticoagulant reagents such as ethylenediamine tetra-acetic acid (EDTA) or heparin, and serum samples are collected in tubes with a clot accelerator such as thrombin. Serum does not contain platelets and other coagulating proteins, thus allowing proteomic analysis of a greater variety of proteins. While most biochemical analysis are performed on serum, plasma obtained by using EDTA as an anticoagulant can be used for downstream proteomic, genomic, and metabolic analyses (Paskal et al., 2018; Mendi et al., 2018; Hsieh et al., 2006). The optimal storage temperature for each blood component depends on the specific analyte or marker that will be measured. Generally, blood components are cryostored at low (-20°C) or ultralow (-80°C) temperatures to preserve their integrity and stability (Paskal et al., 2018; Mendi et al., 2018).

Urine samples are easily collected and provide a source for DNA, RNA, proteins, and metabolites that can be measured after minor or no processing (Paskal et al., 2018; Mendi et al., 2018). Urine composition changes during the day and according to the donor's disease state (Bernini et al., 2011; Playdon et al., 2016). Collection time and first, midstream, or last part of urine depend on the intended research and analytical endpoints and should be documented (Bernini et al., 2011; Betsou et al., 2013). Urine biospecimens can be stored long term at ultralow temperatures or used immediately for diagnostic and prognostic purposes using genomic, proteomic, and metabolomic techniques (Playdon et al., 2016).

Additional types of body fluids that can be biobanked include cord blood, saliva, breast milk, and cerebrospinal fluid (Paskal et al., 2018). The preanalytical parameters that influence fluid quality must be carefully chosen depending on the intended research; these include type of primary collection tube, precentrifugation and postcentrifugation time delay and temperature, centrifugation conditions, and long-term storage duration and temperature

(Paskal et al., 2018; Betsou et al., 2010; Bernini et al., 2011; Betsou et al., 2013; Lehmann et al., 2012).

Cellular models

Tissues can be further processed to extract derivatives such as cells. Human cancer-derived cell lines have been widely used in the past as preclinical models because of their high-throughput capacity for pharmaceutical drug screening (Goodspeed et al., 2016). The HeLa cell line was the first cellular model established in 1951 at Johns Hopkins Hospital. HeLa cancer cells were obtained without the donor's consent and have been in widespread use worldwide for research (Masters, 2002). Increasing numbers of immortalized cell lines have been established within the last decades, and official cell line biobanks have arisen to guarantee the origin and quality of cellular model systems (Coppola et al., 2019). Despite some considerable success achieved with cell lines, such conventional preclinical model systems have shown limited clinical applicability, mainly owing to lack of heterogeneity and poor physiological relevance and translatability. In particular, immortalized cancer cell lines lack stromal cells, and interactions between tumor cells and the extracellular matrix (ECM) (Aboulkheyr et al., 2018). Three-dimensional culture models, such as patient-derived tumor organoids (PDTOs), have emerged as robust preclinical models showing multiple advantages in comparison with conventional 2D tumor models (Voest and Bernards, 2016). Phenotype-genotype correlations have shown that PDTOs resemble the original tumor and hold great promise in personalized cancer therapy, specifically for gene-drug correlation studies, preclinical drug screening, modeling the dynamics of drug resistance, and prediction of drug response and patient outcome (Vlachogiannis et al., 2018; Beshiri et al., 2018; Pauli et al., 2017; Sachs et al., 2018; Yan et al., 2018; Jacob et al., 2020; Lee et al., 2018).

PDTO culture methods vary depending on the tumor type. The most standard technique for their generation consists in mechanical and/or enzymatic digestion of a piece of tumor tissue into smaller pieces and subsequent embedding into a 3D matrix. A commonly used ECM is Matrigel. Matrigel contains several basement membrane proteins of animal origin, such as laminin, entactin, heparin sulfate proteoglycan, and collagen IV; its composition can vary from lot to lot, rendering its use inappropriate for harmonizing standard operating procedures (Blondel and Lutolf, 2019; Aisenbrey and Murphy, 2020). Other promising products for tissue engineering and 3D cell models are synthetic hydrogels (Unal and West, 2020; Naahidi et al., 2017).

Freezing living cells damages cellular membranes, so cryoprotective agents, such as dimethyl sulfoxide (DMSO), must be used for cryostorage to facilitate viable cell

recovery. Fetal bovine serum (FBS) is widely used in combination with DMSO in conventional cryopreservation media. However, since FBS contains a mixture of growth factors and cytokines and its composition varies between batches, its use for biobanking should be avoided (Coppola et al., 2019). Maintaining a living cell biobank requires a highly complex regulatory, administrative, and laboratory infrastructure. Therefore it is fundamental that living biobanks collect and effectively store high-quality biospecimens that add value to support translational and clinical research (Bolck et al., 2019).

Blood and other fluids may also be processed to separate specific cellular components. Centrifugation of anticoagulated blood elicits the separation of the red blood cell fraction and the buffy coat layer containing white blood cells and platelets (Paskal et al., 2018; Perry et al., 2019). Peripheral blood mononuclear cells (PBMCs) present in the buffy coat layer comprise lymphocytes, monocytes and macrophages. Blood cell components can be cryopreserved as viable cells for future cell sorting, cell immunophenotyping, immortalization and immunooncology assays (Mendy et al., 2018). Immunooncology is based on modulating the expression of immune checkpoint receptors and ligands for modulating tumor antigen presentation. Cancer immunotherapy is showing extensive clinical benefit for treating many cancers, as the innate and adaptive immune system can be harnessed for optimizing treatment response and clinical outcome. Extensive clinical and translational research is currently focusing on developing and validating predictive biomarkers for response to immunooncology therapies as well as biomarkers prognostic of disease outcome (Allard et al., 2018; Gwin et al., 2019).

Nucleic acids: DNA and RNA

Fresh or frozen tissue is the most suitable sample for research on nucleic acids, as it yields high-quality DNA and RNA for a wide variety of genomic analysis. Anticoagulated blood is preferred for nucleic acid extraction and should be stored at ultralow temperatures if extraction cannot be performed immediately (Perry et al., 2019). Whereas DNA is a very stable molecule, RNA is a labile molecule that is prone to degradation, so a standard procedure to avoid RNA degradation is essential. Different RNA yields are achieved depending on the biospecimen, and RNA quality is usually measured by using the RNA integrity number, which represents electrophoretic measurements of whole RNA integrity. DNA is stable at 4°C for several week and ideally is stored at -20°C. RNA must be stored at -80°C to prevent degradation. All samples should be cryopreserved in small aliquots and repetitive thaw-freeze cycles should be avoided (Caixeiro et al., 2016; Shabikhani et al., 2014; Strom, 2019).

DNA biobanking efforts and the rapidly decreasing costs of next-generation sequencing have enabled comprehensive genetic profiling of tumor samples and uncovered novel oncogenes and tumor suppressor genes with altered mutation frequencies or profiles. This has led to the identification of new drug targets and has been leveraged for developing personalized diagnostic tests. Correlating the genomic profiles to the transcriptome status of cells and tissues is the focus of intense research and may provide an even more complete view of the tumor landscape and contribute to identifying novel gene signatures as potential biomarkers of disease (Hudson et al., 2010; Olivier et al., 2019). In addition, tumor cells acquire epigenetic marks, such as DNA methylation and histone modifications, that can be detected in both fluid and tissue biospecimens. Epigenetic changes have been proposed as biomarkers for cancer detection, tumor prognosis, and prediction of treatment response (Olivier et al., 2019; Dumitrescu, 2018; Nakagawa and Fujita, 2018).

Protein and other subcellular components

Proteins can be extracted from tissues and blood plasma and are more stable than nucleic acids, especially RNA. Both fresh frozen or fixed (FFPE) tissue biospecimens are suitable for proteomic analyses, some of which require only small amounts of starting material. Biospecimen processing for proteomic studies usually incorporates first a lysis step and heating of the sample, which is essential for de-crosslinking FFPE samples (Mendy et al., 2018; Doll et al., 2019). Proteins can be localized in different subcellular compartments and can be further modified posttranslationally. Consequently, protein abundance and activity do not always correlate with gene or transcript expression. It is therefore important to directly measure protein amount and posttranslational modifications (PTMs), which are invisible to genomic studies, to obtain a clear overview of the disease landscape (Liu et al., 2016). Abundance of specific proteins can be measured by using antibody-based methods, and unbiased, large-scale whole-proteome profiling can be performed by using mass spectrometry (MS). Phosphorylation is one of the most important and commonly studied PTMs. Phosphoproteomics can be applied to protein samples to determine the identity and quantity of phosphorylation sites and to help determine kinase activity and aberrant signaling pathways, which are commonly altered in cancer. Proteomics can be implemented in clinical research for profiling tumor-specific expression of proteins and PTMs, which has the potential for uncovering novel biomarkers and improving prognostic and predictive power in the clinic (Doll et al., 2019).

Mitochondria are intracellular organelles found in cells that have been isolated from blood or tissues. Mitochondria

are essential for energy generation and central to understanding susceptibility to pathologies with a metabolic etiology as well as pathologies associated with aging including cancer and cardiac and neurodegenerative diseases. Optimized methods for processing, storing, and quality control of metabolic samples are becoming more standardized. Metabolomic techniques can detect many classes of organic compounds, including lipids, amino acids, sugars, biogenic amines, and organic acids. The most common technologies in metabolomics are nuclear magnetic resonance (NMR) spectroscopy, gas chromatography mass spectrometry (GC-MS), and liquid chromatography MS (LC-MS). Research on mitochondrial genetics, cellular bioenergetics and metabolomics holds promise for developing metabolism-based clinical tests and metabolotherapies (Hill et al., 2019; Wishart, 2016; Li et al., 2017).

Exosomes are a particular type of nanosized extracellular vesicles with intracellular biogenesis that can be detected in fluid biospecimens, including blood, urine, and saliva. Exosome research has been the focus of some controversies, which might be explained in part by the current lack of consensus on isolation methods and characterization criteria. Standardized isolation, characterization, and storage methods for exosomes are yet to be established. Translational and clinical research on exosomes is ongoing and, given time, might prove exosomes to be valuable clinical tools, specifically as potential noninvasive cancer biomarkers, drug delivery vehicles, and therapeutic targets (Di Meo et al., 2017; Mora et al., 2015).

Quality assurance of biospecimens

Successful biobanking needs high-quality specimens, and quality is the most importance for biobanking operations. Heterogeneity in biospecimen collection, processing, and storage constitutes a major source of variability for the intended analyses and study goals (Bevilacqua et al., 2010). The management of biobanks requires comprehensive quality management policies that adapt to the legal framework, guarantee the best practices, and comply with strict technical requirements, especially for biospecimen processing methods and biospecimen quality control methods (Mendy et al., 2018). These are necessary to ensure that biospecimens collected for clinical or research purposes are of consistently high quality. Harmonization of preanalytical annotations and standard operating procedures for biobanking have been implemented (Campbell et al., 2018; Betsou et al., 2010; Lehmann et al., 2012) and are continuously being adapted to provide the basis for improving reproducibility of molecular data and for designing multicenter clinical trials in which biospecimens need to be exchanged among different collection centers (Bevilacqua et al., 2010; Abdaljaleel et al., 2019).

Biobanking in the era of precision medicine

Precision medicine is an emerging approach for disease prevention and treatment that takes individual variability in genes, environmental factors, and lifestyle into account. Availability of biomarkers for disease diagnosis and prediction of patient prognosis and therapy promises personalized medicine. In the oncology field, the premise of precision oncology is to identify treatments that target the molecular characteristics of an individual's tumor. This kind of targeted treatment will eventually lead to more effective treatments with fewer side effects (Zatloukal and Hainaut, 2010). The precision oncology approach has initially focused on the genomic changes in a tumor but is now also taking RNA and proteins into account to better guide patient care. A biobank that supports precision medicine involves patient care and would require the highest standards for operations as well as rigorous quality assurance and quality control (Liu and Pollard, 2015). Medical research in the era of precision medicine is based on the analysis of samples with clinical data. The better characterized high-quality samples become and are linked to clinical data and available through biobanks, the faster research will advance and affect the delivery of health-care. Therefore there is a growing requirement for biobanks to have increased capacity and sufficient informatics capabilities in order to ensure these demands are met. Modern biobanking is shifting its focus from "only" sample-driven to data-driven strategies. However, in order to fulfill the opportunities promised by precision medicine, challenges remain on the road ahead. In the past two decades the role and value of biobanks have become an integral part of precision medicine, and many new biobanks have been established all over the world to support the dramatic development in diseases prevention, prediction, diagnosis, and treatment. (Fig. 7.1).

Ethical issues

Ethical issues are commonly present in many of the aspects of biobanking as human specimens are involved and the individuals' autonomy can be at risk. Data protection and privacy are fundamental human rights which need to be protected (Lunshof et al., 2008). In the era of precision medicine where more "identifiable information" is generated more specimen and information is shared, this becomes even more of an ethical challenge. There is an increasing need for the appropriate handling and protection of sensitive patient data. In 2018, the EU General Data Protection Regulation (GDPR) dramatically increased organization's responsibility for data protection and the privacy of customers (Lawlor et al., 2018). A patient informed consent according to the international conventions and guidelines in

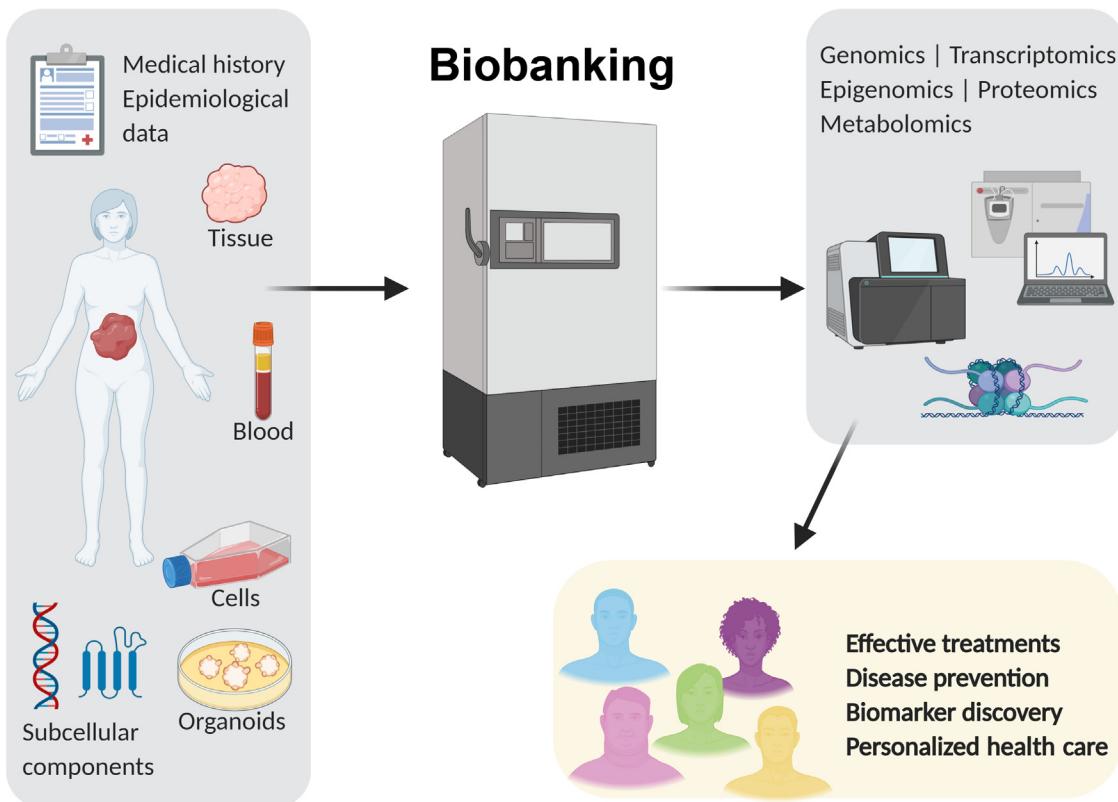


FIGURE 7.1 Tissue biobanking from biospecimen collection to personalized therapy approaches. Credit: Created with BioRender.com.

research ethics is to guarantee the voluntary participation in research and address privacy issues in research (Commission, 2013). Many institutions have their own informed consent in order to allow samples to be used for research. Informed consent respects individuals' autonomy and vulnerability. Special care and attention must be paid to specific groups of donors like children, elderly, mentally deficient persons, severely injured patients and when specific cultural, ethnical or traditional backgrounds are involved (Gurwitz et al., 2009; Mandy et al., 2018; Kim and Milliken, 2019).

Data ownership is of course a very important issue as multiple stakeholders are involved in a biobank: patient, institution that is hosting the biobank-infrastructure, funding agencies, medical personal involved in specimen collection, ethics, and review board. It has been proposed that the institution that is hosting the biobank should hold the administration right for the use of the resource. Harmonization in the entire process is needed as biobanking is becoming more important in the era of precision medicine.

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Chapter 8

Animal models: value and translational potency

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Abstract

Modern biomedical research that aims to translate research findings into novel therapies to benefit patients relies to a large extent on animal models of human pathophysiology. However, success stories of translational research—that is, preclinical research that successfully predicts a positive outcome of a clinical trial—are scarce. Therefore we address here the current state of preclinical disease modeling as well as actions that have been taken to improve the translational value of animal models.

Keywords: Animal models; pathophysiology; disease mechanisms; preclinical randomized controlled trials; modeling of care

What is the value of animal models? Pathophysiological concepts

The majority of translational research relies on preclinical animal models. However, given the enormous number of examples of failed translation, that is, phase II or phase III clinical trials that were not able to reproduce the beneficial effect of preclinical findings (O'Collins et al., 2006; Perrin, 2014; Prinz et al., 2011), the translational value of animal models has been questioned. In particular, rodent models have been accused of falsely modeling human disease conditions.

Nonetheless, many animal models are geared to replicate pathophysiological conditions found in patients. An ideal animal model of a human disease is characterized by similarities between animal and human in terms of (1) pathophysiology, (2) phenotypical and histopathological characteristics, (3) predictive biomarkers for course or prognosis, (4) response to therapies, and (5) drug safety or toxicity (Perrin, 2014; Prabhakar, 2012).

Four types of animal models are used in preclinical research: (1) disease induction models, (2) xenograft

animal models, (3) inbred strains, and (4) transgenic models (Prabhakar, 2012). The rodent stroke model of middle cerebral artery occlusion is a typical disease induction model. Xenografting or transplantation of organs or tissues from one species into another is often used in cancer research. “Humanized” mice are another example of xenograft models. Inbred animals are genetically homogenous, allowing investigation of pathobiology with small sample sizes (Prabhakar, 2012). By using methods of molecular biology, specific genes are either deleted (knock-out), mutated or overexpressed in transgenic animals, mainly mice. Often, these models are combined. For example, disease induction models in transgenic mice are often used to investigate the contribution of specific genes in diseases.

Rodent models of cerebral ischemia are good examples of animal models that replicate human pathophysiology well (Astrup et al., 1977; Heiss, 2011). The ischemic penumbra is defined as the area surrounding the core of the ischemic lesion. While physiological cascades are compromised, this area of brain tissue can potentially be rescued by medical intervention. This concept was first described in animal models (Astrup et al., 1977; Heiss, 2011) and has since been found to be relevant for human stroke pathophysiology (Dirnagl et al., 1999; Donnan et al., 2008; Mergenthaler et al., 2004; Mergenthaler and Meisel, 2012). The same has been found true for the concept of stroke-induced immunodepression. While the pathophysiological concept was initially described in animal models (Chamorro et al., 2012; Prass et al., 2003), clinical trials have been able to replicate this concept in human stroke pathophysiology (Chamorro et al., 2012; Harms et al., 2008; Mergenthaler and Meisel, 2012), although therapeutic protocols making use of this concept are still under development (Mergenthaler and Meisel, 2012).

Likewise, animal models of cancer—in particular, genetically engineered mouse models—have significantly contributed to the understanding of tumor biology and cancer pathophysiology. In particular, advances in genetic engineering have allowed modeling the manifold genetic defects underlying many forms of cancer (Cheon and Orsulic, 2011). Likewise, the concept that several mutations in the genome might be required for tumor development as well as prototypic oncogenes has been established by the use of mouse models (Cheon and Orsulic, 2011). However, similar to the situation in stroke (Dirnagl and Fisher, 2012) mouse models in preclinical cancer research have yet to prove their translational capacity (Cheon and Orsulic, 2011).

What is a good animal model for translational research?

It is clear that there is no single ideal animal model of human disease conditions. Likewise, the design of pre-clinical experimental studies at present offers substantial room for improvement. While this topic has recently received significant attention, many of the proposed remedies for the translational roadblock have yet to prove themselves in translational studies and the design of clinical trials. Among other issues, considering the complex characteristics of the animal models as well as of the human disease state is essential in selecting an appropriate model for preclinical studies. Three aspects are often not considered in preclinical studies: the heterogeneous nature of disease, the presence of comorbidities, and appropriate outcome measures (Mergenthaler and Meisel, 2012).

Several approaches to improve translation from animals to the clinic have been suggested. Before clinical trials are started, preclinical investigations should be performed in multiple experimental setting involving different small and large animals modeling different disease states, including the characterization of the optimal therapeutic window, optimal administration routes, and schemes as well as dose-response curves (Xiong et al., 2013). Furthermore, preclinical studies need to reflect the clinical scenarios, including relevant treatment windows and outcome parameters. For example, drug administration at onset or even before injury, as performed in many preclinical studies investigating disease mechanisms, is of minor relevance for therapy.

Most preclinical research in stroke or traumatic brain injury (TBI) suffers from short-term studies demonstrating treatment effects 1–7 days after the event (Xiong et al., 2013). Investigations on long-term outcome, weeks to months after injury, are still scarce. By contrast, primary endpoints of clinical phase III trials have to focus on relevant long-term outcome measures.

Disease modeling focused on pathophysiological research is invariable an oversimplification of the clinical situation. For example, stroke patients often suffer from a variety of other diseases, such as hypertension, diabetes mellitus, or chronic obstructive pulmonary disease, which are commonly not modeled. Beyond the comorbidities patients have before stroke onset, patients are often affected by several poststroke complications, such as infection or depression, which are also usually either not modeled or not considered. The same holds true for other disease models, such as TBI. Moreover, stroke patients receive a myriad of treatments, including medication and general care such as nursing and physiotherapy, among others. Although stroke unit care is efficient, without any doubt, we do not know which individual pieces of treatment are of relevance. Nevertheless, modeling of care is probably one prerequisite for successful translation of treatment strategies of complex disorders such as stroke (Mergenthaler and Meisel, 2012).

Modeling comorbidities

Most investigators disregard the fact that most patients are not young or middle-aged males without any comorbidities (Howells et al., 2010; Sena et al., 2010). One fundamental criticism of animal research is that most models do not consider age (Howells et al., 2010), which is one of the most relevant cofactors of outcome for most non-communicable disorders (Howells et al., 2010; Lozano et al., 2012). However, young to middle-aged inbred rodents of one gender and of homogeneous genetic backgrounds are typically used for preclinical animal studies. Ideally, preclinical animal studies should include animal populations of mixed gender, advanced age, and with various comorbidities, such as diabetes mellitus, hyperlipidemia, hypertension, obesity, or other risk factors that are relevant for the respective human disease. Such an approach would model the human etiology of most diseases more closely. In many cases, such models are readily available (Howells et al., 2010). In addition, experimental animal populations should become increasingly complex as a therapeutic intervention advances in the translational pipeline (Fig. 8.1). The concept of establishing a framework as well as funding schemes to enable such preclinical randomized controlled trials (pRCTs) has been suggested in many medical disciplines, including oncology (Cheon and Orsulic, 2011) and stroke (Bath et al., 2009; Dirnagl and Fisher, 2012; Mergenthaler and Meisel, 2012).

Modeling care of patients

Many successful therapeutic strategies rely on “intensified care” of critically ill patients in the acute phase of the disease on dedicated and highly specialized hospital wards.

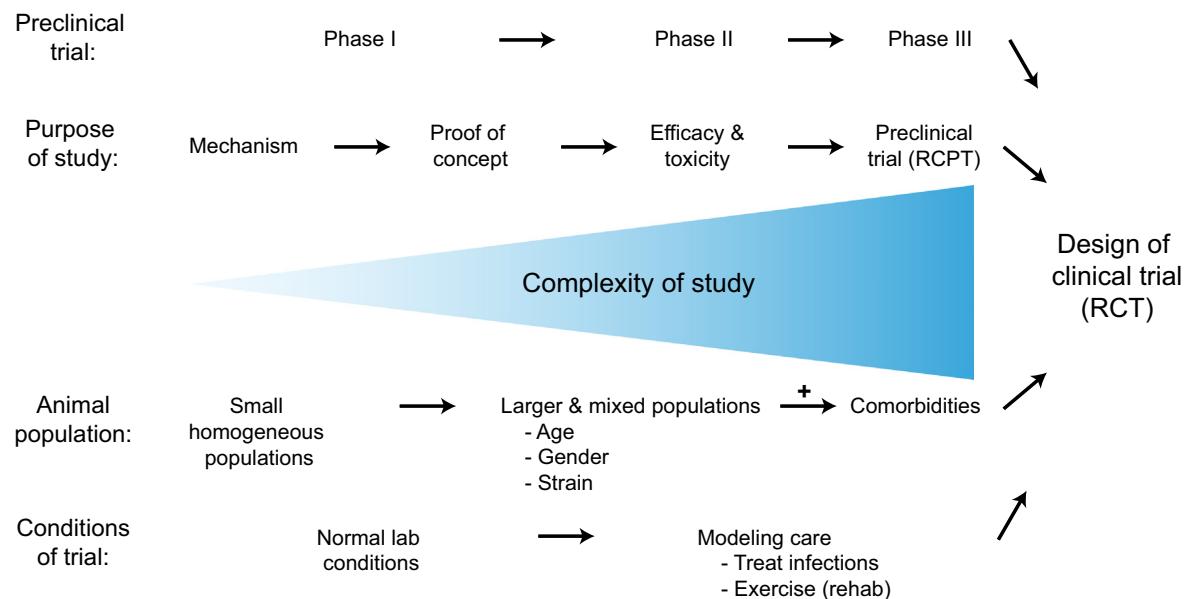


FIGURE 8.1 The preclinical trial phases of translational research. As therapeutic agents or concepts advance in development, the experimental setting increases in complexity. It ranges from small cohorts to investigate novel mechanisms to large mixed populations with possibly multiple comorbidities and additional modeling of stroke care. The final stage of preclinical development is the conduct of a randomized-controlled preclinical trial, ideally in a stroke unit setting. Randomized clinical trials commence after this process has been completed and is based on evidence gained in preclinical testing. Reproduced from: Mergenthaler, P., Meisel, A., 2012. Do stroke models model stroke? *Dis. Model. Mech.* 5, 718–725.

Acute care is usually complex and committed to optimize physiological parameters. Including such a strategy in preclinical modeling would aid in better modeling clinical care of patients as well as the associated complications.

In patients with cerebral ischemia, stroke units are prepared to treat the clinical condition as well as potential complications (Donnan et al., 2008). Infections have largely been neglected in preclinical stroke research (Meisel and Meisel, 2011; Meisel et al., 2005), although they heavily influence stroke outcome (Mergenthaler and Meisel, 2012; Westendorp et al., 2011). While preventive antibacterial treatment not only prevents infections, it also improves survival and neurological outcome after experimental stroke compared with placebo treatment (Meisel et al., 2004). Phase IIb trials have successfully proven this experimental concept (Chamorro et al., 2005; Harms et al., 2008; Schwarz et al., 2008) by demonstrating that prevention of infection is effective in stroke patients (van de Beek et al., 2009). Thus basic research findings and preclinical modeling preceded the development of this new treatment approach (Mergenthaler and Meisel, 2012).

A novel approach to preclinical research would include modeling the acute, subacute, and chronic phases of the disease. Clinical and empirical evidence indicates that intensified and specialized treatments are beneficial for long-term outcome. Thus taking “care” of patients should be reflected in future preclinical trials. In summary, pre-clinical trials as the foundation for future clinical trials should include large and complex cohorts of animals and

should include gender-mixed, aged animals from different strains, ideally with different comorbidities, and model care of (hospitalized) patients. Furthermore, complex long-term outcome analyses should be performed to evaluate the success of a novel therapeutic concept or pharmacological agent (Fig. 8.1).

What is the translational value of animal models?

Recurrent failure to translate promising treatment strategies in animal models into the clinic has challenged the value of animal research for predicting the effectiveness of treatment strategies in humans. Thus animal models of human disorders are more and more condemned, and they are often considered meaningless or at best as imprecise for the human setting. However, all medical areas employ models that have advantages and limitations. Animal models have been used successfully to define basic pharmacokinetic properties as well as to investigate safety and toxicity issues (McGonigle and Ruggeri, 2014).

One example for this approach is the following. Amyotrophic lateral sclerosis (ALS), a devastating neurodegenerative disorder, is characterized by a progressive degeneration of motor neurons leading to a generalized paralysis, respiratory insufficiency, and death usually within 3–5 years. Stem cell transplantation has emerged as a promising approach for ALS patients. Rather than motor neuron replacement, current approaches consider

mesenchymal or neural stem cells as supporters for motor neurons, delaying neurodegeneration. Although some ALS models suggest that stem cell–based approaches might delay motor neuron degeneration, current strategy in the field is focused not proving on efficacy but on demonstrating safety in preclinical models aiming at quick translation to the clinical setting investigating efficacy in patients. The main argument for this approach is the rather poor understanding of ALS pathobiology (Thomsen et al., 2014). However, whether or not this safety-focused approach in translation is successful or not remains to be demonstrated.

Even preclinical studies aiming at toxicity analysis might fail in predicting safety for humans. For example, the immunomodulatory humanized agonistic anti-CD28 monoclonal antibody TGN1412, which was developed for autoimmune disorders such as multiple sclerosis and rheumatoid arthritis, was tested successfully for safety in various animal models, including mice. However, in the first human phase I trial, TGN1412 caused a severe systemic inflammatory response syndrome due to a cytokine storm, resulting in a disastrous outcome with multiorgan failure for the study participants, despite the fact that the dose used was 500 times lower than the dose that had been found to be safe in animal studies (Suntharalingam et al., 2006).

Drug discovery begins with target identification and validation and then proceeds with identification and development of candidate therapeutic agents. At each step of this process, which often requires more than 12 years, animal models are needed (Whiteside et al., 2013). However, only 15% of novel drugs that are successfully tested in animal models pass early clinical trials, and approximately half of those that survive phase III are finally approved for clinical practice by the regulatory authorities (Ledford, 2011).

Extrapolation of preclinical findings into the clinical settings might also depend on the substances under investigation. For example, animal models mimicking airway susceptibility in different lung disorders have been demonstrated to be predictive for the human situation for anesthetic drugs such as halothane, isoflurane, propofol, and ketamine but not for lidocaine, morphine or muscle relaxants. Among other reasons, variability between species in different receptor distributions and drug affinities might account for the different predictability of the pre-clinical models (Habre and Petak, 2013).

Animal models of human tumors are considered indispensable for drug discovery and development. The commonly used ectopic and orthotopic xenografts models, primary human tumorgraft models, genetically engineered models, or various multistage carcinogen-induced models, all have different strengths and weaknesses (Cheon and Orsulic, 2011; Heyer et al., 2010). These models should

be used as sophisticated biological tools at specific stages of drug development in a hierarchical manner of increasingly complex modeling (Fig. 8.1) of the diversity of human cancers (Ruggeri et al., 2014).

One approach to test the predictive power of animal models is conducting reverse translational studies investigating known effective treatment strategies of human disorders in appropriate animal models. Temozolamide is a good example of a successful forward and reverse translational approach for the treatment of glioblastoma. A systematic review and metaanalysis of temozolamide in animal models of glioblastoma predicted clinical efficacy. This treatment is effective in reducing tumor volume and improving survival clinically as well as in experimental models of malignant glioma. The reported efficacy for treatment has not significantly changed since publication of the seminal phase III temozolomide trial demonstrating efficacy in glioblastoma patients (Stupp et al., 2005), although evidence suggests a publication bias overemphasizing its therapeutic efficacy (Hirst et al., 2013).

Genetic mouse models of Huntington's disease (HD) should help to identify and prioritize the most promising treatment strategies to be tested in clinical trials (Menalled and Brunner, 2014). Many neural circuits affected by HD are evolutionary conserved. More than a dozen genetic mouse models express a mutation similar to that responsible in HD with many variations in CAG length of the Huntington gene. These models mimic the human genetic insult with different phenotypic aspects of HD (Menalled and Brunner, 2014).

Numerous transgenic or surgically induced pig models of neurodegenerative disorders have been established to develop cell replacement strategies. Defining the optimal cell dose, immunosuppression protocols and testing new cell delivery devices were a prerequisite for designing human clinical trial protocols in neurodegenerative disorders such as ALS, stroke, spinal cord and TBI, HD, Alzheimer's disease, and Parkinson's disease. In contrast to other animal models, fully or partially major histocompatibility complex–matched pig strains model the human situation, thereby better modeling host–versus-graft and graft–versus-host reactions of cell and tissue replacement strategies (Dolezalova et al., 2014).

In neuropathic pain research, the effect size of successful pain treatment in animal models is almost twice that in clinical trials. Correspondingly, the number needed to treat, which reflects the number of individuals that must be treated to see one successful treatment outcome, is almost half in animal compared to clinical pain trials. Among other issues, placebo effects in clinical trials, which are absent in animal research, are significant confounders. Effect sizes of at least 60% pain relief in animal models are required to predict clinical efficacy (Whiteside et al., 2013).

Psychiatric disease is not directly translatable to animal models. For example, even transgenic mouse models of neuropsychiatric disorders cannot fully represent the broad spectrum of symptoms, including confusion or suicidal thoughts. However, these models serve to explore psychiatric disorders by unraveling disturbances of neural circuits underlying disease-relevant phenotypes, in particular how environmental and (epi-)genetic factors interact to shape behavioral phenotype and predispositions to psychiatric disorders (Donaldson and Hen, 2014). Traditionally, in psychiatric animal models, abnormal animal behavior was created, phenotypically resembling the aspects of mental disorders. Reverse translation using knowledge about the mechanisms of human disorders has been used to identify and develop animals that have the molecular and cellular abnormalities found in these diseases (Malkesman et al., 2009). For example, depression has been modeled in mice having point mutations in the mitochondrial DNA polymerase (Kasahara et al., 2006), and glutamate receptor 6 knock-out mice have a high face and predictive validity for mania (Shaltiel et al., 2008).

Lost in translation has become a very popular paraphrase for the obstacles encountered in translational research. Three reasons for the lost in translation problem have been suggested. First, small differences in the models might lead to vast differences in the results, which has been attributed to the chaotic behavior of the models and termed the *butterfly effect*. Second, the effect size is decreasing from biochemical models over cell and tissue cultures to animal experiments to human studies, which seems to be unexpected according to the “princess and the pea” story. Finally, the so-called two cultures of pre-clinical and clinical research are different (Ergorul and Levin, 2013; Mergenthaler and Meisel, 2012).

Remedies for failed translation: improving preclinical research

Improving models

To improve the quality of translational biomedicine, it has been suggested that the process of preclinical research should become more like clinical research. For example, applying rules similar to those used by regulatory agencies for clinical trial has been suggested also for preclinical studies. Using methods such as systematic reviews and metaanalyses have become more and more popular in animal research to identify robust treatment effects. Commonly accepted “futility” and “stopping” rules in clinical research have become increasingly accepted in preclinical research. These approaches have been demonstrated to improve the predictive value of animal research (Perel et al., 2007).

An ideal animal model will meet all three of the following criteria: face validity, predictive validity, and construct validity. Face validity refers to the phenomenological similarity between the model and its corresponding disorder. Predictive validity refers to the ability of the model to have comparable biomarkers and treatment responses as the human disorder. Construct validity reflects the degree to which a model measures what it claims to be measuring (Willner, 1986).

To improve construct validity, it has been proposed that therapeutic interventions should be tested in animal models of central nervous system (CNS) disorders under conditions of greater environmental enrichment. One limitation of current research is that most animal studies are performed under caging conditions with sedentary, unstimulated animals that have unlimited access to food. An enriched environment that stimulates the sensory system, cognition, and physical exercise have been demonstrated to affect outcome significantly (McOmish et al., 2014).

To improve translational power, the use of more humanized models has been suggested (Ergorul and Levin, 2013). Immunodeficient mice that have been engrafted with human primary hematopoietic cells and tissues generating a functional human immune system in these mice are a well-established example of humanized mice. These models have been successfully used in investigating infectious diseases, autoimmune disorders, and tumors (Shultz et al., 2012).

Recent exciting findings in stem cell biology have opened the door to novel approaches in disease modeling using human model systems. Terminally differentiated human somatic cells may be reprogrammed to an induced pluripotent stem cell (hiPSC) state in order to then differentiate these cells into any cell type of interest (Lee and Studer, 2010). These developments might revolutionize investigations of human disorders, in particular those affecting the CNS (Philips et al., 2014). Patient-derived hiPSCs can be differentiated in specific neuronal subpopulations, such as cortical neurons (Zhang et al., 2013) or striatal medium spiny neurons (Philips et al., 2014), which are affected in brains of patients suffering from HD. Obviously, brain cells are usually not directly accessible as primary material for study disease mechanisms or for specific treatment. Furthermore, in recent years, organoid technologies have revolutionized experimental biomedical research (Schutgens and Clevers, 2020). In the context of brain research, human brain organoids (Lancaster, 2020; Lancaster et al., 2013) have provided for the first time a human model system with the prospect of studying developmental aspects and disease mechanisms in a brain-like model system. Thus far, they have mostly been used for studying brain development and developmental disorders (Marton and Pasca, 2020). Using hiPSC technology, specific cell differentiation, organoid technologies, and refined

genomic editing tools (Hendriks et al., 2020), correction of mutations are feasible, and specific treatment is conceivable (Kaye and Finkbeiner, 2013). Although it has been suggested that failure in clinical trial could have been predicted at least in some cases using human pluripotent stem cell-based model systems (Antonic et al., 2018), translational success using these models has yet to be established.

Finally, cell-based models cannot reflect the complexity of an organism. For example, investigating systemic effects of local disease, such as poststroke pneumonia, requires animal models (Prass et al., 2003) to complement mechanistic cellular modeling. Another example is the blood-brain barrier (BBB), a highly selective permeability barrier separating the blood from the brain extracellular fluid. Although sophisticated in vitro models of the BBB have been developed in the last decade, drug transport across the BBB and brain-specific drug delivery strategies remain challenging for development of successful treatment strategies (Bicker et al., 2014). Enzymes usually cannot pass the BBB. However, local enzyme replacement therapy in the brain by intrathecal application is a promising strategy for the treatment of patients with metabolic disorders caused by the absence or malfunction of enzymes involved in cerebral metabolism. For example, repeated injections of a recombinant enzyme into the spinal fluid (intrathecal) corrects enzyme deficiency and normalizes lysosomal storage in a canine model of mucopolysaccharidosis (Dickson and Chen, 2011).

Improve rigor of preclinical studies

The lack of reproducibility of preclinical studies and the failure of translation to the clinic have attracted attention for several years (Howells et al., 2014; Ioannidis, 2005; Macleod et al., 2014; Perrin, 2014; Prinz et al., 2011). One important reason is the publication bias toward reporting positive results due to difficulties or missing incentives in publishing negative results (Dirnagl and Lauritzen, 2010; Dwan et al., 2013). Moreover, experimental design (Ioannidis et al., 2014; Neumann et al., 2017), including statistics (Schlattmann and Dirnagl, 2010), has been challenged as a quality problem in preclinical trials. For example, definition and declaration of statistical approaches and endpoint measures need to be performed before preclinical trials are finally analyzed or even started (Dirnagl and Lauritzen, 2011). Whereas clinical trial registries are widely accepted as good clinical research practice, preclinical trial registries are rather uncommon and may need to be established (Dirnagl, 2020). Thereby, posthoc analyses generating hypotheses in an exploratory manner can be clearly distinguished from a primary hypothesis that has been tested in a confirmatory approach. A priori power calculations and sample size considerations, randomized assignment, to groups

and blinding for treatment groups are further important issues that are well established in clinical research but not in preclinical research (Button et al., 2013).

Finally, it has been suggested that bringing the rigor and quality of study design expected in clinical trials to preclinical trials will improve translational success (Dirnagl and Fisher, 2012; Ioannidis et al., 2014; Macleod et al., 2014). This includes better knowledge about the drug and thorough target assessment before starting a preclinical trial (Emmerich et al., 2020). For example, pharmacokinetics might be different between mutants and wild-type mice (Menalled et al., 2010). Confirmation of research findings includes replication of preclinical research in independent laboratories (Fig. 8.2). Using different models will increase the robustness of the observed findings in treatment effects (Menalled and Brunner, 2014).

Endpoint measures are of great importance in preclinical research as well as in clinical research. Therefore endpoints in preclinical research should follow those used in clinical research as closely as possible. For example, HD

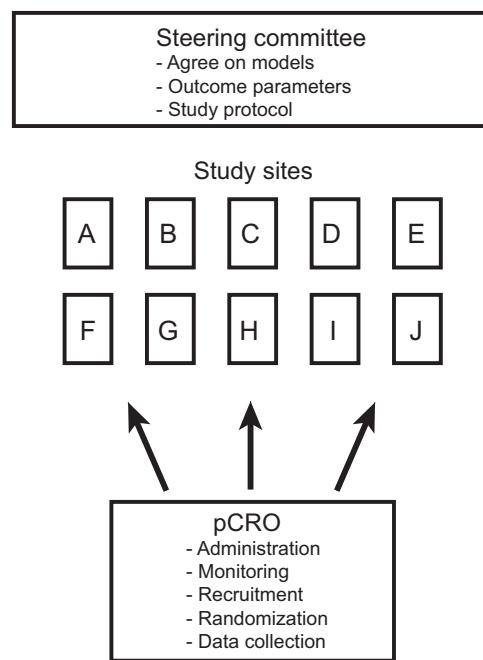


FIGURE 8.2 Modeled after randomized controlled clinical trials, the final stage of preclinical testing is to conduct a randomized controlled preclinical trial (RCPT). A steering committee agrees on the intervention to be tested and all related aspects (e.g., models, outcome parameters). All administrative matters are centrally organized by a preclinical research organization and include objective criteria for the recruitment of study sites, the modes of randomization, collection of the data from the study sites, and central monitoring of all aspects of the trial. Ideally, all study sites are capable of performing the same experiments (i.e., they have access to the same models and equipment). All aspects of the RCPT are monitored by an independent organization. *Reproduced from: Mergenthaler, P., Meisel, A., 2012. Do stroke models model stroke? Dis. Model. Mech. 5, 718–725.*

is characterized not only by motor symptoms but also by cognitive and psychiatric symptoms appearing years before the loss of motor control. These complaints often have a large impact on the patient's quality of life. Although survival is an important outcome measure also in clinical trials, caution is required in translating preclinical findings into clinical findings. In contrast to animals even in preclinical research, survival in patients depends not only on the specific intervention under investigation but also on general care as well as ethical and religious issues leading to end-of-life decisions.

Specific suggestions for improving the predictiveness of preclinical stroke research have been based on accepted standards of clinical research (Bath et al., 2009; Dirnagl, 2020; Macleod et al., 2009; Mergenthaler and Meisel, 2012). To improve internal validity, good clinical research avoids any kind of bias, in particular selection bias (biased allocation to treatment groups), performance (biased care of treatment groups apart from intervention under study), assessment (biased rating due to knowledge of treatment assignment), and attrition (biased handling of protocol violation and loss in follow-up).

Preclinical research in the final stages of translation into clinical trials should follow the guidelines of clinical research by (1) improving internal validity by predefined inclusion/exclusion criteria and primary endpoint(s), randomization, blinding for treatment allocation, and outcome assessment intention-to-treat analysis; (2) improving external validity by studying pathophysiology and treatment strategies in animals of both sexes, old age, and with comorbidities, using disease-related appropriate dosing and treatment windows for the drug under investigation; (3) replicating pivotal findings; (4) publishing negative as well as positive results; focusing on long-term functional outcome, and using metaanalyses of preclinical studies; (5) establishing registries of preclinical studies; and (6) conducting international multicenter phase III preclinical trials (Dirnagl and Endres, 2014; van der Worp et al., 2010). Moreover, preclinical trials need a standardized and humanized modeling of general as well as disease-specific patient care (Mergenthaler and Meisel, 2012).

Summary

In summary, many well-defined animal models for human disease are employed in modern preclinical and pathophysiology-driven research. However, the scientific community across all fields of modern biomedicine has become aware of weaknesses in current preclinical animal modeling. Here, we have outlined several strategies that have already been set into action to overcome the translational gap that is common to all current preclinical modeling of human disease.

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Chapter 9

Biomarkers in the context of health authorities and consortia

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Abstract

While multiple publications have described the use of biomarkers for personalized medicine in early stages, only limited numbers of biomarkers have progressed to use in patient care, owing to limitations in the scientific evidence, the development resources required, and regulatory hurdles. This chapter highlights the development pathway and regulatory requirements to bring biomarkers as in vitro diagnostic tests and as companion diagnostic tests to the market. Further, collaborations of stakeholders in consortia to qualify biomarkers as drug development tools will be highlighted.

Keywords: In vitro diagnostics; companion diagnostics; consortia; biomarker qualification process; health authorities; C-Path Initiative; Innovative Medicines Initiative

Introduction

Biomarkers have played a crucial role in recent decades in life sciences and medical research. Searching PubMed (PubMed 2020) in December 2020 for the terms “biomarkers” and “medicine” revealed more than 326,000 results. In addition, the concept of tailoring the treatment for each patients using biomarkers in combination with drugs, typically known as personalized medicine (more than 84,000 PubMed hits in December 2020) and precision medicine (more than 59,000 PubMed hits in December 2020), has been a key area of research from basic life sciences to applied medicine. However, if we look at the impact on patients, the picture is very different.

In the ideal case, the concept of combining biomarker readouts with decisions on selecting the right drug is known as companion diagnostics. In companion diagnostics a diagnostic test linked to a specific drug is performed to determine whether a specific patient will benefit from treatment with this particular drug. A biomarker and a

drug candidate are developed with a close interplay, and the test eventually comes to the market as companion diagnostics to support medical decisions involving the associated drug. A look at the numbers of approved and available companion diagnostics in the United States in December 2020 (U.S. Food and Drug Administration 2020a) reveals only 44 available companion diagnostic tests, which is a disappointing number on first sight.

This chapter offers insights into the reasons and regulatory processes that lead to such a perceived discrepancy between the number of biomarkers in early research and translational medicine and the rather limited number of diagnostic tests, which affect patient care directly as companion diagnostics. This chapter also shows how biomarkers influence patient care beyond companion diagnostics—for example, as complementary in vitro diagnostic tests (IVDs) or lab-developed tests (LDTs). In addition, the potential of biomarkers to support drug development, as drug development tools, will be explained, and the role of consortia to develop biomarkers in a noncompetitive space will be highlighted.

From biomarkers to diagnostic tests to improve patient care

The vast majority of biomarkers are known in the early research phases of life sciences and translation sciences. On one hand, this can be explained by the general funnel of life sciences and technology development in many areas, starting with many opportunities and candidates in the discovery phase, more and more candidates dropping out during the different phases of development, and ending up with few candidates finally making it to the market or patient. However, beyond this general development funnel, the significant investments necessary to bring

biomarkers as IVDs to the market and especially the oversight by regulatory authorities are significant gatekeepers. Since IVDs can lead to decisions that may have a negative impact on patient health, IVDs are regulated worldwide by national authorities: the U.S. Food and Drug Administration (FDA) in the United States, notified bodies (and in some cases the European Medicines Agency) in the European Union, the PMDA in Japan, the CFDA in China, Health Canada in Canada, and the Australian TGA in Australia, just to name a few.

Depending on the type of diagnostic test and the risk associated with the test, different regulatory and development pathways exist to bring the test to the market. While this chapter focuses on the U.S. and EU regulations, the regulatory authorities in other countries have established very similar regulatory pathways to allow companies to create submission packages, which contain very similar data and evidence.

For bringing biomarkers as diagnostic tests to the market, three different approaches can be considered, which will be explained in more detail in the following sections:

1. IVDs: These are standalone diagnostic tests that can be used for patient management
2. Companion diagnostic tests (CDx): These are diagnostic tests that are directly linked to a specific drug and support the management of patients with respect to this specific drug.
3. CLIA lab tests (often referred to as homebrew tests): These tests are developed in a diagnostic lab or a hospital lab and performed only in this lab.

For the use of biomarkers to support drug development, an additional regulatory pathway has been established, the so-called Qualification of Drug Development Tools or Biomarker Qualification. Hereby, biomarkers are developed that are not linked to a specific drug but help in the development of new drugs, for example for safety monitoring during clinical trials. Owing to this broad and rather generic approach, companies and academia work together in consortia to qualify biomarkers.

In vitro diagnostic tests

The most common pathway to bring biomarkers to patient care is the development of an IVD based on a biomarker. While a biomarker primarily describes a relation between a state and an intervention or abnormal situation, an IVD is a device or a specific assay for a diagnostic device implementing this biomarker. The IVD needs to be well characterized with respect to its technical performance and limitations as well to its performance with clinical samples, and IVDs need to be developed and manufactured under strict quality control. National competent authorities regulate the manufacture, commercialization,

and use of IVDs. Typical examples of IVDs are tests to support the diagnosis of diseases to screen patients, or to stage the severity of a disease. While these tests are often not linked directly to a specific drug (in contrast to CDx), they often support the treatment with medicine indirectly, such as speeding up the diagnosis and referral of patients or enabling more precise diagnoses and thus the selection of the most appropriate treatment option. In addition, tests for the monitoring of therapeutic drug levels to adjust drug doses for each patient—often used in the fields of transplantation, neurology, and antibiotics treatment—are typically treated not as CDx but as IVD tests.

In vitro diagnostic tests and US regulations

In the United States the FDA Center for Devices and Radiological Health (CDRH) oversees the development, manufacture, and commercialization of IVDs. Depending on the risk assessment, the intended use of the device, and the intended target user, different regulatory pathways exist. Therefore the first step of regulatory review is a risk assessment of the IVD, which classifies test into three different risk categories ([U.S. Food and Drug Administration 2020b](#)):

- For low- and medium-risk IVDs, the test will be reviewed under the 510(k) premarket notification ([U.S. Food and Drug Administration 2020c](#)). If an equivalent IVD had been approved before (e.g., using a different device platform), the review process focuses on reviewing the analytical performance of the new IVD such as accuracy, precision, analytical sensitivity, and specificity. Further, the equivalence to the already approved IVD has to be demonstrated, using clinical samples. The review process can be as short as 30–90 days. If no equivalent test had been approved previously, the pathway is called the 510(k) de novo premarket notification ([U.S. Food and Drug Administration 2020d](#)). While the review process is similar to the 510(k) process, the manufacturer also has to present clinical data for the evaluation of the clinical performance of the test. In addition the FDA releases a regulation for this type of test if necessary and identifies any special controls required for future 510(k) submission of equivalent devices. Most diagnostic tests are submitted under the 510(k) and 510(k) de novo pathway. A list of devices that have been cleared is available to the public ([U.S. Food and Drug Administration 2020e](#)).
- High-risk IVDs need to undergo the premarket approval process (PMA). The PMA process is the most stringent review process of the FDA to evaluate the safety and effectiveness of high-risk IVDs, which are considered to be of substantial importance in

preventing impairment of human health or present a substantial potential risk to safety ([U.S. Food and Drug Administration 2020f](#)). Manufacturers have to provide extensive documentation about the technical aspects and analytical performance of their tests and run clinical studies to demonstrate the safety and effectiveness of the test in the relevant indications. However, before running clinical studies to evaluate the new IVD, the sponsor has to submit an Investigational Device Exemption (IDE) to the FDA with analytical data demonstrating that the device performs according to the claims before putting patients into potential danger in the clinical study. Generally, the PMA review process is very detailed and cumbersome for the sponsor. While it can be as short as 180 days, requests for new data and establishing a review panel often extend the timeline to 1–2 years. After approval, further data about the safety and efficacy have to be collected and reported. Manufacturers of IVDs that fall into the high-risk category therefore often consider a specific PMA approval pathway, the so-called single-site PMA (ssPMA), for which the test will be commercialized only by a single laboratory. In this case the manufacturer needs to submit less clinical and analytical evidence, and the review process is shorter. While ssPMAs have been originally established for CDx in rare indications, more and more manufacturers of standalone IVDs take the ssPMA as a way to accelerate commercialization while compiling all evidence for a subsequent PMA submission. The FoundationOne CDx, a next-generation sequencing oncology panel, is an example of a test (in this case a CDx) that has been brought to the market as single-site PMA safety ([U.S. Food and Drug Administration 2020g](#)). Usually, IVDs in the oncology area and CDx tests are brought to the market under the PMA pathway. A publicly accessible database of PMA-cleared medical devices and IVDs is available on the FDA website ([U.S. Food and Drug Administration 2020h](#)).

- All previous described regulatory pathways require that the approved diagnostic test be performed in a laboratory or at least in an environment with a laboratory director overseeing the tests. If point-of-care tests are performed without a lab oversight, for example in the office of a general practitioner, the manufacturer of an IVD test has to apply for a CLIA Waiver on top of the regular 510(k) or PMA submission ([U.S. Food and Drug Administration 2020h](#)). The manufacturer needs to provide additional user study, clinical data, and technical evidence to demonstrate that the device can be operated by users with limited training and that the device provides correct results, like laboratory tests, and will not cause any harm in the hands of an

untrained user. CLIA Waiver applications are considered challenging in the industry, and the failure rate is approximately 50%.

In vitro diagnostic tests and Europe regulations

In Europe the oversight of diagnostic tests is in a transition process, as the European Parliament adopted the new Regulation (EU) 2017/746 on in vitro diagnostic medical devices, also known as the in vitro diagnostic regulation (IVDR), in 2017 with a transition period until 2022 ([European Parliament 2017](#)). Previously, the in vitro diagnostic directive (IVDD) ([European Parliament 1998](#)) was in force, which gave most responsibility to the manufacturer. The manufacturer had to establish a quality system and processes following the ISO 13485 guidance, and these processes and quality system were monitored by a so-called notified body such as the TÜV Süd ([ISO 2016](#)). However, the diagnostic tests could be brought to the market under the CE (Conformité Européenne) label without any regulatory review by a central regulatory authority. Instead, the manufacturer declared that it had followed its processes for development and manufacturing and that the diagnostic test performed according to the package insert. Consequently, diagnostic tests were brought rapidly to the market, sometimes without real clinical data. Yet in a number of cases the performance was inadequate or could even cause risk to health, which triggered the enforcement of the new legislation. The new IVDR follows a risk-based review approach similar to that of the FDA regulations. For medium- and high-risk tests, the notified bodies, which had to requalify for their new increased responsibility, reviewing the nonclinical and clinical data before a test can be commercialized under the CE label. In summary the European regulations have come a lot closer to the U.S. FDA regulation; however, different notified bodies still review the data instead of a single competent authority like the FDA.

Companion diagnostics

Companion diagnostics can be considered the ideal implementation of the concept of personalized medicine or precision medicine, as a CDx can do the following ([U.S. Food and Drug Administration 2018a](#)):

- identify patients who are most likely to benefit from a particular therapeutic product;
- identify patients who are likely to be at increased risk for serious side effects as a result of treatment with a particular therapeutic product; or
- monitor response to treatment with a particular therapeutic product for the purpose of adjusting treatment to achieve improved safety or effectiveness.

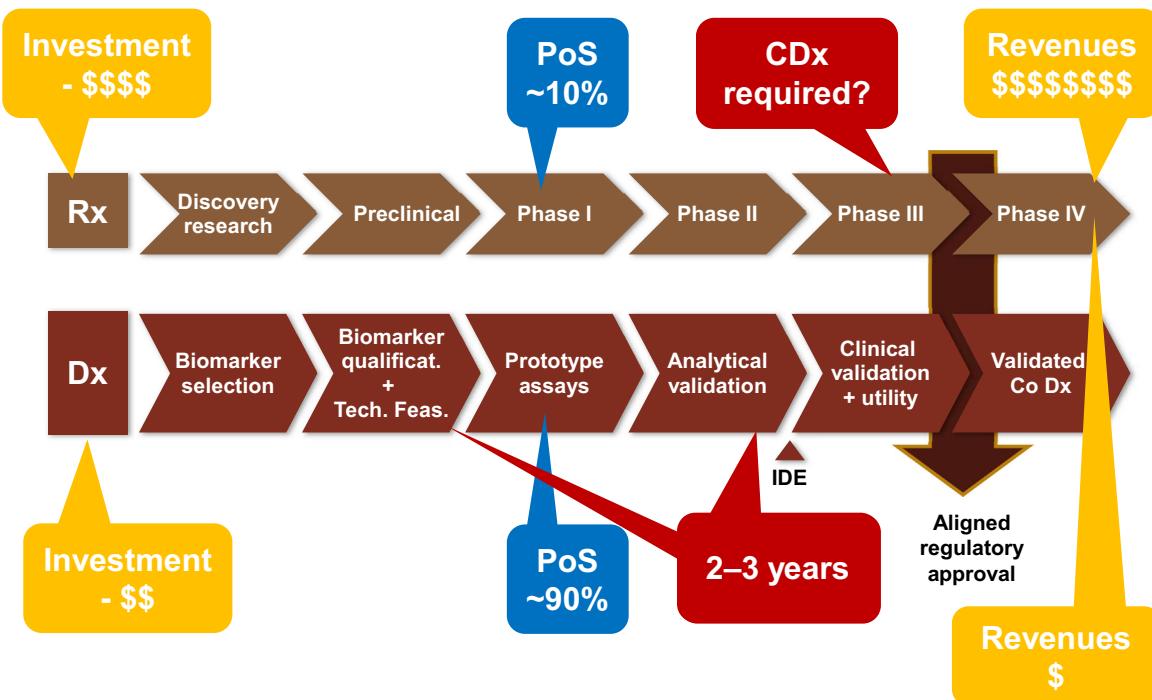


FIGURE 9.1 Ideal coordinated development process of a drug (Rx) and a diagnostic test (Dx) and bubbles showing challenges and discrepancies between the drug and test development processes. PoS, Probability of success; CDx, companion diagnostics.

In December 2020, 47 different CDx had been approved by the FDA ([U.S. Food and Drug Administration 2020a](#)). The numbers of biomarkers behind these tests are lower, as several tests use the same biomarker but implemented with different technologies. Overall, the number of tests is lower than the hype around personalized medicine suggests, which can be attributed primarily to the complexity of the development process, financial and development risks, regulatory hurdles, and challenges in the development process, which will be discussed in the following:

While the guidance of the FDA ([U.S. Food and Drug Administration 2014a](#)) states that a 510(k) as well as a PMA regulatory pathway is possible (based on the risk), a reality check of the approved CDx shows that the PMA pathway is the de facto standard for CDx. Thus significant evidence needs to be generated for the test as well as for the interplay between test and drug and, most important, for the clinical outcome of the test combined with the drug. This may include multiple-arm clinical trial designs (test-positive with placebo and active drug treatment as well as test-negative with placebo and active drug treatment). Before the test is used in clinical trials to manage patients, an IDE needs to be filed, which implies significant development work very early in the test development. The review process requires close collaboration between the CDRH reviewing the test dossier and the FDA Center for Drug Evaluation and Research reviewing the drug dossier. In Europe the review process needs to

be coordinated between the EMA reviewing the drug dossier and a notified body reviewing the test dossier. For a drug manufacturer the requirement of a companion diagnostic test is a significant risk; if the test will not be authorized, the drug manufacturer may not be authorized to launch the drug despite a positive decision concerning the drug.

[Fig. 9.1](#) shows the ideal codevelopment process of a drug (Rx) and of a test (Dx) along the different stages of drug and test development leading to a synchronized review of the competent authorities. Such ideal timing presents multiple challenges between the manufacturer of the drug and the manufacturer of a test, shown in the colored bubbles. The red bubbles show the challenges of synchronizing the development of diagnostic tests and drugs. Only the results of phase III clinical trials will determine whether a companion diagnostic test is required. Because it takes approximately 2–3 years to develop a test that can be used in a phase III clinical trial (starting with a qualified biomarker), significant upfront investment for the test development is required without knowing whether the test will be required at all. Based on the evidence of preclinical data or other similar CDx programs, drug manufacturers need to decide early whether they want to invest in the test development or they take the risk of not developing a CDx. During the last few years an interim solution has been established for many CDx programs. Instead of embarking on a full CDx development, manufacturers develop a single lab clinical trial

assay for the drug phase III study and prepare to launch the test for a SPMA in case the test is required for the commercialization of the drug. Subsequently, a full CDx kit can be developed, and the equivalence to the single-site test can be shown with samples collected during the phase III study with the possibility of launching a full CDx solution later.

Because drug manufacturers are typically not developing IVD tests, they need to partner with professional IVD test manufacturers. These collaborations have proven to be difficult, owing to different financial incentives (yellow bubbles) and different probabilities of success (blue bubbles). Test development requires moderate resources (compared to resources required for drug development), the probability of success is high, but the revenues are very limited. On the other hand, drug development requires significant resources with very limited probability of success but a high revenue potential. These discrepancies render the collaboration of IVD and drug manufacturers difficult, especially because the test will not be useful without a drug and the drug manufacturer does not want to share the revenues with the test manufacturer. Negotiations for this difficult collaboration can be challenging, and drug manufacturers prefer not to require a CDx in contrast to the ideal dream of personalized medicine.

Lab tests (CLIA test or lab-developed test)

In Europe and the United States, labs can develop their own tests. These tests are known as LDTs, homebrew tests, or CLIA tests (CLIA refers to the Clinical Laboratory Improvement Amendments of the FDA). The laboratory director has full oversight and responsibility over these tests, in particular for their analytical performance and clinical performance. While the lab can offer a worldwide testing service using these tests on their own premises, these tests cannot be sold as kits to other laboratories. The quality and performance of these tests have not been reviewed by independent authorities, and customers need to be able to rely on the lab's quality assurances. The LDTs route is a very efficient way for labs to bring innovative tests to the market or bring self-developed tests onto the market (e.g., using mass-spectroscopy systems) when no commercial test kit exists.

While clinical laboratories have a general oversight through national authorities (e.g., the Centers for Disease Control and Prevention in United States), tests have been brought to the market with controversial performance. One of the most prominent cases was the multi-billion-dollar company Theranos, which developed its own devices and promoted LDTs for multiple markers using a droplet of blood. However, investigations revealing that these LDTs performed very poorly to the downfall of

Theranos ([Wikipedia 2020](#)). Because of cases like these, the FDA has announced several times that it will oversee LDTs; however, this oversight has not yet been implemented.

Drug development tools

While the development of diagnostic tests and companion diagnostic tests is a highly competitive field, biomarkers to support drug development are often considered non-competitive, in particular if biomarkers are used to monitor safety. Because these biomarkers are supposed to work independent of specific drugs, a lot more evidence about the performance and limitations of these biomarkers is required compared to IVD and CDx tests. Therefore pharma companies, diagnostic companies, and regulatory agencies have teamed up to create sufficient evidence and qualify these biomarkers to support drug development. This section highlights some of the key efforts in this area.

The critical path initiative

The stagnation of medical product development has been pointed out in numerous contexts, in particular in an impactful report from the FDA back in 2004 ([U.S. Food and Drug Administration 2018b](#)) entitled "Challenge and Opportunity on the Critical Path to New Medical Products." Consequently, public and private stakeholders joined forces to identify the gaps in drug development and to find promising ways to make the medical product development process more efficient, predictable, and productive. The consultation process led to the critical path opportunities list with a special emphasis on creating better evaluation tools for drugs, in particular biomarkers.

With the release of the critical path opportunities list, the FDA aimed to increase awareness of the need for collaboration. Most of the listed opportunities imply considerable resources; therefore single entities (regulatory authorities, single companies, universities, or other government agencies) cannot achieve the stated objectives on their own. Collaboration means openly sharing data to meet the needs of the individual projects within the critical path infrastructure. As one of the first projects of the Critical Path Initiative, the C-Path Institute was founded in 2005 as a neutral ground and catalyst for programs. The first project hosted by the C-Path Institute, the Predictive Safety Testing Consortium (PSTC), initially brought 16 pharmaceutical and biotechnical companies together to exchange data and methodologies and led recently to the first ever submission of newly qualified kidney toxicity biomarkers.

New regulatory pathways for qualification of biomarkers and drug development tools

As a first reaction to the emergence of genomics datasets as potential tools for diagnostic and prognostic purposes, the FDA had to define clear submission processes for drug manufacturers, consortia, and collaborators, which were originally known as voluntary genomics data submission (VGDS) processes. The VGDS process was subsequently renamed the voluntary exploratory data submission (VXDS) process to reflect an evolution and extension to biomarker panels of different kinds, including proteins, metabolites, and imaging types of markers. In addition, the VXDS process allowed health authorities to give regulatory approval for the use of the biomarkers in specific defined contexts.

Subsequent to the first couple of biomarker data submissions under the VXDS guidance, the EMA and the FDA released guidelines to applicants with a well-defined biomarker qualification process incorporating what had been learned from the first successful biomarker qualification as well as lessons learned from several ongoing biomarker qualifications.

The guidance of the EMA ([European Medicines Agency 2009](#)) defines the framework and process how biomarkers are reviewed and could finally obtain regulatory approval. The guidance defines the “CHMP Qualification Opinion on the acceptability of a specific use of the proposed method (e.g. use of a novel methodology or an imaging method) in a research and development (R&D) context (nonclinical or clinical studies), based on the assessment of submitted data,” which is a 190-day formal review process with the goal of regulatory approval. The guideline also defines a “CHMP Qualification Advice on future protocols and methods for further method development towards qualification, based on the evaluation of the scientific rationale and on preliminary data submitted,” which is a useful tool to obtain informal advice at the beginning or during the conduct of biomarker qualification programs. The guidance also proposes the content and formats for presenting data when submitting a biomarker qualification application.

The FDA has released a similar guideline ([U.S. Food and Drug Administration 2014b](#)), called the Qualification Process for Drug Development Tools. In contrast to the EMA, the FDA guideline covers not only biomarkers, but also patient-reported outcomes and animal models. The recommended format and content for biomarker qualification submissions are similar to those of the EMA guideline.

Examples

Until recently, none of the newly discovered entities for monitoring the side effects of drugs had a clear path to

full validation for acceptance by health authorities. In the context of the PSTD, new biomarkers of drug-induced kidney injury were initially negatively affected by skepticism linked to their lack of considerable historical biological evidence, owing to their steady state, even though they had a higher sensitivity than the current clinical standards blood urea nitrogen (BUN) and serum creatinine. In light of the known weaknesses of the standards, the use of the new biomarkers in preclinical and clinical practice provided a body of evidence that was large enough to facilitate interpretation and knowledge of limitations. The PSTD compiled a package for the seven biomarkers consisting of different proposed context of uses and data supporting these intended uses and submitted these to the EMA and the FDA for regulatory endorsement. Following the FDA and joint approval of seven new renal safety biomarkers ([European Medicines Agency 2008](#)), the use of these biomarkers in clinical practice or in nonclinical studies and in translational early clinical studies became attractive for drug manufacturers to add further safety measures and to demonstrate that their drug is safe in humans following mixed preclinical safety signals. Subsequently, several consortia (IMI-SAFE-T), the PSTD, and the PhRMA Biomarkers Consortium worked together to create more evidence for the utility of the new kidney biomarkers in clinical drug development studies. These efforts were rewarded by a broader context of qualified use of the biomarkers to monitor kidney safety in clinical drug studies ([U.S. Food and Drug Administration 2018c](#)).

Besides the first successful biomarker qualification of kidney biomarkers, the EMA and FDA have approved several markers in various disease areas for different levels of implementation in clinical studies. Both the EMA ([European Medicines Agency 2020](#)) and the FDA ([U.S. Food and Drug Administration 2020j](#)) have published lists of qualified biomarkers for different diseases and their intended use in drug development. Besides safety biomarkers, biomarkers to enlarge patient populations in studies focused on diagnosis and prognosis of diseases have also been qualified. For example, a number of biomarkers to enroll patients in a prodromal Alzheimer state into clinical trials on the basis of CSF proteins and PET and NMR imaging have been qualified, as have biomarkers (total kidney volume) to enrich clinical trials with fast-progressing cases.

Collaborations for biomarkers and drug development tools

Public-private partnerships (cooperative R&D agreements)

Collaboration (sharing of research and development data, samples, expertise, and resources) between pharmaceutical

companies and federal agencies such as the FDA is dictated via what is known as a cooperative R&D agreement (CRADA) contract. By definition a CRADA is a legal document describing the terms by which one or more federal parties and one or more private parties join together to conduct specified research or development. In particular, the research protocol, the resources each party will contribute to the research or development effort (personnel, services, facilities, equipment, etc.), and documentation of the financial support are specified in the contract. The private CRADA partner must be able to make significant intellectual and/or technical resource contributions that would not otherwise have been available to the federal agency. Among the issues that are typically addressed in the CRADAs are confidentiality, dissemination of research results so as to appropriately protect proprietary materials, and the intellectual property rights of the federal party and of the private CRADA partner.

In the field of biomarkers and drug development, one CRADA has drawn a lot of attention recently: the validation of preclinical biomarkers of safety, which aimed to identify the process and analysis standards by which pre-clinical safety biomarkers can be validated for their use in regulatory decision making for new drug therapies. Together with the FDA, Novartis conducted preclinical studies with ten reference compounds to evaluate the degree to which a panel of plasma or urine biomarkers (genomics and proteins) predicts renal toxicity before the current standards (serum creatinine and BUN) and possibly even being prodromal ([Novartis 2008](#)). To increase the evidence of the opportunities and limitations of the kidney biomarker, Novartis shared the data with the PSTC consortium that was the basis of the first successful regulatory biomarker qualification.

Consortia

The call for consortia was triggered by the intense and broad challenges described in the FDA's critical path opportunities report. Consortia were created to combine the expertise, resources, and data of their members. This is especially necessary considering the amount of clinical trial data that needs to be reassembled for rare events or qualifying new assays across databases of pooled samples from numerous studies. Because a biomarker is considered applicable for decision with regulatory impact only after it has been shown to be valid or qualified in a large or diverse set of samples and compounds, many pharmaceutical companies have decided to participate in such an effort to have access to a critical mass of samples. At the time of this writing, joining consortia is the only way to validate and qualify a biomarker for regulatory purposes outside of the limited context of use for one specific drug, such as biomarkers for organ toxicity. Also, joining a

consortium means accelerating the generation of a dataset, which will support the biomarker qualification. In the context of the creation of a consortium, several points need to be taken into serious consideration:

- Consistency with applicable antitrust regulations
- Fulfillment of legal requirements such as membership, operations, management, and intellectual property
- Clearly stated missions and goals that cannot be achieved by individual entities
- Definition of the parties' rights and obligations (new and preexisting intellectual property, governance, confidentiality)
- Subscription to common and strict project management ground rules, full commitment to a common goal, and compliance with agreed-upon timelines and plans.

The Critical Path Institute's Predictive Safety Testing Consortium

Although the high-level frame for biomarker qualification was set through the VGDS and VXDS processes, the type of data and amount of biological evidence needed to support a qualification as "known valid biomarker" were not specified. This black-and-white approach—either broadly validating a biomarker or keeping it as an exploratory biomarker—has discouraged sponsors. A new opportunity to validate a biomarker for a specific context of use (fit-for-purpose qualification) was proposed in 2007 by the PSTC in the framework of the first biomarker submission for qualification. This new context was endorsed by the PSTC group working on drug-induced kidney injury markers, which were submitted to the FDA and EMA in July 2007 with claims that the markers in question were either superior or added value to BUN and serum creatinine. The first level of usefulness of these new markers in drug development was expected to be translational between toxicology studies and the first safety trials in healthy subjects and patients. The PSTC is a precompetitive consortium formed under the C-Path Institute (Tucson, Arizona) and was launched in early 2006. The PSTC consists of 19 pharmaceutical companies, one patient organization, and several academic institutions. and it focuses on defining or testing a safety biomarker qualification process for markers of drug-induced injuries in different target organs of high interest that often lead to drug development failures (liver, kidney, vascular system, muscles, etc.). While the PSTC initially focused on the preclinical qualification of safety biomarkers for kidney, liver, carcinogenicity, and vascular systems, which led to the first successful qualification of kidney biomarkers, the consortium teamed up with the Foundation for the National Institutes of Health (FNIH) to run qualification studies for kidney biomarkers in human to extend the FDA and EMA approved context of use of the kidney biomarkers as translational tools to bring potentially nephrotoxic drug into human

studies and evaluate their safety and efficacy profile in humans ([U.S. Food and Drug Administration 2018c](#)).

The PSTC collaboration model has been very successful. Subsequently multiple additional consortia were founded with the C-Path Institute as the driver ([Critical Path Institute Website 2020](#)). Some examples are as follows:

- The Critical Path for Alzheimer's Disease
- The Polycystic Kidney Disease (PKD) Outcomes Consortium
- The Transplant Therapeutics Consortium (TTC)
- The Type 1 Diabetes (T1D) Consortium.

The innovative medicines initiative in Europe

Although the critical path initiative was mainly driven by the U.S. health authorities, government, and federal institutions, the European side, including the European Commission, the EMA, and the European Federation of Pharmaceutical Industry and Associations (EFPIA), started a comparable program several years later, named the Innovative Medicines Initiative (IMI).

IMI projects ([IMI Website 2020](#)) are conducted by public-private consortia comprising a variety of stakeholders, in particular small and medium-sized enterprises (SMEs), academia, research centers, patient groups, public authorities (including regulators), and the research-based pharmaceutical industry. In contrast to the PSTC, public funding is reserved exclusively for the first group (SMEs and academia) to operate cost-effectively, whereas big pharma companies will bear their own costs and contribute in kind. This is a unique public-private partnership between the European Community (represented by the European Commission) and the pharmaceutical industry (represented by the EFPIA). IMI has a total budget of €3.3 billion for the period 2014–2024. As the biggest public-private partnership in life sciences, IMI funds innovative projects addressing the causes of delay or bottlenecks in biopharmaceutical R&D.

IMI currently has multiple ongoing projects with more in the pipeline. A number of projects focus on biomarkers, such as the following:

- TransBioline ([TransBioline 2020](#)) and the predecessor SAFE-T: Qualification of safety biomarkers for drug development
- LITMUS: Qualify biomarkers that diagnose, risk stratify, and/or monitor NAFLD/NASH progression and fibrosis stage
- RHAPSODY: Project for Precision Therapy and Prevention of Diabetes
- TRISTAN: Translational Imaging in Drug Safety Assessment.

A number of additional consortia have biomarker identification, qualification, and development as primary or

secondary objectives. The most extensive program focusing on biomarkers is the SAFET-T consortium ([SAFE-T 2020](#)) and its successor TransBioLine ([TransBioLine 2020](#)). The goal is the development and qualification of biomarkers for liver, kidney, and vascular safety. While the PSTC consortium focused on the preclinical qualification of safety biomarkers, the TransBioLine consortium focuses on the clinical qualification of biomarkers and has been conducting numerous clinical studies to compile evidence of the potential and limitations of the kidney, liver, and vascular safety biomarkers. Since clinical biomarker qualification programs are very resource intensive, TransBioLine and SAFE-T have teamed up with several other initiatives and networks, such as the PSTC consortium, the Center for Drug Safety Science at Liverpool University, and the Spanish DILI registry.

The objectives of the Innovative Medicines Initiative are very similar to the FDA Critical Path Initiative (with the added incentive of financial support). Indeed, the IMI intends to complement the FDA Critical Path Initiative rather than competing with it. For example, the PSCT and SAFE-T consortium have started to work together and combine resources for the qualification of safety biomarkers. Other consortia are looking for partners worldwide, as the qualification of biomarkers is a very resource-intensive task.

The PhRMA Biomarkers Consortium

In the PhRMA Biomarkers Consortium ([PhRMA Biomarkers Consortium Website 2020](#)), which was founded in October 2006, U.S. government organizations and private companies are working to identify and validate new biomarkers for use in the prevention and detection of disease. Companies share early, nonproprietary information on biomarkers. This collaboration is supported by the FNIH, the National Institutes of Health, the FDA, and the Pharmaceutical Research and Manufacturers of America. The consortium covers multiple projects, such as the following:

- Clinical Evaluation and Qualification of Translational Kidney Safety Biomarkers, which collaborates with the PSTC consortium and the IMI TransBioLine consortium
- The Autism Biomarkers Consortium for Clinical Trials
- Longitudinal Proteomic Changes in CSF from ADNI: Towards Better Defining the Trajectory of Prodromal and Early Alzheimer's Disease
- Evaluation of the Utility of Adiponectin as a Biomarker for Predicting Glycemic Efficacy
- Developing Endpoints for Clinical Trials in Community Acquired Bacterial Pneumonia (CABP) and Acute Skin and Skin Infections (ABSSSI)

- Biomarkers Consortium—High Definition Single Cell Analysis of Blood and Tissue Biopsies in Patients with Colorectal Cancer Undergoing Hepatic Metastasectomy
- Biomarkers Consortium—Sarcopenia as a Valid Biomarker for Identifying Individuals at Risk of Disability.

Overall, consortia have proven to be able to compile adequate evidence for the qualification of biomarkers and drug development tools, as is seen in the increasing lists of qualified biomarkers by FDA and EMA.

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Chapter 10

Human studies as a source of target information

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Abstract

An important option for identifying and validating key drug targets is the analysis of data from human studies pointing to targets other than the one for which a particular drug has been developed. As is shown in this chapter, this approach is a particularly powerful component in the toolbox for target identification or validation, as those targets are human ones and thus validated in the best test system possible: the human body. This approach is also termed *reverse pharmacology* or *reverse transcription* and covers results derived from the use of both small organic molecules and natural compounds contained in traditional (“herbal”) medicines. More details on reverse translation starting from human data are given in this chapter.

Keywords: Reverse translation; reverse pharmacology; target identification; target validation; clinical studies

An important option for identifying and validating key drug targets is the analysis of data from human studies pointing to targets other than the one for which a particular drug has been developed. As is shown in this chapter, this approach is a particularly powerful component in the toolbox for target identification or validation, as those targets are human ones and thus validated in the best test system possible—the human body. This approach is often termed *reverse pharmacology* or *reverse transcription* and covers results derived from the use of both small organic molecules and natural compounds contained in traditional (“herbal”) medicines. Biologicals (antibodies) are very target-specific; therefore reverse transcription beyond the establishment of a function of the target apart from the anticipated one is rare. New indications for antibodies typically reflect the known functions of the target, such as their immunosuppressant activities. For example, ustekinumab has been approved for more than one disease: Crohn’s disease and severe psoriasis.

Using old drugs for new purposes: baclofen

The first example is archetypical, in that an unexpected side effect of a particular drug has subsequently been looked at closely and used as a lead observation for the profiling and establishment of a potentially useful target.

Baclofen was developed as a skeletal muscle relaxant and is being used in this capacity, especially in patients with lumbago, spastic disorders (including multiple sclerosis, vertebral trauma with paraplegia, or stroke sequelae), or cramps. It is used orally or even intrathecally. In an entirely different setting, the related pharmaceutical activity of baclofen (gamma-aminobutyric acid receptor type B agonism, or GABA_B receptor agonism) has been used as a proof-of-principle tool to validate a human target. Reflux esophagitis is very common in Western societies, and its major treatment, acid secretion inhibition by proton pump inhibitors (PPIs), such as omeprazole, is effective in only about two-thirds of patients (Coron, Hatlebakk, and Galmiche, 2007; Scarpellini et al., 2016a.). Thus mechanisms other than acid damage have been increasingly investigated as potential causes of PPI-resistant disease. Subsequently, transient lower esophageal sphincter relaxations (TLESR) have been found to be important contributing factors in this regard, and researchers have begun to seek out agents to suppress them. It became obvious that GABA_B receptor agonism seemed to represent an effective principle, at least in animals such as ferrets or dogs. How should this hypothesis be tested in humans? Should researchers wait until a new compound is developed that specifically targets GABA_B receptors in the lower esophageal sphincter to find out whether this mechanism is important in humans as well? In this particular situation an elegant solution was discovered and consequently tested: Baclofen had been used for many years in patients, but no one had done studies on esophageal reflux. Given the medicinal use of this compound, there was no ethical

hurdle to test its effects on human esophageal reflux; therefore experiments were performed in both adults and children. Although larger randomized trials show the limitations of effects in adults, effects in children seem promising, even for therapeutic purposes (for a review, see [Sifrim and Zerbib, 2012](#)). Baclofen is still considered to be a choice for refractory reflux to reduce reflux episodes ([Scarpellini et al., 2016b](#)).

More important than the use of baclofen in reflux treatment, which is not very feasible at least in adults because of its main muscle relaxant and central nervous effects, is the fact that those studies clearly show the following:

- GABA_B receptors do exist and function in the lower esophageal sphincter of humans.
- GABA_B receptors in the lower esophageal sphincter can be reached by compounds in humans.
- Activating GABA_B receptors in the lower esophageal sphincter can reduce TLESR in humans.
- Reducing TLESR by baclofen in humans can reduce esophageal reflux episodes ([Figs. 10.1 and 10.2](#)) and PPI-resistant reflux symptoms ([Koek et al., 2003](#)). Baclofen may even be used clinically in children.

The reader might think the approach failed because baclofen was not highly successful in clinical practice in adults, but this reflects a gross misunderstanding of the approach. It was clear from the beginning that baclofen would not become the ideal new drug to treat TLESR and PPI-resistant reflux. This would be almost impossible, as baclofen was developed to treat spinal cord-related disorders and thus was designed to penetrate into this tissue, which is probably not necessary in TLESR treatment. Its pharmacokinetics was chosen to treat neuromuscular disorders, which tend to fluctuate, and a longer half-life may be desirable in TLESR treatment. Baclofen served only as

a clinically available test compound for which all prerequisites for human use had been fulfilled because of its clinical use in another human indication. It was thus extremely useful for validating the GABA_B receptors in the lower esophageal sphincter as effective drug targets in human disease, and it conceivably raised considerable interest in this target on the part of drug companies. Obviously, baclofen is not ideal for this indication (as it was designed for something else), but investors in industry and funding bodies in academia gained strong confidence in this target on the grounds of the baclofen experience in human studies. Investing millions of U.S. dollars in the lead optimization process is now a relatively safe decision, or at least one that is much less speculative than situations in which target properties in humans can only be extrapolated from information such as gene screens or animal experiments. Even the lead identification phase could potentially be reduced or eliminated, as baclofen—depending on intellectual property issues—might be able to serve as a lead compound.

So far, several companies have been working on this target. A GABA_B receptor agonist may reach the market that has been specifically designed for GABA_B receptors in the lower esophageal sphincter in humans and thus will be less reminiscent of baclofen's actions elsewhere (muscle relaxation). This research led to the development of a peripherally restricted, novel GABA_B receptor agonist, lesogaberan, which unfortunately did not meet its goal to be clearly superior to PPIs ([Shaheen et al., 2013](#)). With TSLERs identified as important biomarkers by baclofen, other receptors were looked at acting in a similar way. Mavoglurant, a selective metabotropic glutamate receptor five antagonist also acting on the lower sphincter, induced a significant decrease of TLESR compared to baclofen ([Rouzade-Dominguez et al., 2017](#)). Thus baclofen has

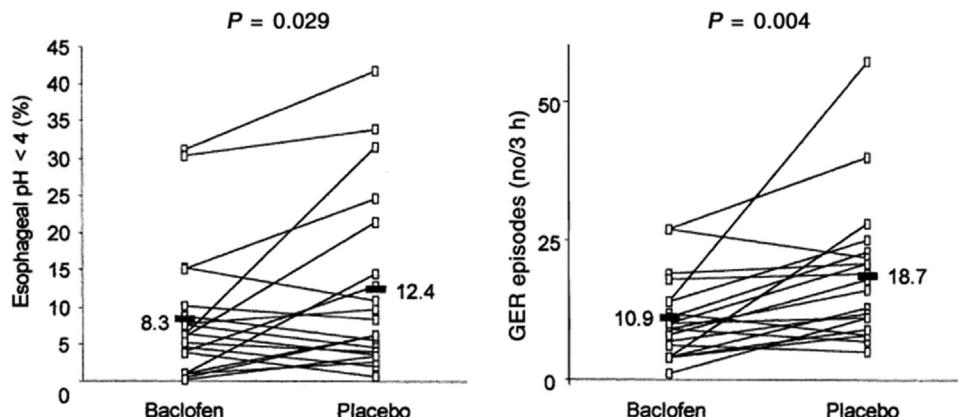


FIGURE 10.1 The effect of baclofen on esophageal acid exposure and on the number of gastroesophageal reflux (GER) episodes. Baclofen significantly reduced the total reflux time and the number of gastroesophageal reflux episodes during the postprandial period. *Reproduced from van Herwaarden, M.A., Samsom, M., Rydholm, H., Smout A.J., 2002. The effect of baclofen on gastro-oesophageal reflux, lower oesophageal sphincter function and reflux symptoms in patients with reflux disease. Aliment. Pharmacol. Ther. 16(9), 1655–1662 (van Herwaarden et al., 2002) with permission from Wiley Publishers.*

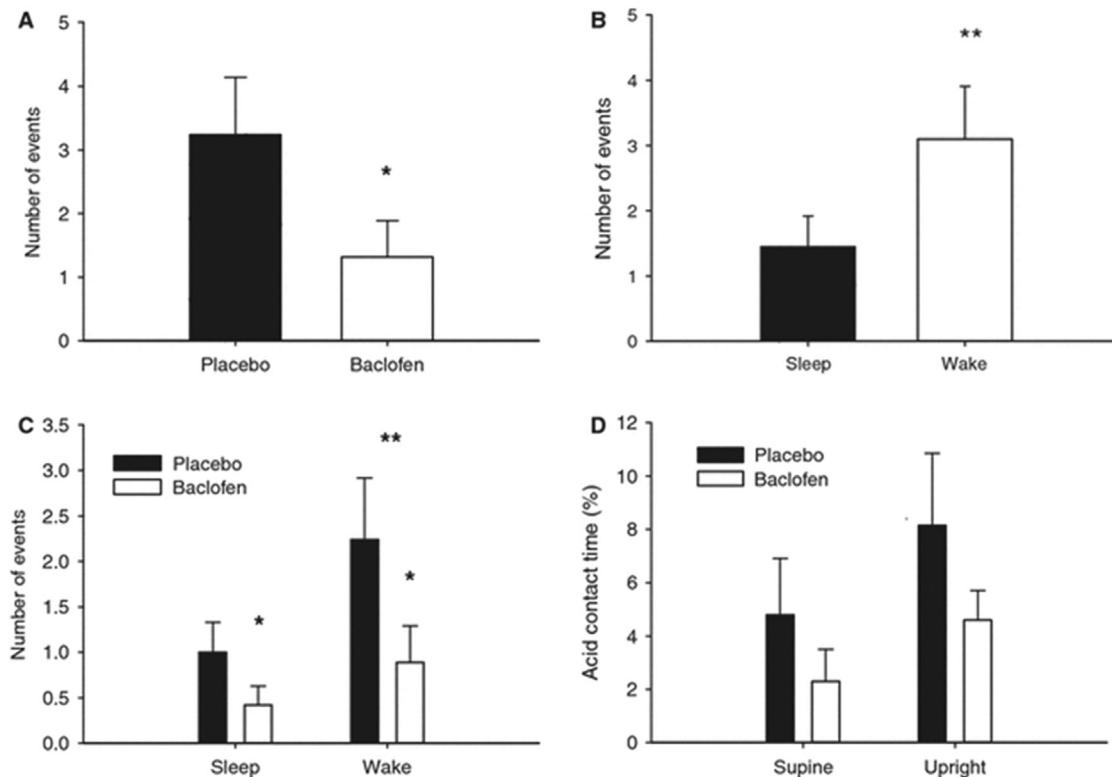


FIGURE 10.2 Reflux outcomes. (A) Number of reflux events in the distal esophagus for the placebo (black bars) and baclofen (white bars) conditions. (B) Number of reflux events during sleep and wakefulness. (C) Number of reflux events during sleep and wakefulness for the placebo and baclofen conditions. (D) Acid contact time (ACT) in the distal esophagus in the upright and supine positions. Data are presented as mean \pm standard error. * $P < 0.05$ placebo versus baclofen. ** $P < 0.05$ sleep versus wake. Reproduced from Orr, W.C., Goodrich, S., Wright, S., Shepherd, K., Mellow M., 2012. The effect of baclofen on nocturnal gastroesophageal reflux and measures of sleep quality: a randomized, cross-over trial. *Neurogastroenterol Motil.* 24, 553–559 (Orr et al., 2012) with permission from Wiley Publishers.

not (yet) been replaced in its exceptional use for PPI-refractory gastroesophageal reflux disease (Roman and Mion, 2018). Interestingly, the lead compound, baclofen, has been studied further and may be used in refractory reflux inducing chronic cough (Xu et al., 2013; Dong et al., 2019), rumination (Pauwels et al., 2018), or hiccups (Chang and Lu, 2012).

This example of experiences from human trials reflecting back to drug discovery and development belongs to a category that is probably best termed *target validation* in humans by a test compound unrelated to an existing drug development program in the particular area.

This means that baclofen as a test compound was not an early but imperfect result of the drug discovery program in the reflux disease area; this category is thus distinct from the category of compounds that are imperfect (e.g., because the half-life is too short for rationalistic use), are novel, and have never been used in humans. It is possible to test such a compound on the grounds of a limited safety package under the guidance of the experimental investigational new drug (eIND) rules by the U.S. Food and Drug Administration or the European Medicines Agency (see Chapter 18, The Pharmaceutical

R&D Productivity Crisis: Can Exploratory Clinical Studies Be of Any Help?).

The scientific question behind this example in the context of this book is “Can this concept be generalized and become a standard tool for drug discovery and development?” The clear answer is to ask yet another question in all drug discovery projects: “If a particular target has been discovered, for example, by chance findings in animals or by genetic approaches, is it possible to test the system in humans using a test compound that can easily be applied to humans because it has already been approved for other purposes?” If the answer is yes, the next question would be “Can the effects in question be discerned or separated from the main effects for which the compound had been developed?” In other words: “Are toxic effects or pharmacokinetic properties compatible with testing in a different system that may require, for example, prolonged exposures or exposures at higher doses?”

The main purpose of this book—the creation of a scientific backbone for translation in medicine—consists of algorithms such as this one that could be used reproducibly and transparently to increase the translational output.

The different categories in this context are discussed at the end of this chapter.

Serendipity: sildenafil

The one of the most prominent examples in the category of serendipity findings is sildenafil. Although it was originally developed as an antianginal or antihypertensive agent, it became obvious during early clinical trials in healthy male volunteers that it had an additional, unexpected effect on erectile function. It would be redundant to summarize the subsequent revolution in this therapeutic area, with congener development, debates about lifestyle drugs, reimbursement, and so on. The main point is the fact that the early clinical trials revealed unexpected results, led to modification of the approval process, and were reflected back into drug discovery. In this case, drug development was mainly restricted to me-too compounds with minor but distinct advantages (toxicity, half-life) over the index compound.

How should we capitalize on this principle? In a scientific context, this is probably the most unreliable and non-systematic—thus nonscientific—approach to obtaining data for reverse drug development from clinical experiences. The main reason is that this chance finding depends on what is called serendipity. By definition it is impossible to establish rules to increase the likelihood of serendipity. Pharmaceutical history is full of examples of serendipitous findings, including the discovery of penicillin and, in particular, psychotropic drugs such as chlorpromazine or imipramine (Ban, 2006). The dependence on serendipity of discoveries in the central nervous system area reflects the tremendous translational difficulties in this context due to the lack of appropriate animal models and robust cross-species biomarkers. Still today, this area seems to be prone to serendipitous findings, such as the recently reported use of serotonergic drugs to treat refractory schizophrenia (Lowe et al., 2018). As this dependence on serendipity is seen to be critically inhibiting progress, modern psychopharmacotherapy should be based on genomic and personalized medicine, thereby moving away from serendipity (Thibaut, 2019).

Is there no way to utilize serendipity? The only reasoning in this regard reflects the fact that the flexibility, open-mindedness, and sensitivity of clinical (and preclinical) researchers toward unexpected findings should be increased. Teaching examples of serendipitous findings might create awareness and inspiration, which could serve as prerequisites to the willingness to sense “strange” results and interpret them correctly. On the other hand, safety findings and unwanted effects will occur unexpectedly in many instances. Defense mechanisms may exist that keep researchers from recognizing such shortcomings, thereby jeopardizing long-term work and big expenditures. Safety issues (see Chapter 22, Pharmaceutical Toxicology)

may represent good examples of attempts to avoid being taken by surprise or becoming a victim of negative or inverse serendipity. If the safety program undertakes to test sensitive and specific biomarkers as early as possible in the developmental program of a drug, surprises will be reduced. On the other hand, the interpretation of safety signals such as increases in liver enzymes (transaminases) requires skills, experience, and open-mindedness, which are key to positive dealing with serendipitous findings.

These examples show that the grid by which serendipitous findings can be detected and thus retained can be tailored and tuned to increase the likelihood of detecting valuable, though unexpected, findings, especially in safety-related issues. This approach may be called *facilitated serendipity* Fig. 10.3).

This includes the following:

- Heating the pot to increase emanations: In other words, utilizing multifaceted trials and multiple read-out strategies, provocation tests, and challenging patients as early as possible as the true target population rather than healthy volunteers, who may be more robust and thus might not exhibit signs of toxicity.
- Increasing the sensitivity of the sensors, that is, making use of better, more sensitive biomarkers, especially for safety issues. Why do liver transaminases ALAT and ASAT, which were introduced into medical practice more than half a century ago with early utility reports from the 1950s, still represent the standard test biomarkers for liver toxicity assessment?

From a scientific standpoint, however, serendipity is the least structured and controlled area in translational medicine. It is not very likely that this will ever change.

Reverse pharmacology

The third paradigm is a far-reaching, backward-oriented translational drug discovery process. The term *reverse*

Heating the pot



More and better sensors
("metal detector")



FIGURE 10.3 Facilitated serendipity.

pharmacology has been utilized especially for this approach. It is based on the fact that many major drug discoveries have been derived from natural products, from which either the drug itself or a lead compound that was refined in the subsequent drug discovery process was isolated.

Historic examples are manifold. They include morphine, which was isolated from poppy plants—specifically from opium, the primary extract—or digoxin, which was isolated from *Digitalis lanata*, woolly foxglove. Even more famous is the use of *Salix alba* extracts containing salicylic acid, which was the starting point for the most successful drug synthesis ever: that of acetylsalicylic acid by Felix Hoffmann in 1897. The most impressive development any drug ever had (Bayer aspirin) was simply a retrograde translational approach starting from observations made by the use of natural products. Salicylic acid from salix and other plants had been used for at least 3500 years by almost all developed cultures; the first known documentation of this is from ancient Egypt and describes a myrtle extract for pain relief. Acetylsalicylic acid was gentler on the stomach than its progenitor and easy to synthesize. In the early twentieth century, acetylsalicylic acid was advertised as an efficient painkiller that did not affect the heart (in contrast to painkillers used at that time). Since then, as a result of clinical observations, we have discovered that acetylsalicylic acid is a potent platelet inhibitor and saves thousands of lives by protecting individuals from heart attacks. This addition of a new indication has inspired other companies to find other platelet inhibitors, such as clopidogrel or prasugrel, which are currently on the market. In this sense, acetylsalicylic acid can also be placed in the category of retrograde translation discussed at the beginning of this section, in that it became a model or test compound to demonstrate and validate a very important target in humans, namely, the platelet in the prophylaxis of heart attacks.

One might think that the approach described in this section is quite outdated and that modern rationalistic technology for drug development has replaced those natural products with small, smartly designed organic chemicals. This is untrue, and one of the clues to resuscitating successful translation may still come from this angle. The most famous recent example is the market approval of exenatide, a glucagon-like peptide type 1 (GLP-1) analog. This compound served as model compound for innovation in the treatment of the current epidemic of diabetes mellitus, a safe and reliable compound to reduce blood glucose and improve metabolic control in type II diabetics. It was developed synthetically from a compound called exendin-4, which had been isolated from one of the only two venomous lizards in the world, the Gila monster, after wide screens of chemical structures and biological effects of lizard venoms. The key idea in the search for an antidiabetic compound in lizard venom was the “clinical”

observation that victims bitten by the lizard may develop pancreatitis, and it was assumed that the venom somehow overstimulated this organ, causing its inflammation. Subsequently, researchers discovered that the active ingredient could be isolated, and its blood glucose-lowering potency seemed to result from its similarity to a human hormone, GLP-1, which stimulates insulin secretion from the pancreas. It took another 15 years to bring the related drug to the market, but it all started from observations of the impact of lizard venom on humans, retrograde translation, preclinical design, and clinical development of the drug exenatide. Its success has come from its unique feature of reducing HbA1C as a measure of glucose control and weight reduction in the same place, with a modern congener—liraglutide—having received one of the rare market approvals to treat obesity (Pastor and Tur, 2019). Insulin supplementation or sulfonylurea drugs are accompanied by weight gain, the last thing one wants to see in obese type II diabetics. The so-called incretin-analogs (or GLP1-analogs) such as exenatide have to be applied parenterally, as they are peptidic drugs. In attempting to overcome this disadvantage, orally applicable drugs inhibiting the degrading enzyme (dipeptidyl peptidase type IV or DPP IV) for endogenous GLP1 were successfully developed. The resulting DPP IV inhibitors (“gliptins,” e.g., sitagliptin) have been introduced into the modern treatment of diabetes mellitus type 2 and represent important principle at present, as they do not induce weight gain or hypoglycemia—the two main down sides of former, insulinotropic therapies. These novel drugs seem to be safe even in the elderly, vulnerable population (Strain et al., 2013; Bethel et al., 2017) underscoring their innovative potential. The superiority of DPP IV inhibitors over the older sulfonylurea drugs has been underlined by a metaanalysis clearly demonstrating higher event-free survival rates for DPP IV inhibitors (Rathmann et al., 2013; see Fig. 10.4).

This example demonstrates that facilitated serendipity may not only precipitate directly useful results, but may also open wide avenues of innovation. The systematic search for drugs from animal venoms has been successfully performed for other areas as well, such as anticoagulation (e.g., hirudin from leeches and bivalirudin as a novel derivative or ancrod from snake venoms, captopril, the first ever inhibitor of the renin-angiotensin system derived from a venom by the poisonous Brazilian viper, *Bothrops jararaca*). Those developments were triggered by the observation that the wounds of victims of animal venoms fail to clot, an easily available, very obvious observation. Because animal venoms need to have strong biological effects, at least if they are to be poisonous to humans, systematic screening has been and is being performed by many companies and academic institutions. The main limitation is the fact that venoms are designed

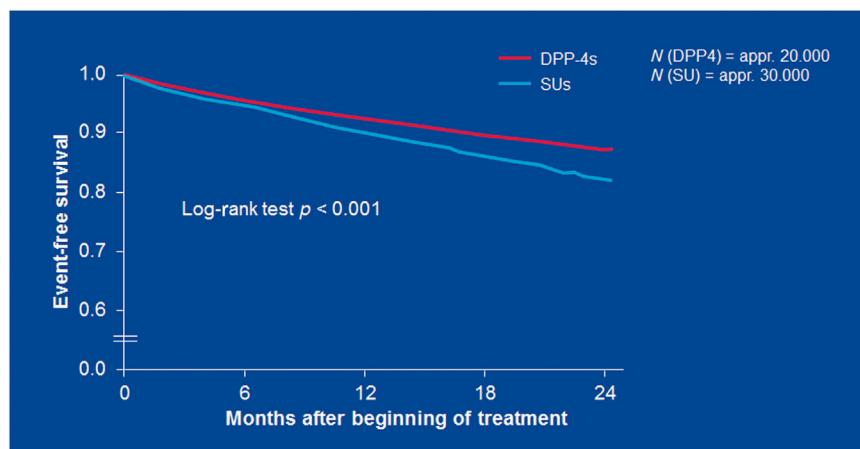


FIGURE 10.4 Kaplan-Meier curves for event-free (macrovascular events) survival over 24 months show significantly fewer endpoint and better event-free survival of patients on DPP IV inhibitors (DPP-4s) versus sulfonylureas (SU). Data are from an IMS analysis. Modified from Rathmann, W., Kostev, K., Gruenberger, J.B., Dworak, M., Bader, G., Giani G., 2013. Treatment persistence, hypoglycaemia and clinical outcomes in type 2 diabetes patients with dipeptidyl peptidase-4 inhibitors and sulphonylureas: a primary care database analysis. *Diabetes Obes. Metab.* 15, 55–61 by kind permission from John Wiley and Sons.

for rapid and short action (for defense and hunting purposes), so treatments for chronic illnesses might only occasionally be derived. Still, there are snake venom approaches aiming at a cure for cancer (Waheed et al., 2017), which seems quite distant from what snake venoms are originally made for.

This shortcoming must not exist for a by far larger source of organic compounds with potential medical importance, namely, those derived from plants. Some plant-derived principles may have begun their career in human treatment, owing to initial intoxication experiences with subsequent dose-ranging trials in early humankind, which made them usable. An even larger asset of natural compounds may act only if used chronically with little or no immediate effects. Such compounds will be much harder to detect than those with immediate actions. This is a fascinating area in which shamanism meets scientific medicine. Undoubtedly, there are medicinal uses of plant extracts, including those cited above. It is also absolutely feasible to assume that there is a lot more to discover from plant ingredients. This approach is being increasingly promoted and advertised by Indian scientists who claim that thousands of years of Ayurveda medicine should have produced useful evidence to be explored by reverse pharmacology. Traditional Chinese Medicine has followed a similar path. There is evidence for *Rauwolfia* use in hypertension (reserpine has been in use for many years, but it is essentially an outdated drug, owing to its side effects) or *Boswellia serrata* (incense resin) in rheumatic diseases or inflammatory bowel disease due to boswellic acids. Even Ayurvedic pharmacoepidemiology has been proposed as a novel research area (Vaidya et al., 2003; Debnath et al., 2017). There is very wide-reaching, cross-cultural evidence that various plants contain anti-tumor necrosis factor alpha (TNF- α) activity (Ichikawa et al., 2007; Mbiantcha et al., 2018). TNF- α is a major mediator in cancer disease, and herbal drugs would certainly garner a lot of interest.

The latter example points to a very scientific way to explore the efficacy of plant extracts and separate true effects from shamanism, which is the biggest threat to the rational utilization of herbal medicines. This transcultural approach depends on the fact that distant cultures would use the same or similar plants for the same diseases if they produce true effects. This means that different societies would independently come to the same medical uses of plants if their observations of biological effects were objective. This is one of the objectives of the *Journal of Ethnopharmacology*, and systematic comparisons of, for example, Chinese, Indian, and Native American habits in this regard (like the one cited previously) could yield valuable results.

It should be mentioned that all drug developments starting from plant preparations are hampered by the difficulties of isolating one or a few active ingredients and by the difficulties of standardizing the composition in regard to the active compounds, especially if they are yet unknown. It is assumed that India and China will be leading countries in the exploitation of this route of reverse pharmacology, which shares important translational aspects.

These examples of backward translational activities indicate five principal approaches in this regard, which are summarized in Table 10.1.

Utilization of the side effects of marketed drugs is amenable to systematic, pharmacoepidemiologic approaches and may become an important addendum in the drug discovery toolbox if properly employed. Using novel drug candidates as eINDs is another option. In general, if drug development has been done in an area in which the target is still largely unknown, as has been the case in obesity drug development in many instances, taking suboptimal compounds rapidly into humans can be very helpful, especially if confidence in a new target can be considerably increased by this test compound. However, proprietary problems (target validation can be used by all competitors) and the relatively modest time gain by the limitation of the toxicology

TABLE 10.1 Evidence from human experiences that can guide drug discovery and development (reverse pharmacology).^a

Approach	Example	Strength	Weakness	Scientific value
Using “old” test compound developed and already marketed for other indication	Baclofen for treatment of reflux disease	No hurdle to human testing as already used	Side effects to be used as main target may be too weak versus main effect and dosing into useful ranges, thus limited	++
Using newly developed test compound for same indication, which is, however, suboptimal as drug	Examples within proprietary protection	Lower hurdle to human testing if used as eIND, limited toxicology testing, may deliver valuable target validation data in humans	May delay development of better drug if decision could be taken without further target validation	++
Serendipity finding in clinical development or use	Sildenafil	Redirection of development still possible	May be overlooked, especially in regard to safety findings	+++ if discovered and correctly interpreted
Natural compounds from venoms and “toxic” plants/mushrooms	Exenatide, digoxin, morphine	Strong biological activities of venoms if poisonous to humans	Mostly acute actions, long-term effects need distant extrapolation	++++
Natural compounds from plants	Salicylic acid	Longstanding, traditional experiences, cross-cultural independent observations may provide objective evidence	Hard to isolate active ingredient, sometimes multiple active ingredients, standardization of dose, shamanism as threat	+−? for newer approaches

^aFor categorization, see text.

package may be offset by the limitation of the respective trial to short exposure times (a maximum of 7–10 days).

Serendipity findings are most resistant to scientific approaches. As was noted previously, increasing the challenges during early human trials and increasing the quality and number of relevant biomarkers in conjunction with open eyes and minds may facilitate serendipitous findings, but this is still unreliable. In particular, such attempts should be maximized for the safety aspects of drug development.

The feedback route starting from natural compounds seems especially viable for strong biological effectors, as seen in venoms or toxic plants (and mushrooms). This route is being systematically exploited and has been successful even in the present day. Exploiting longstanding experiences with nontoxic plants is a cross-cultural task and seems very ambitious given the lack of standardization and multiple ingredient problems inherent to this approach. If the old successes, such as acetylsalicylic acid, could be repeated with new compounds, this area would certainly gain momentum. So far, the majority of attempts have come from researchers in India and China.

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Chapter 11

Target profiling in terms of translatability and early translation planning

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Abstract

If a target gains attention as a potential drug action site, it is of paramount importance to estimate its translational relevance and feasibility. Nowadays, the key question is not target identification (in scholarly terms, one could say that by the time of this writing, all potential targets have been identified in by the human genome cloning project) but target validation. Thus the critical assessment of a potential target is the most relevant and crucial assessment in the pharmaceutical industry, and a lot of this has to do with translational issues. It has become obvious to many in the field that the selection of targets with strong translational validation is key to success. Translational predictivity scoring as described in this chapter may be one of the potential keys for risk estimation and portfolio balancing.

Keywords: Translational risk; predictivity scoring; portfolio balancing; target selection; industrial success; translational success

If a target gains attention as a potential drug action site, it is of paramount importance to estimate its translational relevance and feasibility. Given that 20,000–30,000 human genes have been cloned and that the variable gene products (mostly proteins) may add up to more than 200,000, for example by alternative splicing, it is obvious that there is still no shortage of potential targets. Nowadays the key question is not target identification (in scholarly terms, one could say that all potential targets have been identified by the human genome cloning project) but target validation. Thus the critical assessment of a potential target is the most relevant and crucial assessment in the pharmaceutical industry, and a lot of this has to do with translational issues.

One dimension of this assessment is the so-called druggability, meaning the accessibility of a target to an intervention by pharmaceutical agents. Enzymes may be easily druggable, as they are soluble proteins in many instances. Another prerequisite for druggability is their functional expression

allowing for the assessment of a quantitative structure activity relationship. Druggability also depends on the required effect direction: Activators are harder to find than inhibitors. In addition, a target may be accessible to a known protein (antibody) in vitro, but if it represents an intracellular structure, this target is not druggable if antibodies are the only known interacting partners. It may still be druggable if it may be accessible by small organic chemicals, for example, lipophilic kinase inhibitors easily crossing cell membranes. Receptors within the brain that can be attacked only by proteins may be hard to reach. Receptors requiring hydrophilic compounds may lead to water-soluble but poorly absorbed compounds that would require parenteral therapy, just to name some basic chemical druggability criteria. However, such aspects of medicinal chemistry, though very important in the druggability assessment of a target, will not be further debated here, as this book is focused on translation.

It has become obvious to many in the field that the selection of targets with strong translational validation is key to success. If one would need serial brain slicing to detect the beneficial effect of a drug, for example, against Alzheimer's disease (AD), such a target could lead to success in animal models of the disease, but human testing would have to rely on entirely different testing methods (e.g., imaging or functional testing). These tests would certainly be closer to the desired clinical effect but may not conclusively be predictable from the histological findings in animals.

If—at the time of translational assessment—there are no biomarkers that could bridge the gap from animal to man, the result of the assessment could be to invest in biomarker development, and, if success is sensed in this undertaking, the target would gain enough translational power to be heavily investigated in a full-blown drug R&D project. As such biomarkers would probably benefit various targets, an integrated view of such an investment would be necessary. This means that several companies plus academia should team up to

develop such biomarkers (or basic understanding of a given disease such as Alzheimer's) by joint funding, a powerful but rarely realizable strategy called "precompetitive research".

This example shows that translational assessment of targets—whether drug, device, or diagnostic test targets—needs to be done from day one of a given project. It is therefore strongly recommended to establish a plan for translation (translational medicine plan) as soon as a novel target is discussed for the first time. This certainly applies to commercial ventures, but in academia too, translational assessment should become a constant challenge that cannot be done early enough in any given project. To limit translational failures in projects funded by tax money, this assessment should become an essential part of funding applications in the public domain, and funding agencies should routinely require this. Nowadays, funding applications routinely contain sections on "exploitation," translation, or result dissemination; this typically relates just to a short list of standard technology transfer or publication strategy aspects that do not imply that particular attention has been paid to the translational assessment beforehand in a structured way (see below).

If we accept that translational profiling of a target is a key asset in the biomedical development processes and that it should commence very early on, the main question relates to the dimensions this profiling should have. Obviously, these dimensions depend on the stage of the developmental process.

Essential dimensions of early translational assessment

The main dimensions of translational assessment are summarized in Table 11.1. As soon as a novel potential target is being described, the area of clinical interest should be stated for which a drug should be developed. This is important because it is the only way to define the unmet clinical need as the main driver for innovation and—as has become nearly as important in recent years—reimbursement. Obviously, current drug treatment of arterial hypertension is almost ideal; thus unmet medical need seems low. This may be reflected by the fact that truly innovative antihypertensive agents have not been introduced for decades, while approaches based on medical devices (e.g., renal denervation by renal artery ablation) have gained considerable interest, though success appears to be limited (Stavropoulos et al., 2020). Meanwhile, treatment options for many malignancies and neurological disorders (e.g., AD) are still poor, and unmet medical need is huge.

This assessment also implies that competitors are identified in the generic and proprietary domains. Patent expiries have a strong negative impact, as expensive development programs cannot be refinanced. Cost pressure by reimbursement or insurance mechanisms needs to be extrapolated to the expected market approval time,

TABLE 11.1 Translational assessment dimensions.

Area of clinical interest

Unmet clinical need
Competitors—generic or proprietary—and patent expiry
Cost pressure by reimbursement or insurance mechanisms

Starting evidence

In vitro evidence, including animal genetics
In vivo evidence, including animal genetics (e.g., knock-out, overexpression models)
Animal disease models
Multiple species

Human evidence

Genetics
Model compounds
Clinical trials

Biomarkers for efficacy or safety prediction

Biomarker grading
Biomarker development

Concept for proof-of-mechanism, proof-of-principle, and proof-of-concept testing

Biomarker strategy
Surrogate or endpoint strategy

Personalized medicine aspects

Disease subclassification, responder concentration
Pharmacogenomics

Chemical tractability^a

Lead identification
Lead optimization

IP position and patent expiry^a

^aAlthough not directly linked to translation, these dimensions are essential contributors to project feasibility.

Source: Modified with kind permission from Springer Nature (Wehling, M., 2009. Assessing the translatability of drug projects: what needs to be scored to predict success? *Nat. Rev. Drug Discov.* 8, 541–546).

which contains an assessment of willingness-to-pay for innovation in the health-care community.

From the scientific, translational point of view, obtaining the starting evidence for the validation of applicable and translatable targets is the biggest scientific challenge in this regard. In vitro and animal in vivo evidence for potentially useful effects will be collected, experiments involving multiple species will be compared, and potential human effects will be extrapolated. Animal models for normal physiology and—even more important—pathology (disease models) will be tested, and genetically engineered or genetically inbred animals (e.g., spontaneously hypertensive rats) will be utilized. Certainly, at the very early stages, even those animal experiments are not yet available, and translation assessment remains truly speculative.

From the first day of discussion of a novel target, human evidence for its validation is the most relevant source. Human genetics should be searched meticulously for clues to the genetic impact, as experiments by Mother Nature may guide translational efforts. Genetic association studies are most important in this context, although their reliability or predictivity may be very limited. An example is the polymorphism of leukotriene A4 hydrolase or 5-lipoxygenase-activating protein (called FLAP), which, according to the Icelandic DECODE Consortium, may be relevant to ethnic differences in myocardial infarction incidences ([Helgadottir et al., 2006](#)). The contributions of the different constituents of the 5-lipoxygenase pathway to arterial thrombotic diseases have been quantified in a Spanish population in between ([Camacho et al., 2012](#)). Related studies have sparked interest in drug development with FLAP or other contributors to this pathway as targets, and this has been investigated by several drug companies. Clinical applications in the important areas of cardiovascular disease and cancer for which evidence of pathophysiological contributions of this pathway is available are yet missing ([Steinhilber and Hofmann, 2014](#)). Meanwhile, as is found for the relevance of many genome-wide association studies, the contribution of the 5-lipoxygenase pathway to the pathogenesis of myocardial infarction has been confirmed, but its effect size seems to be small ([Gammelmark et al., 2016](#)), possibly explaining the translational failure of this approach. So far, zileuton is the only marketed drug in this context, but it was approved for the treatment of asthma (United States only). Licofelone has been profiled for the treatment of osteoarthritis, but clinical results were disappointing, and its development was discontinued. Model compounds can be very useful for target validation if they have generated evidence of the importance of a target. As was mentioned above, baclofen used as a muscle relaxant produced effects on TLESR and thus provided strong evidence for GABAB receptors as potential targets in reflux disease treatment. It should be pointed out that this is the main reason for the development of me-too compounds. The first approved drug aiming at a particular target makes translation relatively reliable and safe for its congeners—with the important exception of clinical safety, which may reflect multiple effectors other than the main target, for example, allergies. Still, the seductive reliability of translation for congeners is spurring innovation, and increased reliability of translation for unrelated, innovative developments would be desirable to discourage me-too approaches. In certain cases, however, pharmacokinetic or pharmacodynamic (PK/PD) optimization by second- or third-entry congeners has been considered a successful innovation. Take, for example, tiotropium versus ipratropium in treatment of chronic obstructive pulmonary disease. Tiotropium and other long-acting antimuscarinic drugs (LAMA, e.g., umeclidinium) can be applied once daily rather than four times daily and is more efficacious than ipratropium, but it acts via the same bronchial muscarinic receptors ([Matera et al., 2020](#)).

Clinical trials as part of developmental processes are often published before market approval and are the main source of human evidence.

Biomarkers for efficacy or safety prediction are the major vehicle for translational processes. As specified in the next (Chapter 12, Biomarkers), the availability of cross-species, easily accessible, accurately measurable, and validated biomarkers will largely determine the success of a translational program. Biomarker grading or quantitative predictivity assessment is an essential part of translation planning from very early days. The linear strategy of drug development is not successful if such biomarkers are lacking. A prominent example is drug development in psychiatric sciences (such as treatment for schizophrenia), in which animal models are absent or very weak. The lack of models and thus cross-species (including human) biomarkers has resulted in clinical empiricism rather than rational drug development. Biomarker development is an important element for improving the validity of available biomarkers, and strategies for biomarker development need to be planned from the early stages.

Proof-of-mechanism, proof-of-principle (PoP), and proof-of-concept (PoC) testing needs to be conceptualized at early stages as well. This includes determining the biomarker strategy (which biomarker to use when) and, at later stages, defining surrogate or clinical endpoints to test efficacy. The PoP study design will be the key gating trial for translational processes, and early assessment of the feasibility, validity, and size of such a trial is elementary. For grading purposes, early conceptualizations of even the final endpoints in the last phase III trial may be instrumental if multiple projects are competing for limited resources. If one project will ultimately have to comprise an expensive and long mortality trial because accepted surrogates [e.g., low-density lipoprotein (LDL) cholesterol for statin development] do not exist, another project with an accepted surrogate endpoint may be preferable. Personalized medicine aspects such as disease subclassification, responder concentration, and pharmacogenomics need to be covered as early as possible. This concept addresses the key question of which patients are the most appropriate for a given drug, as classified by disease subtype, stage or severity, and the patient's pharmacogenetic setting. The blockbuster concept is being replaced by a niche buster concept, which exactly reflects this dimension. The current success of drug development in oncology is driven mainly by this responder concentration. The genetic characterization of a malignancy and thus its subclassification will lead to dependence on druggable targets to be hit ([Said et al., 2020](#)). In terms of catch phrases, personalized medicine is on its path to “targeted therapies” or “precision medicine,” meaning specific treatment of genetically defined subpopulations of malignancies. While these strategies are still applied to groups of

patients (e.g., ALK-positive non-small-cell lung cancer to be treated by alectinib or lorlatinib), the next stage will be a truly individualized treatment of cancers by variable drug cocktails (Jørgensen, 2019) designed to hit as many genetic features of an individual patient's tumor as possible. This would lead to different treatments for almost all patients, and first reports on successful application of this strategy are available (Kyr et al., 2019).

As was noted at the beginning of this section, chemical tractability, lead identification issues, and lead optimization issues are very important for a drug project, but they are not directly linked to translation. In an overall assessment, however, they are essential contributors to project feasibility. The same holds true for the IP position (patent strength, freedom to operate, patent expiry).

A translatability scoring instrument: risk balancing of portfolios and project improvement

If translation planning is performed early and if those major dimensions are graded accordingly, the risk of embarking on development of a given target can be estimated comparatively

and competitively. The same holds true for assessment of in-licensing opportunities, and investments should be guided by such objective rules. Developing and validating a scoring system to grade the evidence and facts in a translational planning document should be a future goal of the new scientific approach to translational medicine. A similar scoring system has been developed to grade biomarkers (see Chapter 12, Biomarkers), and this would become an essential part of the overall scoring of the translational potential of a given target.

A translatability scoring instrument is described in Table 11.2 (Wehling, 2009). The individual scores are chosen between 1 and 5, multiplied by the weight factor, and then divided by 100. Any score above 4 is indicative of fair to good translatability. If no data are available, no score will be given in this line. Stoppers (such as no evidence or very weak evidence for animal effects at large) will be rated zero and render the project untranslatable. The biomarker grading score should correspond to the one described in (Chapter 12, Biomarkers), divided by 10.

This score should show the relative importance of translational parameters and help in the translatability assessment. In the pharmaceutical setting, unmet medical need will certainly be very important, but translation can be successful even without any unmet medical need. Me-too drugs have a

TABLE 11.2 Scoring the translatability of an early project.

	Score 1–5	Weight (%)	$\sum (\text{score} \times \text{weight}/100)$
Starting evidence			
In vitro including animal genetics		2	
In vivo including animal genetics (e.g., knock-out, overexpression models)		3	
Animal disease model		3	
Multiple species		3	
Human evidence			
Genetics		5	
Model compounds		13	
Clinical trials		13	
Biomarkers for efficacy/safety prediction			
Biomarker grading		24	
Biomarker development		13	
Concept for proof-of-mechanism, proof-of-principle, and proof-of-concept testing			
Biomarker strategy		5	
Surrogate/endpoint strategy		8	
Personalized medicine aspects			
Disease subclassification, responder concentration		3	
Pharmacogenomics		5	
Sum		100	

Source: Reproduced with kind permission from Springer Nature (Wehling, M., 2009. Assessing the translatability of drug projects: what needs to be scored to predict success? Nat. Rev. Drug Discov. 8, 541–546).

very low translational risk, and scores are very high for them. Yet, due to market considerations, this approach may not be feasible although not jeopardized by translational risk.

The same holds true for chemical tractability (translation can be performed even with suboptimal compounds; see eINDs in (Chapter 10, Human studies as a source of target information) and IP issues. That chapter describes in more detail the overall assessment of a novel target, whereas the score given here is focused on translational items only. It is obvious that arithmetic scoring alone is insufficient, as there could be stoppers (no effects or weak effects in animal experiments at large) that would not be detected by this score.

Obviously, translational dimensions of target validation will change according to the stage of the developmental procedure. With the important transition from animal to human testing, translation of PK/PD modeling will be a major task. Extrapolation of essentials in PK/PD modeling, such as half-life time, volume of distribution, absorption rate, or route of elimination from various species to humans, is described elsewhere, but successful translation in this area is a prerequisite for dose-finding studies. Dose-finding studies are the ultimate necessity for designing human trials that are fit to test the target in humans in a meaningful way, meaning that the doses that are chosen yield the correct exposure to the drug. Thus this piece of translational extrapolation is an essential part of target validation as a process.

Traditionally, drug metabolism and pharmacokinetics (DMPK) studies in animals that are the only source of predictive data for the transition to humans and the corresponding early human dose-finding trials (single ascending dose studies and multiple ascending dose studies) are not seen as central components of the translational process. This separation, being variably expressed among different drug companies, is not logical, and DMPK studies should be integrated into translational medicine efforts.

As a drug project approaches phase II, either the target has been validated by congener or model compounds in humans or the PoP study has revealed that the compound works in principle. This means that the efficacy assessment is based on biomarkers close to the surrogates or endpoints in patients suffering from the disease to be treated. Because those biomarkers are not good enough to become surrogates or clinical endpoints (see Chapter 12), surprising efficacy failures are still not uncommon after this phase.

At this stage, a human target is almost fully validated and just awaits PoC validation representing the last phase before the pivotal phase III (market approval) trial. If the underlying concept is correct, the compound affecting the target in question will ultimately treat the disease as measured by surrogates or clinical endpoints. The large phase III trial is then confirmatory in terms of target validation only and mainly covers safety aspects. These are dominated by

the unknown (or almost unknown) extra-target activities of a compound, such as liver or muscle toxicity that is not linked to the target receptor. In this sense, target validation is finalized in the PoC stage, and thus efficacy translation has been completed successfully if the compound works at this level. It is important to note that the currently large number of antibody new molecular entities (NMEs) is very specific and extra-target (often also called off-target) issues are rare, yet on-target issues may be crucial, meaning that the target interaction (e.g., immune suppression) produces not only desired effects (e.g., flare reduction in autoimmune diseases) but also undesired target effects (e.g., increased infections or tumor rates). However, it is important to note that efficacy validation of a target as a major task of translational medicine is only half the battle. Toxicology or safety translation is usually seen as target related and is consequently neglected; thus accessible toxic effects represent a minority of potential safety issues (as was stated previously). One of the biggest challenges to the drug industry is setting up non-specific test batteries in animal and early human testing to better predict toxic off-target effects. This translational aspect has started to yield considerable novel safety biomarkers or strategies for screening, for example, for novel renal safety biomarkers (Barreto et al., 2018). Established procedures are mandatory screening for human ether-a-go-go related gene (hERG) channel effects (potential of QT interval prolongation and thus proarrhythmic effects) and electrocardiogram changes in early human trials (see Fig. 11.1).

As the science behind translational medicine progresses, scoring will be only one of the possible approaches to judging objectively on translatability issue; in the end it should be rendered obsolete. Peer review and consensus processes would then regain weight as peers and reviewers are being raised to the expert level. At the time of this writing, translational experts are still rare, and consensus processes still seem to lack objectivity in this area.

The translatability scoring instrument reflects the decreasing risk along the developmental path, earlier projects being necessarily more risky than later ones. Thus it may be wise to apply it preferably to compare projects at a similar stage of development. Thereby, drug companies but also public funding agencies or venture capitalists may risk-balance their portfolios in different strata (e.g., preclinical, early/late human). The average risk curve as depicted in Fig. 11.2 shows the progressive decline of risk along the R&D development, with a major value deflection point at human PoC (some authors use this abbreviation for animal testing as well). If human PoC is positive, the value of a project may increase by 100-fold; thus any license holder should aim at earning this value increase and make the best possible prediction of the likelihood of success.

This fact mandates not only the translatability assessment before a speedy rush to PoC, but also the smart use of knowledge on weaknesses of a given project as

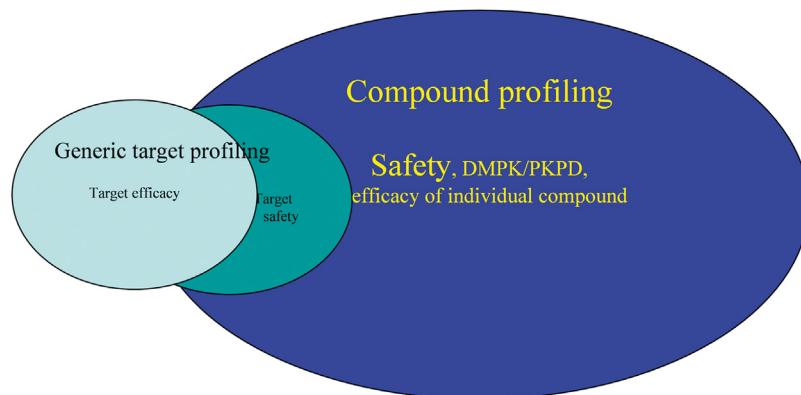


FIGURE 11.1 The target validation process is attached to the overall translational profiling of a compound, safety concerns being the biggest challenge at present. The generic target validation aspects of receptor-mediated efficacy and receptor-related safety concerns are, in most instances, embedded or close to the translational profiling of a given compound. The generic nature of target validation is important for congener compounds within the program of the company hosting the project and for its competitors as well, if published in the public domain, for example, in clinical trial registries, patent applications, or even journals or books. *DMPK*, drug metabolism and pharmacokinetics, *PKPD* pharmacokinetics-pharmacodynamics.

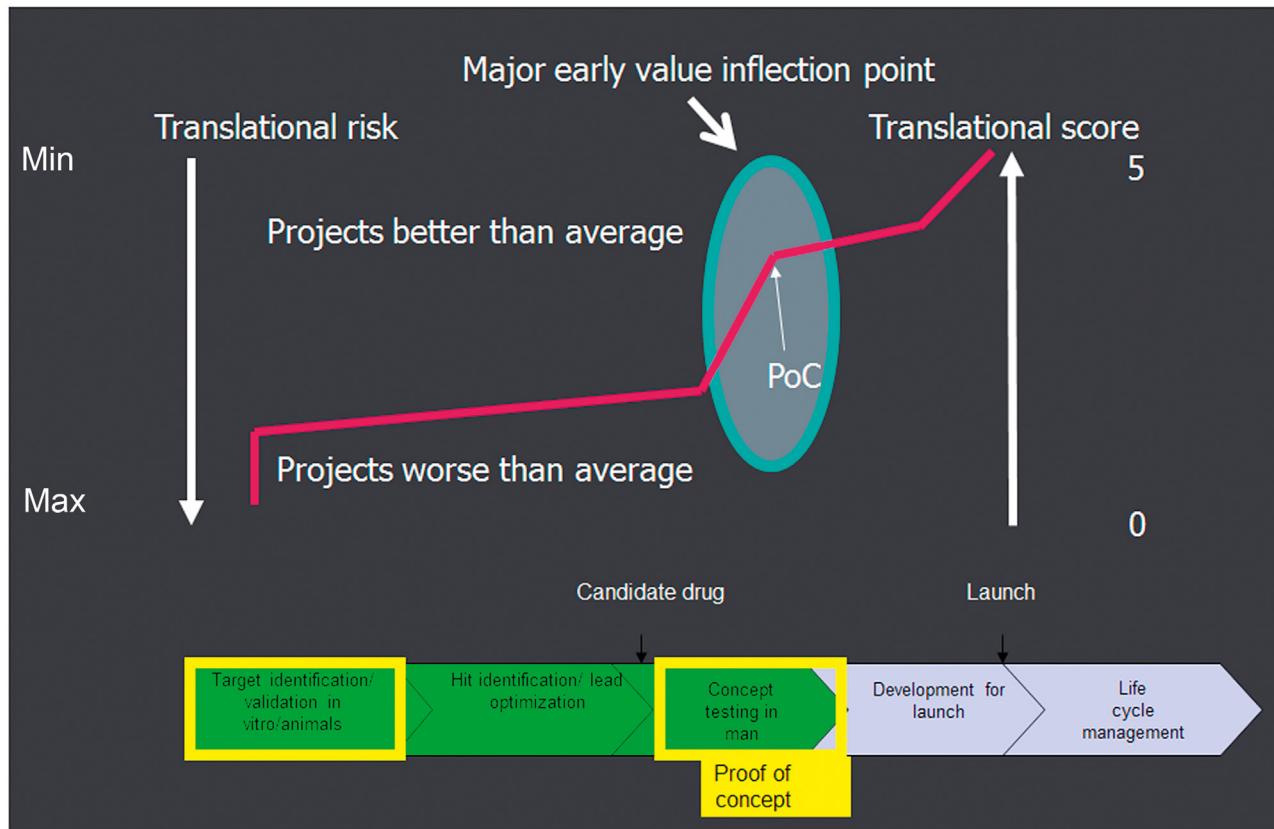


FIGURE 11.2 “Natural” course of translational risk in the pseudolinear drug R&D process. The scores from the translatability assessment instrument are depicted as right-hand ordinate, the inverse level of risk on the left-hand ordinate. Min: minimal; Max: maximal. With kind permission from Springer Nature (Wehling, M., 2009. Assessing the translatability of drug projects: what needs to be scored to predict success? *Nat. Rev. Drug Discov.* 8, 541–546).

identified by the scoring approach. If the score is low, for example, owing to the lack of viable and predictive biomarkers, the money should be spent to develop the biomarkers first before frustration emerges from failed PoC.

In 2017 the authors published an update of the scoring instrument (Wendler and Wehling, 2017). The background were data on 116 NMEs approved by FDA from 2012 to 2016 that were analyzed for the relative

contribution of various parameters in the score to final success or failure. A special emphasis was placed on the contribution of biomarkers, in particular companion biomarkers, study strategies, and animal models in the translational process. In total, six therapeutic areas were analyzed and compared for the contribution of these translationally relevant parameters. The original score weights (Wehling, 2009) had been derived mainly for cardiovascular drugs; they were now compared with oncological, psychiatric, antiviral/antibacterial/antifungal, and monogenetic orphan drugs. These assessments showed that companion diagnostics are important in every field with the exception of psychiatrics. Animal models did not essentially contribute in psychiatrics and antivirals, if compared with the other fields. These findings resulted in increased weights for animal models, biomarkers, and personalized medicine in oncology and decreased weights of animal models, biomarkers, and personalized medicine for psychiatrics. Here, the weights for model compounds, clinical trials, and surrogate or endpoint strategy were increased. For antiviral drugs weights of in vitro data and personalized medicine were increased, and those for animal models were decreased. For antibacterial and antifungal drugs, weights for animal models and personalized medicine were increased, as were weights for genetics and personalized medicine for monogenetic orphans (Table 11.2). As was stated in the original publication, the relative weights of contributions to the translational process should be adapted to experience, stage of the project, and disease area. The proposed weight factors for the therapeutic areas may still be modified according to the stage of the project and personal experiences.

Case studies: applying the translatability scoring instrument to real-life experiences

The translatability assessment tool as described in the preceding (Chapter 10, Human studies as a source of target information), has been applied and evaluated in retrospective case studies (Wendler and Wehling, 2012). The detailed individual score results are given in Table 11.3.

The drugs were assessed starting from a literature search at a fictive date before the phase III trial was commenced. The question to be answered was as follows: “Which translational risk had been taken at the transition from phase IIb to phase III, and how did it correlate with the outcome in terms of market approval?”

One drug, gefitinib, which failed at the first attempt, was assessed twice because new essential biomarker data helped to improve responder concentration.

Dabigatran as treatment for atrial fibrillation was developed at relatively low risk because several features

of the drug, such as safety and the effect on coagulation, had already been investigated in earlier studies. Translatability scores were high for the items’ model compounds, clinical trials, biomarker grading, and surrogates in Table 11.4. This represents the development of a new therapeutic indication of an already-approved drug, with a much lower risk than the development of a new drug for a new application. The overall translatability score, which was 3.77 (Table 11.4), is thus plausible and indicates mean to fair translatability. This drug was subsequently approved for this indication. All other drugs with scores greater than 3.5 were also subsequently approved (ipilimumab, varenicline, gefitinib with improved biomarker), whereas those with lower scores failed (vilazodone, latrepirdine, semegacestat, torcetrapib, gefitinib/first attempt).

The gefitinib story is remarkable as an example of improved biomarkers that were discovered only after the first phase III trial had been started. Several studies showed that only patients with activating mutations in the epithelial growth factor receptor (EGFR) responded well to this treatment (e.g., Lynch et al., 2004). When the novel biomarker (activating EGFR mutation) was introduced, results were improved by far, and the drug was approved. This was reflected in the translatability scoring instruments at the level of clinical trials, biomarkers, and disease subclassification (personalization), as can be seen in increased scores in Table 11.4 after the introduction of this biomarker. Both drugs (latrepirdine, semegacestat) against AD failed. The major implication of the assessment of these two potential AD drugs is the fact that the etiology of AD is not yet understood, and amyloid- β -targeted therapies are likely to attack an epiphomenon. This finding has been confirmed by a multitude of failed, expensive drug developments addressing amyloid- β clearance (Huang et al., 2020). This gap of knowledge is reflected mainly by the lack of a biomarker placed more proximally in AD etiology. The scores for biomarker-related items are 0 for both compounds in Table 11.4; that could be seen as a stopper.

Further studies are needed to analyze the pathogenesis of the disease and to develop suitable biomarkers before any drug should be developed.

The development of torcetrapib was driven by the hypothesis that increasing levels of high-density lipoprotein (HDL) cholesterol should be cardioprotective for the patients. This was derived from epidemiological data, for example, from the famous Framingham study that showed a negative correlation between cardiovascular risk and HDL cholesterol (Gordon et al., 1977). Unfortunately, this biomarker had never been proven to be protective if manipulated by drugs, in contrast to LDL cholesterol, which is one of the best surrogate parameters. Almost any intervention that lowers LDL cholesterol was beneficial

TABLE 11.3 Modified weight factors (in percent) for the items of the translatability score in the different disease areas.

	Original = cardiovascular	Oncology	Psychiatric	Antiviral	Antibacterial/fungal	Monogenetic orphans
Aspect						
Starting evidence						
In vitro data, including animal genetics	2	2	1	9	3	2
In vivo data, including animal genetics	3	<i>1</i>	1	1	5	2
Animal disease models	3	5	1	1	5	2
Data from multiple species	3	<i>1</i>	1	1	1	2
Human evidence						
Genetics	5	5	4	4	4	20
Model compounds	13	<i>10</i>	26	12	12	2
Clinical trials	13	<i>11</i>	26	12	12	10
Biomarkers for efficacy and safety prediction						
Biomarker grading	24	28	10	23	22	23
Biomarker development	13	<i>17</i>	5	12	11	12
Proof-of-mechanism, proof-of-principle and proof of concept testing						
Biomarker strategy	5	5	7	5	5	8
Surrogate or endpoint strategy	8	2	12	7	7	7
Personalized medicine aspects						
Disease subclassification and responder concentration	3	7	2	9	9	9
Pharmacogenetics	5	6	4	4	4	4
Sum	100	100	100	100	100	100

Deviations from the original = cardiovascular score by more than two points are in italic.

Source: From Wendler, A., Wehling, M., 2017. Translatability score revisited: differentiation for distinct disease areas. *J. Transl. Med.* 15, 226. Open access.

for patients. Such evidence was (and still is) lacking for HDL cholesterol, which in Table 11.4 got low biomarker scores, rendering the project high to intermediate risk. It finally failed, yet the insufficiency of the biomarker was not fully acknowledged, but off-target effects (increase in blood pressure) blamed for the excess mortality were observed. It soon became clear that the blood pressure increase was not intense enough to increase mortality if a beneficial effect by the target effects would have been present (Nissen et al., 2007). Despite this clinical failure, which is the ultimate evidence against translational success, a congener drug, dalctrapib, was taken into phase III. It also failed although this drug, being devoid of off-target effects, at least did not increase mortality. In the scoring system the score went down from 1.95 for torcetrapib to 1.06 for dalctrapib as the item's "model

compounds, clinical trials, biomarker strategy" turned to 0 and "biomarker strategy, surrogate/endpoint strategy" to only 1 (from 5 and 4, respectively). Thus there were stoppers (0), so the expensive phase III trial was performed at the highest possible risk. This assessment was done prospectively and, unfortunately, proved to be correct. This drug did not reach the market, and anacetrapib, though producing weak cardioprotective effects (Grabie et al., 2019), was also not approved.

With the exception of this last case, the cases presented were retrospective ones; the prospective validation of the tool is still missing. This would probably take 10–15 years, given the slow cycle time in drug research and development. However, the results are referenced and plausible. Therefore it is hoped that this structured approach to translatability assessment would be adapted

TABLE 11.4 Assessment of translatability for eight drugs [according to Wehling (2009)].

Compound	Dabigatran	Ipilimumab	Gefitinib	Gefitinib ^a	Vilazodone	Latrepirdine	Semegacestat	Torcetrapib [37] ^b	Varenicline [37]
Aspect									
Starting evidence									
In vitro data, including animal genetics	0.1 [4]	0.06 [38]	0.1 [6, 7, 39, 40]	0.1 [6, 7, 39, 40]	0.08 [41]	0.06 [42–44]	0.1 [12]	0.1	0.1
In vivo data, including animal genetics	0.15 [4]	0.06 [45] [46]	0.15 [6, 7, 14]	0.15 [6, 7, 14]	0.12 [41, 47, 48]	0.06 [42, 49]	0.15 [12, 50]	0.15	0.15
Animal disease models	0.09 [51]	0.06 [1, 52]	0.09 [1]	0.09 [1]	0.06 [41, 47, 48, 53]	0.06 [49]	0.03 [18]	0.12	0.15
Data from multiple species	0.15 [4]	0.09 [46]	0.06 [54]	0.06 [54]	0.06 [41, 55]	0.03 [42]	0.15 [12]	0.03	0.15
Human evidence									
Genetics	0.05	0.05	0.05	0.25 [8–10, 22, 28]	0.05	0.05	0.25 [56–59]	0.05	0.05
Model compounds	0.52 [60]	0.52 [61]	0.13 [54]	0.13 [54]	0.65 [62–64]	0.13 [42, 43, 65, 66]	0.13		0.65
Clinical trials	0.52 [31, 67]	0.65 [68–74]	0.26 [24, 25, 75–78]	0.65 [8, 9, 24, 25, 75–81]	0.26 [15, 82]	0.13 [15, 42, 83]	0.39 [50, 84, 85]	0.26	0.52
Biomarkers for efficacy and safety prediction									
Biomarker grading	0.96 [35, 36]	0.96 [5]	1.2 [24, 25]	1.2 [8, 9, 24, 25]	0.72 [26, 27]	0	0 [23]	0.48	1.2
Biomarker development	0.26	0.52 [5]	0.13 [24, 25]	0.65 [8–10, 22, 28]	0.13 [26, 27]	0	0 [23]	0.26	0.52
Proof-of-mechanism, proof-of-principle, and proof-of-concept testing									
Biomarker strategy	0.2 [35, 36]	0.2 [5]	0.05 [24, 25]	0.25 [8–10, 22, 28]	0.15 [26, 27]	0	0 [23]	0.1	0.25
Surrogate or endpoint strategy	0.4	0.4 [5]	0.24 [24, 25]	0.32 [8–10, 22, 28]	0.24 [26, 27]	0	0 [23]	0.16	0.32
Personalized medicine aspects									
Disease subclassification and responder concentration	0.12	0.03	0.03	0.15 [8–10, 22, 28]	0.03	0.03	0.03	0.09	0.03
Pharmacogenetics	0.25	0.05	0.05	0.25 [8–10, 22, 28]	0.05	0.05	0.05	0.15	0.05
Sum	3.77	3.65	2.54	4.25	2.6	0.60 (0)	1.28 (0)	1.95	4.14

^aAfter the development of the pivotal biomarker (EGFR mutation status).

^bNumbers in brackets refer to citations in the paper; though they are not referenced here, this should underline that those scores are based on data in the literature.

Source: From Wendler, A., Wehling, M., 2012. Translatability scoring in drug development: eight case studies. *J. Transl. Med.* 10, 39.

by the key stakeholders. As has been said, it is open for modifications regarding the items and the (better) weighing factor, and may be developed to be more specific for different therapeutic areas. In cancer projects, for example, the weight of personalization appears to be much higher than has been proposed here. Conversely, the uniform score allows for transtherapeutic comparisons that are becoming increasingly important. At least a common understanding seems to be gaining ground in that gut-feeling approaches are outdated and structured, transparent, reproducible, and citable procedures should be instituted at large.

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Chapter 12

Biomarkers

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Abstract

The most generic definition of biomarkers is based on their role as descriptors or measures of biological systems. Thus anything that one can use to describe or measure features of a biological system is a biomarker. Biomarkers may be obvious measures such as blood pressure or heart rate in humans or less obvious measures such as hair color or cell cycle stages, gene polymorphisms, life or death, and ability to fertilize and produce offspring. In biomedical sciences it is helpful to focus the definition of biomarkers on their operational value, as critically selecting what to look at or measure is indispensable if scientific approaches to drug or medical device development are to succeed. The types of biomarker and, their classification and usability are described in this chapter.

Keywords: Biomarkers; biomarker classification; scoring system for biomarkers; biomarker development; biomarker case studies

Defining biomarkers as very important contributors to translational science

If one defines the aim of drug or medical device development as the prevention, palliation, or cure of diseases, there is an obvious need to measure the impact and relate it to disease stage, types, and severity. This need is the basis for the development of the concept of biomarkers. As with translational medicine in general, biomarkers are anything but new; they have been used at all times by scientists trying to describe biological systems. The most generic definition of biomarkers is based on their role as descriptors or measures of biological systems (Table 12.1, definition 1). Thus anything that one can use to describe or measure features of a biological system is a biomarker. Biomarkers may be obvious measures such as blood pressure or heart rate in humans or less obvious measures, such as hair color or cell cycle stages, gene polymorphisms, life or death, and ability to fertilize and produce offspring. In biomedical sciences it is helpful to focus the definition of biomarkers on their operational value, as

TABLE 12.1 What is a biomarker?

1: Generic definition: Biomarkers describe or measure biological systems
2: Operational definition for therapeutic development: A biomarker relevant to treatment development describes or measures
Disease
Type
Subtype
Severity
Activity
Stage
Impact of intervention on key biological variables
Linked to disease, pathophysiology, or outcome
Linked to safety

critically selecting what to look at or measure is indispensable if scientific approaches to drug or medical device development are to succeed. Under these operational auspices the definition is limited in that biomarkers must reflect biological effects induced by or linked to disease and/or therapeutic interventions and must be the main tools for predicting and describing their efficacy and safety (Table 12.1, definition 2; for a further discussion, see Frank and Hargreaves, 2003; Strimbu and Tavel, 2010; Sarma et al., 2020). Biomarkers with potential relevance to drug or medical device development are likely to be used if they are easily accessible and tightly linked to disease and its modification by intervention (for classification and grading, see Subchapters “Classes of Biomarkers”, “Development of Biomarkers” and “Predictivity Classification of Biomarkers and Scores”). In this context, serum parameters (e.g., the primary product of an enzyme that is inhibited by the drug), generic

markers of disease severity [e.g., high-sensitivity C-reactive protein (CRP) and other markers in inflammatory processes (Schonbeck and Libby, 2004; Pereira et al., 2014; Dulai et al., 2019)], histology (especially in cancer), and particularly imaging [e.g., plaque morphology in drugs affecting atherosclerosis, liver fat content as a safety biomarker, positron emission tomography (PET) (Bao et al., 2017) or neuroimaging in Alzheimer's disease (Dekkers et al., 2020; Leuzy et al., 2014)] are frequently used as biomarkers. Biomarkers may be as obvious as low-density lipoprotein (LDL) cholesterol measurements in the development of lipid-lowering drugs or arterial blood pressure in the development of antihypertensive agents, but at preclinical stages and, in particular, at the critical transition phase to human trials, they may not be easily identifiable. Rather, a large program may be necessarily devoted to the discovery and validation of novel markers because the existing ones are not good enough. “Omics” approaches (e.g., genomics, metabolomics)

may generate huge panels of biomarkers; as stated in Chapters 2 and 3 in greater detail, the huge number of measures (“spots”) aligning into patterns may create more problems (mainly statistical in nature) than solutions to discovery of reliable drug effects (Bilello, 2005; Scala et al., 2019). The validation process of biomarkers resembles the drug development process and is described in Subchapter “Development of Biomarkers”.

How should biomarkers be used? Fig. 12.1 illustrates the three key questions to be answered during the drug discovery or development process. They are related to three types of testing:

1. Proof-of-mechanism (PoM) testing: Does the biomarker hit the target?
2. Proof-of-principle (PoP) testing: Does the biomarker alter the mechanism? In other words, does the biomarker change the parameters related to the disease, regardless of whether it can be proven that the disease is beneficially affected?

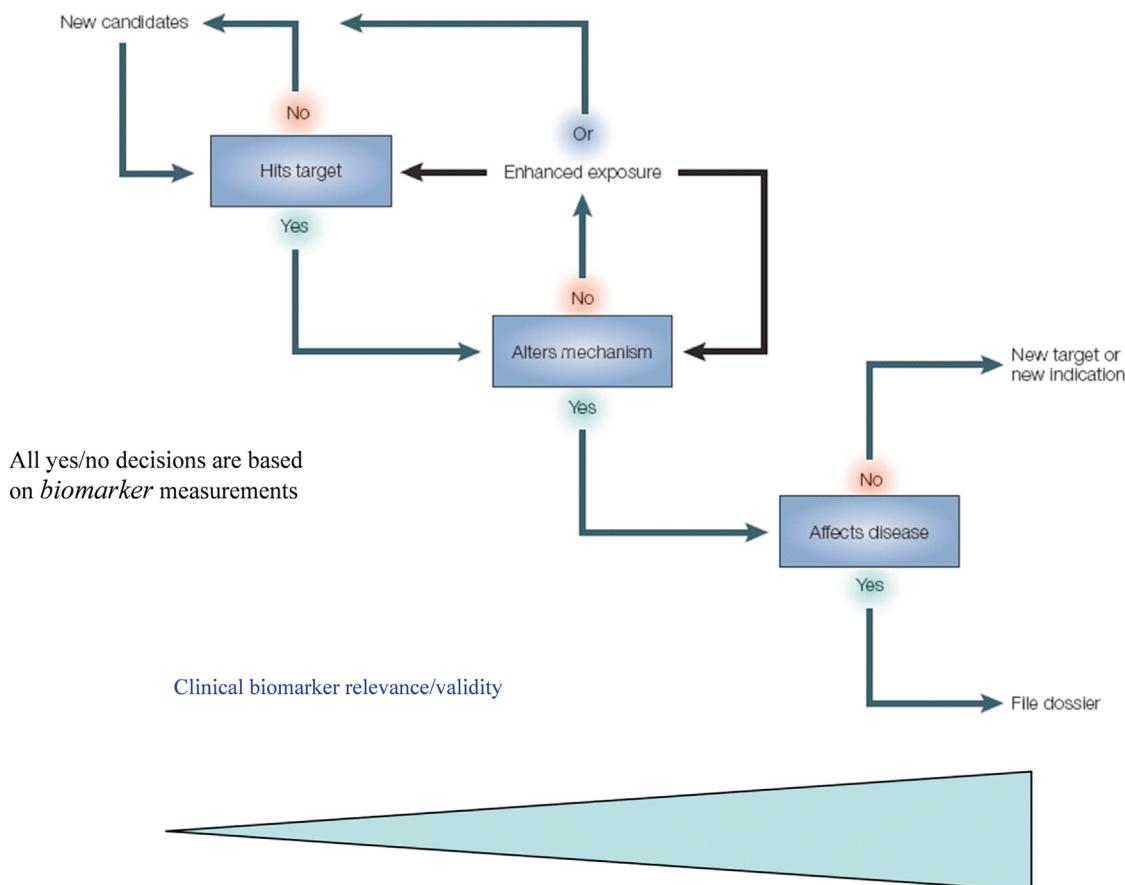


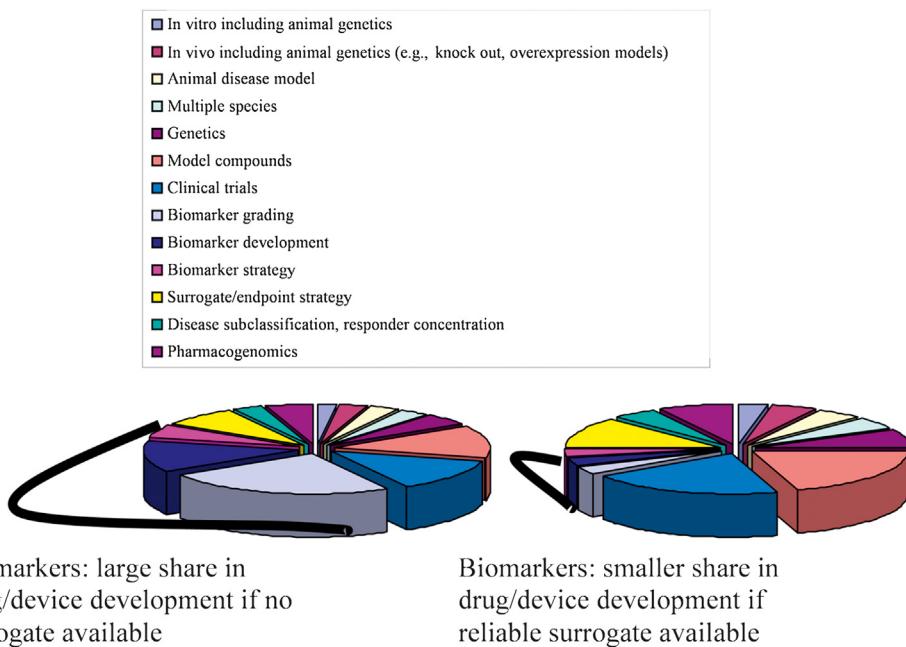
FIGURE 12.1 Impact and remits of biomarkers in drug development. The essential questions to be answered in the drug development process are related to PoM testing (Does the biomarker hit the target?), PoP testing (Does the biomarker alter the mechanism? In other words, does it change the parameters related to the disease, regardless of whether it can be proved that the disease is beneficially impacted?), and PoC testing (Does the biomarker affect the disease? In other words, does the drug actually treat the disease as needed?). Modified from Frank R., Hargreaves R., 2003. Clinical biomarkers in drug discovery and development. *Nat. Rev. Drug. Discov.* 2, 566–580, adapted by permission from Springer Nature, © 2003.

3. Proof-of-concept (PoC) testing: Does the biomarker affect the disease? In other words, does the drug actually treat the disease and benefit the patient?

All yes-or-no, program-modifying decisions are based on appropriate biomarker measurements. The most obvious biomarker at the first level is a direct product of the receptor or enzymatic activity to be affected by the drug, for example, leukotriene LTB4 for inhibitors of 5-lipoxygenase. A typical biomarker for the second level would be closely linked to the disease; for example, generic inflammatory markers would be used if inflammatory bowel disease treatment were the aim of drug development. Finally, biomarkers for the last stage would directly describe the clinical endpoints by which disease activity is clinically assessed, for example, biomarkers that describe the clinical symptoms of inflammatory bowel disease (stool frequency and consistency), histology, complications (fistulas, need for surgery), or even disease-related mortality. This example demonstrates that the clinical relevance and validity of biomarkers increase at each stage of development and that they increasingly resemble or are identical to the clinically used categories for disease grading, staging, or activity assessment. Those criteria represent biomarkers as well. If translation from preclinical to clinical stages is one of the core processes determining the success or failure of drug or medical device developments, then biomarkers are the core of the translational process. The validity of translational projections is closely tied to the predictive value, robustness, reproducibility, and accessibility of the central biomarker(s) on which go-or-no-go as well as program-modifying decisions in project developments are based. The correct choice and assessment of biomarkers therefore may be seen as the key contribution of translational medicine in general. Successful or failed translational processes, as described in Chapter 32, have a lot to do with the choice and assessment of biomarkers. Take, for example, the aborted development of torcetrapib (discussed in greater detail in Chapter 32). There has been considerable interest in raising high-density lipoprotein (HDL) cholesterol levels, and this CETP inhibitor was a powerful tool for achieving this effect. Unfortunately and unexpectedly, mortality was increased when torcetrapib was used. Apparently, HDL cholesterol as such is not a good biomarker for describing and predicting the beneficial intervention in this area. Looking back, it seems that HDL functionality (as measured by nitrosylation), rather than merely its concentration, should have been determined and would probably have predicted the drug to fail at the cost of patient lives. Of course, the nontarget effects of the drug, especially on blood pressure and aldosterone production, have been blamed for the failure, but this has been belied by testing the direct congener, dalcetrapib, which also failed ([Schwartz et al., 2012](#)).

Thus the inherent value of a biomarker or a panel of biomarkers is the most precious asset in translational processes. There are areas of biomedical research in which the quality of biomarkers is weak in general, at least when it comes to animal or human translation. In particular, this holds true for psychiatric drug developments, as animal models for complex psychiatric syndromes without clear morphological changes are lacking. This applies to common disorders such as schizophrenia and depression as well.

The opposite is true for antibiotic agents. Because the pathogens may be treated *in vitro* (either directly in cultures or, in the case of viruses, grown in cell cultures), efficacy translation is very predictive even before animal experiments have been conducted. Obviously, efficacy prediction from test tube results to humans is quite good because the biomarker, microbial growth, is very closely tied to the disease induced (e.g., in bacterial angina lacunaris, the bacteria are killed and the disease is healed). Although *in vivo* conditions can be different from test tube conditions, especially with regard to target accessibility (e.g., central nervous system infection, the blood-brain barrier), *in vivo* growth and growth inhibition are generally in line with *in vitro* results. The main problem remaining is the challenge of safety issues, which are not different from those in other projects, as safety for small molecule antibiotics is mostly unrelated to the test tube but entirely dependent on the host organism. Apparently, many novel antibiotic drugs “die” even after market approval (e.g., trovafloxacin, grepafloxacin, and telithromycin in Australia), mostly as a result of safety concerns. Cardiovascular projects are claimed to have a similarly positive scoring, but going by the very rare recent market approvals in this segment, this figure in the plot seems to be overrated. Accepting the pivotal role of quality biomarkers for successful translation, it is crucial both to develop assessment strategies for biomarkers to define their predictive quality and, if gaps or suboptimal biomarkers become identified, to promote the development of novel biomarkers. The latter is pertinent to almost all projects in which novel targets are addressed. In general, little is known about how those targets are linked to the disease and whether biomarkers other than the clinical endpoints can be trusted as the basis of decisions on heavy investment into late-stage clinical trials (especially phase III trials, which can cost up to several hundred million U.S. dollars). There are few situations in which surrogates (a term that is defined later in the chapter) or clinical endpoints are easy to obtain and can be utilized early in clinical development. Take, for example, LDL cholesterol (unlike HDL cholesterol), which is an accepted surrogate for lipid-lowering agents (i.e., proof of efficacy can be given by a simple blood test rather than by clinical endpoints such as myocardial infarction



Biomarkers: large share in drug/device development if no surrogate available

Biomarkers: smaller share in drug/device development if reliable surrogate available

or death). Thus there was no need for the development of biomarkers for early efficacy testing, as this one could be determined in subjects in all clinical trials, including early-stage trials. The situation is quite different in the development of other antiatherosclerotic drugs, which are mainly aimed at inflammatory processes (e.g., FLAP inhibitors or canakinumab, a monoclonal antibody targeting interleukin-1 β). The ultimate proof of efficacy will be derived only from huge clinical trials on morbidity (cardiovascular events, such as myocardial infarction, stroke) or even mortality. This approach resulted in positive endpoint data for canakinumab (Ridker et al., 2017), although high drug costs prevented the company from filing for market approval in the atherosclerotic indication.

Obviously, biomarkers for earlier clinical stages of development will have to be defined that provide the best evidence that a major endpoint trial is reasonably likely to yield positive results. At present, combined biomarker panels comprising imaging and serum inflammatory parameters are seen as favorites in this therapeutic area (atherosclerotic diseases). This example should show that investing in biomarker development and choice can vary considerably and may excessively increase the risk of a drug project. The feasibility check on a given drug project should therefore include the biomarker status—the extent to which predictively powerful biomarkers are available or need to be developed. Development not only costs money but also carries considerable risk of failure, as is the case in all developmental projects.

Fig. 12.2 summarizes the central role of biomarkers in drug or medical device projects. The investment depends

on the availability of good surrogates, which diminish biomarker work dramatically but are rarely available.

Classes of biomarkers

Because the definition of biomarkers used in this book is very broad, classification of biomarkers seems desirable. It is obvious that the function and value of a biomarker can be very different; for example, death or life is a very clear and serious biomarker, whereas other biomarkers such as appetite are quite diffuse and hard to standardize and measure. Some biomarkers just describe mechanistic aspects of physiology or pathophysiology, such as blood pressure or heart rate, and—by themselves—are not the ultimate goal of treatment, which is reduction of cardiovascular events such as myocardial infarction or stroke. However, we know that having an effect on those mechanistic biomarkers is—at least in general—associated with altered clinical outcomes. Other biomarkers that have been thought to be closely linked to disease severity have been disappointingly vague or weak predictors of outcome (e.g., uric acid).

Thus the inherent value of a biomarker as a translationally relevant predictor is widely variable, and its assessment is an obvious—if not the most important—task in translational medicine. Attempts in this direction have been made early on, for example, by Rolan et al. (2003), who classified the biomarkers by their mechanistic character, such as genotype or phenotype or target occupancy (see Table 12.2).

This approach consists of a seven-point classification based on the location of the biomarker in the sequence of

events from underlying subject genotype or phenotype through to clinical measures. The classification has shortcomings; for example, the type 0 biomarker relating to a subject's genotype or phenotype is traditionally considered a covariate rather than a biomarker. Similarly, the type 6 biomarker, a clinical scale, can be regarded as a measurement of a clinical endpoint, and this is not covered by all biomarker definitions.

Previous to Rolan and colleagues' classification, the National Institutes of Health (NIH) biomarker definition working group ([Biomarkers Definitions Working Group, 2001](#)) simplified the existing biomarker spectrum into only three categories: biomarkers per se, surrogates (which are closely linked to endpoints and can substitute for them), and clinical endpoints ([Table 12.3](#)).

TABLE 12.2 Proposal for a biomarker classification.

Type 0: Genotype or phenotype
Type 1: Concentration
Type 2: Target occupancy
Type 3: Target activation
Type 4: Physiologic measures or laboratory tests
Type 5: Disease processes
Type 6: Clinical scales

From Rocchi, A., Khoudigian, S., Hopkins, R., Goeree, R., 2013. Surrogate outcomes: experiences at the common drug review. *Cost. Eff. Resour. Alloc.*, 11, 31, reprinted by permission from John Wiley and Sons, © 2003.

The [Biomarkers Definitions Working Group \(2001\)](#) published a paper in *Clinical Pharmacology & Therapeutics*, in which the following explanations are given:

Biological marker (biomarker): A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. Biomarkers may have the greatest value in early efficacy and safety evaluations such as in vitro studies in tissue samples, in vivo studies in animal models, and early-phase clinical trials to establish “proof of concept.” Biomarkers have many other valuable applications in disease detection and monitoring of health status. These applications include the following: Clinical endpoint: A characteristic or variable that reflects how a patient feels, functions, or survives. Clinical endpoints are distinct measurements or analyses of disease characteristics observed in a study or a clinical trial that reflect the effect of a therapeutic intervention. Clinical endpoints are the most credible characteristics used in the assessment of the benefits and risks of a therapeutic intervention in randomized clinical trials. Surrogate endpoint: A biomarker that is intended to substitute for a clinical endpoint. A surrogate endpoint is expected to predict clinical benefit (or harm or lack of benefit or harm) based on epidemiologic, therapeutic, pathophysiologic, or other scientific evidence. . . . Although all surrogate endpoints can be considered biomarkers, it is likely that only a few biomarkers will achieve surrogate endpoint status. The term surrogate endpoint applies primarily to endpoints in therapeutic intervention trials; however, it may sometimes apply in natural history or epidemiologic studies. It is

TABLE 12.3 Use of biomarkers for decision making: NIH Biomarkers Definitions Working Group Consensus Language.

	Definition	Additional Information
Biomarker	A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention	Categories: <ul style="list-style-type: none"> • Disease relevant (diagnosis, prognosis) • Mechanistic (pharmacological activity)—linked to drug • Safety^a • Efficacy^b
Surrogate	A characteristic that is intended to substitute for a clinical endpoint, often thought of as a clinical endpoint	<ul style="list-style-type: none"> • Should predict clinical benefit (or harm or lack of benefit or harm) • Based on epidemiological, therapeutic, pathophysiological, or other scientific evidence
Clinical endpoint	A characteristic or variable that reflects how a patient feels, functions, or survives	<ul style="list-style-type: none"> • Disease characteristics observed in a study or a clinical trial that reflect the effect of a therapeutic intervention

^aExpected to predict clinical benefit or harm based on epidemiological, therapeutic, pathophysiological, or other evidence.

^bNote that biomarkers are “evaluated,” not “validated.”

Extracted and modified from Biomarkers Definitions Working Group, 2001. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clin. Pharmacol. Ther.* 69, 89–95.

important to point out that the same biomarkers used as surrogate endpoints in clinical trials are often extended to clinical practice in which disease responses are similarly measured. . . The term surrogate literally means “to substitute for”; therefore use of the term surrogate marker is discouraged because the term suggests that the substitution is for a marker rather than for a clinical endpoint. The use of surrogate endpoints to establish therapeutic efficacy in registration trials is an established concept that has been addressed in regulation that enables the US Food and Drug Administration (FDA) to grant accelerated marketing approval for certain therapeutics (Food and Drug Modernization Act of 1997, 1997). (Reprinted by permission from Macmillan Publishers Ltd: Clinical Pharmacology & Therapeutics, 69: 89–95, © 2001.)

A list of examples of potential surrogates is as follows:

- Blood pressure
- Intraocular pressure (glaucoma)
- HbA1c (diabetes mellitus)
- Psychometric testing
- Tumor shrinkage (cancer)
- ACR criteria (rheumatoid arthritis)
- Pain scales (pain)
- LDL cholesterol
- HIV particle concentration (AIDS)

These surrogates may fulfill the criteria mentioned previously and have been shown to work in practice. It may be surprising that other parameters, such as blood glucose (diabetes mellitus) or CRP (inflammatory processes), are not listed. They lack specificity, are too variable, or simply do not predict hard endpoints such as death or major cardiovascular events well enough. The most prominent negative example of a former surrogate that caused patients to die was the number of ventricular premature beats (VPBs). In the cardiac arrhythmia suppression trial the antiarrhythmic drugs encainide and flecainide did indeed suppress VPBs, but patients died earlier (Echt et al., 1991). This was apparently not a sufficient surrogate, and we now know that the proarrhythmic effects of many antiarrhythmic drugs far outweigh their benefits on VPBs.

On the other hand, a big leap (one might even say a quantum leap) occurred in AIDS research with the discovery of the close correlation between HIV particle concentration (viral RNA or p24 antigen as direct measures) (Ledergerber et al., 2000) in plasma and morbidity or mortality. With the establishment of this correlation in expensive outcome trials, development of HIV drugs has become fast and reliable and has contributed to the tremendous success drugs have brought about in treating this disease. It is currently possible to develop a new HIV drug within 3–5 years rather than 8–10 years, as lengthy morbidity or mortality trials are no longer required, and efficacy can be proven in relatively small trials of, for example, 50 participants.

Unfortunately, the acceptance of surrogates across regulatory authorities or health technology assessment institutions is not consistent. Rocchi et al. (2013) showed a variable extent of surrogate acceptance for different therapy areas and for different institutions. As can be seen in Fig. 12.3, HIV drugs have always been approved on the base of surrogate markers, whereas for cardiovascular disease or diabetes drugs, this was true in only two-thirds of cases.

The acceptance by different institutions varies widely, as shown in Fig. 12.4. The Centers for Disease Control and Prevention does not accept any surrogates, while the European Medicines Agency and FDA accept them in the majority of examples.

The use of biomarkers in drug development according to these definitions is depicted in Fig. 12.5. In this model, it is obvious that biomarkers represent everything but the clinical endpoint and serve more or less as weak surrogates (biomarkers in the narrow sense) or good surrogates for the clinical endpoint. This is the case for both efficacy and safety concerns. The major concern according to this model is the fact that some effects, especially at the safety level, may be missed because the appropriate biomarkers have not been measured (Fig. 12.6).

In my opinion and that of others, the classification of the Biomarkers Definitions Working Group is too simple. The first category, “biomarker,” deserves a more detailed subclassification. Biomarkers are still distant from surrogates and endpoints, which are studied only at the late or even final stages of the drug development process. Biomarkers are the key instruments of translational medicine. They are ideally first established in animal disease models and then used in the equivalent human diseases to support early confidence in the efficacy and safety of

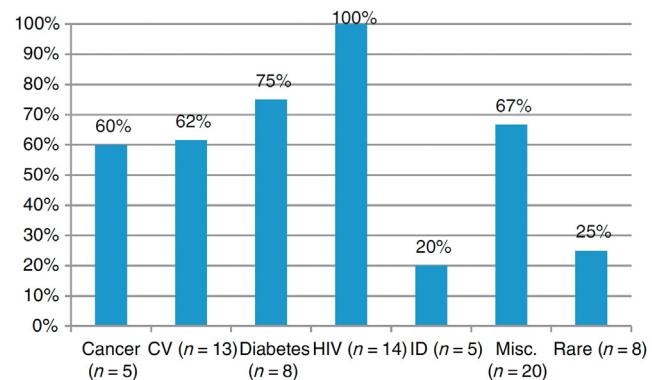


FIGURE 12.3 Percentage of drug recommendations with surrogate acceptability by therapeutic area. Y-axis: Percent of recommendations using surrogate outcomes. X-axis: Therapeutic area. CV =, Cardiovascular disease; HIV =, human immunodeficiency virus; ID =, infectious disease. From Rocchi, A., Khoudigian, S., Hopkins, R., Goeree, R., 2013. Surrogate outcomes: experiences at the common drug review. *Cost. Eff. Resour. Alloc.* 11, 31, Open Access.

	CDR	HC	FDA	EMA	NICE	PBS	SMC
Saxagliptin (HbA1c)	No (e2)	N/S	Yes (e)	Yes (e1)	N/A	N/S	No (ref)
	No (e2)	N/S	Yes (e)	N/S	N/A	N/S	No (e2)
Sitagliptin/Metformin (HbA1c)	N/S	N/A	N/S	Yes (e1)	N/A	N/A	N/S
	N/S	N/S	Yes (used)	Yes (e)	N/A	No (ref)	No (e2)
Ambrisentan (6MWD)	No (e2)	N/A	N/S	Yes (used)	N/A	N/S	No (e2)
	No (e2)	Yes (e1)	N/A	Yes (e)	N/A	N/S	N/A
Sildenafil (6MWD)	No (e2)	N/A	N/S	Yes (used)	N/A	N/S	No (e2)
	No (e2)	Yes (e1)	N/A	Yes (e)	N/A	N/S	N/A
Tadalafil (6MWD)	No (ref)	N/A	Yes (used)	No (e1)	N/A	N/S	N/A
	No (e2)	N/A	No (e2)	N/A	N/A	N/S	N/A
Treprostинil (6MWD)	No (e1)	N/A	Yes (e)	Yes (e)	Yes (used)	No (e1)	N/S
	N/S	N/S	Yes (e2)	Yes (guid)	Yes (e1)	Yes (e)	N/S
Adefovir (composite)	No (e1+e2)	N/A	N/S	Yes (used)	Yes (used)	N/A	N/S
	No (e2)	No (e1)	Yes (e)	Yes (guid)	Yes (e1)	Yes (e)	N/S
Entecavir (composite)	No (e2)	N/A	Yes (e1)	Yes (e)	Yes (e1)	Yes (e)	N/S
	No (e1+e2)	N/A	N/S	Yes (used)	Yes (used)	N/A	N/S
Telbivudine (composite)	No (e2)	No (e1)	Yes (e)	Yes (guid)	Yes (e1)	Yes (e)	N/S
	No (e1)	N/A	N/A	Yes (guid)	N/S	N/S	N/S

FIGURE 12.4 Comparison of international agencies: concerns with surrogate outcomes. Y-axis: Drug submission. X-axis: Agency. *No: no (e2) = implicit no “evidence 2”; no (ref) = implicit no “reference”; no (e) = explicit no “evidence 1”; no (e1 + e2) = explicit no “evidence 1” and implicit no “evidence 2”; Yes: yes (e1) = implicit yes “evidence 1”; yes (e2) = implicit yes “evidence 2”; yes (used) = implicit yes “used before”; yes (ref) = implicit yes “reference”; yes (e) = explicit yes; Not identified: N/S = no statement; N/A = not applicable; Red shade = negative statements of surrogate acceptability; Green shade = positive statements of surrogate acceptability; HbA1c, hemoglobin A1c; 6MWD, 6-minute walk distance; composite = histology, virology, serology; SVR, sustained virological response; CDR, Common Drug Review; HC, Health Canada; FDA, Food and Drug Administration; EMA, European Medicines Agency; NICE, National Institute for Health and Clinical Excellence; PBS, Pharmaceutical Benefit Scheme; SMC, Scottish Medicines Consortium. From Rocchi, A., Khoudigian, S., Hopkins, R., Goeree, R., 2013. Surrogate outcomes: experiences at the common drug review. *Cost. Eff. Resour. Alloc.* 11, 31, Open Access.

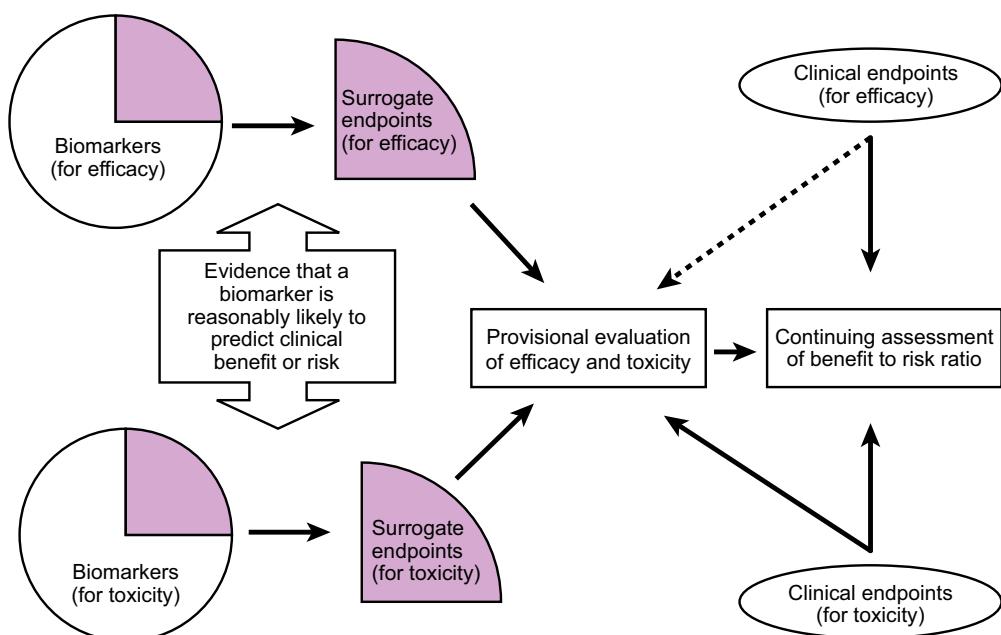


FIGURE 12.5 Conceptual model of the relationship of biomarkers, surrogate endpoints, and the process of evaluating therapeutic interventions according to the NIH biomarker definition group. From Biomarkers Definitions Working Group, 2001. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clin. Pharmacol. Ther.* 69, 89–95, © 2001.

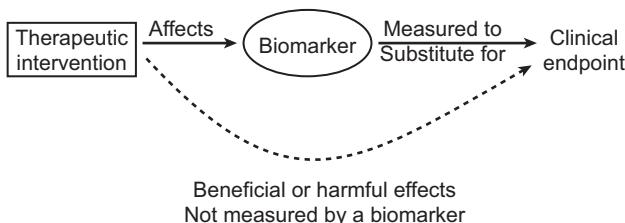


FIGURE 12.6 Conceptual model of the use of biomarkers in drug development. Important efficacy and especially safety aspects may be missed if biomarker selection is not optimal. *From Biomarkers Definitions Working Group, 2001. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin. Pharmacol. Ther.* 69, 89–95, reprinted by permission from John Wiley and Sons, © 2001.

a new drug or intervention. It is obvious that their predictive value can range from almost useless (e.g., if one has to obtain serial brain slices, which will never become a human biomarker) to that of a surrogate (e.g., LDL cholesterol, one of the few surrogates accepted by the FDA), with most cases being somewhere in between.

Thus a subclassification of the predictive value of biomarkers seems desirable and should be considered a major task in biomarker development. Describing the mechanistic dimension of a biomarker, as done by [Rolan et al. \(2003\)](#), does not solve the problem of defining predictive biomarkers versus biomarkers without predictive potential, as there may be potent and weak biomarkers in all subcategories.

Another important set of attributes of biomarkers relates to their prospective use. The major divide is between efficacy and safety; biomarkers are commonly attributed to one area or the other. A third operational category has to do with the increasing importance of personalized medicine issues: the right drug for the right patient. In this context, biomarkers have been specifically developed to predict responsiveness in a given patient; the most prominent example is HER2/neu expression as prerequisite for successful trastuzumab therapy in breast cancer treatment. The FDA has posted a rapidly growing list of valid genomic biomarkers in the context of approved drug labels, now listing 404 entries ([U.S. Department of Health and Human Services Food and Drug Administration, 2020](#); see [Table 12.4](#)). This list demonstrates the rapidly increasing importance of these patient-oriented, personalized medicine-type biomarkers. Of course, they aim at increasing efficacy and safety and thus could be easily categorized into either of the two areas of prospective use.

In addition, the FDA has issued detailed documentation regarding the submission procedure for biomarkers ([U.S. Department of Health and Human Services Food and Drug Administration, 2011](#)) guiding the format of regulatory information in drug or device approval processes. It thus generalizes the requirements for valuable

biomarkers that could now be any measure with proper documentation and evaluation.

Another classification of biomarkers is driven by technology. It is simply a description of what kind of measurement has to be performed to obtain the biomarker. The main groups worth mentioning are summarized in [Table 12.5](#), which compiles major categories and prominent examples. More recently, [Califf \(2018\)](#) categorized biomarkers by multiple dimensions taking into account those aspects discussed above; thereby, he summarized the efforts by the [FDA-NIH Biomarker Working Group \(2016\)](#) and gives biomarkers the following attributes:

1. Diagnostic
2. Monitoring
3. Pharmacodynamic/response
4. Predictive
5. Susceptibility/risk
6. Prognostic
7. Safety
8. Surrogate
9. (Future) digital
10. (Future) complex composite

This chapter shows that biomarkers may be classified by various approaches. Under the auspices of translational medicine, it is most important to classify them according to their predictive power. If this dimension is added to the simple NIH definition and if endpoints are understood as biomarkers as well, a useful classification could emerge. The technical classification is important in terms of methodological planning and investment. In the context of biomarkers, the related terms *risk factor* and *risk marker* should be used wisely and specifically. In general, they describe classified biomarkers that have a proven value on top of their basic function as biomarkers.

A *risk factor* is linked to a disease because of a causal relationship to major elements of the disease. In turn, this implies that therapeutic intervention affecting this biomarker has been shown to improve clinical outcomes. Typical examples are arterial hypertension or LDL cholesterol. Not only is there a correlation between those parameters and morbidity or mortality, but intervention (by drugs) has been proven to change the parameter and clinical outcome in parallel.

A *risk marker* is associated with the disease (epidemiologically) but need not be causally linked; it may be a measure of the disease process, and as such, it may represent only an epiphenomenon. Intervention aiming at this biomarker does not lead to improved clinical outcomes because there is no causal relationship between disease and marker. A prominent example unmasked by a failed drug is HDL cholesterol (see Chapter 32, Translational Science in Medicine: Putting the Pieces Together), which

TABLE 12.4 Mod. from the FDA's list of valid genomic biomarkers in the context of approved drug labeling.

Drug	Therapeutic Area ^a	Biomarker ^b
Abacavir	Infectious diseases	HLA-B
Abemaciclib (1)	Oncology	ESR (hormone receptor)
Abemaciclib (2)	Oncology	ERBB2 (HER2)
Ado-trastuzumab emtansine	Oncology	ERBB2 (HER2)
Afatinib	Oncology	EGFR
Alectinib	Oncology	ALK
Alpelisib (1)	Oncology	ERBB2 (HER2)
Alpelisib (2)	Oncology	ESR (hormone receptor)
Alpelisib (3)	Oncology	PIK3CA
Amifampridine	Neurology	NAT2
Amifampridine phosphate	Neurology	NAT2
Amitriptyline	Psychiatry	CYP2D6
Amoxapine	Psychiatry	CYP2D6
Amphetamine	Psychiatry	CYP2D6
Anastrozole	Oncology	ESR, PGR (hormone receptor)
Arformoterol (1)	Pulmonary	UGT1A1
Arformoterol (2)	Pulmonary	CYP2D6
Aripiprazole	Psychiatry	CYP2D6
Aripiprazole lauroxil	Psychiatry	CYP2D6
Arsenic trioxide	Oncology	PML-RARA
Articaine and epinephrine (1)	Anesthesiology	G6PD
Articaine and epinephrine (2)	Anesthesiology	Nonspecific (congenital methemoglobinemia)
Ascorbic acid, PEG-3350, potassium chloride, sodium ascorbate, sodium chloride, and sodium sulfate	Gastroenterology	G6PD
Atezolizumab (1)	Oncology	CD274 (PD-L1)
Atezolizumab (2)	Oncology	Gene signature (T-effector)
Atezolizumab (3)	Oncology	EGFR
Atezolizumab (4)	Oncology	ALK
Atomoxetine	Psychiatry	CYP2D6
Avatrombopag (1)	Hematology	F2 (prothrombin)
Avatrombopag (2)	Hematology	F5 (factor V Leiden)
Avatrombopag (3)	Hematology	PROC
Avatrombopag (4)	Hematology	PROS1
Avatrombopag (5)	Hematology	SERPINC1 (antithrombin III)
Avatrombopag (6)	Hematology	CYP2C9
Avelumab	Oncology	CD274 (PD-L1)
Azathioprine (1)	Rheumatology	TPMT

(Continued)

TABLE 12.4 (Continued)

Drug	Therapeutic Area ^a	Biomarker ^b
Azathioprine (2)	Rheumatology	NUDT15
Belinostat	Oncology	UGT1A1
Binimetinib (1)	Oncology	BRAF
Binimetinib (2)	Oncology	UGT1A1
Blinatumomab	Oncology	BCR-ABL1 (Philadelphia chromosome)
Boceprevir	Infectious diseases	IFNL3 (IL28B)
Bosutinib	Oncology	BCR-ABL1 (Philadelphia chromosome)
Brentuximab vedotin (1)	Oncology	ALK
Brentuximab vedotin (2)	Oncology	TNFRSF8 (CD30)
Brexipiprazole	Psychiatry	CYP2D6
Brigatinib	Oncology	ALK
Brivaracetam	Neurology	CYP2C19
Busulfan	Oncology	BCR-ABL1 (Philadelphia chromosome)
Cabozantinib	Oncology	RET
Capecitabine	Oncology	DYPD
Carbamazepine (1)	Neurology	HLA-B
Carbamazepine (2)	Neurology	HLA-A
Carglumic acid	Inborn errors of metabolism	NAGS
Cariprazine	Psychiatry	CYP2D6
Carisoprodol	Rheumatology	CYP2C19
Carvedilol	Cardiology	CYP2D6
Ceftriaxone (1)	Infectious diseases	G6PD
Ceftriaxone (2)	Infectious diseases	Nonspecific (congenital methemoglobinemia)
Celecoxib	Rheumatology	CYP2C9
Ceritinib	Oncology	ALK
Cerliponase alfa	Inborn errors of metabolism	TPP1
Cetuximab (1)	Oncology	EGFR
Cetuximab (2)	Oncology	RAS
Cevimeline	Dental	CYP2D6
Chloroprocaine (1)	Anesthesiology	G6PD
Chloroprocaine (2)	Anesthesiology	Nonspecific (mongenital Methemoglobinemia)
Chloroquine	Infectious diseases	G6PD
Chlorpropamide	Endocrinology	G6PD
Cisplatin	Oncology	TPMT
Citalopram (1)	Psychiatry	CYP2C19
Citalopram (2)	Psychiatry	CYP2D6
Clobazam	Neurology	CYP2C19
Clomipramine	Psychiatry	CYP2D6

(Continued)

TABLE 12.4 (Continued)

Drug	Therapeutic Area ^a	Biomarker ^b
Clopidogrel	Cardiology	CYP2C19
Clozapine	Psychiatry	CYP2D6
Cobimetinib	Oncology	BRAF
Codeine	Anesthesiology	CYP2D6
Crizanlizumab-tmca	Hematology	HBB
Crizotinib (1)	Oncology	ALK
Crizotinib (2)	Oncology	ROS1
Dabrafenib (1)	Oncology	BRAF
Dabrafenib (2)	Oncology	G6PD
Dabrafenib (3)	Oncology	RAS
Daclatasvir	Infectious diseases	IFNL3 (IL28B)
Dacomitinib	Oncology	EGFR
Dapsone (1)	Dermatology	G6PD
Dapsone (2)	Dermatology	Nonspecific (congenital methemoglobinemia)
Dapsone (3)	Infectious diseases	G6PD
Darifenacin	Urology	CYP2D6
Dasabuvir, ombitasvir, paritaprevir, and ritonavir	Infectious diseases	IFNL3 (IL28B)
Dasatinib	Oncology	BCR-ABL1 (Philadelphia chromosome)
Denileukin diftitox	Oncology	IL2RA (CD25 antigen)
Desflurane	Anesthesiology	Nonspecific (genetic susceptibility to malignant hyperthermia)
Desipramine	Psychiatry	CYP2D6
Desvenlafaxine	Psychiatry	CYP2D6
Deutetrabenazine	Neurology	CYP2D6
Dexlansoprazole	Gastroenterology	CYP2C19
Dextromethorphan and quinidine	Neurology	CYP2D6
Diazepam	Neurology	CYP2C19
Dinutuximab	Oncology	MYCN
Docetaxel	Oncology	ESR, PGR (hormone receptor)
Dolutegravir	Infectious diseases	UGT1A1
Donepezil	Neurology	CYP2D6
Doxepin (1)	Psychiatry	CYP2D6
Doxepin (2)	Psychiatry	CYP2C19
Dronabinol	Gastroenterology	CYP2C9
Drospirenone and ethinyl estradiol	Gynecology	CYP2C19
Duloxetine	Psychiatry	CYP2D6
Durvalumab	Oncology	CD274 (PD-L1)

(Continued)

TABLE 12.4 (Continued)

Drug	Therapeutic Area ^a	Biomarker ^b
Duvelisib	Oncology	Chromosome 17p
Efavirenz	Infectious diseases	CYP2B6
Elagolix	Gynecology	SLCO1B1
Elbasvir and grazoprevir	Infectious diseases	IFNL3 (IL28B)
Elexacaftor, ivacaftor, and tezacaftor	Pulmonary	CFTR
Eliglustat	Inborn errors of metabolism	CYP2D6
Elosulfase	Inborn errors of metabolism	GALNS
Eltrombopag (1)	Hematology	F5 (Factor V Leiden)
Eltrombopag (2)	Hematology	SERPINC1 (antithrombin III)
Eltrombopag (3)	Hematology	Chromosome 7del
Eltrombopag (4)	Hematology	Chromosome 13del
Emapalumab-lzsg	Hematology	PRF1, RAB27A, SH2D1A, STXBP2, STX11, UNC13D, XIAP (hemophagocytic lymphohistiocytosis)
Enasidenib	Oncology	IDH2
Encorafenib	Oncology	BRAF
Enfortumab vedotin-ejfv	Oncology	NECTIN4
Entrectinib (1)	Oncology	ROS1
Entrectinib (2)	Oncology	NTRK
Erdafitinib (1)	Oncology	FGFR
Erdafitinib (2)	Oncology	CYP2C9
Eribulin (1)	Oncology	ERBB2 (HER2)
Eribulin (2)	Oncology	ESR, PGR (hormone receptor)
Erlotinib	Oncology	EGFR
Erythromycin and sulfisoxazole	Infectious diseases	G6PD
Escitalopram (1)	Psychiatry	CYP2D6
Escitalopram (2)	Psychiatry	CYP2C19
Esomeprazole	Gastroenterology	CYP2C19
Estradiol and progesterone (1)	Gynecology	PROC
Estradiol and progesterone (2)	Gynecology	PROS1
Estradiol and progesterone (3)	Gynecology	SERPINC1 (antithrombin III)
Eteplirsen	Neurology	DMD
Everolimus (1)	Oncology	ERBB2 (HER2)
Everolimus (2)	Oncology	ESR (hormone receptor)
Exemestane	Oncology	ESR, PGR (hormone receptor)
Fam-trastuzumab deruxtecan-nxki	Oncology	ERBB2 (HER2)
Fesoterodine	Urology	CYP2D6
Flibanserin (1)	Gynecology	CYP2C9

(Continued)

TABLE 12.4 (Continued)

Drug	Therapeutic Area ^a	Biomarker ^b
Flibanserin (2)	Gynecology	CYP2C19
Flibanserin (3)	Gynecology	CYP2D6
Fluorouracil (1)	Dermatology	DPYD
Fluorouracil (2)	Oncology	DPYD
Fluoxetine	Psychiatry	CYP2D6
Flurbiprofen	Rheumatology	CYP2C9
Flutamide	Oncology	G6PD
Fluvoxamine	Psychiatry	CYP2D6
Formoterol (1)	Pulmonary	CYP2D6
Formoterol (2)	Pulmonary	CYP2C19
Fosphenytoin	Neurology	HLA-B
Fulvestrant (1)	Oncology	ERBB2 (HER2)
Fulvestrant (2)	Oncology	ESR, PGR (hormone receptor)
Galantamine	Neurology	CYP2D6
Gefitinib (1)	Oncology	EGFR
Gefitinib (2)	Oncology	CYP2D6
Gilteritinib	Oncology	FLT3
Givosiran	Gastroenterology	CPOX, HMBS, PPOX (acute hepatic porphyria)
Glimepiride	Endocrinology	G6PD
Glipizide	Endocrinology	G6PD
Glyburide	Endocrinology	G6PD
Golodirsen	Neurology	DMD
Goserelin	Oncology	ESR, PGR (hormone receptor)
Hydralazine	Cardiology	Nonspecific (NAT)
Hydroxychloroquine	Infectious diseases	G6PD
Ibrutinib (1)	Oncology	Chromosome 17p
Ibrutinib (2)	Oncology	Chromosome 11q
Iloperidone	Psychiatry	CYP2D6
Imatinib (1)	Oncology	KIT
Imatinib (2)	Oncology	BCR-ABL1 (Philadelphia chromosome)
Imatinib (3)	Oncology	PDGFRB
Imatinib (4)	Oncology	FIP1L1-PDGFRα
Imipramine	Psychiatry	CYP2D6
Indacaterol	Pulmonary	UGT1A1
Inotersen	Neurology	TTR
Inotuzumab ozogamicin	Oncology	BCR-ABL1 (Philadelphia chromosome)
Ipilimumab (1)	Oncology	HLA-A

(Continued)

TABLE 12.4 (Continued)

Drug	Therapeutic Area ^a	Biomarker ^b
Ipilimumab (2)	Oncology	Microsatellite instability, mismatch repair
Irinotecan	Oncology	UGT1A1
Isoflurane	Anesthesiology	Nonspecific (genetic susceptibility to malignant hyperthermia)
Isoniazid, pyrazinamide, and rifampin	Infectious diseases	Nonspecific (NAT)
Isosorbide dinitrate	Cardiology	CYB5R
Isosorbide mononitrate	Cardiology	CYB5R
Ivacaftor	Pulmonary	CFTR
Ivacaftor and lumacaftor	Pulmonary	CFTR
Ivacaftor and tezacaftor	Pulmonary	CFTR
Ivosidenib	Oncology	IDH1
Ixabepilone (1)	Oncology	ERBB2 (HER2)
Ixabepilone (2)	Oncology	ESR, PGR (hormone receptor)
Lacosamide	Neurology	CYP2C19
Lansoprazole	Gastroenterology	CYP2C19
Lapatinib (1)	Oncology	ERBB2 (HER2)
Lapatinib (2)	Oncology	ESR, PGR (hormone receptor)
Lapatinib (3)	Oncology	HLA-DQA1
Lapatinib (4)	Oncology	HLA-DRB1
Larotrectinib	Oncology	NTRK
Ledipasvir and sofosbuvir	Infectious diseases	IFNL3 (IL28B)
Lenalidomide	Hematology	Chromosome 5q
Lenvatinib	Oncology	Microsatellite instability, mismatch repair
Lesinurad	Rheumatology	CYP2C9
Letrozole	Oncology	ESR, PGR (hormone receptor)
Lidocaine and prilocaine (1)	Anesthesiology	Nonspecific (ongenital methemoglobinemia)
Lidocaine and prilocaine (2)	Anesthesiology	G6PD
Lidocaine and tetracaine (1)	Anesthesiology	G6PD
Lidocaine and tetracaine (2)	Anesthesiology	Nonspecific (congenital methemoglobinemia)
Lofexidine	Anesthesiology	CYP2D6
Lorlatinib (1)	Oncology	ALK
Lorlatinib (2)	Oncology	ROS1
Luspatercept—aamt	Hematology	HBB
Lusutrombopag (1)	Hematology	F2 (prothrombin)
Lusutrombopag (2)	Hematology	F5 (factor V Leiden)
Lusutrombopag (3)	Hematology	PROC
Lusutrombopag (4)	Hematology	PROS1
Lusutrombopag (5)	Hematology	SERPINC1 (antithrombin III)

(Continued)

TABLE 12.4 (Continued)

Drug	Therapeutic Area ^a	Biomarker ^b
Mafenide	Infectious diseases	G6PD
Meclizine	Neurology	CYP2D6
Meloxicam	Anesthesiology	CYP2C9
Mepivacaine (1)	Anesthesiology	G6PD
Mepivacaine (2)	Anesthesiology	Nonspecific (congenital methemoglobinemia)
Mercaptopurine (1)	Oncology	TPMT
Mercaptopurine (2)	Oncology	NUDT15
Methylene blue	Hematology	G6PD
Metoclopramide (1)	Gastroenterology	CYB5R
Metoclopramide (2)	Gastroenterology	G6PD
Metoclopramide (3)	Gastroenterology	CYP2D6
Metoprolol	Cardiology	CYP2D6
Midostaurin (1)	Oncology	FLT3
Midostaurin (2)	Oncology	NPM1
Midostaurin (3)	Oncology	KIT
Migalastat	Inborn errors of metabolism	GLA
Mirabegron	Urology	CYP2D6
Mivacurium	Anesthesiology	BCHE
Modafinil	Psychiatry	CYP2D6
Mycophenolic acid	Transplantation	HPRT1
Nalidixic acid	Infectious diseases	G6PD
Nebivolol	Cardiology	CYP2D6
Nefazodone	Psychiatry	CYP2D6
Neratinib (1)	Oncology	ERBB2 (HER2)
Neratinib (2)	Oncology	ESR, PGR (hormone receptor)
Nilotinib (1)	Oncology	BCR-ABL1 (Philadelphia chromosome)
Nilotinib (2)	Oncology	UGT1A1
Niraparib	Oncology	BRCA
Nitrofurantoin	Infectious diseases	G6PD
Nivolumab (1)	Oncology	BRAF
Nivolumab (2)	Oncology	CD274 (PD-L1)
Nivolumab (3)	Oncology	Microsatellite instability, mismatch repair
Nivolumab (4)	Oncology	EGFR
Nivolumab (5)	Oncology	ALK
Nortriptyline	Psychiatry	CYP2D6
Nusinersen	Neurology	SMN2
Obinutuzumab	Oncology	MS4A1 (CD20 antigen)

(Continued)

TABLE 12.4 (Continued)

Drug	Therapeutic Area ^a	Biomarker ^b
Olaparib (1)	Oncology	BRCA
Olaparib (2)	Oncology	ERBB2 (HER2)
Olaparib (3)	Oncology	ESR, PGR (hormone receptor)
Olaratumab	Oncology	PDGFRA
Omacetaxine	Oncology	BCR-ABL1 (Philadelphia chromosome)
Ombitasvir, paritaprevir, and ritonavir	Infectious diseases	IFNL3 (IL28B)
Omeprazole	Gastroenterology	CYP2C19
Ondansetron	Gastroenterology	CYP2D6
Osimertinib	Oncology	EGFR
Ospemifene (1)	Gynecology	CYP2C9
Ospemifene (2)	Gynecology	CYP2B6
Oxcarbazepine	Neurology	HLA-B
Oxymetazoline and tetracaine (1)	Anesthesiology	G6PD
Oxymetazoline and tetracaine (2)	Anesthesiology	Nonspecific (congenital methemoglobinemia)
Palbociclib (1)	Oncology	ESR (hormone receptor)
Palbociclib (2)	Oncology	ERBB2 (HER2)
Paliperidone	Psychiatry	CYP2D6
Palonosetron	Gastroenterology	CYP2D6
Panitumumab (1)	Oncology	EGFR
Panitumumab (2)	Oncology	RAS
Pantoprazole	Gastroenterology	CYP2C19
Parathyroid hormone	Inborn Errors of metabolism	CASR
Paroxetine	Psychiatry	CYP2D6
Patisiran	Neurology	TTR
Pazopanib (1)	Oncology	UGT1A1
Pazopanib (2)	Oncology	HLA-B
Peginterferon alfa-2b	Infectious diseases	IFNL3 (IL28B)
Pegloticase	Rheumatology	G6PD
Pembrolizumab (1)	Oncology	BRAF
Pembrolizumab (2)	Oncology	CD274 (PD-L1)
Pembrolizumab (3)	Oncology	Microsatellite instability, mismatch repair
Pembrolizumab (4)	Oncology	EGFR
Pembrolizumab (5)	Oncology	ALK
Perphenazine	Psychiatry	CYP2D6
Pertuzumab (1)	Oncology	ERBB2 (HER2)
Pertuzumab (2)	Oncology	ESR, PGR (hormone receptor)
Phenytoin (1)	Neurology	CYP2C9
Phenytoin (2)	Neurology	CYP2C19

(Continued)

TABLE 12.4 (Continued)

Drug	Therapeutic Area ^a	Biomarker ^b
Phenytoin (3)	Neurology	HLA-B
Pimozide	Psychiatry	CYP2D6
Piroxicam	Rheumatology	CYP2C9
Pitolisant	Psychiatry	CYP2D6
Ponatinib	Oncology	BCR-ABL1 (Philadelphia chromosome)
Prasugrel (1)	Cardiology	CYP2C19
Prasugrel (2)	Cardiology	CYP2C9
Prasugrel (3)	Cardiology	CYP3A5
Prasugrel (4)	Cardiology	CYP2B6
Primaquine (1)	Infectious diseases	G6PD
Primaquine (2)	Infectious diseases	CYB5R
Probenecid	Rheumatology	G6PD
Procainamide	Cardiology	Nonspecific (NAT)
Propafenone	Cardiology	CYP2D6
Propranolol	Cardiology	CYP2D6
Protriptyline	Psychiatry	CYP2D6
Quinidine	Cardiology	CYP2D6
Quinine sulfate (1)	Infectious diseases	G6PD
Quinine sulfate (2)	Infectious diseases	CYP2D6
Rabeprazole	Gastroenterology	CYP2C19
Raloxifene	Oncology	ESR (hormone receptor)
Raltegravir	Infectious diseases	UGT1A1
Ramucirumab (1)	Oncology	EGFR
Ramucirumab (2)	Oncology	RAS
Rasburicase (1)	Oncology	G6PD
Rasburicase (2)	Oncology	CYB5R
Regorafenib	Oncology	RAS
Ribociclib (1)	Oncology	ESR, PGR (hormone receptor)
Ribociclib (2)	Oncology	ERBB2 (HER2)
Risperidone	Psychiatry	CYP2D6
Rituximab	Oncology	MS4A1 (CD20 antigen)
Rivaroxaban	Cardiology	F5 (factor V Leiden)
Ropivacaine (1)	Anesthesiology	G6PD
Ropivacaine (2)	Anesthesiology	Nonspecific (congenital methemoglobinemia)
Rosuvastatin	Endocrinology	SLCO1B1
Rucaparib (1)	Oncology	BRCA
Rucaparib (2)	Oncology	CYP2D6

(Continued)

TABLE 12.4 (Continued)

Drug	Therapeutic Area ^a	Biomarker ^b
Rucaparib (3)	Oncology	CYP1A2
Rucaparib (4)	Oncology	Homologous recombination deficiency
Sevoflurane	Anesthesiology	RYR1
Simeprevir	Infectious diseases	IFNL3 (IL28B)
Siponimod	Neurology	CYP2C9
Sodium nitrite (1)	Toxicology	G6PD
Sodium nitrite (2)	Toxicology	Nonspecific (congenital methemoglobinemia)
Sodium phenylbutyrate	Inborn errors of metabolism	ASS1, CPS1, OTC (urea cycle disorders)
Sofosbuvir	Infectious diseases	IFNL3 (IL28B)
Sofosbuvir and velpatasvir	Infectious diseases	IFNL3 (IL28B)
Sofosbuvir, velpatasvir, and voxilaprevir	Infectious diseases	IFNL3 (IL28B)
Succimer	Hematology	G6PD
Succinylcholine	Anesthesiology	BCHE
Sulfadiazine	Infectious diseases	G6PD
Sulfamethoxazole and trimethoprim (1)	Infectious diseases	G6PD
Sulfamethoxazole and trimethoprim (2)	Infectious diseases	Nonspecific (NAT)
Sulfasalazine (1)	Gastroenterology	G6PD
Sulfasalazine (2)	Gastroenterology	Nonspecific (NAT)
Tafamidis	Cardiology	TTR
Tafenoquine	Infectious diseases	G6PD
Talazoparib (1)	Oncology	BRCA
Talazoparib (2)	Oncology	ERBB2 (HER2)
Tamoxifen (1)	Oncology	ESR, PGR (hormone receptor)
Tamoxifen (2)	Oncology	F5 (factor V Leiden)
Tamoxifen (3)	Oncology	F2 (prothrombin)
Tamoxifen (4)	Oncology	CYP2D6
Tamsulosin	Urology	CYP2D6
Telaprevir	Infectious diseases	IFNL3 (IL28B)
Tetrabenazine	Neurology	CYP2D6
Thioguanine (1)	Oncology	TPMT
Thioguanine (2)	Oncology	NUDT15
Thioridazine	Psychiatry	CYP2D6
Ticagrelor	Cardiology	CYP2C19
Tipiracil and trifluridine (1)	Oncology	ERBB2 (HER2)
Tipiracil and trifluridine (2)	Oncology	RAS
Tolazamide	Endocrinology	G6PD
Tolbutamide	Endocrinology	G6PD
Tolterodine	Urology	CYP2D6

(Continued)

TABLE 12.4 (Continued)

Drug	Therapeutic Area ^a	Biomarker ^b
Toremifene	Oncology	ESR (hormone receptor)
Tramadol	Anesthesiology	CYP2D6
Trametinib (1)	Oncology	BRAF
Trametinib (2)	Oncology	G6PD
Trametinib (3)	Oncology	RAS
Trastuzumab (1)	Oncology	ERBB2 (HER2)
Trastuzumab (2)	Oncology	ESR, PGR (hormone receptor)
Tretinoin	Oncology	PML-RARA
Trimipramine	Psychiatry	CYP2D6
Umeclidinium	Pulmonary	CYP2D6
Upadacitinib	Rheumatology	CYP2D6
Ustekinumab	Dermatology and gastroenterology	IL12A, IL12B, IL23A
Valbenazine	Neurology	CYP2D6
Valproic acid (1)	Neurology	POLG
Valproic acid (2)	Neurology	Nonspecific (urea cycle disorders)
Vemurafenib (1)	Oncology	BRAF
Vemurafenib (2)	Oncology	RAS
Venetoclax (1)	Oncology	Chromosome 17p
Venetoclax (2)	Oncology	Chromosome 11q
Venetoclax (3)	Oncology	TP53
Venetoclax (4)	Oncology	IDH1
Venetoclax (5)	Oncology	IDH2
Venetoclax (6)	Oncology	IGH
Venetoclax (7)	Oncology	NPM1
Venetoclax (8)	Oncology	FLT3
Venlafaxine	Psychiatry	CYP2D6
Vincristine	Oncology	BCR-ABL1 (Philadelphia chromosome)
Voriconazole	Infectious diseases	CYP2C19
Vortioxetine	Psychiatry	CYP2D6
Voxelotor	Hematology	HBB
Warfarin (1)	Hematology	CYP2C9
Warfarin (2)	Hematology	VKORC1
Warfarin (3)	Hematology	PROS1
Warfarin (4)	Hematology	PROC

^aTherapeutic areas do not necessarily reflect the CDER review division.

^bRepresentative biomarkers are listed based on standard nomenclature as per the Human Genome Organization (HUGO) symbol and/or simplified descriptors using other common conventions. Listed biomarkers do not necessarily reflect the terminology used in labeling. The term Nonspecific is used when labeling does not explicitly identify the specific biomarker(s) or when the biomarker is represented by a molecular phenotype or gene signature, and in some cases the biomarker was inferred based on the labeling language.

From U.S. Department of Health and Human Services Food and Drug Administration, 2020. Table of pharmacogenomic biomarkers in drug labeling. <https://www.fda.gov/drugs/science-and-research-drugs/table-pharmacogenomic-biomarkers-drug-labeling>. (Accessed 17 February 20).

TABLE 12.5 Categorization of important biomarkers by technical condition and type of measure.

Omics
Proteomics or peptidomics
Genomics or pharmacogenomics
Metabolonomics
Histomics
Genetic markers
Serum markers, defined serum constituents (e.g., enzymes, proteins, electrolytes)
Histology
Microbiology
Functional tests
Cardiovascular parameters (e.g., blood pressure)
Endothelial function
ECG
EEG
Neurological tests
Psychometric tests
Joint or muscle function tests
General: weight, fever, well-being
Imaging
X-ray, conventional, + contrast or staining
Tomography, magnetic imaging, X-ray, + contrast, staining, or receptor-staining
Functional imaging (e.g., fMRI)
PET
Ultrasound or Doppler, + contrast enhancement (microbubbles)
Endoscopy
Clinical endpoints, for example,
Death
Major cardiovascular events
Any disease-related measure by which treatment effect is thought to indicate disease modification according to normal clinical standards (what is normally used in daily clinical practice to find out whether a patient has improved). Listings are incomplete.

in epidemiological analyses is clearly linked to cardiovascular risk but does not yield therapeutic success in all interventional settings (torcetrapib is the dramatic exception). There is no doubt that HDL cholesterol is a risk marker, but its role as a risk factor needs further clarification and is not established at present.

Development of biomarkers

Biomarkers may not be readily available when it comes to translational processes, and important decisions need to be based on biomarkers in drug, diagnostic test, or medical device development. One task is the assessment of existing biomarkers, but if the assessment shows that current biomarkers are insufficient for the given task, this frequently leads to the need for the development of new ones. If biological material (blood, urine, tissue) is available—as in almost all cases—omics and genetic/pathway approaches are the ones that are tested most frequently. In general, omics tend to produce multiple readouts, such as hundreds of up-or-down-regulated proteins (proteomics are the most prominent example), and it is very challenging to test such biomarkers and establish their utility. This contrasts to genetic or expression testing aiming to identify major receptor/signaling pathways as drug targets, for example, in oncology. Although the relative contribution of a given receptor mutant or signaling pathway may be overestimated, the answer is often yes or no and so is much easier to interpret.

What are the ingredients necessary for basing go-or-no-go decisions on biomarkers that have just been found to correlate with disease parameters and/or therapeutic interventions? In other words, which properties have to be established and proven for a biomarker to, for example, become an FDA-approved genomic biomarker, as shown in Table 12.4?

There have been several different proposals that are partially specific to the targets to be measured. [Pepe et al. \(2001\)](#) proposed a five-step scheme for the development of an oncologic biomarker ([Fig. 12.7](#)). In principle, the process resembles a drug development process, from preclinical stages through early- and late-stage clinical testing. Translation is a key element in this process, as one has to prove that biomarker differences are specific and reliable.

[Vasan \(2006\)](#) aligned key elements of biomarker development to these stages of biomarker development. After a biomarker is detected, it will be tested in small groups of patients (similar to PoP testing) and in retrospective studies; finally, prospective studies of its prognostic value, including its prognostic value for interventions, will be established. Phases 4 and 5 in this model are analogous to PoC testing in drug development. In the result segment of this process, basic requirements for valid and finally effective biomarkers are described: assay precision, reliability and sensitivity, reference limits, intraindividual or interindividual variation, and finally receiver operating characteristic (ROC) analyses and number-needed-to-screen analyses.

As specified further in the chapter on statistics (Chapter 27, Translational science biostatistics), there are defined tests to attach key figures to a given biomarker and thereby describe some of its important features. Key

Preclinical exploratory	PHASE 1	Promising directions identified
Clinical assay and validation	PHASE 2	Clinical assay detects established disease
Retrospective longitudinal	PHASE 3	Biomarker detects disease early before it becomes clinical and a "screen positive" rule is defined
Prospective screening	PHASE 4	Extent and characteristics of disease detected by the test and the false referral rate are identified
Cancer control	PHASE 5	Impact of screening on reducing the burden of disease on the population is quantified

FIGURE 12.7 Proposed stages of biomarker development for the detection of early cancer. From Pepe, M.S., Etzioni, R., Feng, Z., Potter, J. D., Thompson, M.L., Thorquist, M., et al., 2001. Phases of biomarker development for early detection of cancer. *J. Natl. Cancer Inst.* 93, 1054–1061, reprinted by permission from Oxford University Press.

elements of biomarker profiling aim to answer the following questions: What is the normal distribution of values of this biomarker? How do they differ between healthy individuals and patients (how well do they discriminate between health and disease, and is there an essential, obscuring overlap)? How useful is this biomarker for disease progression prediction? The last question will be answered in most cases not by identifying a particular cutoff line (although this would be desirable) but by gradual increase of risk with a slurred border zone of transition. Again, LDL cholesterol may serve as an example in that we know that risk reduction may be achieved even into ranges that have been termed normal up to the present. There is no threshold at which the risk becomes zero. Accordingly, for treatment guidelines, cutoff lines (treat if above, do not treat if below) will be derived from consensus processes that comprise scientific evidence plus cost-effectiveness estimates and social aspects such as compliance. Fig. 12.8 illustrates the ROC curves for prostate-specific antigen (PSA) concentrations and prostatic cancer detection at different points in time before diagnosis.

As one can see, sensitivity and specificity are highest if determined at the time of diagnosis, whereas increasing time intervals between sampling and cancer diagnosis lead to decreases in both parameters. This biomarker development sequence, in general, is reminiscent of the development of any established, clinically used laboratory test, such as the test for blood glucose measurement. In line with items developed for cardiovascular biomarkers (Vasan 2006), development of biomarkers for all areas generally aims at the following key aspects:

- Assay standardization, meaning that methods are established and described in detail
- Assay reproducibility, accuracy, and availability (e.g., is the required antibody commercially available?)

- Knowledge of the distribution of biomarker values in the general population and in select subpopulations
- Abnormal levels, both in general and in subpopulations (e.g., women, children, older adults)
- Correlation between biomarker levels and known disease risk factors
- Increased utility (whether great or incremental) of the new biomarker over currently established biomarkers
- Knowledge of the relation between the new biomarker and mechanisms of disease initiation or progression
- Knowledge of the relationship between the biomarker and outcome prediction
- A superior multiple readout strategy using the new biomarker in combination with known biomarkers for overall testing for the disease
- Ability to predict the therapeutic course of action or the response to an agent by the new biomarker
- Cost-effectiveness

These questions will recur with modifications and additions when biomarker assessment approaches are examined later in the chapter. The Pepe model is focused not on drug or medical device development-related biomarkers but rather on oncologic disease markers (which can also be used to describe drug effects).

Another model or scheme that is more drug development-oriented is a proposal for pharmacogenomic biomarker development by the FDA (U.S. Food and Drug Administration, 2005; Fig. 12.9). The key elements of this proposal are essentially similar to the ones described previously, but this scheme aims at supporting drug development by novel markers under the premises of personalized medicine. It is the model still underlying the regulatory approval process for biomarkers as discussed previously (U.S. Department of Health and Human Services Food and Drug Administration, 2011, results

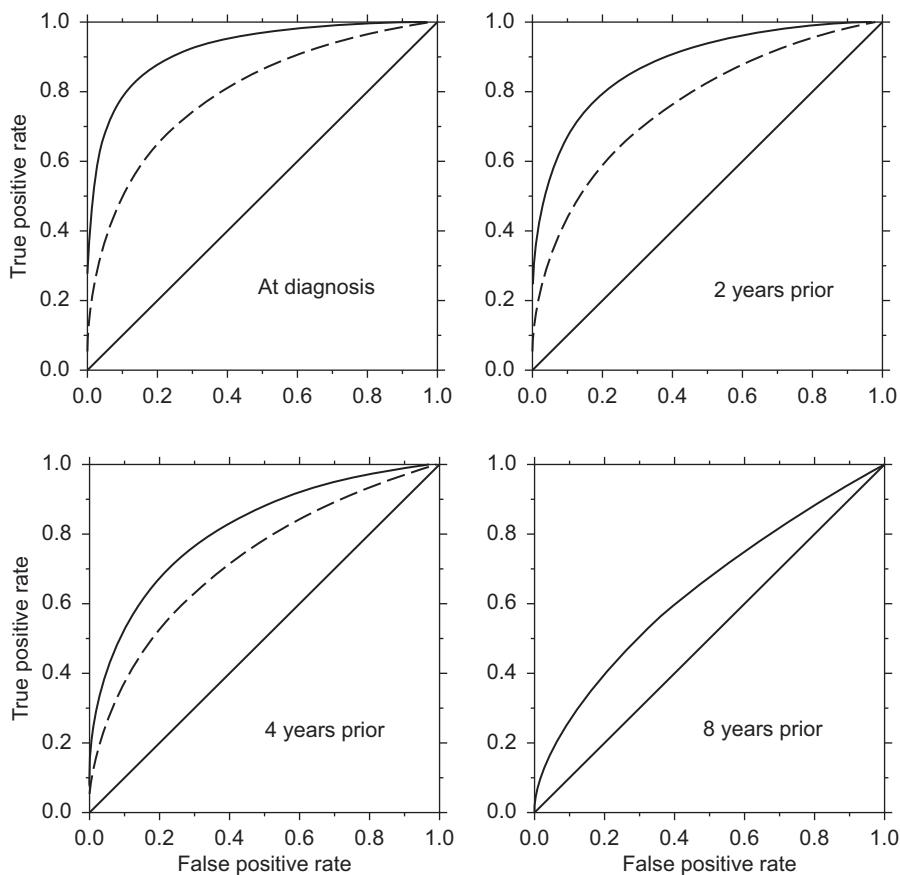


FIGURE 12.8 Receiver operating characteristic (ROC) curves for total prostate-specific antigen (PSA) and ratio of free to total PSA at various times before diagnosis. The curved solid lines represent total PSA, the curved dashed lines represent the PSA ratio, and the straight solid lines represent the line of identity. The figure shows that at time of diagnosis, PSA determinations (in particular, ratio determinations) have a high sensitivity and specificity. This is indicated by an almost rectangular ROC approaching 1 on the ordinate at small abscissa values. That means that despite low false positive tests (high specificity), high sensitivity (true positive tests) is achieved. In other words, if a test shows positive results, prostate cancer is very likely to exist; if the test shows negative results, prostate cancer is very unlikely to exist. Thus cancers that exist are not likely missed, nor are cancers suspected if they do not exist. This strength of the test is increasingly lower if done at increasing time intervals before diagnosis. From Pepe, M.S., Etzioni, R., Feng, Z., Potter, J. D., Thompson, M.L., Thorquist, M., et al., 2001, Phases of biomarker development for early detection of cancer. *J. Natl. Cancer Inst.* 93, 1054–1061, reprinted by permission from Oxford University Press.

shown in Table 12.4). It introduces staging terms describing the developmental stage of a novel biomarker:

- Pharmacogenetic test: An assay intended to study interindividual variations in DNA sequence related to drug absorption and disposition (pharmacokinetics) or drug action (pharmacodynamics), including polymorphic variation in the genes that encode the functions of transporters, metabolizing enzymes, receptors, and other proteins.
- Pharmacogenomic test: An assay intended to study interindividual variations in whole-genome or candidate gene, single-nucleotide polymorphism maps, haplotype markers, or alterations in gene expression or inactivation that may be correlated with pharmacological function and therapeutic response. In some cases the *pattern or profile of change* is the relevant biomarker rather than changes in individual markers.
- Valid biomarker: A biomarker that is measured in an analytical test system with well-established performance characteristics and for which an established scientific framework or body of evidence exists that elucidates the physiologic, toxicologic, pharmacologic, or clinical significance of the test results. The classification of biomarkers is context-specific. Likewise, validation of a biomarker is context-specific, and the criteria for validation will vary with the intended use of the biomarker. The clinical utility

(e.g., predict toxicity, effectiveness, or dosing) and use of epidemiology/population data (e.g., strength of genotype-phenotype associations) are examples of approaches that can be used to determine the specific context and the necessary criteria for validation.

- Known valid biomarker: A biomarker that is measured in an analytical test system with well-established performance characteristics and for which there is widespread agreement in the medical or scientific community about the physiological, toxicological, pharmacological, or clinical significance of the results.
- Probable valid biomarker: A biomarker that is measured in an analytical test system with well-established performance characteristics and for which there is a scientific framework or body of evidence that appears to elucidate the physiological, toxicological, pharmacological, or clinical significance of the test results. A probable valid biomarker may not have reached the status of a known valid marker for any of the following reasons:
 - The data elucidating its significance may have been generated within a single company and may not be available for public scientific scrutiny.
 - The data elucidating its significance, although highly suggestive, may not be conclusive.
 - Independent verification of the results may not have occurred (U.S. Food and Drug Administration, 2005).

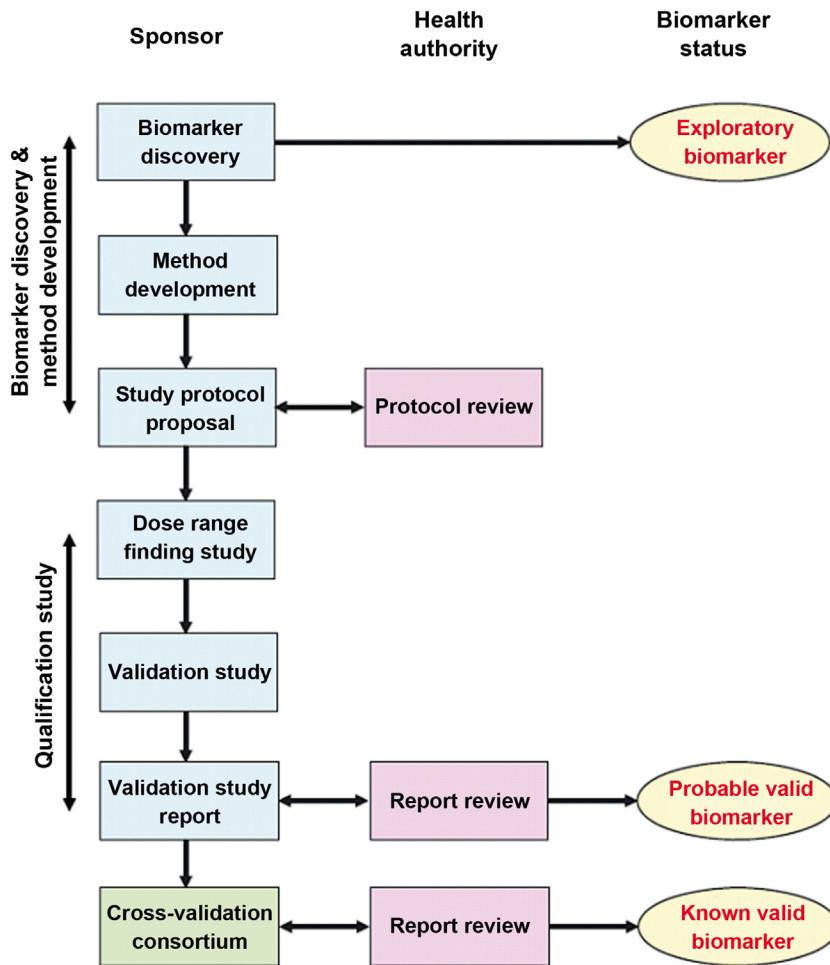


FIGURE 12.9 Schematic summary of the FDA recommendation for pharmacogenomic biomarker development (U.S. Food and Drug Administration 2005). Although principally independent from the drug development progress, it aims at facilitating it by developing novel biomarkers. From Marrer, E., Dieterle, F., 2007. Promises of biomarkers in drug development—a reality check. *Chem. Biol. Drug. Des.* 69, 381–394, reprinted by permission from John Wiley and Sons.

Though probably somewhat biased for the use of genetic markers, as shown by the first two terms, this proposal conveys generalizability and may serve as a template for biomarkers from other sources as well. At least the categories “known valid biomarker” and “probable valid biomarker” seem useful, although their definition borders are not sharp and easily measurable in all cases. As was noted in the definition for known valid biomarkers, this assessment is based on consensual processes that are accepted in the scientific community. But is this acceptance merely by common usage, or is it substantiated by data? Could widespread agreement be a result of company sponsoring?

These critical questions should point to the fact that an objective (or as objective as possible) assessment is considered superior to interindividual consensus agreements (a gut-feeling consensus). Fig. 12.10 exemplifies the process of biomarker development for chronic graft rejection biomarkers. It summarizes the principal stages of drug development and codevelopment of a novel biomarker. After identification in discovery processes, this biomarker will be profiled and optimized as a useful, predictive biomarker; further tested in clinical trials to concentrate the patients (stratification) who may benefit most; used for adaptive trial designs

(trial designs that will be changed while the trial is running, for example, the number of patients included may be changed on the basis of biomarker tests); and finally coapproved with the drug. That means that the approval of the drug includes the necessity to test the novel biomarker before its use, for example, HER2 testing with the use of trastuzumab. The evaluation of the novel biomarker will follow the FDA process for pharmacogenomic biomarker submissions (U.S. Food and Drug Administration, 2005; U.S. Food and Drug Administration, 2011). In the case of chronic graft rejection biomarkers as depicted in Fig. 12.10, the NIH-led Clinical Trials in Organ Transplantation program (which is similar to the NCI Cancer Biomarker Project) facilitates the process. This program announced that CXCL9 may be a valuable stratification biomarker (among others) for kidney transplant rejection (Hricik et al., 2013), which could be useful in guiding clinical decision processes (Safa et al., 2017). The Biomarkers Consortium, a private-public partnership founded in 2006 managed by the Foundation for the National Institutes of Health supports and organizes biomarker development (Buckman et al., 2007; Wilson et al., 2007). The Biomarkers Consortium plays a central role in this plot and frequently announces new data on biomarker

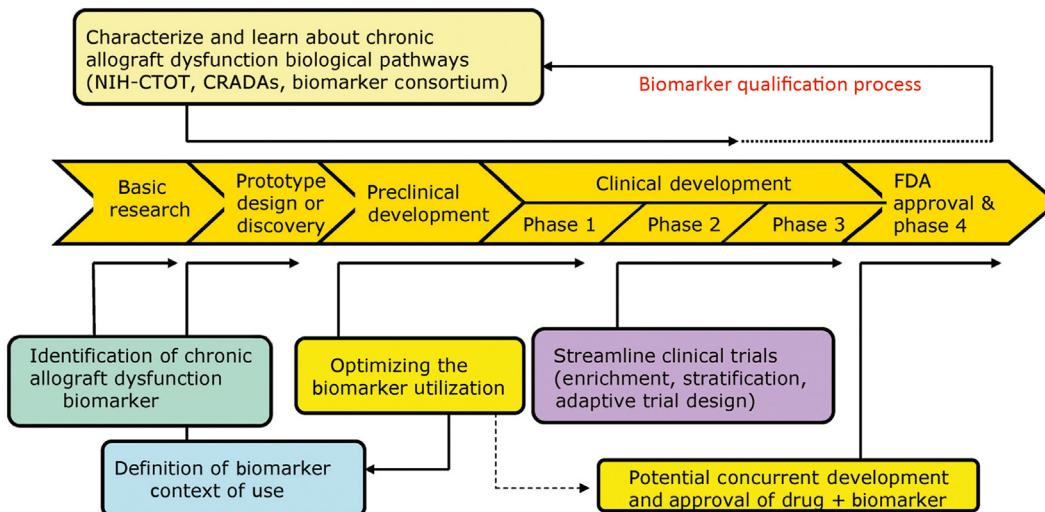


FIGURE 12.10 Scheme of the biomarker development for chronic graft dysfunction (renal transplant rejection) as an example. Its development parallels drug development, which is depicted in the central arrow. The graph shows the potential impact of novel biomarkers on drug development (streamlining clinical trials by patient concentration and stratification) and points to the possibility of codevelopment of drugs and biomarkers, resulting in concurring approval (i.e., the drug is given only when the novel biomarker reaches certain values). The qualification process involves the Biomarker Qualification Pilot Process of the FDA (see text) and, specific to this area, the NIH-CTOT (the Clinical Trials in Organ Transplantation study sponsored by the National Institutes of Health) and CRADAs (cooperative research and development agreements). *From Burckart, G.J., Amur, S., Goodsaid, F.M., Lesko, L.J., Frueh, F.W., Huang, S.M., et al., 2008. Qualification of biomarkers for drug development in organ transplantation. Am. J. Transpl. 8, 267–270, open access, reprinted by permission from John Wiley and Sons.*

qualification studies, for example, on adiponectin as a biomarker to predict glycemic efficacy (Wagner et al., 2009), or on biomarkers for drug-induced liver injury (Bonkovsky et al., 2018). The NCI Cancer Biomarker Project has identified the following scientific components for detecting and developing biomarkers in the cancer area through the [Early Detection Research Network \(2008\)](#):

- Biomarker development laboratories
- Biomarker reference laboratories
- Clinical validation centers
- Data management and coordinating centers
- Informatics centers

In a similar attempt, the Predictive Safety Testing Consortium (PSTC) was launched by the FDA and the C-Path Institute in March 2006 to assess new preclinical and clinical biomarkers for nephrotoxicity, hepatotoxicity, vascular injury, and genotoxic and nongenotoxic carcinogenicity. The PSTC presented 23 biomarkers for nephrotoxicity assessment to the FDA for qualification consideration in June 2007 (Burckart et al., 2008). Subsequently, the FDA accepted seven of them as valuable biomarkers for renal safety issues (Dieterle et al., 2010). The status of this topic was reviewed by McDuffie (2018). The PSTC recently issued detailed guidance on the analytical validation of assays used in the qualification of biomarkers (Critical Path Institute 2019).

In Europe, with some delay, biomarker consortia have been founded as well. The Biomarker for Cardiovascular Risk Assessment across Europe (BiomarCaRE) consortium

is an EU-funded organization including partners from academia and industry; it focuses on the identification and development of cardiovascular risk assessment biomarkers, such as NTproBNP or troponin (BiomarCaRE 2020). Another initiative from the IMI EU organization is the Translational Safety Biomarker Pipeline (TransBioLine) (Innovative Medicines Initiative 2017).

These activities demonstrate that biomarker development for efficacy and—maybe even more important—safety assessment has become a vital and essential part of drug development and underpins the translational efforts of industry and public institutions in the biomedical arena, especially the FDA, with biomarkers being considered the single most important vehicle of translational success.

Predictivity classification of biomarkers and scores

Biomarkers and their development are the backbone of successful translation. A major challenge in this context is the assessment of biomarker quality. It is obvious that a biomarker may just reflect nonspecific alterations of broad physiological responses, and its alterations could be induced by a manifold of influences. Thus this marker is quite non-specific and is prone to disturbances that may or may not have anything to do with the disease and the related intervention. Such a marker might be, for example, fever, CRP, or headache; those markers reflect inflammation at large (fever or CRP) or the state of feeling unwell (headache).

One can easily understand that such markers at least as standalone biomarkers are not very helpful for designing and developing new drugs. These changes could have been induced by infections or psychological stress that might not have any relation to the intervention in question.

On the other hand, there are biomarkers that are tightly correlated with outcome and highly specific for a given condition, and they may still be very simple and straightforward. Tumor size serves as an example from oncology. If diagnosis has been proven by histology, any change in tumor size is a relevant biomarker for regression or progression and thus for the natural or interventional course of the disease. If size is determined by imaging techniques, as usual, the translational power is high, and drug effects may easily be followed across species borders. Failures of translation would have to do with tumor biology and animal models, but tumor size as such reliably measures disease progression or regression and thus therapeutic effects.

Although those two examples are located at the extremes of the spectrum of biomarker validity, there are more complex and unclear biomarker situations in which the assessment of the predictive value of a given biomarker is much more difficult. A prominent example is the problem of early biomarkers for beneficial drug effects on atherosclerotic plaque progression. To begin with, there are no good animal models for human atherosclerosis, which takes 30–50 years to develop, and conditions of arterial damage in animal models are very different from those in humans. Animal models of atherosclerosis (for a review, see [Emini Veseli et al., 2017](#)) tend to be heterogeneous, so regression of atherosclerosis in an animal model may not indicate that the same happens in human pathology. More troublesome is the fact that some species, namely, rodents, seem to be protected from ischemic endpoints relating to atherosclerosis. Thus even if atherosclerotic lesions develop in rodents, their importance for endpoints may be different from that of humans. Therefore the important goal is to find biomarkers that indicate antiatherosclerotic effects across species borders. Markers of inflammation, which is inherent to atherosclerotic processes, were seen as favorable biomarkers. They include high-sensitive CRP, which had been shown to correlate to cardiovascular events in humans but has been shown to have drawbacks and is still under critical discussion ([Ben-Yehuda, 2007; Peikert et al., 2019](#)). Current thinking may even support the hypothesis that mild inflammation from independent sources could accelerate atherosclerosis ([Grufman et al., 2014](#)). Because this marker is still controversial in humans, related effects in insufficient animal models may be even more misleading and translationally irrelevant. Another route of assessment of atherosclerotic disease utilizes imaging techniques that are easily transferable and directly show atherosclerotic plaques. As detailed in the discussion of typical cardiovascular biomarkers, the main problem with imaging biomarkers is the assessment of

plaque stability, because the leading problem is not its size but its potential rupture. Thus plaque size, location, and maybe composition (fat content) can be visualized by imaging (including *in vivo* staining), but it is still hard to predict which plaque will rupture ([Briley-Saebo et al., 2007](#)). Modern techniques are further developed in the hope to improve prediction, such as [18-F] fluorodeoxyglucose PET/computed tomography ([Mehta et al., 2012](#)) or optical coherence tomography ([Sugiyama et al., 2018](#)). This example should draw our attention to a relevant area of biomarker development in which clearly predictive biomarkers are still lacking and major efforts are being undertaken to develop them. The Biomarkers Consortium ([Biomarkers Consortium, 2020; Briley-Saebo et al., 2007](#)) has included related concepts in its portfolio. These examples should also demonstrate that biomarkers can range from very strong and predictive (not requiring any developmental investment) through unsatisfactory to dismal or useless. Although this judgment seems straightforward for the examples given previously, an objective, interindividually reliable, reproducible, standardized, and valid process should be developed by which the value of a biomarker is assessed. If we accept that there should be a standardized assessment procedure in place, we need to define its remits.

In fact, the only true remit of this assessment is an answer to the question “How much does this biomarker or a biomarker panel (if they are concomitantly applied) contribute to the translation of preclinical findings into human use in terms of both efficacy and safety?” A simpler terminology would be *predictive value*, implying that a predictivity score should be aligned to a given biomarker. This predictive value is reflective of the pivotal role a biomarker plays in translational medicine. Thus the *standardized assessment of the predictive biomarker value* would be a major remit of the science of translation in medicine to which this book is devoted.

In the discussion of biomarker classification, some attempts to structure the universe of the large spectrum of potential biomarkers have been presented, including the technology-oriented mechanistic approach ([Rolan et al., 2003](#)) and the three-level approach by the NIH ([Biomarkers Definitions Working Group, 2001](#)). Although the first approach does not relate to the predictive value, the latter at least separates surrogates and clinical endpoints from the bulk of unclassified markers. Certainly, the predictive value of surrogates is thought to be close to 100% (almost all predictions from this type of marker into clinical endpoints had been correct), and the endpoints do not require prediction because they represent the top end of development.

The proposal for pharmacogenomic biomarker development by the FDA ([U.S. Food and Drug Administration, 2005](#); new guidance [U.S. Department of Health and Human Services Food and Drug Administration, 2011](#))

goes one step further and introduces “valid biomarkers,” subdivided into “known” and “probable” biomarkers, and provides simple definitions that, however, lack quantification. They rely on consensus (see Table 12.3) and thus require a good deal of gut-feeling assessment.

However, there are only a handful of surrogates, and situations in drug or device development in which they may be used early on are rare. Thus the vast majority of biomarkers require a true predictivity assessment, which is outlined in the following discussion. Vasan (2006) already presented a list of questions to be answered for a given biomarker, which were described earlier in the chapter. Although those items represent major features of laboratory test development such as accuracy, reproducibility, stability, correlation to disease, and many others, the list lacks the major translational aspects (e.g., in how many species other than humans is the biomarker present and validated). In addition, it does not provide a score by which the overall value can be determined quantitatively. Although quantification of some variables such as stability or accuracy will be arbitrary, at least in part, some attempt of overall utility scoring should be undertaken to be enable weighing biomarkers against each other. Notably, quantification has its limits, and measures such as knock-outs or knock-ins—that is, overruling of the other parameters—need to be installed in such an approach.

Here, the first and—to the best of the author’s knowledge—so far only attempt to provide a quantitative predictivity scoring system for biomarkers is described (Wehling, 2006a, Table 12.6). The 10 items in the scoring

system are related to animal data, human data, the proximity of the biomarker to the disease (e.g., causal relation, the disease constituent involved in pathophysiology), test parameters (sensitivity, specificity as integrated into predictivity, which has nothing to do with the overall predictive value of the biomarker but is a statistical measure of the test quality), and feasibility aspects (accessibility). The grading scale ranges from (0)1 to 5(6), with 0 being a knock-out feature and 6 being a knock-in feature, which indicates that the marker may be close to being a surrogate. The extreme scores overrun the other item scores. Following is an explanation of the 10 points of the scoring systems and how each point is scored [modified from Wehling (2006a), reprinted by kind permission from Springer Science and Business Media]:

- Animal or in vitro testing: Are there data at this level? Yes = 1; animal plus human data = 5; only human data = 3. This item should underscore the fact that human data on this particular biomarker are highly relevant and reflected by a high score. The lowest score of 1 is given if at the time of assessment all considerations rely on animal or in vitro data only. It is obvious that in this situation, predictivity is merely speculative. A score of 0 would be given if no animal or in vitro data are available. This is rare but could reflect observational, nonsystematic material that is not sufficient to qualify a measure as a biomarker.
- How many species have been tested positively? $\leq 1 = 1$; $2 = 3$; $\geq 3 = 5$. Obviously, if cross-species variation is low and several species show the same change with regard to the biomarker, scoring should be higher than in cases in which only one species or model shows expected effects.
- Are the animal models sufficient to reflect human disease? If there is no human validation in terms of disease or mechanistic similarity, the score is 1; grading goes up to 5 if homology is perfect, for example, when certain bacterial infections in which the in vitro testing is very similar to human testing because the infectious agent is the same. This decision should reflect the amount of scientific evidence as judged by the number of different groups involved, the consistency of the data, and the level of publication (whether the journal is peer-reviewed).
- Are there corresponding clinical data—including genetics or histology—to judge the validity of the animal or in vitro data? This is asking not whether there are clinical studies with the biomarker in interventional studies but whether there is evidence that the animal model or in vitro testing in general correlates with similar diseases or related conditions. Answering this question affirmatively does not mean that the biomarker has already been tested in the same disease; it means that there is evidence that the model is relevant.

TABLE 12.6 Predictive profiling of biomarkers.

Biomarker Profile		
1	Animal or in vitro	
2		How many species?
3		Model sufficient to describe human disease
4		Corresponding clinical data classification
5	Human	
6		Data classification
7	Pivotal disease constituent	
8	Predictability	
9	Accuracy or reproducibility	
10	Accessibility	

Modified from Wehling, M., 2006a. Translational medicine: can it really facilitate the transition of research “from bench to bedside”? *Eur. J. Clin. Pharmacol.* 62, 91–95, reprinted by kind permission from Springer Science and Business Media.

The score is 1 for a singular study, 2 for multiple studies; if the biomarker is correlated with intervention from other projects on similar targets, the score is 2 for a singular study, 3 for multiple studies; if the biomarker is correlated with interventional endpoint change from other projects on similar targets, the score is 4 for a singular study, 5 for multiple studies.

- Are human data on this biomarker available that directly relate to the project or disease to be treated? Yes = 5; no (only animal data are available) = 1. This again underpins the importance of human data in this context. This is a yes-or-no item; no intermediate scores are allowed.
- Human data classification: Does the available clinical material show a meaningful correlation? If the biomarker is correlated with the disease, the score is 1 for a singular study, 2 for multiple studies; if the biomarker is correlated with intervention, the score is 2 for a singular study, 3 for multiple studies; if the biomarker is correlated with interventional endpoint change, the score is 4 for a singular study, 5 for multiple studies, *6 if it is a surrogate*. This score should be doubled to underscore that this pivotal feature has been derived from human studies.
- Does the biomarker represent a pivotal disease constituent? In other words, is it essential to the pathophysiology of the disease to be assessed? No (it is likely to represent an epiphenomenon) = 1. A score up to 5 is given if it is a solitary, standalone contributor to the disease (e.g., absence of singular enzymes in monogenetic diseases, supplementation of gene product or its derivatives fully restores health).
- What is the statistical predictability, which in a test describes its sensitivity and specificity (false positive and negative results)? >60% = 1; >75% = 2; >85% = 3; >90% = 4; >95% = 5. If a precise percentage is not available, grade according to an estimate.
- What is the accuracy or reproducibility of the biomarker assay? Poor (e.g., SD > 70%) = 1; a score of up to 5 is given if the accuracy or reproducibility is high (SD < 15%).
- How accessible is the specimen? This is an important issue, as a specimen that is hard to obtain limits the use of a biomarker. Easy access (e.g., a by-product such as peripheral blood that requires no legal procedures or additional animal use) = 5; expensive animal work (e.g., monkey work), legal concerns, parameter measurement that has a relatively high impact on humans (e.g., muscle or liver biopsies) = 1; inaccessibility in humans = 0, which is a knock-out (e.g., serial brain slices).

The final score may be interpreted as follows, regarding the predictive value of the biomarker:

- <30 = a low-value biomarker; it may just serve as a PoM marker.

- 31–40 = a medium-value biomarker; it may serve as a PoP marker.
- 41–50 = a high-value biomarker; this score is desirable for PoP markers, and a score of >50 (maximum 55) means that the biomarker is close to a PoC marker.
- A score of 60 or higher means that the biomarker is a PoC or even a surrogate marker.
- A score of 0 should prevent the biomarker from being considered to be able to promote the translational aspects of drug or device development.

Any single component described in the scoring system can overrun the scoring if it is considered to be overwhelmingly important. Thus important parts of the scoring remain consensus decisions, although this should be limited to the reading of the score items. Of course, for example, the causal relationship of a biomarker to the disease may be more or less important, and scoring may thus be partially subjective. Subjectivity cannot be entirely excluded, and experts need to be involved in this process regardless.

This scoring system reflects two main assumptions: First, biomarkers that have no human validation are very weak, at least at that stage, because the human system is the ultimate test system for successful translation. The scoring system conveys the operational guidance to rapidly progress into human material, which could be genetics, retrospective analyses from tissue banks, results from other studies, and much more. The second assumption is that it is very important that the biomarker be tied into the disease process to become highly predictive. These two key features are supplemented by typical test parameters such as accuracy and (statistical) predictivity.

It is obvious that this proposal is only an initial attempt to give labels to biomarkers and to spur developmental aspects. Clearly, the scoring of a biomarker may be dramatically increased if, for example, a clinical trial shows its value or if the third and fourth species seem to be in line with the first and second. Thus this grading system should also be inspiring in the sense that a bad biomarker needs the hypothetical studies A, B, and C to become a better one if results are positive. On the other hand, in some cases, one is surprised how limited the justification of the use of frequently used biomarkers (e.g., CRP in atherosclerosis) is when challenged by a structured approach like the one described previously.

Case studies

As a first step in its ongoing validation process, this scoring system has been applied to biomarkers in the cardiovascular area. Table 12.7 shows the results of applying the proposed grading scheme for biomarkers to frequently used imaging procedures in the assessment of atherosclerotic lesions. They include magnetic resonance imaging (MRI)

TABLE 12.7 Predictive profiling of atherosclerosis imaging biomarkers according to the grading scheme described in the text.^a

	Biomarker profile	Total plaque burden (MRI)	Carotid plaque volume (MRI, US)	Coronary plaque volume (IVUS, MRI)	Stenosis (X-ray angiography, CT, MRI, IVUS, SPECT, PET)	Intra-plaque lipid core (MRI, US strain analysis), pre-ORION	Intra-plaque lipid core (MRI, US strain analysis), post-ORION	Intra-plaque thrombus (MRI, US)	Calcium scoring (X-ray, CT, US [for animals])	Intima media thickness (US)
1	Are animal or in vitro data available?	5	5	5	5	1	5	1	5	5
2	How many species have been tested positively?	3	3	3	5	1	5	1	5	5
3	Are the animal models sufficient to reflect human disease?	2	2	2	2	2	2	2	3	3
4	Is there corresponding clinical data?	2	5	5	5	2	5	2	2	5
5	Are human data available?	5	5	5	5	1	5	1	5	5
6	Human data classification	4	10	10	10	2	10	2	4	10
7	Does the biomarker represent a pivotal disease constituent?	2	3	3	3	2	3	3	2	3
8	What is the statistical predictability?	1	2	3	3	1	3	2	4	3
9	What is the accuracy or reproducibility of the assay?	3	4	3	4	2	4	2	4	3
10	How accessible is the specimen?	3	4	1	1	4	4	4	3	5
	Score	30	43	40	43	18	46	20	37	47

^aWehling (2006a). The table depicts the individual scores and sums for frequently used biomarkers in the field. IVUS, Intravascular ultrasound; MRI, magnetic resonance imaging; PET, positron emission tomography; SPECT, single photon emission computed tomography; US, ultrasound.

Modified from Wehling, M., 2006b. Translational science in medicine—implications for the pharmaceutical industry. Int. J. Pharm. Med. 20, 303–310, reprinted by permission from Adis International, Wolters Kluwer Health.

techniques, computer tomography, ultrasound techniques, intima media thickness determination, X-ray techniques, PET techniques, single photon emission computed tomography, and intravascular ultrasound techniques. All these methods are assessed in **Table 12.7** according to their capacity to predict the beneficial effects of antiatherosclerotic drugs in translational approaches. As can be seen, the scores range from low (18) to high (47). Intima media thickness received the highest score because it has been shown in several human studies to predict cardiovascular outcome and the beneficial effects of interventions (for a review, see [Hurst et al., 2007](#)). It was termed *surrogate*, although this is not generally accepted because its determination is subjective and reproducibility is limited. Meanwhile, its reliability has been even further studied, but correlations to cardiovascular events seem to be nonlinear and follow models that are difficult to assess ([Simonetto et al., 2020](#)). Thus it did not surmount the limit of 50 to be considered a surrogate or to at least come close to it.

At the other end, determination of intraplaque lipid core, mainly by MRI in carotid arteries, scored lowest in this set of biomarkers. It has been postulated that this parameter can predict plaque instability and thereby identify the so-called vulnerable patient. The vulnerable patient is threatened by his or her unstable plaques, which may suddenly rupture, causing coronary thrombosis and myocardial infarction and death. At the time of initial assessment (early 2005), this hypothesis was driven almost exclusively by animal evidence. There were no human data clearly showing that in humans the extent of the lipid core of a plaque was predictive. However, this changed dramatically when the ORION (outcome of rosuvastatin treatment on carotid artery atheroma: a MRI observation) trial was published ([Underhill et al., 2008](#)). This trial for the first time showed that intervention with rosuvastatin beneficially affected the lipid core; this represents the first human data on this type of intervention. If the assessment was done including ORION data (column “post-ORION” next to column “pre-ORION” in **Table 12.7**), the scores relating to human data (items 1, 2, 4, 5, 6, and 9 in **Table 12.7**) dramatically increase, thereby elevating the total score to 46. This is the second best score in the table, exceeded only by intima media thickness. Plaque regression by this statin and other LDL-lowering strategies has been confirmed by subsequent studies (e.g., [Shirahama et al., 2018](#)).

This example demonstrates that assessment of biomarkers can unveil major gaps in the strength of a biomarker. The generation of data that fill the gap, as in this case, measurably changes the grading, which thus reflects successful biomarker development. The given example of scoring various imaging biomarkers in atherosclerosis does seem to identify stronger and weaker biomarkers, which could be valuable in making decisions based on such

biomarkers. If the grading process is further validated by its practical use, it may become a constant reminder to push biomarker development, because successful drug or device development relies on predictive biomarkers. Biomarker choice and development in certain areas could become more expensive than drug development, but this could represent a way to optimize costs because lower rates of late attrition could compensate or even overcompensate for increased biomarker development costs.

The proposed grading is an initial step in raising translational medicine to the rank of a science; it requires further refinement and development. Validation may be derived only from experiences with its practical impact. Researchers in this field will have to decide whether they want to continue with the merely subjective biomarker assessment approaches highlighted here—such as the gut-feeling approach—or whether a structured approach such as the 10-step scoring system is superior. The author strongly believes that there is no alternative to structured, scientific approaches if translational medicine is to develop past the level of wishful thinking to become an essential, validated tool.

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Chapter 13

Genetics, molecular biomarkers, and artificial intelligence to improve diagnostic and prognostic efficacy

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Abstract

Recent advancements in high-throughput “omics” technologies have open the possibility of obtaining a huge amount of data on DNA, RNA, microRNA (mRNA), noncoding RNA (ncRNA), proteins, peptides, and metabolites with limited cost and resource employment. These resources have paved the way for more precise and personalized patient care, which has brought great strides in disease treatment, as clinicians are able to select therapeutic courses that have been tailored to a patient’s specific set of biomarkers. However, the discovery of new biomarkers, especially using proteomics and genetics studies, required not only well-defined and standardized protocols to generate reliable data, but also standards or other materials for guaranteeing the internal quality control procedures of the whole analysis workflow. Finally, statistical analysis, currently based mainly on artificial intelligence and machine learning approaches, are error-prone procedures and require pipelines optimized to avoid overfitting errors.

Keywords: Biomarkers; proteomics; genetics; artificial intelligence; disease diagnosis; disease prognosis

Introduction

Accurately diagnosing, monitoring, and treating disorders requires continuous advances in biomarker discovery and development of rigorous methods for their measurements in body fluids. In recent years, several new technologies have been developed in laboratory diagnostic testing, and they offer unprecedented prospects for translating early and accurate diagnoses of human disorders into improvements in clinical care (Plebani and Laposata, 2006). Indeed, technological advancements in high-throughput “omics” technologies, such as mass spectrometry, microarrays, and

sequencing technologies [such as next-generation sequencing (NGS)], have enormously improved our possibilities of obtaining accurate information on DNA, RNA, microRNA (mRNA), noncoding RNA (ncRNA), proteins, peptides, and metabolites with limited cost and resource employment (Borga et al., 2019). However, in contrast to the past situation in which the researchers’ focus was on generating good-quality data by which valid conclusions could be drawn, a new era has emerged in translational medicine that is based on the integrative analysis of omics results. The large amount of generated data and the growing availability of clinical health data have paved the way to a more precise and personalized patient care (Hodson, 2016). The term *precision medicine* refers to preventive medicine or tailored treatments based on the careful understanding of individual genes, biomarkers, demographic, and lifestyle features (Wang et al., 2016). The possibility of obtaining such advancement is also due to the recent proliferation of big datasets, emblematic of precision medicine, that make it feasible to study stratified variation and clinical outcomes at scale (Data et al., 2018). Interestingly, precision medicine has allowed overcoming many limitations on the appropriate interpretation and utilization of laboratory information, given the complexity of large volumes of data and numbers, potentially creating confusion for practicing physicians.

While the field is complex, presenting several areas of research and development, we would like to focus on some relevant issues:

- Sources of errors in clinical proteomic and genetic studies
- Bioinformatics tools for proteomic or genetic studies

- Artificial intelligence (AI), machine learning (ML), and deep learning (DL) techniques to improve the clinical utility of proteomics or genetics studies
- Applications of proteomics and genetics algorithms for diagnosis or prognosis of tumors

Source of errors in clinical proteomics studies

Clinical application of proteomics is based on the concept that a multimarker approach should be more accurate and reliable in identifying a disease state. However, proteomic pattern diagnostics, sometimes called *proteomic profiling*, presents several challenges.

Current proteomic technology enables the construction of a biomarker discovery pipeline from some essential process components: candidate discovery, qualification, verification, and biomarker validation (Gisbert and Chaparro, 2019). In particular, the discovery phase represents how the differential expression of specific proteins between states is first defined. This process results are particularly sensitive to errors, owing to poor study design, nonstandardized processes, and wrong data analyses.

As underlined by Ransohoff, the introduction of discovery-based research, in which high-throughput technologies allow the measurements of multiple proteins, mutations, and gene expression without a specific hypothesis, threatens the validity of obtained data (Ransohoff, 2005). The study design is the essential feature of small studies because it minimizes problems caused by chance (random errors) and systematic biases. Well-driven small studies can effectively answer essential questions and demonstrate a proof-of-principle about a molecular marker. Systematic biases are types of errors that occur nonrandomly during the analysis, while unknown and unpredictable changes can cause random errors. Problems caused by systematic biases and chance could arise at any point of the preanalytical, analytical, and postanalytical phases and are shared across them. A large sample size does not directly address biases, although it can reduce statistical uncertainty by providing a smaller confidence interval around a result and reducing random error. Both sources of errors could be carefully minimized by (1) choosing an appropriate study design and (2) selecting adequate standard operating procedures (SOPs). SOPs are defined by international scientific organizations, such as the Human Proteome Organization (HUPO). HUPO fosters the development of new technologies and techniques and defines protocols to standardize proteomics analyses and achieve uniformity of results. Different laboratories can use the data obtained interchangeably.

Preanalytical conditions, such as collection practices, sample handling, and storage conditions, may differ from

institution to institution, thus influencing the protein present in biological fluids. Hence multicenter studies better demonstrate the real efficiency of profiling proteins reliably.

Analytical variables, such as inconsistency in conditions of instruments, could result in poor reproducibility and high measurement imprecision. The use of control material after calibration and throughout a run ensures good instrument performance and reliable data. However, a limited number of studies have focused on how internal and external quality control, an essential and quality-related feature, should be incorporated into experimental proteomic protocols (Toghi Eshghi et al., 2018; Lebert et al., 2015; Horan et al., 2019). Reproducibility studies that are performed with adequate control materials are, in fact, prerequisites for the safe introduction of the new techniques in clinical laboratory practice. However, the studies that have evaluated this topic showed several limitations in the applicability of internal and external quality control analyses for proteomic studies (Horan et al., 2019; Maia et al., 2020). Indeed, several challenges remain unsolved and require further efforts, such as the complexity of the production of new reference materials and difficulties associated with achieving their homogeneity and stability (Kiełbasa et al., 2016).

Finally, postanalytical variables, such as biological variability and reference ranges as well as decision limits, represent other key issues for a correct interpretation of the laboratory data.

Current limitations and open questions relate to within-class biological variability. It may concern possible diurnal variation in protein expression, making time standardization of sample collection mandatory. For any individual, many analytes fluctuate based on time of day, fasting state, or age. Although these fluctuations may not be clinically relevant, they do add an additional level of complexity in elucidating disease-induced protein changes from changes due to biorhythmic fluctuations. An evaluation of the effects of gender, age, pathophysiological conditions, and benign diseases is also important in understanding other possible effects on protein profiling expression.

Source of errors in next-generation sequencing

NGS technologies produce high amounts of raw data, but they also generate artifacts and errors despite being much faster and cheaper. Some of these errors may mimic biological signals (mutation), thus leading to incorrect conclusions. In NGS, two types of errors may happen: experimental and computational.

In a typical NGS workflow, experimental errors can be introduced in all steps prior to sequencing, including sample processing, DNA isolation, and polymerase chain reaction (PCR) amplification (Chen et al., 2017).

In the context of genomics and molecular pathology, computational, mathematical, and statistical tools are used to collect, organize, and analyze large and complex genetic sequencing data and related biological data. When executed in a predefined sequence to process NGS data, a set of bioinformatics algorithms is collectively referred to as a bioinformatics pipeline (Roy et al., 2018; Kadri, 2018).

Computational errors may happen in every postsequencing NGS pipeline step. A typical NGS pipeline consists of quality control of raw sequence reads, alignment to a reference genome/assembly, postalignment quality control, identification of mutation (variant calling and genotyping), postvariant call, and data storage. It is more important than ever that laboratories strive to develop pipelines with the highest possible performance standards and validate them as stringently as possible to reduce errors. In their paper, Kadri S and colleagues briefly discuss the standard methods used for preprocessing data and variant calling and outline in detail various other bioinformatics modules that can be incrementally added to a bioinformatics pipeline to detect emerging and more complicated biomarkers in parallel with existing workflows (Kadri, 2018).

Bioinformatics and computational tools for clinical proteomic studies

As a result of the copious amount of data produced from proteomic analysis, the use of bioinformatics tools is required to sift through the data to identify proteins of interest from raw mass spectrometric data. However, a further step is needed before the research insights of proteomics can be translated into clinical practice is to identify and characterize any single peak detected in the proteomic profile. Again, bioinformatics represents a fundamental tool to identify proteins (e.g., Mascot database) (Perkins et al., 1999) and protein fragments through databases available for specific proteins and posttranslational modifications, such as Pfam (Punta et al., 2012), Swiss-Prot, and UniProt (Wu et al., 2006). Several other tools have been developed in recent years, most of them by independent researchers (Cox and Mann, 2008).

Bioinformatics and computational tools for next-generation sequencing

NGS assays for genomic and transcriptomic biomarkers include whole-genome sequencing, whole-exome sequencing, RNA sequencing, and genomic or gene expression panels (Feliubadaló et al., 2017; Ascierto et al., 2013; Byron et al., 2016). NGS-based gene panel tests, in which only clinically important genes are examined, have been developed to obtain genomic data in a timely and cost-effective manner (Feliubadaló et al., 2017; Ascierto et al.,

2013; Byron et al., 2016). At present, the NGS-based gene panel test is the first choice for individual cancer patient care (Feliubadaló et al., 2017; Ascierto et al., 2013; Byron et al., 2016).

NGS can enable the simultaneous analysis of a broad spectrum of genomic alterations, such as mutations, copy number variation, translocations, and fusion in multiple genes (Moorcraft et al., 2015).

NGS technology allows clinical laboratories to generate high amounts of data per instrument run (thousands of molecules in a parallel shape). Therefore this amount of data generated required computational and bioinformatics skills to manage, analyze, and interpret them. Data analysis consists of primary analysis, secondary analysis, and tertiary analysis.

The primary analysis consists of detecting and analyzing raw data, the generation of sequencing reads, and scoring base quality. FASTQ files are the output of primary analysis. Data analysis is a secondary analysis and includes the reads alignment against the reference human genome and variants calling. This process may result in sequence alignment errors, such as errors in sequencing or gaps between the sequenced data and the reference genome causing misalignment problems; indeed, it is essential to understand the different between a real variation and a misalignment (Pereira et al., 2020). The output of the secondary analysis is the binary alignment/map and sequence alignment/map files (Li et al., 2009). Some user-friendly software is available to view the alignments, such as the Integrative Genomics Viewer (Thorvaldsdóttir et al., 2013). The tertiary analysis consists of variant annotation, filtering, and data visualization tools; these steps are made possible by combination with different software. The output of the variant calling is a VCF file (Pereira et al., 2020).

Several commercial software programs have been developed to assist in interpretation and selection of variant in a diagnostic context, such as VarSeq/VSClinical (Golden Helix), Ingenuity Variant Analysis (Qiagen), Alamut Software Suite (interactive biosoftware), and VarElect (Stelzer et al., 2016).

Artificial intelligence, machine learning, and deep learning techniques to improve the clinical utility of clinical proteomics and genetics

Data science and, in particular, AI, ML, and DL tools have become leverage that enhances the comprehensive of complex datasets of integrative omics data, such as proteomics or genomics data. These data are merged with demographic and other individuals features (e.g., imaging or medical records) come from different sources (Gruson et al., 2019). In these analyses, results obtained from

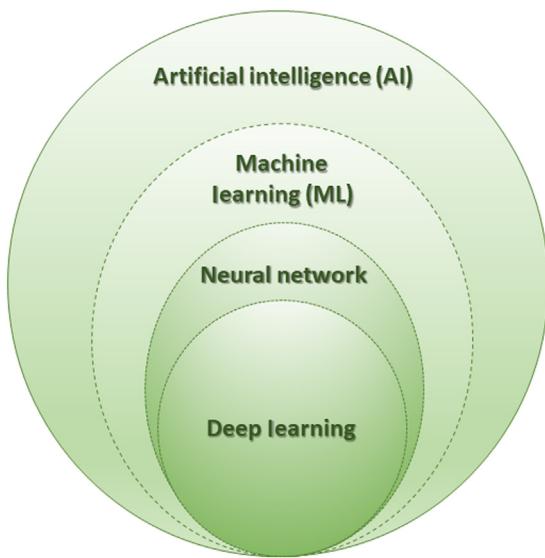


FIGURE 13.1 Artificial intelligence (AI) and machine learning (ML). ML is a subset of AI, which includes all the approaches used to learn from data. Thus deep learning (DL) is a further subset of ML and includes models and algorithms that imitate the architecture of complex biological neural networks in the brain.

proteomics or NGS bioinformatic pipelines as well as integrated data are used.

AI and ML may be useful for improving the medical decision making process by the development of expert systems or neural networks (NNWs) capable of assisting the clinician diagnostic process (Lippi and Plebani, 2020). Following recent definitions, AI refers to a field of data science dedicated creating systems performing tasks that usually require human intelligence. ML is a subset of AI, which include all the approaches used to learn from data. Thus DL is a further subset of ML and includes models and algorithms that imitate the architecture of complex biological NNWs in the brain (Jakhar and Kaur, 2020) (Fig. 13.1).

The advancements in AI techniques has allowed overcoming the problem of the high dimensionality of the obtained dataset from integrative omics, which usually contains a large number of variables (features) as compared to the low number of subjects. Most of the statistical and computational methods currently used for omics studies are suitable to guarantee results' reproducibility, avoiding overfitting issues by several computational and noncomputation techniques (Ransohoff, 2005; Khan et al., 2019; Hayes et al., 2012; Annalisa et al., 2008).

Machine learning: supervised and unsupervised learning

The two useful broad categorizations of the techniques used in ML are *supervised* learning techniques and *unsupervised* learning techniques (Annalisa et al., 2008). The

two techniques are easily distinguished by the presence of external subjects' labels (patients' classification):

- The supervised learning techniques find the features that are correlated with the specific patient's group, thus creating predictive algorithms for these labels.
- In contrast, the unsupervised learning techniques find those features that are correlated across all the samples and operate independently of any external labels (e.g., pathological and healthy subjects).

These two ML methods are generally used to answer different types of questions. In supervised learning, a typical goal is to obtain a set of variables (a process also known as *features selection*) that can be used reliably to make a diagnosis, predict future outcome, predict future response to pharmacologic intervention, or categorize that patient as part of a class of interest. In unsupervised learning, the typical application is to find either a completely novel cluster of peptide or proteins with putative common (but previously unknown) expression or, more commonly, to obtain clusters or *groups of features* that appear to have patterns of *similar expression*.

The most used techniques for unsupervised learning are principal component analysis and clustering determination. In contrast, multiple logistic regression, classification and regression trees (CARTs), support vector machines, and NNWs are widely used as supervised learning techniques.

The superiority or inferiority of multiple logistic regression, support vector machines, and NNWs to each other has not yet been demonstrated, although some important differences can be elucidated for logistic regression. Multiple logistic regressions present the advantage of providing a mathematical algorithm (equation) containing predictors (e.g., age, biomarkers) and their corresponding coefficients. Thus through use of this equation it is possible to directly derive the patient's probability of developing the disease. Differently, the results produced by support vector machines and NNWs define the patient outcome, so they solely predict the presence or absence of the disease. For this reason, many researchers considered support vector machines and NNWs as "black boxes" rather than multiparameter approaches. Differently, the CART approach allowed to derive readable algorithms, that can be used for visually and explicitly represent decisions and decision making. Therefore clinicians can read the tree, follow the algorithm and find the predicted response by themselves.

Techniques to avoid overfitting issues

Despite the employing of these computational methods in data analysis, the overfitting problem remains unsolved and afflicts any approaches listed in the preceding

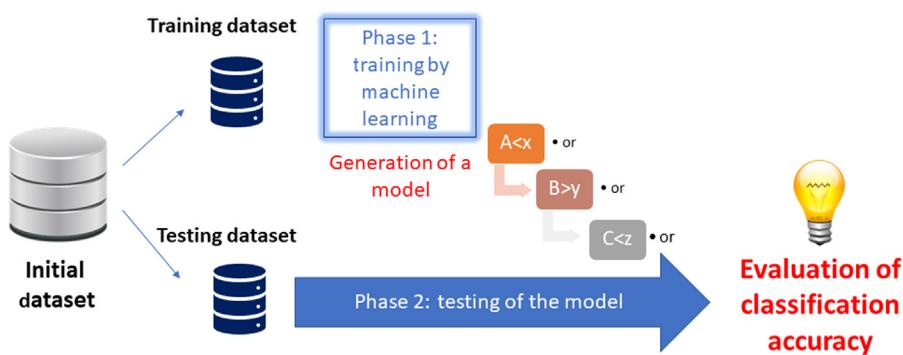


FIGURE 13.2 The pipeline for generation of a machine learning model. Initially, available data are randomly split into a training dataset and a testing dataset. The training dataset is used to generate (train) a ML algorithm (phase 1). The model is then evaluated for its accuracy using the testing dataset (phase 2).

sections. By the term *overfitting*, researchers generally mean the probability of finding a discriminatory pattern of features completely by chance. This probability becomes high when large numbers of variables are assessed for a small number of outcomes. The problem can be partially overcome by splitting the database into a *training set* (a randomly chosen patient subgroup is used) and a *validation set* (the remaining patients are used to assess the classification accuracy) (Ransohoff, 2004). However, when the dataset contains a limited number of subjects, dividing the database into training and validation datasets is not a convenient choice (Fig. 13.2). For this reason, *cross-validation* is applied to assess algorithm performance without dividing the database. The two most used cross-validation methods are the *k*-fold cross-validation and the leave-one-out cross-validation.

Examples of the applications of genetics and molecular markers for diagnostic or prognostic purposes

A widely held viewpoint of research on biomarkers of disease is that no single marker can provide high enough discrimination between cases and noncases for clinical applications and that the use of multiple markers, combined in some type of algorithm, will be necessary to produce the required level of predictive power. Furthermore, several applications of genetics and NGS are currently available for defining people at risk for disease and for optimizing pharmacological therapies.

The following sections describe some examples of the application of these technological advancements on different diseases.

Ovarian cancer

It is now recognized that ovarian cancer is a clinical heterogeneous disease, with many histotypes and different genetic mutations among these histotypes (Huang et al., 2012). Therefore it is unlikely that any single marker will be sufficiently sensitive to provide an optimal initial

screen (Badgwell and Bast, 2007). The use of multiple markers for the differential diagnosis of pelvic masses represents a clinical setting within the management of ovarian cancer, in which significant improvements could be achieved in the near future.

The serum tumor marker CA 125 can be used to predict the presence of a malignancy in women with a pelvic mass. However, Kirchhoff et al., upon differential cDNA screening of human epididymal tissues, observed that the human epididymal secretory protein E4 gene expression was upregulated in epithelial ovarian carcinomas (Kirchhoff et al., 1990). As a result of that evidence the derived protein HE4 has been extensively studied as biomarker for ovarian cancer. Several publications have demonstrated the superiority of HE4 over CA 125, especially in patients with early-stage disease, in which HE4 has been shown to have greater sensitivity than CA 125 alone (Plebani and Melichar, 2011).

Recently, some multimarker strategy-based risk indexes have been developed. An example is the Risk of Ovarian Malignancy Algorithm (ROMA), which was introduced as a predictive index and basically takes into account the serum concentrations of both biomarkers (CA 125 and HE4) and the patient's premenopausal or postmenopausal status (Karlsen et al., 2012). ROMA performances were initially demonstrated to be superior to CA 125 or HE4 alone in detecting ovarian cancer in patients with pelvic mass by a large prospective study (Karlsen et al., 2012). Recently, Karlsen and colleagues proposed a modified version of the ROMA, the Copenhagen Index (CPH-I), in which the menopause status is replaced by age (Karlsen et al., 2015). In the same large multicentric study, comprising 1060 patients with benign ovarian masses and 550 patients with ovarian cancer, the same authors demonstrated that CPH-I and ROMA performed equally with similar sensitivities and specificities (Karlsen et al., 2015).

Multimarker panels have also been used for ovarian cancer diagnosis. An example of a proteomic-derived diagnostic test panel is OVA1, which was cleared by the U.S. Food and Drug Administration (FDA) in September 2009

to assist physicians in triaging women with suspected pelvic masses ([Zhang et al., 2013](#)). The OVA1 is an in vitro diagnostic multivariate index assay for assessing ovarian cancer risk in women diagnosed with ovarian tumor prior to a planned surgery. OVA1 analyzes five serum biomarkers (CA 125, transthyretin, ApoA1, β -2 microglobulin, transferrin) by immunometric assays, and the results are combined through an algorithm to yield a single-valued index within the range of 0–10. A menopausal status-dependent cutoff is used to classify a patient into the high-risk group or the low-risk group. In comparison to CA 125, the OVA1 panel provides an increase in sensitivity (94%), while specificity widely decreased to 35%. This allowed the researchers to obtain an elevated negative predictive value of 92.9%, which is a clinically important measure to assure physicians and patients that the risk of malignancy will be low for patients who have a negative result by OVA1 ([Zhang, 2012](#)).

In another recent study, a series of 177 protein biomarkers from the Olink Oncology II and Inflammation panels were analyzed in 180 women with benign tumor, borderline tumor, early (stage I) ovarian cancer, or late (stage II-IV) ovarian cancer, and a statistically significant difference in levels between benign tumors and cancer was found for eight proteins ([Leandersson et al., 2020](#)). A model was then derived using the ML technique LASSO using the combination of six biomarkers (HE4, CA125, ITGAV, CXCL1, CEACAM1, IL-10RB) and age (sensitivity 0.86 and specificity 0.82, AUC 0.868), although the model has not yet been independently validated ([Leandersson et al., 2020](#)).

Genetic studies

Breast cancer (BC) is known to be a heterogeneous matrix of molecular subtypes characterized by significant variability in overall prognosis. As an example, *BRCA1* and *BRCA2* gene mutations are responsible not only for 90% of hereditary BC cases and but also for the majority of hereditary ovarian cancer cases ([Mehrgou and Akouchekian, 2016](#)).

Some BC tests evaluate germline mutations that can affect treatment decisions and prognosis as well as future risks. Other tests research somatic mutations to determine prognosis and treatment options. These last tests represent a major area of research and clinical implementation using gene panels of BC mutations that can help in decision making, such as for defining adjuvant chemotherapy (after surgery) to reduce the chances of recurrence or for determining treatment with targeted biological drugs and combinations of therapies ([Rutgers et al., 2019](#)).

The two broad molecular profiling NGS tests approved by the FDA that interrogate the most number of genes at once are the FoundationOne CDx (F1CDx) test and the MSK-IMPACT test ([FDA, Food and Drug Administration; Cheng et al., 2015](#)).

F1CDx can help to identify patients who may benefit from treatment with targeted therapies for a variety of cancer types. This test provides profiling information on a number of different cancer mutations that may help in the clinical management of oncology patients. F1CDx is used in BC to help decide if a patient might be a candidate for treatment with Herceptin (trastuzumab), Kadcyla (ado-trastuzumab-emtansine), or Perjeta (pertuzumab).

MSK-IMPACT is a 468-gene oncopanel intended to detect gene mutations and other critical genetic aberrations in both rare and common cancers.

The test is intended to provide information on somatic mutations (point mutations and small insertions and deletions) and microsatellite instability (MSI) for use by qualified healthcare professionals in accordance with professional guidelines ([Cheng et al., 2017](#)).

Recent advances in NGS technologies, have led to the development of a broad range of NGS multigene panels for BC. This implementation in diagnostic enables maximization of health benefits for the patient, early detection of tumor early, and an increase in the survival rate ([Catana et al., 2019](#)).

Other non-*BRCA* genes have been identified as inducing BC, such as *ATM*, *CHEK2*, *PALB2*, *PTEN*, and *TP53*.

Chan and colleagues have developed and validated the 35-gene hereditary cancer panel to detect clinically relevant germline mutations associated with hereditary risk for common cancer types (breast, ovarian, colorectal, pancreatic, prostate, uterine, and stomach cancers and melanoma) by new-generation sequencing ([Chan et al., 2020](#)). The test performance is assessed across a board range of variants in the 35 genes to support clinical use. This validation study confirmed high sensitivity (99.9%) and high specificity (100%) across a total of 4820 variants. The reproducibility and repeatability are 99.8% and 100%, respectively. Therefore these genes can be used to detect clinically relevant germline mutations associated with hereditary risk for certain common cancer types ([Chan et al., 2020](#)).

Furthermore, Tabaldi and colleagues, using NGS analysis with a panel of 94 cancer predisposition genes on germline DNA from an Italian case series of 70 patients with male breast cancer, have identified three pathogenic variants in the *BRCA1* gene and six in the *BRCA2* gene. Besides these alterations, they found six additional pathogenic or likely pathogenic variants in *PALB2*, *CHEK2*, *ATM*, *RAD51C*, *BAP1*, and *EGFR* genes. These results suggest that novel genes could be involved in MBC susceptibility, shedding new light on their role in cancer development ([Tedaldi et al., 2020](#)).

Prostate cancer

Prostate cancer is the first leading cause of cancer in men and the most common malignant neoplasm of the urinary

tract (Siegel et al., 2020). The current recommendations for PCa diagnosis are based on the guidelines of the European Association of Urology, which consist in analyzing the concentration of prostate-specific antigen (PSA) as well as conducting a digital-rectal examination (Mottet et al., 2017). Prostate cancer, like ovarian and BC, has been demonstrated to be a heterogeneous disease at genomic levels by Barbieri and Tomlins (Barbieri and Tomlins, 2014). Therefore in addition to the well-established biomarkers PSA, other multimarker strategies (including or not including PSA) appear particularly promising for this type of tumor. The studies involving new biomarkers for PCa diagnosis can be subdivided by the evaluated matrix, namely serum/plasma, urine, or other biological fluids.

Through proteomic profiling, Neuhaus et al. studied the seminal plasma of 125 patients: 70 with prostate cancer, 21 with benign prostate hyperplasia (BPH), 25 with chronic prostatitis, and 9 healthy controls. They first identified three panels of peptides: (1) 21 peptides differently expressed in prostate cancer/BPH versus inflammatory and healthy prostate; (2) 5 peptides, differently expressed in prostate cancer and BPH; and (3) 11 peptides, differently expressed in organ-confined or advanced disease (Neuhaus et al., 2013). They applied the support vector machine to construct algorithms based on the panels of peptides found and split the database into training and testing datasets to avoid overfitting problems. By combining the 21 and 5 peptide panels, they obtained 83% sensitivity and 67% specificity for diagnosing prostate cancer versus other conditions, although these performances were as high as those obtained by PSA (87% sensitivity and 59% specificity). They obtained better results (AUC = 0.99) for the identification of advanced versus organ-confined diseases.

Another study compared the low-molecular-weight proteomic (peptidomic) profiles of PCa subjects obtained by urine and serum (Padoan et al., 2018) and compared results with prostate-specific antigen (PSA) serum levels and prostate cancer antigen 3 (PCA3) score. The PCA3 score, commercialized by Progensa, calculates the ratio of the mRNA of PCA3 versus PSA mRNA in urine (Capoluongo et al., 2014). When MALDI-TOF/MS urinary and serum peptidomic profiles were compared, only 43 features were overlapping in the m/z 1000–4000 even if, in that m/z range, peptides should be free to pass the glomerular filtration. Furthermore, results showed that urine presents worse sensitivity results in discriminating PCa in patients presenting lower urinary tract symptoms. Finally, univariate or multivariate analyses of urine and serum proteomic profiles do not allow amelioration of diagnostic performances of PSA or PCA3 alone (Capoluongo et al., 2014), despite the issue of reproducibility of MALDI-TOF/MS results (Padoan, 2018).

A series of biomarker panel for PCa has been developed and has been approved by the FDA. One of them is the prostate health index (PHI), which is based on the determination of total PSA, free-PSA, and the precursor 2 pro PSA isoform and reports the probability of disease calculated by the PHI. This test has been proposed for reducing unnecessary biopsy and for predicting high-grade PCa. This test was approved by the FDA in 2012. The indication of test is for predicting the necessity of repeating the biopsy after an initially negative biopsy. In addition, several other studies proposed new diagnostic algorithms, such as 4Kscore (serum), SelectMDX (urine), MiPS (urine), and ExoDX Prostate IntelliScore urine exosome test (Porzycki and Ciszkojewicz, 2020).

Genetics studies

MSI is characterized by mutations in repetitive DNA sequence tracts, caused by a failure of the DNA mismatch repair system to correct these errors. Furthermore, in May of 2017, the FDA granted accelerated approval of an immunotherapy-based anti-PD-1 cancer treatment (pembrolizumab) for patients whose cancers have MSI or dMMR (Hempelmann et al., 2018). An example of use of NGS-based gene panel in the context of prostate cancer is represented by Hempelmann JA and colleagues' study, which evaluated the performance characteristics of two NGS (Hempelmann et al., 2018). The performance of MSI detection methods, MSIplus (18 markers, sensitivity of 96.6% and specificity of 100%) and MSI by Large Panel NGS (> 60 markers; sensitivity of 93.1% and specificity of 98.4%) were also evaluated (Hempelmann et al., 2018). Afterward, each NGS method is compared with the performance of the most widely used five-marker MSI-PCR (sensitivity of 72.4% and a specificity of 100%) detection system (Hempelmann et al., 2018).

The widely used five-marker MSI-PCR panel has inferior sensitivity when applied to prostate cancer, while NGS testing with an expanded panel of markers performs well. In addition, NGS methods offer advantages over MSI-PCR, including no requirement for matched nontumor tissue and an automated analysis pipeline with a quantitative interpretation of MSI-status.

MSIplus is probably most appropriate as first-line MSI screening in a low-prevalence population. MSIplus is an amplicon-based NGS assay, which takes less time, uses less sample material, and is much lower cost than Large Panel NGS. Given the ever-increasing demand for clinical MSI testing following the FDA's tumor agnostic approval of pembrolizumab for MSI-positive cancers, a low-cost, relatively fast, and highly sensitive/specific assay for MSI is urgently needed. MSIplus fulfills all these criteria and is now validated for both colorectal and prostate cancer (Hempelmann et al., 2018).

Pancreatic cancer

Pancreatic cancer is one of the most fatal malignancies worldwide, ranking fourth among the leading causes of cancer death for both males and females, with diagnosis at late stages carrying a dismal prognosis (Siegel et al., 2020). Unlike other types of cancer, few risk factors have been associated with this malignancy. Cigarette smoking and chronic pancreatitis in addition to other metabolic factors, such as obesity and diabetes or metabolic inflammation, have been considered the most relevant risk factors for this tumor (Basso et al., 2014).

Since PDAC is characterized by late-onset symptoms, rapid progression, and death, a positive prognosis is mostly associated with an early diagnosis, even if the disease remains almost silent until the lesion becomes clinically relevant (Gheorghe et al., 2020). Currently, the only broadly accepted biomarker of PDAC is CA 19-9, and in a recent meta-analysis, the median sensitivity and specificity were demonstrated to be 79% and 82%, respectively (Khomia et al., 2020). However, CA 19-9 can be elevated in other malignant conditions, such as colorectal, lung, and liver cancer, or even benign diseases, such as cholestasis, pancreatitis, or systemic lupus erythematosus (Khomia et al., 2020). Therefore a single tumor marker has low diagnostic value in PDAC. Combinations of multiple biomarkers and unique analysis algorithms can be applied to overcome these limitations.

Machine learning-derived biomarkers

Diabetes mellitus (DM) has been demonstrated to be associated with pancreatic cancer (PDAC), especially when of new onset. Padoan et al. studied serum peptidome profiling of PDAC patients, compared to that of DM or healthy subjects and chronic pancreatitis (ChrPa) and gastric cancer patients (Padoan et al., 2013). They used both single-marker and multimarker strategies, applying univariate statistical analyses as well as CART analyses. Results were compared with the established biomarker for PDAC, which is CA 19-9. The best classification accuracy was obtained for distinguishing diabetic patients from PDAC and ChrPa patients, achieving a cross-validated (leave-one-out cross-validation) percentage of classification rate of 65.4%. Univariate analyses, performed in the same study, showed that serum Apo-A1 and complement C3 were altered in PDAC patients. These two candidate biomarkers were determined by a nephelometric assay, and the results were validated by using multiple logistic regressions. A logistic model containing Apo-A1 and C3 underlined that they performed better than CA 19-9 alone in discriminating PDAC patients with respect to DM patients.

Another example is represented by the diagnostic algorithm proposed by Kim and colleagues, which combines a panel of six biomarkers (ApoA1, CA125, CA19-9,

CEA, ApoA2, and TTR) through an RF classification algorithm method that increased the diagnostic accuracy of PDAC to 95% (Kim et al., 2020). This algorithm achieved better performances of each single biomarker at a limited cost with respect to other, more expensive combinations of markers (Kim et al., 2020) and was validated by the same authors using a different dataset of 60 PDAC cases and 191 negative subjects (healthy controls).

Several other biomarkers that are detectable in blood or pancreatic juice have been described for PDAC diagnosis. Most of them have not yet been evaluated for their diagnostic performances (Gheorghe et al., 2020).

Genetics studies

In a recent study, Zarkavelis and colleagues used a targeted NGS panel encompassing genes that commonly mutate in pancreatic cancer in a patient population managed with either nab-paclitaxel regimens or targeted compounds modulating the epidermal growth factor receptor (EGFR)/AKT/mTOR axis. In the study population, KRAS, TP53, SMAD4, and CDKN2A were identified as being the most prevalent mutations with the exception of an intriguingly lower incidence regarding KRAS mutants. The coexistence of KRAS and TP53 mutations seemed to adversely affect the outcome of patients whether treated with targeted therapy containing either an EGFR TKI or a PI3K/Akt/ mTOR inhibitor or cytotoxic drugs (Zarkavelis et al., 2019). This multimarker tumor panel approach allowed the researchers to obtain multiple types of information at the same time, including mutations involved in the tumor, therapy effect, and outcome of patients.

Conclusions

The increasingly advocated use of multimarker strategies for improving the early detection, diagnosis, prognosis, and monitoring of human diseases represents a major challenge, including the identification of valuable reporting strategies. Proteomics and other omics sciences, generates massive quantities of data and requires appropriate data-mining strategies to discover the most meaningful items in these data.

The future of biomarker panels involves cooperation and collaboration between different professionals, such as basic researchers, clinicians, laboratory workers, and bioinformaticians, to translate potentially powerful technologies into clinical practice.

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Chapter 14

Cardiovascular translational biomarkers: translational aspects of hypertension, atherosclerosis, and heart failure in drug development in the digital era

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Abstract

Biomarkers are essential in the development of drugs against cardiovascular diseases. Conventional biomarkers are biochemical or imaging biomarkers, but the advent of new technology has made it possible to track a host of vital and behavioral parameters everywhere and at any time. In this chapter, three indications (hypertension, atherosclerosis, and heart failure) are profiled against the use of major biomarkers in drug development, and derived learnings are applied to a fourth one in the form of a case study involving atrial fibrillation.

Keywords: Cardiovascular biomarkers; imaging biomarkers; tracking of vital parameters; arterial hypertension; atherosclerosis; heart failure

What is new?

While longevity has substantially increased on a global scale (OECD 2020) and medical innovation has improved survival rates for the most common deadly diseases (Nabel and Braunwald, 2012), in the Western world, these exact same killers remain prevalent and hold firm to their leading positions in our death statistics (WHO 2018). Coronary artery disease and stroke still account for the majority of deaths but they now occur at later ages and with often multiple events. For drug developers this creates a dilemma, because event rates in clinical trials have shown a decrease (Nabel and Braunwald, 2012) while sample sizes had to increase thereby, making trials ever larger and more costly to conduct. The expanding use of smart wearable technology in combination with the almost ubiquitous presence of smartphones represents an

opportunity that has increasingly been gaining a foothold in clinical trials as well as in routine clinical care (Batalik et al., 2020). In this chapter we contrast three indications (hypertension, atherosclerosis, and heart failure) and apply derived learnings to a fourth one in the form of a case study involving atrial fibrillation.

Specifically, the advent of new technology has made it possible to track a host of vital and behavioral parameters everywhere and at any time (Torous et al., 2017). This development provides unprecedented opportunities to collect more data (in particular, at more time points longitudinally or even continuously) and data of higher quality (from participants in their natural environment and following their normal routines instead of measurements performed at the study site) (Babruk et al., 2019). Other expected advantages include higher adherence and transparency and the opportunity to increase the recruitment and retainment of study participants by offering them more convenience (trials@home initiative; Joint, 2020).

Smart wearable technology is already used by millions of people to track parameters such as activity and sleep and in professional settings such as in sports and coaching (Babruk et al., 2019). Meanwhile, the introduction of this technology into the medical field is already happening. An increasing number of devices and apps are being certified as medicinal products by the U.S. Food and Drug Administration (FDA) and by the European process for obtaining a CE mark. This is a key requisite for use in clinical trials because of the medical and regulatory implications, including accuracy, precision, reproducibility, calibration, and traceability as well as compliance with

protocols and with predetermined analyses (Torous et al., 2017).

As an example, the increasingly stringent, evidence-based treatment goals for patients with arterial hypertension (Whelton et al., 2017), triggering the need for more rigorous monitoring, and the low rate of treatment compliance in this patient group lend themselves to more frequent testing of newly developed biomarkers in the broadest sense, including digital biomarkers that allow both aspects to be addressed (Boehme et al., 2019).

Increasingly, these technologies and protocols are being used in clinical trials, although very few can already be considered validated. Applying the FDA's BEST criteria (FDA-NIH Biomarker Working Group 2020), in essence digital biomarkers are available, and the categorization can be applied to every category outlined, that is, diagnostic and monitoring biomarkers (e.g., detection of atrial fibrillation), pharmacodynamic/response biomarkers (e.g., activity tracking under treatment), predictive and prognostic biomarkers (e.g., multiparameter early detection for heart failure decompensation), and safety biomarkers (e.g., detection QT prolongation).

However, in line with the innovative step, the degree of validation of digital biomarkers is moderate at best in most cases. Following one recent classification of biomarkers (Rock Health 2020) into (1) approved (known measure and clinical outcome), (2) original (known measure, unknown clinical outcome), or (3) novel (unknown measure and clinical outcome), no digital biomarker falls into the first, or highest, category. A further challenge, particular to this field, is the fast-paced evolution in technological advances, which makes it difficult or even impossible the transfer of knowledge from one study to the next as the device or technological possibilities will have evolved in the meantime (Babruk et al., 2019). Clearly, much research is needed to better validate already identified digital biomarkers. Digital biomarkers are defined in this chapter as digital solutions that involve a wearable, portable, or implanted sensor device with remote data transfer.

For this chapter we apply a broad biomarker definition (FDA-NIH Biomarker Working Group 2020) that includes genetic or traditional soluble biomarkers to the same extent as digital biomarkers (Califf 2018; Coravos et al., 2019). Of note is the fact that the same biomarker could be a diagnostic biomarker and a surrogate endpoint and thus through the combination with digital means, offers a new dimension that has so far been little explored. For example, blood pressure is used to diagnose arterial hypertension, serving as one of the few established and clinically validated surrogate endpoints, while new ways of its continuous measurement allows the derivation of new signals, such as pulsus paradoxus or blood pressure variability, that can serve as proxies for airway obstruction (Truebel et al., 2019) and provide a new dimension of cardiovascular health (Parati et al., 2020), respectively.

Hypertension

Introduction

Hypertension is one of the most common cardiovascular disorders; according to guidelines dated 2017, around 60%–70% of the population over the age of 60 years are affected (Egan et al., 2010). In 2017 the American Heart Association (AHA) and the American College of Cardiology (ACC) suggested lower diagnostic thresholds for systolic blood pressure (≥ 130 mmHg) and diastolic blood pressure (≥ 80 mmHg) and adapted treatment goals for arterial hypertension, leading to even higher prevalences around the globe (Dorans et al., 2018), not only potentially leading to more disorders due to hypertension but potentially also leading to more side effects of new treatment paradigms (Anker et al., 2020).

Hypertension is often regarded as a single disorder, but this is not the case. Instead, it is a complex and multifactorial disease resulting from the interaction of multiple environmental and genetically determined homeostatic control mechanisms (Takahashi and Smithies, 2004), and its etiology can be primary or secondary. The exact cause of primary (or essential) hypertension is unknown but includes an increased activity of the renin-angiotensin-aldosterone system (RAAS) and increased activity of the sympathetic neural system (Staessen et al., 2003). The central role of the kidneys in blood pressure control is generally accepted, and high levels of sodium intake are associated with high blood pressure (Chien et al., 2008). Secondary hypertension can have several causes, including primary renal disease, endocrine disorders, or drug-induced causes.

Hypertension is now defined as a systolic blood pressure of 130 mmHg or more and a diastolic blood pressure of 80 mmHg or more (Whelton 2018). Antihypertensive drugs are still among the most prescribed drugs by clinicians (Egan et al., 2010). Despite all the available treatment options, disease management is still not adequate. Only 50% of patients with hypertension have their blood pressure regulated properly ($< 140/90$ mmHg) (James et al., 2014), and compliance with therapy for this (mostly) asymptomatic condition is often low (Wang and Vasan, 2005). It is expected that these numbers will rise as a result of the aging of the population and changed guidelines (Dorans et al., 2018). The generally low treatment compliance of patients with arterial hypertension can be partially explained by the absence of symptoms (hypertension is sometimes called the “silent killer”) and side effects of therapy that interfere with the quality of life. In the end, hypertension remains one of the most important risk factors for stroke and heart disease (Greenland et al., 2003).

Clearly, the combination of increasingly tight blood pressure goals and the typically poor therapy adherence in

patients with arterial hypertension call for new technologies that allows more frequent measurements and testing, including biomarkers that address both aspects (Boehme et al., 2019).

Animal models of hypertension

The use of animal models that mimic the human hypertensive situation may offer valuable information in understanding the different routes of pathophysiology and studying new therapeutic interventions. In experimental hypertension research, several animal models exist (Mondritzki et al., 2018), and rats are the most commonly used animals (Dornas and Silva, 2011). Animal models can be classified in two large groups: animals with primary hypertension and animals with secondary hypertension. Among animals with primary hypertension, genetically modified animals are most popular. One of the most used models, a phenotype-driven model derived from the Wistar strain, is the spontaneous hypertensive rat (SHR). In this model, rats develop hypertension at 4–6 weeks after birth. The Dahl salt-sensitive rat is another example of a phenotype-driven model. These rats are derived from Sprague–Dawley rats and become hypertensive with a normal salt diet. Transgenic rat models are also available: TGR(mREN2)27 (REN2) transgenic rats show overexpression of renin and therefore develop hypertension (Mullins et al., 1990).

Other nongenetic animal models of primary hypertension include the cold model and the stress model. Different kinds of sensory stimulation, such as flashing lights or loud noises, can stress the animal, thus causing hypertension (Zimmerman and Frohlich, 1990). Also, cold temperature (around 5°C) leads to hypertension in animals within 3 weeks (Sun et al., 2002). Since these stress-induced models reflect an exaggerated “white coat”

response (Nuredini et al., 2020), their relevance for translational drug development can be called in question.

Secondary hypertension may be induced via surgery, for instance, in the 2K1C model (2 kidneys, 1 clip). In this model, unilateral constriction of the renal artery is accomplished, which will lead to a chronic increase in blood pressure within 2 weeks. This procedure can be performed in different kinds of animals (Wiesel et al., 1997; Mondritzki et al., 2018). But there are also pharmacological options to create secondary hypertension. The DOCA-salt method is widely used. In this method a mineralocorticoid (deoxycorticosterone acetate), such as a subcutaneous slow-release tablet, is administered to the animal (Terris et al., 1976), which in turn leads to increased RAAS activity and subsequent hypertension. (See Table 14.1.)

Another important factor regarding the evaluation of animal models of hypertension, besides and independent of the underlying causal effect of raised blood pressure, is the applied methodology to measure blood pressure. While noninvasive methods are easy to apply (e.g., tail-cuff measurements in rats or dogs), they are notoriously flawed by the handling of the animals (e.g., taking measurements once the animal is sedated) or with invasive measurements using catheters or simply the need to warm the tail in order to take an adequate reading. In essence, stress-induced hypertension through handling while taking the blood pressure measurement in animals leads to a phenomenon similar to that in humans referred to as the “white coat effect” (Nuredini et al., 2020), in which the act of taking a simple measurement negatively affects the dimension one is trying to measure. Here, new developments, especially the introduction of advanced telemetric systems, allow for the acquisition of high fidelity data in free-roaming animals, better resembling their true physiological state (Vatner 1978; Mondritzki et al., 2018). This development is paralleled by advancements in the human clinical

TABLE 14.1 Example of animal models of hypertension and their characteristics.

Animal model	Characteristic features
Primary hypertension	
REN2 rat	Overexpression of the renin gene
Spontaneously hypertensive rat	Increased sympathetic tone
Dahl salt-sensitive rat	Fluid retention by salt administration
Cold/stress model (rat/mouse)	Stressor-induced hypertension
Secondary hypertension	
DOCA-salt hypertension (rat, pig)	Salt retention and impaired renal function give rise to fluid retention
1-clip 2-kidney (rat, dog)	Increased renin levels
Aortic banding	High aortic pressures prior to the banding area

Biomarker categories in arterial hypertension trials (1960–2020; n = 327)

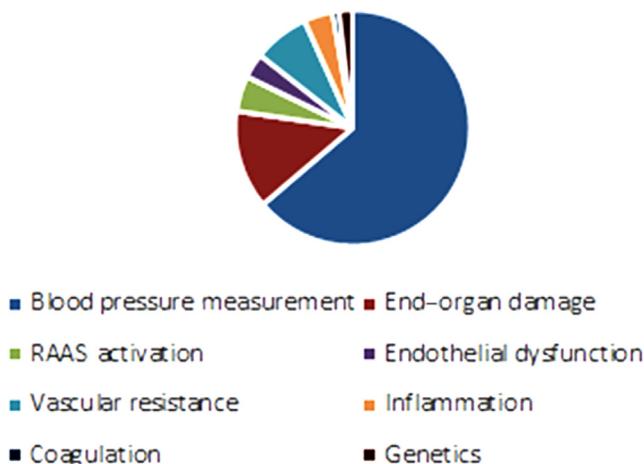


FIGURE 14.1 Relative distribution of different biomarker categories used in interventional trials investigating patients with arterial hypertension.

setting, such as continuous blood pressure sensors for ambulatory use that have been newly approved by regulators (see [Biobeat 2019](#)).

Biomarkers of hypertension

[Fig. 14.1](#) illustrates different biomarker types that have been used in clinical trials in patients with arterial hypertension. Measurement of pressure have been evolving over the years from invasive and direct to indirect measurements as 24-hour ambulatory measurement ([Whelton et al., 2017](#)) and now even continuous methods are finding their way into the market ([Biobeat 2019](#)).

Besides the measurement of blood pressure, including its variability during the day (e.g., regarding a nightly decline), the assessment of end-organ damage, such as in the heart (left ventricular hypertrophy), the kidneys (microalbuminuria), the brain (stroke), the eyes (retinal damage), or the vascular system (increased vascular wall thickness and arterial stiffness), investigators looked at a number of other aspects to understand the relationship with, for example, inflammatory changes or coagulopathies associated with hypertension.

It is also possible to execute supportive studies on hypertension, for instance, to obtain myocardial wall thickness with magnetic resonance imaging (MRI) or echocardiography to assess left ventricular (LV) hypertrophy. Other options include measurements of pulse wave velocity in the aorta by means of carotid-femoral pulse wave analysis to estimate vascular stiffness. Ultrasound examination of carotid intima media thickness can be used to measure vascular wall thickness. Furthermore, urine samples can be investigated for microalbuminuria,

retinal photographs can be made to evaluate retinal changes ([Touyz and Burger, 2012](#)), and high-sensitivity C-reactive protein (hsCRP) has been employed to assess inflammatory changes. Overall, we continued to use the biomarker categories as they had been subdivided in previous editions, but we would like to point out that current biomarker concepts address the predictive potential more holistically, that is, integrating different biomarker categories into one score ([Berezin 2019; Currie and Delles, 2016; Schulte and Zeller, 2020](#)).

Blood-borne biomarkers

Renin-angiotensin-aldosterone system and sympathetic-adrenergic system activity

Renin is a component of the RAAS and is released during renal hypoperfusion or increased sympathetic activity ([Nushiro et al., 1990](#)). However, in hypertension, patients are in either a low-renin state or a high-renin state, as proposed by [Laragh and Sealey \(2011\)](#). Low-renin hypertension is a state in which sodium increases to a certain level, so the RAAS system becomes “switched off,” and hypertension develops by volume retention. These patients will mostly benefit from diuretics or calcium antagonists ([Alderman et al., 2010](#)). In a high-renin state, renin is secreted in too high amounts relative to sodium levels, and patients will benefit more from ACE inhibitors. This implies that renin (or plasma renin activity) is not of use to assess severity of hypertension but can help to distinguish what kind of therapy will be useful for the patient ([Turner et al., 2010](#)). In a similar way the sympathetic-adrenergic system (SAS) contributes to the blood pressure regulation and specifically under “stress”

conditions can lead to borderline or manifest arterial hypertension (Hjedahl 1988).

Endothelial dysfunction

Endothelial dysfunction is one of the detrimental effects of hypertension (Quyyumi and Patel, 2010). Healthy endothelial vessel walls become stiffer, and vasorelaxation is diminished. Thus measures of endothelial dysfunction can be used as a marker for hypertension. One of the main causes for endothelial dysfunction is the reduced availability of nitric oxide (NO), resulting in increased oxidative stress in the endothelium (Feng and Hedner, 1990). Endothelial dysfunction can also precede hypertension, and less compliant vessels will establish higher blood pressures (Park and Schiffrin, 2001). Eventually, endothelial dysfunction leads to a proinflammatory, prothrombotic, vasoconstrictive state with increased cell adhesion and oxidative stress (Flammer and Luscher, 2010).

Markers of vascular resistance

NO is a marker of vascular resistance. Increased levels of NO inhibit oxidative stress and promote vasodilation and vascular health and therefore can be used as an estimate for hypertension. Degradation products of NO, such as nitrite or nitrate, can also be used as surrogate measures (Shiekh et al., 2011). Prostacyclin is another marker of vascular resistance and is a member of the prostaglandin family. Prostacyclin is also produced by endothelial cells, determines vasorelaxation, and inhibits coagulation. Its metabolite, 6-ketoprostaglandin F_{1α}, is used mostly as an estimate for prostacyclin bioavailability and correlates with endothelial function (Doroszko et al., 2011; Touyz and Burger, 2012).

Markers of inflammation

Hypertension promotes the release of cytokines and is accompanied by low-grade inflammation (Savioia and Schiffrin, 2007). Therefore markers of inflammation have been studied for their use as biomarkers for hypertension. Individuals with inflammatory activation are thought to be in a more severe hypertensive state, suggesting that inflammation might play a role in the pathophysiology of hypertension (Harrison et al., 2011). Tumor necrosis factor alpha (TNF-α) is an important cytokine released during inflammation. It is secreted by monocytes, macrophages, and vascular cells (Locksley et al., 2001). Its levels are increased in hypertension (Chrysohou et al., 2004), and reduction of blood pressure decreases TNF-α levels (Koh et al., 2003). Interleukin 6 (IL-6) and hsCRP are other proinflammatory cytokines, and they are elevated in patients with hypertension and normalized after

blood pressure reduction (Chae et al., 2001; Ruscica et al., 2020). Cell adhesion also plays an important part in inflammation, providing attachment of inflammatory cells to the tissue. Levels of these cell-adhesive molecules, such as vascular cell adhesion molecule and intercellular adhesion molecule, are increased in patients with hypertension and have been shown to correlate with severity of hypertension (Preston et al., 2002).

Markers of coagulation

Hypertension results in endothelial dysfunction, which is reflected by a prothrombotic state (Braschi 2019). In this regard, factors of coagulation reflect endothelial (dys) function or hypertension. For instance, levels of von Willebrand factor plasma are elevated in patients with hypertension and correlate with severity of endothelial dysfunction (Tabak et al., 2009). Other factors of coagulation such as PAI-1, which inhibits fibrinolysis, or P-selectin, involved in platelet activation, are also increased in hypertension and reduced by antihypertensive treatment (Tabak et al., 2009).

MicroRNAs and genetic variants

MicroRNAs are small, noncoding nucleotides that regulate gene expression at the posttranscriptional level and can regulate multiple downstream genes. Deregulation of microRNA results in diminished function (Jamaluddin et al., 2011). Out of the endothelial wall, multiple microRNAs are expressed. Among them the most valuable ones in hypertension are miR-126, miR-217, miR-15, miR-21, and miR-122 (Batkai and Thum, 2012). Other microRNAs, such as miR-155, are targeting the RAAS system, whereas miR-29b is involved in the fibrotic pathway. MicroRNAs are a source of new potential targets; however, these targets still need to be validated in clinical studies (Touyz and Burger, 2012). Furthermore, certain genetic variants, found in genome-wide association studies, next-generation sequencing studies, or epigenetic analysis have delivered interesting results with respect to rare genomic associations resulting in hypertension but still need to show their effects on the clinical management of patients with arterial hypertension (Russo et al., 2018; Soler-Botija et al., 2019).

Digital biomarkers of hypertension

Technological advances have made it possible for patients to measure their own blood pressure with high technical accuracy at many more time points than was previously possible. By avoiding the setting of the doctor's office for measurement, stress-related artificially high measurements (the white coat effect) can be avoided, and more realistic values in the patient's natural environment and routine can be obtained. The digital data can be

transferred and made available in real time to doctors or coaches and used for further analyses, including training of deep learning machines, involving many more aspects than was possible before.

Arterial blood pressure in clinical practice is most often performed by using the Riva-Rocci method, consisting of an upper arm cuff combined with auscultatory or oscillometric pulse detection. This method is highly validated in the clinical setting, and all current guidelines, such as those of the European Society of Cardiology (Williams et al., 2018) and the AHA/ACC (Whelton et al., 2017), base their recommendations for treatment initiation and therapeutic blood pressure targets on this method alone. The automated sphygmomanometer is the most frequently used device for patient self-monitoring at home. A major drawback is that it is rather cumbersome, can be annoying or even discomforting, can essentially be measured only in centers or (once training has been provided) at home, and does not generate continuous measurements. These aspects combined make compliance a key topic in real-life and clinical trials. A slightly more user-friendly method to measure blood pressure using a sphygmomanometer is with an inflatable cuff around the wrist, for example, in the form of a watch, which would at least in principle make it possible to measure blood pressure also outside of the home and while exercising. However, a number of studies have shown discrepancies between measurements at the wrist, so this is not recommended unless there is a reason that blood pressure cannot be measured at the upper arm (Nerenberg et al., 2018).

New methods of noninvasive blood pressure measurement have emerged in recent years. Among these the most promising are the tonometry, the volume clamp methods, and measurements using a smartphone based on heart rate and modified normalized pulse volume. However, although these technologies might have a role in screening and early diagnostic of hypertension, they generally have an insufficient correlation with actual blood pressure using established methods and have, to the best of our knowledge, not yet been tested in translational or drug development trials. In combination with the upper arm cuff, such a technology might offer the potential to continuously assess the intraday changes in blood pressure, but this remains to be shown.

A different approach has been taken to generate novel digital biomarkers that would allow to predict the response of patients to a blood pressure–lowering therapy (Guthrie et al., 2019). In a proof-of-concept study, vital and behavioral parameters were collected from a digital therapeutic device over 28 days and used to train a deep learning machine. This study showed that treatment response could be predicted with reasonable accuracy. Approaches such as this might in the future allow the use of digital biomarkers that are predictive of therapeutic response/nonresponse in testing novel drug candidates.

Atherosclerosis

Introduction

Atherosclerosis is a chronic inflammatory disease characterized by lipid-containing inflammatory lesions of large and medium-sized arteries, occurring mostly at sites with disturbed laminar flow, such as branch points (Moore and Tabas, 2011). Overabundance of food seems to play a major part, as was indicated by pathology reports comparing, for example, prewar and postwar autopsies or autopsies that were conducted after soldiers died suddenly in combat or after a prolonged period of starvation (Schettler 1983; Thomas et al., 2014). Of interest is the fact that ancient people, despite a lack of food, might also have suffered from atherosclerosis (Thomas et al., 2014), possibly linked to chronic inflammatory states, hinting at a duality in which atherosclerosis is enhanced by inflammatory states while being an inflammatory state itself (Kotsis et al., 2017). Furthermore, genetic influences (Tibaut et al., 2019) have been identified as additional risk factors besides the well-known ones (e.g., smoking, diabetes mellitus, arterial hypertension).

Furthermore, 1 in 250 individuals has a heterozygous mutation (Berberich and Hegele, 2019) that leads to hypercholesterolemia and high levels of low-density lipoprotein (LDL) cholesterol with associated increased risk for atherosclerosis and subsequent cardiovascular diseases.

Via slowly progressive lesion formations, atherosclerosis gives rise to cerebrovascular and coronary artery disease and luminal narrowing of arteries. Infiltrated immune cells, like macrophages, take up cholesterol and are transformed into foam cells. They form a necrotic core in the growing atherosclerotic plaque. In the development of these atherosclerotic plaques, three stages can be distinguished. First, major lipid accumulation occurs, which is accompanied by reduced amounts of smooth muscle cells; this is the vulnerable plaque stage. Fibroblasts will produce collagen to stabilize the plaque. This stage is characterized by a thin fibrous cap, which separates the bloodstream from the lipid core. At this time the fibrous cap is prone to rupture if subjected to strain. Second, this plaque grows more extensively and is now obstructing the vascular lumen; this is the stable plaque stage. Because this plaque contains collagen-producing cells, it is stable, and plaque rupture or thrombosis does not occur. This kind of plaque may lead to signs or symptoms of angina pectoris. The third stage is the erosive plaque stage. This stage is characterized by formation of thrombosis, and acute myocardial infarction may occur at this stage.

Animal models of atherosclerosis

In the study of atherosclerosis the mouse is the most frequently used species (Pasterkamp et al., 2016). However,

heart rate, total plasma cholesterol, sites of atherosclerosis, plaque-instability, and involved genes differ between mice and human (Pasterkamp et al., 2016) and between preclinical experimental setups (Johnston et al., 2018). The most commonly used mice models are those in which genetic ablation of apoE or the LDL receptor is used. Disruption of the apoE gene causes increased levels of circulating cholesterol without any changes in diet and rapid progression of atherosclerosis. An atherogenic diet can further exacerbate plaque growth (Zadelaar et al., 2007). Mice with a LDL receptor knock-out require an atherogenic diet to develop atherosclerotic lesions. Also a double knock-out exists, which is a cross between the ApoE and LDL receptor knock-outs.

These different animal models have limitations. High cholesterol levels are needed to induce atherosclerosis (small lesions); plaque rupture does not occur; and cholesteroyl ester transfer protein, a potential target in humans, is not expressed in mice. In addition to these limitations, it should be stated that new treatment options tested in these models are administered in early stages of lesion formation, whereas in humans, treatment would be started after a cardiovascular event and not in early development of atherosclerotic lesions. Whether large or newly developed animal models, such as in miniature pigs or rabbits (Ludvigsen et al., 2019; Hara et al., 2018), will be able to overcome some of these limitations is under investigation.

Biomarkers for atherosclerosis

While studying the concentration of cholesterol or different lipoproteins (e.g., LDL or high-density lipoprotein) and their relative proportions is relatively easy and well established, these studies do not identify subjects who are prone to a major cardiovascular event (Martinez et al., 2020). Of interest is the fact that some of the biomarkers that are implicated in the risk stratification of subjects with established atherosclerosis are predictive in one vascular bed but not in another. For example, IL6 is associated with carotid artery atherosclerosis, while hsCRP is more indicative of cardiovascular events (Martinez et al., 2020).

A very important feature in patients with atherosclerosis is the switch to plaque destabilization and consequently rupture. Thus a vulnerable plaque can ultimately lead to an atherosclerotic event. Metallomatrix proteases (MMPs) have been implicated as risk markers for instability (Olejarz et al., 2020). For example, MMP9 was found to be elevated in patients with acute coronary syndrome. However, similar to other inflammatory markers, the MMPs lack specificity with respect to the underlying disease origin or organ distribution of atherosclerosis.

Given the many analytical advances that allow for the detection of even smaller concentrations of proteins,

Eslava-Alcon and coauthors in a recent review came to the conclusion that no single marker (including MMP9) has emerged to predict the presence of vulnerable plaques with enough precision and selectivity to be of help in the clinical setting (Eslava-Alcon et al., 2020).

Furthermore, since arterial hypertension, atherosclerosis, and the development of heart failure are related conditions, there is a substantial crossover between biomarkers in these areas. NT-pro-BNP not only is a risk marker for heart failure but also has a predictive value in patients with hypertension in terms of their risk of developing atherosclerosis and subsequent cardiovascular events (Panney et al., 2019).

Imaging (digital) biomarkers in atherosclerosis conditions

Imaging biomarkers in contrast to soluble markers in atherosclerosis have emerged in a diverse number of modalities. They have been tested to evaluate plaques in terms of their degree of vulnerability and to guide therapeutic interventions. Intravascular ultrasound, optical coherence tomography, and near-infrared spectroscopy are three examples of invasive methods in which a probe has to be advanced into the vessel lumen, where the lesion is examined. Coronary computed tomography angiography (CTA), positron emission tomography, and MRI are non-invasive alternatives (Bom et al., 2017). Of note, CTA has been raised to class I recommendation for diagnosis of coronary artery disease in the recent ESC guidelines (Knuuti et al., 2020). At this time, the jury is still out on whether these imaging approaches can help to better identify subjects who are at risk and to target lesions locally (e.g., via prophylactic stenting), for example, as a new treatment option that adds substantial benefit to systemic therapy with lipid-lowering medications (Almeida and Budoff, 2019; Koganti et al., 2020).

Continuously measured digital biomarkers in atherosclerosis conditions

Entering the search terms “atherosclerosis” and “digital markers” (or synonyms) in the NCBI PubMed database yielded 0 hits as of July 2020. However, some technologies are emerging that may make possible the assessment of certain key mechanisms in a patient’s journey from cardiovascular risk factors to early/late atherosclerosis and clinically manifested arterial stenosis and occlusion using digital methods.

No specific digital measure or marker for early atherosclerotic disease is available. The closest to this are score calculators that are available as apps and allow patients to repeatedly determine their predisposition for the disease and assess their probabilities of disease occurrence and

Biomarker categories in atherosclerosis trials (1960–2020; n = 187)

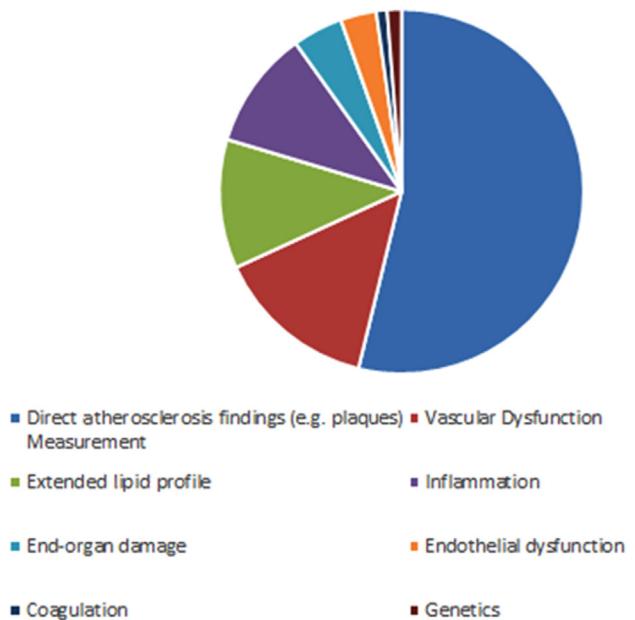


FIGURE 14.2 Relative distribution of different biomarker categories used in interventional trials investigating patients with atherosclerosis.

progression. Such scores include the ASCVD score, the HEART Score, and the vascular age score (Freaney et al., 2020; Khambhati et al., 2018).

Other technologies focus on identifying late-stage events such as acute infarction following plaque rupture rather than detecting or monitoring early stages of the disease (Muhlestein et al., 2015). Wearable electrocardiogram (ECG) devices, such as the Alivecor Kardia Mobile 6L, allow patients to measure their own six-channel ECG. In principle, this should allow them to detect signs of myocardial ischemia and myocardial infarction with the conceptual advantage of being able to seek treatment immediately when symptoms occur and to assess the occurrence of exercise-induced ischemia in real time. However, such technologies have not yet been studied in adequately powered prospective clinical trials; therefore the technologies cannot be considered validated digital biomarkers. Taken together, the continuous measurement of biomarker variables has not found entry in atherosclerotic conditions as we see in hypertension.

An overview of biomarker categories used in interventional trials investigating patients with atherosclerosis is given in Fig. 14.2.

Heart failure

Introduction

Heart failure is a clinical syndrome characterized by an impaired ability of the heart to eject or fill with blood

and support the body with important nutrients. Different medical societies focus in their definitions on certain disease features and a number of biomarkers to follow patients with heart failure emerged (Truby and Rogers, 2020). As a result of an aging population and better treatment options for myocardial ischemia with more patients' acute events, such as myocardial infarction (Nabel and Braunwald, 2012; Truby and Rogers, 2020), there is an increasing prevalence of heart failure. It represents a new epidemic in cardiovascular disease, affecting nearly 6 million people in the United States alone, and this number is expected to rise to 8 million by 2030 (Truby and Rogers, 2020). Especially elderly patients (e.g., more than 10% of people above 70 years old) are affected (Mosterd and Hoes, 2007). Given that heart failure is the fastest-growing cardiovascular condition, the overall healthcare costs are tremendous: In the U.S. healthcare system alone, current estimates are that \$30 billion is spent yearly, and this number is expected to almost double by 2030 (Ziaeian and Fonarow, 2016).

The main causes of heart failure include ischemic heart disease, dilated cardiomyopathy (DCM), valvular heart disease, and hypertensive heart disease (Truby and Rogers, 2020; Ziaeian and Fonarow, 2016). Despite extensive research, mortality and morbidity remain high, and challenges regarding translation of basic science research into humans have been difficult, with many late-stage failures despite positive phase IIa and IIb results.

Animal models in heart failure

Multiple animal models have been applied in different species (Vela et al., 2014) to replicate the human heart failure phenotypes. It is important to recognize that heart failure is not a single disease entity but rather a mixed bag of different diseases. While models of right heart failure induced by pulmonary arterial hypertension (PAH), such as the Sugen and hypoxia rat model, have met all criteria of (1) face validity (does the model resemble the human disease?), (2) predictive validity (does the model show treatment responses and biomarker surrogates similar to those found in humans?), and (3) construct validity (does the model measure what it claims to measure?) (Willner 1986), the same does not hold true for models of heart failure with preserved ejection fraction (Yoon and Eom, 2019).

In fact, one might argue that the advent of multiple new treatment options for right heart failure due to PAH over the last two decades was possible only because validated animal models became available (Happé et al., 2020).

Other heart failure models (Cops et al., 2019) include hypertensive heart disease-induced heart failure characterized by persistent pressure overload, concentric hypertrophy, a normal ejection fraction, and normal or decreased end-diastolic volume. Hypertensive heart disease can induce heart failure without a significant decrease in LV ejection fraction, and diastolic dysfunction is an important contributor in decompensated heart failure.

Animal models of hypertensive heart disease include a canine nephrectomy model (Mondritzki et al., 2018), while models using smaller animals include the SHR (Pfeffer et al., 1982), the TGR(mREN2)27 rat, aortic banding, or genetically modified mice (Molkentin et al., 1998).

Valvular lesions

Valvular lesions can result in heart failure. Aortic stenosis causes LV pressure overload and mitral regurgitation causes LV volume overload. Both conditions will eventually result in fluid retention and fatigue, caused by diastolic dysfunction, LV hypertrophy (LVH), and eventually decompensated systolic failure.

Progressive aortic constriction has been performed in large animals, such as pigs, dogs, and sheep, and strongly resembles the features of human aortic constriction. Transverse aortic constriction (TAC) in the mouse is the most commonly used model of aortic constriction in small animals. In this model, fixed aortic constriction causes an acute increase in LV pressure. This may cause significant postoperative mortality due to acute hemodynamic instability. The model is suitable for research with genetically modified mice. However, TAC in mice is not a progressive disease as it is in humans. For studying progressive

modifications in heart failure, large animal models are more suitable (Charles et al., 2020).

Although few animal models are available to study mitral regurgitation, there are canine models available to study this. This model results in severe contractile dysfunction, LV dilation, and eccentric LVH with myocyte lengthening. In smaller animals, such as rodents, it has only recently been possible to induce mitral regurgitation in a way similar to that achieved in larger animals (Corporan et al., 2020).

Dilated cardiomyopathies

Several models are available to study DCM in large animals. DCM can be induced by coronary ligation in pigs, dogs, and sheep (Blom et al., 2005). Left coronary artery microembolization can be used in dogs and sheep. It is also possible to induce DCM using pacing-induced tachycardia (Kaye et al., 2007), a model in which the degree of dilatation is reversible once the pacing is stopped.

Genetically driven heart failure models

Syrian hamster with genetic modification (Saito et al., 2017) as well as Golden retrievers are well known to develop phenotypes that not only resemble human heart failure but also share features of muscular dystrophy (Yu et al., 2015).

Ischemic heart failure models

Given that rodent models have a number of limitations in terms of their translatability into humans (Haghghi et al., 2003), large animal models still play a considerable role in preclinical development efforts, one of the best models that not only resembles the human disease phenotype but also shows similar patterns with respect to spontaneous deterioration and treatment effects with standard-of-care interventions employs repetitive application of microspheres into the coronary arteries of dogs (Sabbah et al., 2020). The developing ischemic heart failure with reduced ejection fraction has been employed in a number of preclinical development efforts for devices and pharmacological interventions (Sabbah et al., 1993).

Traditional biomarkers of heart failure

When one thinks about the use of a biomarker in the context of heart failure, it is of importance to differentiate the tasks to be accomplished with this biomarker (Wehling 2015):

1. Diagnostic differentiation from other conditions that present with similar symptoms

2. Detection of heart failure relevant comorbidities (e.g. renal or hepatic impairment)
3. Identification of individuals with certain genomic risk factors or metabolic pattern (e.g., CYP metabolism for warfarin or antiplatelet agents)
4. Stratification of certain severity stages, for example, to identify patients who are at risk for readmission or to enrich a clinical trial

Since heart failure is a complex disease, it is difficult to identify “the” perfect biomarker that solves every task to the same extent. It is often necessary to apply a combination of different biomarkers.

Soluble markers for cardiac remodeling and fibrosis

After myocardial injury, cardiac remodeling may occur. Cardiac remodeling involves various biological processes, including apoptosis, hypertrophy of cardiomyocytes, fibrosis, and inflammation. Biomarkers to potentially track these changes include GDF-15, ST2, and galectin-3. These biomarkers represent different aspects of cardiac remodeling and have certain limitations. ST2, GDF15, and galectin-3 are markers that are involved in fibrosis. ST2 is released from cardiomyocytes and fibroblasts upon volume overload and consists of two isoforms. Soluble-suppression-of-tumorigenicity-2 (ST2) is a transmembrane receptor that can also be detected as soluble ST2 (sST2). ST2 has antifibrotic and antihypertrophic effects and acts via IL-33. sST2 is a circulating form of ST2 that acts as a decoy receptor and can bind IL-33 to block its effect. sST2 appears to indicate a poor prognosis in patients with heart failure and was found to be elevated in patients with more progressive cardiac remodeling (Shah and Januzzi, 2010).

GDF-15 is upregulated in cardiomyocytes upon various cardiovascular events, such as ischemia or pressure overload. It is colocalized with macrophages but also circulates through the bloodstream and is not cardio-specific (Rabkin and Tang, 2020). GDF-15 can be regarded as a potential good player, since it has shown to have antiinflammatory and antihypertrophic effects, although its biological function in adverse cardiac remodeling is not fully understood (Wesseling et al., 2020).

Galectin-3 (Gal-3) is released during cardiac stress by activated macrophages in the heart and stimulates fibrosis. It is produced not solely in the heart, but in the entire body. Besides fibrosis, galectin-3 is also involved in tumor growth, metastasis, and inflammation. As a fibrotic factor, galectin-3 was implicated to predict outcome in patients with acute and chronic heart failure (Meijers et al., 2014).

In all fairness, one has to conclude that the specificity and utility of cardiac fibrosis markers is in patients with

heart failure are limited, even when they are used in combination, and so far have added little advantage in studying or treating these patients (Almeida and Budoff, 2019, Rabkin and Tang, 2020). Whether the combination of different markers can substantially increase their utility remains a matter of debate (Almeida and Budoff, 2019). Because of these uncertainties, these biomarkers are currently listed as potentially useful (recommendation grade IIb) in international guidelines (van der Meer et al., 2019).

Soluble markers for myocardial stretch

Myocardial stress is increased in heart failure, due to elevated filling pressures. As a result of elevated intracardiac pressures, natriuretic peptides are released from cardiomyocytes. N-terminal pro B-type natriuretic peptide (NT-proBNP) is now widely used as a diagnostic and prognostic marker in heart failure, and is regarded as the “gold standard.” NT-proBNP plays a very important role in the emergency department to establish the diagnosis of heart failure, with a sensitivity of 92% and a specificity of 84% (Maisel et al., 2011). Normal levels of NT-proBNP (<100 pg/mL) basically exclude significant heart disease. NT-proBNP can also be measured to estimate risk in heart failure: a 50% reduction of NT-proBNP levels led to a 50% reduction in events (Januzzi et al., 2011). Moreover, NT-proBNP levels at discharge are better prognostic indicators, compared to plasma levels during hospital admission. Also the longitudinal measurement of BNPs appears to be associated with changes in clinical status and have found their way into the treatment guidelines of different medical associations (recommendation grade I to IIa depending on exact purpose intended; van der Meer et al., 2019).

Midregional proatrial natriuretic peptide (MR-proANP) is another type of natriuretic peptide that is mainly released from the atrial cardiomyocytes. While some authors view MR-proANP to be an even better predictor than NT-proBNP in a head-to-head comparison (Seronde et al., 2013), others feel that further research is needed (Idzikowska and Zielińska, 2018). Given the lack of data, MR-proANP is currently not reflected to the same degree as BNP or NT-proBNP are (van der Meer et al., 2019). Drawbacks of natriuretic peptide measurements in general are that they are affected by renal function and obesity, renal failure increases plasma levels of natriuretic peptides (Mueller et al., 2005), and obese patients tend to show lower levels of natriuretic peptides via a mechanism that is not completely understood (Das et al., 2005) as well as different predictive values when changes in their plasma concentrations occur (Maisel et al., 2016).

Markers of inflammation

Inflammation is regarded as an important contributor to heart failure. It is known that inflammation after myocardial infarction contributes to increased cardiac remodeling via activation of profibrotic factors (Frangogiannis 2014). C-reactive protein (CRP) is one of the best markers of inflammation and is released after stimulation with IL-6. CRP levels are often elevated in patients who have heart failure or at risk of developing heart failure, and its levels can be associated with higher mortality or rehospitalization for heart failure. A huge drawback of CRP is the lack of specificity for heart failure in general. For example, in the presence of infections, the inflammation associated with heart failure becomes secondary (Lourenco et al., 2010).

In summary, one can conclude that at this point in time, natriuretic peptides yield the largest amount of information in patients with heart failure, while other soluble factors often lack the same degree of specificity and association with the heart failure state.

Digital biomarkers of heart failure

In contrast to the aforementioned approaches, digital technologies offer the potential for a radically different approach to determining the health status of patients with heart failure who are undergoing therapy. In principle, the advantage consists in collecting continuous real-life data, as opposed to the measurement of soluble plasma markers or performing testing protocols at given time points in the artificial environment of a clinic.

Early studies were performed by using implantable devices and monitors with telemonitoring function and provided evidence that outcome prediction is possible in patients with heart failure. Since implantable devices offer the possibility to measure more than one parameter, multiple parameters can be measured and analyzed in a combined manner to improve the predictive value. This generates a score that can be correlated with clinical outcome. As an example, a retrospective analysis of four studies performed using Medtronic CRT-D devices showed that the risk for hospital readmission could be predicted from a set of four predefined criteria, including intrathoracic impedance, atrial fibrillation occurrence, and percentage of CRT pacing (Small et al., 2014).

A recent approach to monitoring the course of disease of patients with heart failure is Cardio-MEMS, an implantable device with remote monitoring function that is connected to a mobile phone via an app (Abraham et al., 2011). It provides data from the pulmonary artery that were previously available only in the inpatient setting or even intensive-care units. This system has been shown to reduce events such as hospitalization by providing early, actionable measures of disease progression. These

data can also be used to monitor the effects of preventive care and to quantify the efficacy of study drugs.

Although these approaches have yielded very promising data (Abraham et al., 2011), the implantation of such devices is a costly and invasive procedure, so they cannot be widely used, and noninvasive devices are needed.

Thoracic impedance measurement using noninvasive approaches is an early tool that has been used to characterize patients with heart failure. However, the results so far have not been conclusive. The miniaturization of sensor technology with digitization has led to the development of accelerometers, which are also available as certified medicinal products. In a study with heart failure patients with preserved ejection fraction, continuous activity measurement was performed by using a triaxis accelerometer (KXUD9–2050, Kionix) and analyzing two outcome measures quantified by area under the curve: average daily activity and hours of activity. A significant difference between treatment groups was found in this study with this parameter. A post hoc analysis showed a significant correlation between the accelerometer-derived measures and established disease markers in heart failure (e.g., hospitalization, orthopnea, diabetes, NYHA class, heart failure–specific quality of life scores, six-minute walk distance, and NT-proBNP). Permutations of such analyses using wearable accelerometer data have been analyzed for prediction of mortality. For example, in a recent UK Biobank (<https://www.ukbiobank.ac.uk>) analysis, real-world data on heart failure from over 96,000 subjects wearing accelerometers, of whom 596 had heart failure, showed a good correlation of the outcome parameters with self-reported activity. Furthermore, since accelerometer data were collected continuously, data revealed that activity was reduced rather uniformly throughout the entire day.

Cardiovascular biomarkers: Quo Vadis?

As we have tried to show, the history of soluble biomarkers in the fields of hypertension, atherosclerosis, and especially heart failure shows a limited number of well-established and validated biomarkers that allow for risk stratification or identification of comorbidities. While in the indications hypertension and atherosclerosis it has been possible to identify predictive surrogate markers (e.g., blood pressure or LDL cholesterol levels and imaging markers of atherosclerotic changes) in heart failure, it still would be desirable for all involved stakeholders to have earlier readouts for earlier risk (e.g., rehospitalization) stratification. So far, the hunt for “the” heart failure surrogate marker (as we have outlined it for GDF-15, ST2, and Gal-3) has not been successful. What could be possible reasons?

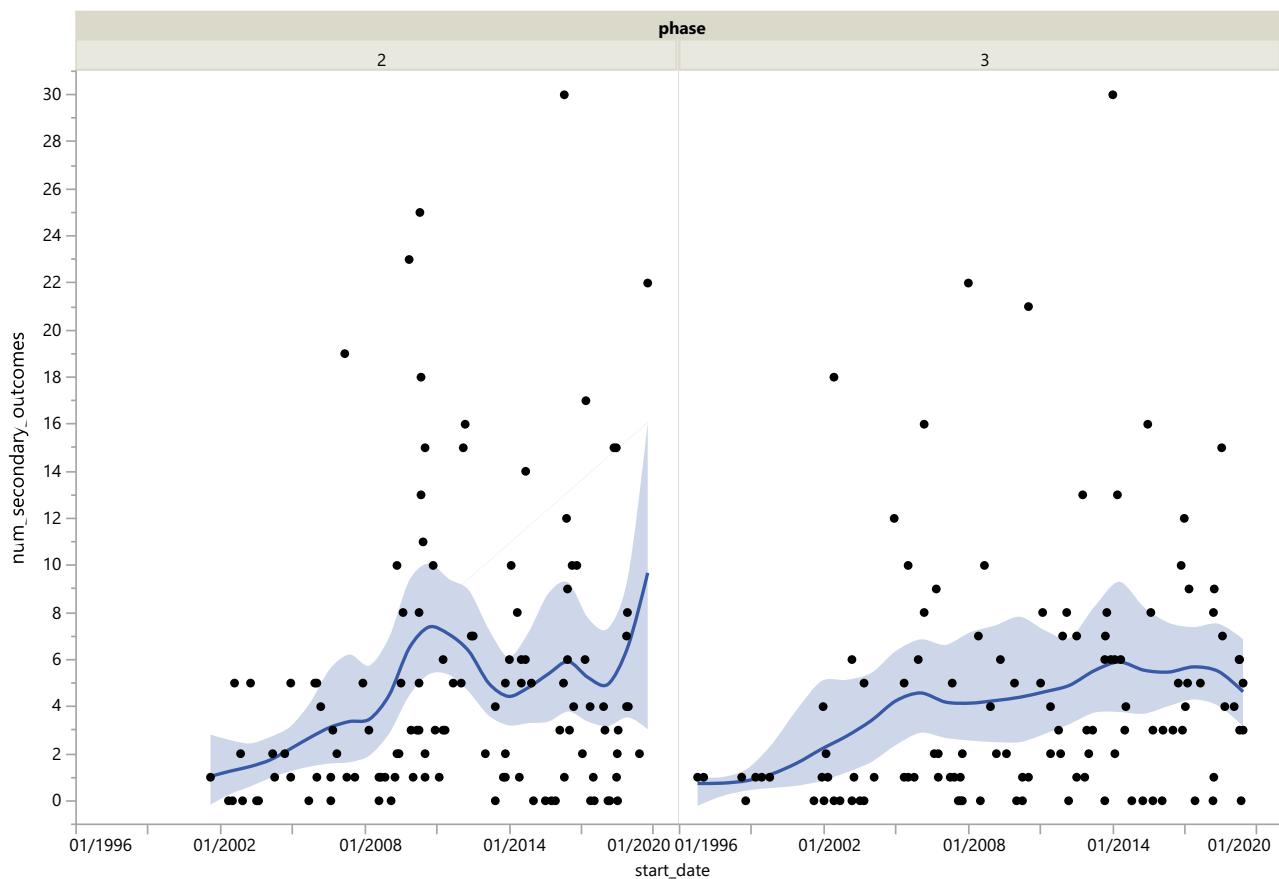


FIGURE 14.3 Trends in complexity of heart failure trials as judged from the number of secondary endpoints. Shown are heart failure trials started between 1996 and the beginning of 2020 registered on clinicaltrials.gov. Numbers of secondary endpoints per trial are overlaid with a spline fit ($\lambda = 0.043$), and the 95% confidence interval is shown as shading. There is a quasilinear increase in secondary endpoints with time. Phase II and III trials show a similar behavior with regard to this phenomenon. Data visualization provided as a courtesy by <https://www.molecularhealth.com>.

1. Have we been looking under the wrong “streetlight”? That is, while it would be nice to measure fibrosis via a soluble marker, is the “streetlamp” to employ instead cardiac MRI (van der Meer et al., 2019)? Overall, it is of interest that the number of additional markers employed to gain further insights increased over time (Fig. 14.3), which is also reflected in the increase in secondary endpoints, though so far without clearly affecting translational success rates.
2. Some authors suggest combining different biomarkers to potentially gain more predictive scores (Rabkin and Tang, 2020). While this could also lead to statistical error propagation, an alternative approach could be to apply multiple similar measurements to thereby decrease the variability. This approach has been applied in a number of indications, for example, diabetes care with continuous blood glucose measurements, chronic kidney diseases with the use of serial measurements of glomerular filtration rate or proteinuria (Inker et al., 2019) or in patients with heart failure through serial measurement of pulmonary artery pressure (Abraham et al., 2011).
3. While cardiovascular genetic biomarkers did not provide the much needed answers, as has occurred in oncology, future big data analysis might rather focus on activities of daily living (e.g., salt intake or the analysis of adherence) to gain information regarding the risk of rehospitalization or potential cardiovascular events in patients at risk (Young et al., 2016).

Case study: atrial fibrillation

Atrial fibrillation is a common disease that can cause thromboembolic strokes. Effective treatment options are available, including cardioversion (medical or electric), rhythm control, and catheter-based interventions (ablation) (Zipes and Libby, 2019). Furthermore, as stroke is the most feared complication, anticoagulation is given in most patients on the basis of their individual risk score (CHA2DS2-VASC-Score ≥ 2). Unfortunately, because atrial fibrillation not diagnosed early enough, many complications occur before treatment is initiated. This may occur because atrial fibrillation frequently presents with few symptoms or is completely asymptomatic (and the

patient therefore does not seek medical help) or it is non-permanent, such as intermittent or paroxysmal (and the arrhythmia is missed when an ECG is performed). The topic is of high importance in patient care and in research and development for novel diagnostic and therapeutic approaches. As of the time of writing, no biomarker is available to predict the likelihood of occurrence of atrial fibrillation. Recently, approximately 450,000 ECGs from the Mayo Clinic were used for artificial intelligence (AI) training and were shown to be able to predict the likelihood of atrial fibrillation occurrence with an accuracy of 90%. If these results are confirmed in prospective clinical trials, this would be a paradigm shift (Attia et al., 2019). Approaches like these could make it possible to perform risk stratifications before the event occurs. In the meantime, early diagnosis of atrial fibrillation is being pursued. The introduction of wearable one-channel ECGs in the last few years has made it possible for patients to measure their own ECG, which presents the opportunity for a new approach to diagnosis of atrial fibrillation (Inui et al., 2020). The patient is now able to measure on a regular basis (e.g., daily), when he or she has symptoms that are suggestive of atrial fibrillation (e.g., palpitations) or in particular circumstances (e.g., while exercising). However, whether this will indeed lead to earlier detection of atrial fibrillation or higher accuracy of detection remains to be demonstrated. So far, the evidence for this is rather anecdotal. The Apple Heart study has laid the groundwork for the large-scale monitoring of patients (Perez et al., 2019). Currently, in the Apple Heart and Movement Study a comprehensive study is running to study whether heart rate and mobility signals, such as walking pace and flights of stairs climbed, relate to hospitalizations, falls, heart health, and quality of life in order to promote healthy movement and improved cardiovascular health (ClinicalTrials.gov Identifier: NCT04198194).

Atrial fibrillation might be the first model indication in which serial or better continuous measurements of a traditional biomarker (here, ECG) will allow for better diagnosis and treatment of the disorder.

Conclusion

Given the increasing prevalence and unmet need in the cardiovascular space, future biomarker discovery activities need to “fish with a wider net” that, for example, includes digital biomarkers, possibly uses trained AI systems, and involves big data and real-world data analysis because the current state with respect to risk prediction and guidance for interventions has not been sufficiently addressed with the markers at hand. Also, serial measurements with well-established markers will need further exploration to overcome the current innovation challenges

in the cardiovascular indication space in order to provide a new biomarker paradigm (Kuhn 1962).

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Chapter 15

Biomarkers in oncology

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Abstract

Modern developments in molecular genetics have led oncologists not only to define tumors according to their anatomical site, stage, and histological type, but also to appreciate the heterogeneity between tumors according to their specific molecular, genetic, or immunological subtype, empowering oncologists to, ideally, provide more individually tailored treatment to particular patients and their specific tumor subtype by using highly targeted therapies. In this chapter, circulating biomarkers and pharmacodynamics markers for target inhibition, among other markers, will be discussed and exemplified with regard to major oncological entities.

Keywords: Oncological biomarkers; markers for target inhibition; lung cancer; PD-1/PD-L1 axis

Modern developments in molecular genetics have led oncologists not only to define tumors according to their anatomical site, stage, and histological type, but also to appreciate the heterogeneity between tumors according to their specific molecular, genetic, or immunological subtype (La Thangue and Kerr, 2011). This empowers oncologists to, ideally, provide more individually tailored treatment to particular patients and their specific tumor subtype by using highly targeted therapies rather than more broad-spectrum and nonspecific cytotoxic agents (Kerr and Shi, 2013).

When we consider some of the first targeted therapies to be clinically approved, such as imatinib in the treatment of chronic myeloid leukemia and the greatly improved 5-year survival rate (Deininger and Drucker, 2003), it would appear that an exciting new day of scientifically eloquent targeted treatments for cancer is upon us. However, the process of developing and using such targeted agents is fraught with difficulty. The large development costs, the potential for serious toxicity, and the fact that only particular subgroups of patients are likely to benefit at all mean

that such treatments are highly scrutinized by regulatory authorities ahead of approval (Raftery, 2009).

Effective biomarkers therefore must be identified and then utilized appropriately to aid in clinical decision making and ensure that targeted agents are given to patients for whom they will be suitably efficacious. For instance, in patients with resected colorectal cancer the QUASAR study clarified mismatch repair deficiency (MMR-D) as a prognostic biomarker, resulting in changes to guidelines regarding use of adjuvant chemotherapy (Kerr and Midgley, 2010).

In addition to aiding clinical decision making in the future, development of biomarkers leads us to retrospectively question the validity of the data derived from previous clinical trials and the conclusions that were drawn. For example, retrospective analysis of the PETACC 3 trial (Van Cutsem et al., 2009) demonstrates a statistically significant difference in (previously unidentified) poor prognosis T4 tumors in just one of the two arms of this trial. Hence by identifying new markers for patient stratification, we may find flaws in the design of previous clinical trials, leading us to question the plausibility of the findings.

It is imperative to develop and validate partner biomarkers in association with new agents from the beginning of the drug development pathway, that is, from phase I trial onwards. Such utilization has made a supportive contribution to dose selection and primary endpoint measurement in early-phase cancer trials. It helps to identify the most appropriate patients, provides proof-of-concept for target modulation, helps to test the underlying hypothesis, helps in dose selection and schedule, and may predict clinical outcomes. Only 5% of oncology drugs that enter the clinical trial development pathway. Use of biomarkers should reduce this high level of attrition and bring forward key decisions (e.g., fail fast), thereby reducing the spiraling cost of drug development and increasing

the likelihood of getting innovative and active drugs to cancer patients ([Sarker and Workman, 2007](#)). Biomarkers may yield information earlier than current imaging techniques and thus provide a potentially more efficient way of following up on patients receiving therapy. Furthermore, they may predict a more biologically appropriate target dosage of a particular agent in order to adjust doses and reduce toxicity without compromising efficacy.

The characteristics of a good biomarker ([Bhatt et al., 2007](#)) are as follows:

1. It should be cost-effective.
2. It should be of low expression or circulation in normal individuals.
3. It should be accessible by noninvasive means such as blood, urine, and so on.
4. It should be accurately reproducible in multiple clinical centers.

Circulating biomarkers

In the absence of biopsy material, a lot of information can still be gathered. For example, biomarkers of antiangiogenic therapy may be cytokines and circulating endothelial cells in peripheral blood. Many surface markers are used to define circulating endothelial cells by multiparameter flow cytometry. [Beerepoort et al. \(2004\)](#) found that patients with progressing cancer have higher levels of mature circulating endothelial cells than do healthy people or patients with a stable disease. [Shaked et al. \(2006\)](#) demonstrated an increase in circulating endothelial progenitors early in the course of the therapy, so sampling at multiple time points is required for interpretation of data. Circulating proteins are vascular endothelial growth factors (VEGFs), basic fibroblast growth factors (bFGFs), endostatin, and thrombospondin 1 (TSP1). In non–small cell lung cancer (NSCLC), VEGF levels are high, whereas TSP1 levels are low, which may trigger and sustain tumor angiogenesis. A high level of serum VEGF at the time of presentation in NSCLC predicts worse survival ([Arkadiusz et al., 2005](#)).

In addition to serum cellular material (circulating cancer cells), tumor antigens (CEA, Ca19-9, PSA etc.), and other molecular targets (circulating polypeptides such as VEGF), research interest around fragments of cancerous genomic material (circulating tumor DNA, or ctDNA) has been steadily increasing.

In contrast to ctDNA, the first report on circulating free DNA (cfDNA) in healthy individuals dates back to 1948 ([Mandel and Metais, 1948](#)).

The practical difficulties of partitioning and differentiating ctDNA from cfDNA or other false-positive genotyping signals in the plasma (such as the clonal hematopoiesis of

indeterminate potential) ([Hu et al., 2018](#)) has been a challenge, but bioinformatics alongside whole-exome and whole-genome sequencing have contributed to retrieving enormous volume of information.

Liquid biopsy was listed as one of ten breakthrough technologies in 2015 ([Standaert, 2015](#)). The quantitative and qualitative analysis of ctDNA holds great potential to become a novel noninvasive, time-efficient, and highly sensitive diagnostic tool in translational oncology practice and is expected to play a key role in precision medicine.

The genetic information resulting from ctDNA analysis could provide entire tumor fingerprinting, including identification of subclones and clone evolution, and is expected and hoped to be superior to the results of localized anatomical tissue samples ([Burrell and Swanton, 2014](#)).

With these features, ctDNA could help in establishing early diagnosis, assess response to treatment, and detecting drug resistance. Most important, it could help in identifying minimal residual disease and real-time dynamic fingerprinting of tumor genome and identifying biomarkers that could lead and strengthen individualized treatment decision making.

This could enable oncologists to not only select the appropriate targeted treatment for each patient, but also to know when each treatment should be introduced to achieve the best possible outcome.

Researchers and clinicians across multiple specialties in Baltimore showed a strong correlation between postoperative ctDNA levels in patients with colorectal cancer and 1-year relapse. They also found that ctDNA was more sensitive and reliable in predicting disease relapse than was the abundantly used tumor-antigen biomarker CEA ([Diehl et al., 2008](#)).

Further studies in patients with NSCLC confirmed that ctDNA quantitation could identify minimal residual disease and was also directly correlated with treatment-related imaging changes ([Newman et al., 2014](#)). In another study in 2016 the quantification of ctDNA was suggested to represent a novel biomarker able to identify minimal disease relapse in postoperative patients with early colon cancer as well as enabling early diagnosis of recurrence after 3 months of adjuvant chemotherapy ([Tie et al., 2016](#)).

At the time of writing, the NCT02887612 (ClinicalTrials.gov) trial in China, which is currently recruiting patients with gastric cancer, is aiming to assess the efficiency of using ctDNA as a mean of early relapse biomarker. Another ongoing (but not recruiting) trial (NCT03145961) aims to explore the use of ctDNA levels as a potential biomarker of monitoring disease progression in early-stage triple negative breast cancer patients after completion of primary treatment to enable early introduction of pembrolizumab as a second-line agent.

The TRACC trial (NCT04050345) is investigating whether detection and quantification of ctDNA can safely lead to identifying minimal residual disease and relapse in patients with early-stage colon cancer, who are likely to benefit from adjuvant chemotherapy while being spared the unnecessary toxic side effects.

Further representative paradigms of ctDNA application in personalized medicine are the plasmaMATCH and OPTIPAL-II trials. The former is trying to reveal actionable biomarkers through analysis of circulating tumor genomic material of patients with advanced breast cancer. The genomic fingerprinting will ultimately guide early decision making for targeted therapies (with fulvestrant, olaparib, neratinib, and experimental agents) for this group of patients.

OPTIPAL-II is exploring the value of ctDNA analysis in identifying metastatic colorectal cancer patients with KRAS, NRAS, or BRAF mutations who could benefit from treatment with anti–epidermal growth factor receptor (EGFR) molecules.

Pharmacodynamic markers: target inhibition

Although noninvasive means of following up on patients would be most appropriate, there is still much interest in obtaining tissue biopsies from patients receiving targeted therapies so that the molecular features of the tumor after exposure can be studied.

Measurement of target inhibition should ideally be undertaken in a tumor before and after a drug is administered, and sometimes more than two biopsies are needed. The tumor needs to be accessible, and the patient must consent to repeated biopsies. This limits the number of patients available to such studies. Normal tissues such as buccal mucosa, peripheral mononuclear cells, blood, and skin can be used to measure the changes in molecular effects. For example, in a trial of the aromatase inhibitor anastrazole, peripheral blood estradiol levels were the endpoint (Ploudre et al., 1995). Similarly, in a trial of metastatic gastrointestinal stromal tumor patients, monocytes were assessed to measure the biological effects of the targeted agent (sunitinib malate), inhibiting the VEGF and vascular endothelial growth factor receptor (VEGFR), and were found to be modulated by treatment, suggesting that these measurements may be effective pharmacodynamics markers for sunitinib (Norden-Zfoni et al., 2007).

Receptor tyrosine kinases (RTKs) play an important role in the regulation of cell proliferation, cell differentiation, and intracellular signaling processes. High RTK activity is associated with a range of human cancers. Therefore inhibition of these receptors represents a potent approach to the treatment of various cancers. EGFR is

one of the RTKs that regulate cell proliferation. EGFR has extracellular ligand-binding domains to which the epidermal growth factor (EGF) binds. Subsequently, their intracellular autophosphorylation domains in the C-terminal region are trans-autophosphorylated, and the catalytic domains of EGFR are activated. Active EGFR recruits and phosphorylates signaling proteins, such as Grb2, which links cell surface receptors to the Ras/mitogen-activated protein kinase (MAPK) signaling cascade. Ultimately, this leads to intranuclear mitogenesis and cell proliferation caused by activation of the Ras/MAPK signaling pathway. A clinical scenario illustrating the importance of the EGFR pathway is high-grade gliomas in which the pathway is commonly amplified. Abnormal signaling from the receptor tyrosine kinase is believed to contribute to the malignant phenotypes that are seen in these tumors. Targeted therapy with small molecule inhibitors of the receptor tyrosine kinase may improve the treatment of these highly aggressive brain tumors. Treatment with the EGFR kinase inhibitors ZD1839 (Gefitinib) with high specificity for EGFR resulted in significant suppression of EGFR autophosphorylation, even with very low levels of the drug (Bin et al., 2003). Patients with tumors with the wild-type KRAS gene achieve disruption of cellular growth signaling through the EGFR pathway when EGFR-targeted therapies are administered. Conversely, tumors with mutated KRAS genes have the ability to continue signaling through the proliferation cascade within the EGFR axis, even when EGFR inhibitor therapy is applied. Thus patients with mutated KRAS genes will not respond to EGFR inhibitors. Examples include cetuximab and panitumumab in colorectal cancer and erlotinib and gefitinib in NSCLC. Such biomarker-guided decision making is now directly implemented in international guidelines and practice for colorectal cancer treatment (Mack, 2009). The prescribing label for such drugs is evolving as more mutations in Ras (Kras and NRas) are identified. This has the effect of decreasing the potential population for treatment, decreasing population risk and toxicity, increasing effectiveness in the eventually treated population, and decreasing cost. These are the ultimate goals of any effective segregating biomarker.

It has taken a long time to discover this differential effect of the EGFR inhibitors, and the discovery necessitated many expensive trials and much retrospective analysis before the biomarker was truly appreciated and refined. We hope that the cultural shift will continue and that in the future, testing and validation of suspected biomarkers will be undertaken prospectively and earlier in the preclinical development stages of drug testing, before any patient is exposed to the drugs. Further validation can then take place during phase I and phase II trials. When such biomarkers are tested in clinical trials, the endpoint

(e.g., the level of expression of protein); the methods of measurement (e.g., mRNA or protein); and the standard operating procedures governing processing, fixing, transport, and storage of the tissue should be clearly defined before the trial starts. The amount of tissue required should also be clearly specified, as this makes a significant difference in terms of ethical and practical applicability. Preclinical studies should guide the magnitude of target inhibition and its impact on tumor growth and the proportion of patients having the effect with a given dose to conclude the recommended dose.

An excellent example of more promptly conducting early-phase trials involving biomarkers is the BRAF V600E inhibitors in melanoma. BRAF is a serine-threonine kinase that activates MAP/ERK kinase signaling to alter gene activation. Following identification of BRAF mutations in 60% of melanoma patients (Miller and Mihm, 2006) and development of BRAF V600E inhibitors, patients were then recruited to phase I trials based on the presence of this mutation. Initial results appear promising, with a reported 81% response rate in the selected patients in the phase I trial (Flaherty et al., 2010).

However, this is just one example, and there are many different ways in which PD markers can be used in a phase I trial of novel agents or targeted therapies. PD measurement can be included as the primary endpoint in trial protocols to assess the magnitude of a biological effect and its relationship to the dose of the drug administered. If there is considerable variation in PD effects among patients, then more patients need to be treated at the same dose level to assess the differences. A detailed PD assessment at all dose levels is necessary for calculating the recommended dose. The phase I trial may be an opportunity to learn about targets if they are not fully defined before the trial starts. Markers that look interesting can be further developed in the study at later stages or in subsequent studies. Where the optimal biological dose has not been fully defined, PD effect may be studied at two different dose levels, that is, the maximum tolerated dose and a lower well-tolerated dose. It is important that preclinical studies define the appropriate target, PD marker, and desired biological outcome, demonstrating, for example, the dose-dependent changes in a signaling pathway in tumors that have responded in the preclinical setting. Sometimes these agents have more than one target or biological effect. Antitumor effects seen in the preclinical studies may not be associated with the same target, so phase I trials are effective in studying those agents. For example, sorafenib was thought to be a specific inhibitor of Raf-1 kinase (Lyons et al., 2001) but was later found to act on several other kinases such as the VEGFR, the platelet-derived growth factor receptor (Wilhelm et al., 2003), and so on and thus was found to be effective against renal cell cancer.

A greater number of patients may be required up front in these elegant trials, compared to a standard design with the primary endpoint being toxicity. For example, for a trial to show a dose response in target inhibition, rising from 40% at one dose level to 90% at a higher dose level (Korn et al., 2001). The biopsy or assay procedures should be clearly explained in the patient information sheet and informed consent document. Maintaining a close link between the clinic and the PD laboratory is clearly very important. Laboratory scientists, radiologists, and molecular pharmacology teams should be involved in protocol writing and development.

Tumor biopsy will give the most direct and relevant information about the target inhibition before and after a treatment but may be quite challenging from a pragmatic or ethical viewpoint. Some tumors such as superficial melanomas, head and neck cancers, and cervical cancers are more easily accessible than others. Therefore alternatives to biopsying the tumor itself have been explored. In a gefitinib phase I trial, skin was biopsied as a marker to assess EGFR tyrosine kinase inhibitor, and inhibition of EGFR activation was seen at all doses in all patients (Baselga et al., 2002).

Preclinical studies should provide some guidance about the tissue that is to be biopsied, the amount needed, and the condition and processing of the tissue. The primary endpoint should be defined up front in cases in which several assays are taken. Assays used to measure PD endpoint in plasma, normal tissue, and tumor samples include ELISAs, immunohistochemistry, Northern and Western blotting, real-time polymerase chain reactions, and gene expression arrays. Most of these are refined and developed for a specific target. Sometimes the pharmaceutical company that is developing the novel agent also develops the assays of PD development. However, often good collaboration between an academic scientific group and the pharmaceutical company will bring about the speediest development of the appropriate assays. If the PD measurement is the primary endpoint of the trial, then the assay needs to be validated to good clinical laboratory practice standards.

The need for such close collaboration and validation of assays has been highlighted by a debate regarding the measurement of HER2 levels. It is well established that HER2 gene expression determines the efficacy of the targeted trastuzumab as adjuvant therapy (Slamon et al., 2001). However, assays involving fluorescence in situ hybridization to measure HER2 indicate that conventional assays are suboptimal, with up to 20% giving an incorrect result (Perez et al., 2002). Thus it is plausible that an inaccurate assay could have been used to influence clinical decision making in patients with breast cancer, which shows

how imperative it is that assays for such biomarkers are sufficiently validated.

Focus on lung cancer

Lung cancer is the fourth most frequently diagnosed yet most lethal cancer worldwide, with 2.1 million new cases and 1.7 million deaths in 2018 (Bray et al., 2018).

The predominant histological type (around 84%) of lung malignancies is NSCLC (squamous or nonsquamous subtype). The type of lung cancer is a great example of how individualized treatment can decrease mortality.

The introduction and use of targeted therapy in the United States are believed to be the main factor for the reduced mortality compared to the incidence of the disease.

During the last decade, the mortality from NSCLC has been decreasing faster than the incidence. Specifically, a 6.3% annual drop in the mortality was observed for the period between 2013 and 2016, while the incidence decreased only by 3.1% annually between 2008 and 2016 (Howlader et al., 2020).

The standard of care for metastatic NSCLC includes screening for actionable driver mutations for EGFR, the B Raf proto-oncogene BRAF, anaplastic lymphoma kinase (ALK), c-ROS-1 oncogene, NTRK, RET, and mesenchymal–epithelial transition (MET).

For tumors lacking a driver mutation, the quantification of programmed death ligand-1 (PD-L1) expression will determine the use of immunotherapy with checkpoint inhibitors either as monotherapy or in combination with chemotherapy.

As was mentioned above, EGFR mutations are known to be involved not only in tumorigenesis but also in growth and metastasis of the cancer cell.

Of all NSCLC cases, it is believed that around 15% harbor at least one EGFR mutation with preference to the female sex and the nonsmokers (Cancer Genome Atlas Research, 2014).

The ligand/receptor binding leads to EGFR dimerization either with itself (autodimerization) or with other tyrosine kinases of the HER/erbB family (heterodimerization), which ultimately leads to ATP-regulated tyrosine autophosphorylation and downward signal transduction, mainly through the activation of the mTOR, MAPK, and PI3K/AKT pathways (Liu et al., 2017).

EGFR-targeted treatment consists mainly by the first- and second-generation tyrosine kinase inhibitors erlotinib/ gefitinib or afatinib, respectively, while osimertinib (a third-generation tyrosine kinase inhibitor) has been recently approved as the first-line treatment for EGFR mutant NSCLC, especially in EGFR exon-19 deletion or exon-21 L858R mutation.

ALK is a transmembrane tyrosine kinase member of the insulin receptor superfamily and reserves significant oncogenic potential via activation of its downstream signaling pathways. Actionable point mutations, rearrangements (ALK-R), and amplifications (ALK-A) have been observed in 3%–7% of NSCLC cases (Du et al., 2018; Takeuchi et al., 2008).

Interestingly, ALK fusion oncogenes are seen more frequently in younger patients with no or light history of smoking (Shaw et al., 2009; Shaw and Solomon, 2011).

Similar to EGFR, the binding of ligands to the relative extracellular epitope of ALK leads to dimerization and autophosphorylation of the intracellular tyrosine kinase.

The activated tyrosine kinase in turn activates phospholipase C gamma (PLC- γ), the MAPK/extracellular signaling regulated kinase (MAPK/ERK), the Janus kinase–signal transducers and activators of transcription (JAK-STAT) and phosphatidylinositol-3-kinase–AKT mouse thymoma (PI3K-AKT) enabling and enhancing cell proliferation, migration, invasion, angiogenesis, and apoptosis inhibition (Katayama et al., 2015).

In the United States, alectinib is currently the first-line option for patients with actionable ALK rearrangements and has also been approved as a second-line treatment for patients who have progressed on the first-generation agent crizotinib.

At the time of writing, the second- and third-generation ALK TKIs ensartinib and lorlatinib are being evaluated and considered as first-line options as alternatives to crizotinib within the eXalt3 and CROWN trials, respectively.

Lorlatinib has shown encouraging results with longer progression-free survival and response in the brain metastatic disease when compared to crizotinib (Shaw et al., 2020).

ROS1 tyrosine kinase receptors such as ALK belong to the insulin receptor family.

Actionable rearrangements of ROS1 are less common in NSCLC, with 1%–2% of all cases, and are seen in genetic translocations between ROS1 and other genes, including CD74, SLC34A2, TPM3, FIG1, and KDELR2 (Bergeron et al., 2012).

ROS1 fusion (similar to ALK) leads to cancer cell growth, proliferation, and survival through the activation of the downstream pathways RAS/RAF/MAPK, PI3K/AKT/mTOR, and STAT-3 (Chu, 2020).

Of interest, ALK and ROS1 retain highly conserved domains, resulting in pronounced structural homology between the two tyrosine kinase receptors.

First-line treatment for ROS1 translocations include the ROS1/tropomyosin receptor kinase (TRK) inhibitor entrectinib or crizotinib, a ROS1/MET inhibitor. Recent evidence from three phase I to II trials (ALKA-372-001, STARTRK-1, and STARTRK-2) suggests that the former

should be the treatment of choice for patients with central nervous system metastatic disease (Drilon et al., 2020b).

BRAF belongs in the MAPK intracellular pathway axis, which has a cornerstone role in cell growth and proliferation through transducing extracellular signals to the nucleus via phosphorylation and activation of the various counterparts.

Mutations in the BRAF protein is found in 3%–5% of NSCLC cases. A high proportion of those harbor the V600 point mutation (similar to melanoma). BRAF V600 mutations are seen across all ethnic groups and most frequently in past or current (at time of diagnosis) smokers (Alvarez and Otterson, 2019).

The phosphorylated BRAF kinase leads to activation of MAP2K ERK 1/2 kinases which in their turn enable activation of various transcription factors in the nucleus, such as FOS, ELK1, and TP53 (Pritchard and Hayward, 2013), and thus promote unchecked proliferation and cell growth.

Patients with BRAF V600 mutation have better prognosis than do patients with non-V600 mutations and were found to respond better to combination TKI treatment with dabrafenib and trametinib targeting BRAF and MEK (MAP2K), respectively (Planchard et al., 2017).

Fusions in the neurotrophic TRK genes (NTRK1, NTRK2, and NTRK3) with ETV6, LMNA, and TPM3 genes (among others) is very rare, found in fewer than 1% of NSCLC cases worldwide. The translated TRK fusion protein is thought to be responsible for transducing growth and proliferation signals and serves as a novel target for small-molecule therapy with the first-generation TRK inhibitors entrectinib and larotrectinib (Haratake and Seto, 2020).

The role of MET factor and the hepatocyte growth factor (HGF) receptor has been well described in embryogenesis, wound healing, and hepatic regeneration.

MET dysregulation in NSCLC, such as MET exon-14-skipping mutations or MET gene amplification, is found in 3%–4% or 1%–6%, respectively, and are thought to promote cell survival and proliferation, angiogenesis, and, as expected, tumor invasion.

For patients with MET exon-14-skipping mutation, degradation of the MET protein is significantly reduced and leads MET factor to act as an oncogenic driver. The U.S. Food and Drug Administration (FDA) has approved the use of capmatinib (GEOMETRY-mono-1 trial) as a selective MET receptor inhibitor.

Besides capmatinib, tepotinib and glesatinib are being evaluated in clinical trials as selective MET inhibitors (Liang and Wang, 2020; Wolf et al., 2020).

RET (a cell membrane tyrosine kinase receptor) rearrangements and formation of fusion proteins with NCOA4, KIF5B, and CCDC6 are found in only 1%–2%

of NSCLC cases and have been linked with younger age and never having smoked (Takeuchi et al., 2012; Wang et al., 2019).

Based on the LIBRETTO-001 and ARROW studies, the FDA approved the use of selpercatinib and pralsetinib, respectively, for patients with actionable RET rearrangement as a first-line treatment options over chemotherapy and immunotherapy combinations (Drilon et al., 2020a).

The PD-1/PD-L1 axis

Programmed death-1 (a member of the CD28 superfamily) is a transmembrane protein that is expressed mainly in activated T and B cells and has a key role in negative feedback upon interaction with its ligands (mainly PD-L1 and PD-L2), hence regulating (through negative feedback) immune responses to various stimulatory events.

Programmed death ligands 1 and 2 (PD-L1 and PD-L2) are widely expressed in healthy tissues and cells and play a significant role in ensuring that immunity homeostasis is achieved (Sun et al., 2018). By contrast, PD-L1 expression in cancer helps malignant cells to evade innate immunity and apoptosis.

The quantification of expression of PD-L1 in cancer cells as well as the presence or absence of driver mutations or rearrangements in the above-mentioned biomarkers determine the therapeutic approach for this group of patients.

Checkpoint inhibitors such as pembrolizumab (anti-PD-1) and atezolizumab (anti-PD-L1) have been approved for use either as single agents or in combination with chemotherapy in EGFR/ALK wild-type NSCLC patients.

For tumors with PD-L1 expression of less than 50% in nonsquamous NSCLC, platinum-based doublet in combination with pembrolizumab is the preferred choice (based on the KEYNOTE 189 phase III trial). A similar approach is followed for squamous NSCLC as a first-line treatment option (phase III KEYNOTE-407).

For patients with PD-L1 expression of more than 50% in the absence of rapidly progressive metastatic disease and with no other actionable driver mutation, single-agent immunotherapy with either pembrolizumab (KEYNOTE-024 trial) or atezolizumab (IMpower 110) is indicated (Hanna et al., 2020).

The KEYNOTE-024 trial compared pembrolizumab monotherapy versus combination chemotherapy in the metastatic NSCLC and showed the superiority of pembrolizumab monotherapy.

Whether pembrolizumab combined with chemotherapy has further additional benefit compared to single-agent pembrolizumab in NSCLC tumors with high PD-L1 expression remains to be evaluated.

Conclusion

Biomarkers are essential to allow identification of novel therapeutic targets, stratify patients according to likely efficacy, and monitor response in order to deliver individually tailored and targeted cancer treatment, as is clearly demonstrated in the management of lung cancer. However, it is imperative that such markers be validated sufficiently before they are utilized to influence clinical practice and that their potential applications be fully understood.

In particular, the prospect of retrospectively applying new biomarker assays to existing trial data to reveal previously unidentified statistical biases and subsequently masked outcomes is fascinating. This was demonstrated eloquently in retrospectively identifying T4 tumors in the PETACC3 trial and uncovering a statistically significant bias between the patients recruited to the two arms of the trial. Hence we are led to question the validity of the outcomes of this trial, and it is interesting to contemplate in how many other trials retrospective analysis with biomarkers could alter and indeed invalidate the outcome.

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Chapter 16

Translational medicine in psychiatry: challenges and imaging biomarkers

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Abstract

Among research disciplines, neuroscience has introduced the most fundamental changes in the way in which mental disease states are conceptualized and pursued. Nowhere is this more obvious than in translational medicine. The slow, arduous empirical process of finding new treatments must be properly incentivized for this work to be done by the current and next generation of psychiatric neuroscientists. Here, the toolboxes such as evolving biomarkers (e.g., imaging, transomic and functional biomarkers) genetics are discussed in relation to translational issues in mental diseases.

Keywords: Mental diseases; imaging biomarkers; transomic biomarkers; antipsychotic drugs; genetics in psychiatry

Biological treatment of psychiatric disorders

Even a cursory look at global morbidity and mortality statistics makes it clear that psychiatric disorders are among the most common and most disabling conditions in medicine. In the World Health Organization statistics measuring the number of years of disability due to illness, four of the top ten disease categories (addiction, depression, schizophrenia, and anxiety disorders) fall into the domain of psychiatry, and, combined, mental disorders cause more disability than any other class of medical illness in Americans between ages 15 and 44 (Insel and Scolnick, 2006). Up to 40% of the population must expect to have a psychiatric condition necessitating treatment at least once in their life, and 20% are being treated at any given time. The direct and especially indirect societal costs (including absenteeism and presenteeism at work) of mental illness are staggering, most prominently so for disorders such as schizophrenia that often have a chronic course and afflict

patients in adolescence or early adulthood, interfering with education and the ability to find employment.

It is widely accepted that mental disorders, although they manifest themselves in the domain of psychology and social behavior, nevertheless have a strong and often predominant biological basis. Many diseases in the purview of psychiatry—especially the most severe ones, such as schizophrenia, bipolar disorder, and depression—are highly heritable, indicating that the major contributor of disease risk is genetic (Meyer-Lindenberg and Weinberger, 2006; Sullivan et al., 2012).

Even for disorders in which the impact of (usually adverse) life events is clear, such as anxiety disorders, depression, or borderline personality disorder, heritability typically still accounts for around 30%–50% of disease risk. This is, in principle, good news in the sense that it suggests a biological approach to these illnesses that has proved powerful in other areas of medicine and that adds to the armamentarium of psychiatry. To address this spectrum of biological, environmental, and individual risk factors, psychiatrists are called upon to choose wisely from available psychotherapeutic, social or rehabilitative, and biological interventions that need to be tailored to the individual patients if the burden of mental illness is to be effectively lessened. In this chapter we will focus on biological interventions.

Given their frequency and severity, it should not come as a surprise that psychiatric or psychotropic medications account for a large proportion of the budget spent on drugs in industrialized nations, and as a group, they are usually among the top two or three spending categories. Antipsychotics, antidepressants, mood stabilizers such as anticonvulsants, and antianxiety agents account for the majority of this expense, reflecting the importance and prevalence of schizophrenia, depression, bipolar disorder,

and anxiety disorders. A large body of evidence in the literature shows that these drugs are effective in treating these illnesses and useful in managing anxiety, impulsivity, and mood in many others. Does this indicate that patients, psychiatrists, and the general public get a satisfactory return on their investment? Most researchers in the field would agree that this is not the case.

For some disorders, such as autism and anorexia nervosa, biomedical treatments for the core symptoms are absent. For others, such as schizophrenia, current treatments are efficacious but only for circumscribed symptom clusters and to a degree that often falls short of recovery. It is clear that conventional and atypical antipsychotics reduce the so-called positive psychotic symptoms of schizophrenia, an indispensable component of the current therapy for this disease, but it is considerably less clear whether they are helpful for cognitive deficits or negative symptoms (Lieberman and Stroup, 2011). Two large-scale trials, the Clinical Antipsychotic Treatment Intervention Effectiveness trial (Lieberman et al., 2005a) and the Cost Utility of the Latest Antipsychotic Drugs in Schizophrenia Study trial (Jones et al., 2006), found that only about one in four patients with chronic schizophrenia continued either a first- or second-generation antipsychotic throughout an 18-month trial, and the data did not show a difference in efficacy between older and newer (so-called second-generation antipsychotic) drugs. A large European trial, the European First Episode Schizophrenia Trial, also found no differences in efficacy, although it did find better adherence to second-generation drugs. However, this trial was open; therefore patient and therapist bias might have contributed to this finding (Kahn et al., 2008). In a 5-year follow-up of 118 schizophrenic patients after their first episode of psychosis, only 13.7% met criteria for full remission lasting 2 years or more. This rate of remission is disappointingly low. In essence, schizophrenia remains a chronic, disabling disorder, just as it was a century ago. The most dramatic confirmation of this contention is the fact that the mortality rate of patients with this illness has not declined in the area of pharmacotherapy and may in fact have increased (Murray-Thomas et al., 2013).

Things may not be much better in the clinical practice of the treatment of depression. Although antidepressants reduce the symptoms of major depressive disorder, these drugs are significantly better than placebo only after 6 weeks of administration. More important, many studies show that patients on medication continue to have substantial depressive symptoms at the end of the trial, and a large proportion of patients require a trial of several drugs, dose adjustment, or comedication.

Insel and Scolnick (2006) reviewed the portfolio of available treatments for schizophrenia and depression and concluded that the number of mechanistically distinct drug classes (in contrast to drugs that have the same

mechanism of action but different side effect profiles and chemical formulation) available to treat these disorders had remained essentially stagnant since the 1950s. This appeared to be a problem of translational success specific to psychiatry, because the number of drug classes used to treat hypertension, which was used for comparison as a complex frequent disorder from internal medicine, was similar in the 1950s but rose by a factor of 10 in the following years. As a consequence, the therapeutic arsenal for these psychiatric disorders lagged behind that for hypertension by almost an order of magnitude. Although investments into research and development have increased considerably throughout the industry and funding for life sciences research in academia has also grown precipitously in many countries, the output of novel substances (the “cures”) has not kept up. It therefore seems reasonable to ask whether there are obstacles to translation and innovation in psychiatry. If the answer is yes, what can be done to overcome them?

Specific challenges of translation in psychiatry

Unknown pathophysiology

A fundamental problem specific to psychiatry is immediately visible: Psychiatry lags behind almost any other branch of medicine in the understanding of the pathophysiology of the illnesses it is called upon to treat. One reason for this is, of course, that the organ in which mental illness plays out is the human brain, whose complexity far exceeds that of most other biological phenomena. However, enormous strides have been made in understanding brain function on the molecular, cellular, and systems levels, which again calls into question why these advances in neuroscience have been so slow in translating into treatment (Insel and Gogtay, 2014; Insel, 2014). One considerable problem here may be the nature of psychiatric disease entities themselves. An illness such as depression or schizophrenia is currently defined by an account of symptoms such as depressed mood, sleep disturbance, or hallucinations, which are elicited from the patient in clinical interview or observed, combined with certain criteria regarding the course of the illness (e.g., specifying a minimum duration of symptoms), impairment of function, and exclusion criteria (e.g., requiring the exclusion of certain somatic illnesses that may present similarly). These diagnostic criteria are manualized and applied internationally, leading to a high degree of reliability that is necessary to, for example, compare incidence rates or conduct a clinical study multinationally. Although they are reliable, psychiatric disease entities such as depression or schizophrenia nevertheless have no biological parameters that establish these diagnoses; for example, there is no

laboratory test for schizophrenia or bipolar disorder (Kapur et al., 2012). This means that the biological validity of these phenotypes is likely to be low, in the sense that it is very likely that several as yet incompletely characterized biological processes could underlie the clinical entity that we now call depression. Seen this way, psychiatric disorders are closer on a categorical level to dyspnea or blindness in the sense that these are also direct clinical observables that are biologically strongly heterogeneous and not on the level of, for example, left ventricular failure or macular degeneration, disorders that can cause these clinical conditions but can be biologically verified or excluded. This should not be construed to say that psychiatric disease entities are arbitrary or even somehow not "real." Not only do they cause enormous suffering, as detailed previously, they also exhibit very high heritability, showing a clear biological foundation that, however, we still have to successfully parse.

This state of affairs creates considerable problems for translation. Modern translational medicine starts from an account of the molecular pathophysiology of the illness and uses it, through the techniques discussed in this text, to discover and validate new treatments. This necessitates the identification of drug targets and the screening of candidate substances *in vivo* and *in vitro*, including in animal models, before they can proceed to clinical studies. To the considerable degree that the information about molecular pathophysiology is lacking in psychiatry, this approach will be difficult.

A second problem—one that is almost specific to and definitely characteristic of psychiatry—is that many psychiatric disorders display features that are specific to humans. For example, schizophrenia and bipolar disorder often have an impact on aspects of formal thought and language. Although we believe that we can identify and create states of fear and drug dependence in animal models (Stewart and Kalueff, 2014), this becomes less obvious when we turn to depression and seems intractable for a disease such as schizophrenia. For example, what would be the mouse model for hallucinations?

Stigma and the second translation

Even if a novel treatment modality becomes available, it needs to be accepted by patients and physicians and implemented into routine care. This second translation also faces specific problems in psychiatry, as most psychiatric disorders carry a degree of stigma and public discrimination unlike that of most somatic disorders. Schizophrenia is a notable example of such a stigmatized condition (Mestdagh and Hansen, 2014). This reduces not only the quality of life but also the likelihood that a patient who is given this diagnosis will seek and maintain treatment, leading to worse outcomes, especially in

disorders that are chronic and may require maintenance therapy that carries relevant side effects that necessitate a considerable degree of patient adherence.

Recent data clearly show that service delivery in psychiatry is deficient. The Schizophrenia Patient Outcomes Research Team study demonstrated that evidence-based treatments are severely underused (Lehman et al., 2004). A metaanalysis of psychoeducation showed that although medium effect sizes in preventing relapse can be obtained, at least in the first 12 months (Lincoln et al., 2007), only a minority of patients receive this treatment. An even starker picture is painted by a 10-year follow-up study of depression in Japan (Furukawa et al., 2008), in which the majority of patients received inadequate medical treatment and not a single participant received psychotherapy, despite the fact that clearly cost-effective treatment modalities exist.

New biomarkers for translation in psychiatry

It has already been stated that the search for new treatments must start from an understanding of the molecular pathophysiology of the disorder. That is less obvious than it seems. For the past five decades the biological study of mental illness has been dominated by the study of the action of the available drugs. This research has uncovered many fundamental and important aspects of these disorders, such as the relevance of the dopamine (DA) system, a discovery that was honored by Nobel prizes awarded to Paul Greengard and Arvid Carlsson. However, two main problems with this approach exist for translation. First, understanding how drugs work is helpful only insofar as these drugs target the core pathophysiology of the disorders. For example, understanding how insulin works is certainly useful in understanding aspects of diabetes, but it is unlikely to lead to an understanding of the autoimmune basis of type I diabetes. Second, and more important to the current context, using animal and human models based on currently available drugs to screen candidate substances is essentially a recipe for developing "me-too" compounds. For example, behavioral tests based on a dopaminergic blockade, such as catalepsy, are currently used in the screening process for antipsychotic substances, inducing a bias toward drugs with this mechanism, which they then share with currently available antipsychotics. To move beyond this process, translational research in psychiatry needs a push toward target identification through pathophysiology. What biomarkers can be derived here?

A first point of departure is in genetics. The prior waves of genome-wide association studies have identified numerous frequent genetic variants associated with both

schizophrenia and bipolar disorder, and similar findings are starting to emerge for other diseases, such as autism (Sullivan et al., 2012; Grove et al., 2019). Understanding the mechanism of action of these genetic variants will have a high potential to identify new treatment targets. Second, a novel genome-wide association study (GWAS) has resulted in the identification of rare copy number variants under negative selection that increase the risk of psychiatric disorders by an order of magnitude and affect: for example, in the case of chromosome 15q11.2 deletions, brain structure has a pattern consistent with that observed in first-episode psychosis (Stefansson et al., 2014). Here again, dissection of the contribution of individual genes in these microdeleted regions is likely to considerably advance our understanding of the molecular architecture of psychiatric disorders, especially if combined with “omics” approaches. Because even if the gene product of a given risk gene variant is not by itself druggable, proteomics can be used to identify interaction partners that are. This gene-driven proteomic mining approach is likely to gain considerable traction when the interactomes of several risk genes are considered jointly, because interaction partners shared between risk genes can be expected to have a higher likelihood of being associated with the disorder. First examples of this approach are appearing in the literature. Identification of druggable targets, in turn, can be a point of departure to set in motion the machinery of high-throughput drug discovery that is described elsewhere in this book and that has been instrumental to mechanistically developing new drugs in the somatic medical disciplines.

Finally, neuroimaging enables an approach that is specific to psychiatry. The ability to measure details of the structure and function of the living human brain has been instrumental in advancing our knowledge of the systems that are involved in the pathophysiology of these diseases (Meyer-Lindenberg, 2010). Understanding these neural systems, which, as we will see, are distributed networks processing information connecting areas of prefrontal cortex (PFC) with regions of the limbic system and striatum and midbrain (MB), can aid in drug discovery in three ways. First, by understanding the neural systems implicated in psychiatric disorders, a new generation of veridical animal models can be created. For example, if we find that interactions between the hippocampal formation and the PFC are important for schizophrenia, mimicking this situation in a rodent is a more tractable problem than taking the point of departure from human-specific symptoms such as thought disorder. Second, the explosion of information available about the molecular architecture of the rodent brain and the human brain makes the construction of priors or even the selection of specific molecular targets for drug discovery possible in some instances. Third, an understanding of the neural systems involved in a

given disorder may enable a new generation of go-or-no-go decisions in early drug development by allowing an early statement about whether a novel compound impacts a relevant neural system by using neuroimaging in healthy participants or in patients (Insel and Gogtay, 2014). As we will see, all of these approaches gain considerable additional power from the ability of neuroimaging to identify neural systems that are affected by genetic variation linked to psychiatric disorders. This so-called imaging genetics approach joins genetic and neural systems information to a powerful tool whose capabilities for drug discovery, response prediction, and individualized medicine is starting to become tested. Given the pivotal and specific status of imaging biomarkers in psychiatry, the following discussion provides an overview of the developing field of imaging biomarkers, focusing on the paradigmatic disorder schizophrenia.

Imaging biomarkers in schizophrenia

In the last 20 years, numerous cognitive, functional, morphological, and metabolic anomalies of the brain have been described in schizophrenia patients, suggesting an overall heterogeneous disease entity rather than a circumscribed pathology. The reflection of this biological heterogeneity in interindividual differences of brain structure has more recently been assessed and confirmed through advanced computational modeling (Wolfers et al., 2018). At the same time, major progress has been made with respect to the delineation of a number of core schizophrenia phenomena, especially disturbances of dopaminergic neurotransmission, frontal lobe efficiency, and neural plasticity. This section reviews the current scientific knowledge on magnetic resonance imaging (MRI) biomarkers in schizophrenia. In doing so, it attempts to give special consideration to the neurocognitive domains that are most critically affected by the disorder while examining advances in the visualization of antipsychotic medication effects and the impact of schizophrenia susceptibility genes on the neural system level. Owing to the sheer volume of published results, this overview does not claim to be all-inclusive; rather, it focuses on selected areas of this vital and still-expanding research field.

Structural brain biomarkers

The original pathogenetic theory of Kraepelin postulates that schizophrenia is a progressive neurodegenerative disease state with an atypically early age of onset (Kraepelin, 1919). Over the last several decades this historic assumption and the repeated finding of ventricular enlargement in affected patient populations has stimulated a large number of postmortem studies attempting to shed light on the question of whether a primarily degenerative

process is involved in the development of the disease (Henn and Braus, 1999; Nasrallah, 1982; Stevens, 1982). In the last several decades these studies evidenced a variety of subtle histopathological changes in the brains of schizophrenia patients, especially aberrantly located neurons in the entorhinal cortex (Arnold et al., 1997; Jakob and Beckmann, 1986), smaller perikarya of cortical pyramidal neurons (Benes et al., 1991a,b; Rajkowska et al., 1998), and a reduction in dendritic arborization (Garey et al., 1998; Glantz and Lewis, 2000). These data were complemented by evidence suggesting alterations in volume, density, or total number of neurons in several subcortical structures, especially the basal ganglia, thalamus, hippocampus, and amygdala (Kreczmanski et al., 2007; Walker et al., 2002; Zaidel et al., 1997). However, major neuropathological hallmarks of a neuronal degenerative process were lacking, especially the proliferation of astrocytes and microglia that is typically seen in the context of a neural degenerative process (e.g., reactive gliosis) (Benes, 1999; Longson et al., 1996). This observation led to the formulation of alternative pathogenetic disease models that have postulated a (nonprogressive) prenatal disturbance in neurodevelopment as the main pathogenetic mechanism. One influential hypothesis assumes that schizophrenia emerges from intrauterine disturbances of temporolimbic-prefrontal network formation, resulting in the manifestation of overt clinical symptoms in early adulthood (Benes, 1993; Lewis and Levitt, 2002; Weinberger, 1987). The concept is supported by data showing that candidate susceptibility genes of schizophrenia (Brandon et al., 2004; Sei et al., 2007) and known epigenetic risk factors (Meyer et al., 2007) interfere with neuronal migration processes during central nervous system development.

Since the middle of the 1980s, the availability of MRI has allowed for the noninvasive examination of structural brain biomarkers in schizophrenia patients (see Wright et al., 2000 and Honea et al., 2005 for a comprehensive review). Earlier studies in the field demonstrated global abnormalities in terms of increased ventricular size (Johnstone et al., 1976) and smaller mean cerebral volume (Gur et al., 1999; Ward et al., 1996). Subsequent regional analyses were performed by manually delineating a priori defined regions of interest (ROI) on MRI scans, an approach that yielded one of the most consistent findings in schizophrenia research, a bilateral decrease in hippocampal gray matter (GM) volume (Henn and Braus, 1999). Available metaanalyses suggest that the mean hippocampal GM volume in schizophrenia patients is decreased to 95% of the volume of normal controls (Wright et al., 2000). In contrast, other subcortical structures, such as the basal ganglia, have repeatedly been reported to show a volumetric increase in patient populations. However, this particular observation is most likely explained by the effects of long-term exposure to

neuroleptic agents, as this finding has not been observed in antipsychotic-naïve patients (Chua et al., 2007; Keshavan et al., 1998) and has proven to be reversed when the medication regimen is switched from conventional neuroleptics to atypical antipsychotic substances (e.g., olanzapine) (Lang et al., 2004; Scheepers et al., 2001). A recent metaanalysis of 317 studies investigating brain volume alterations in schizophrenia showed decreases in intracranial and total brain volume as well as GM structures in medicated patients, whereas reductions in the caudate nucleus and thalamus were more pronounced in antipsychotic-naïve patients (Hajjma et al., 2013). This study has shown that longer duration of illness and higher dose of antipsychotic medication at time of scanning were associated with stronger GM volume reductions. This evidence is consistent with longitudinal brain-volumetric change in schizophrenia, which is supported by metaanalyses of region-of-interest studies that have indicated that the disorder is associated with progressive structural brain abnormalities affecting both gray and white matter and ranging from 0.07% to 0.59% in annualized percentage change compared to control subjects (Olabi et al., 2011; Vita et al., 2012). Metaanalysis has also shown that such progressive GM changes are regionally specific, affecting especially the left hemisphere and the superior temporal structures, and are particularly active in the first stages of schizophrenia (Vita et al., 2012). It is interesting to note that relapse duration (but not number of relapses) has been found to be associated with brain volume decreases in schizophrenia, highlighting the importance of relapse prevention and early intervention if relapse occurs as well as the potential utility of MRI-derived biomarkers for such clinical applications (Andreasen et al., 2013). In the last decades the implementation of new automated processing techniques has allowed for the large-scale analysis of structural MRI datasets. Voxel-based morphometry (VBM) is a fully automated method that allows for the unbiased analysis of the structural differences of the whole brain on a voxel-by-voxel basis (Ashburner and Friston, 2000). This method has been shown to be sensitive to subtle regional changes in tissue volume and concentration that are inaccessible to standard imaging techniques (Krams et al., 1999; Maguire et al., 2000; Valfre et al., 2008). This automated approach is superior to conventional ROI analyses in terms of regional sensitivity and reliability of structural findings and is especially helpful in situations in which the pathology does not reflect traditional neuroanatomical boundaries. Further methodological advances, such as the development of fully automated whole-brain segmentation methods, have also facilitated the volumetric analysis of subcortical structures in large cohorts (Meda et al., 2008). One of our own large-scale, automated MRI segmentation studies (Goldman et al., 2008), which was conducted in

221 healthy controls, 169 patients with schizophrenia, and 183 unaffected siblings, demonstrated a bilateral decrease in hippocampal and cortical GM volume in schizophrenia patients compared to the healthy controls. Moreover, evidence of the heritability of cortical and hippocampal volume reductions were derived.

A thorough metaanalysis of VBM studies identified GM reductions in the bilateral superior temporal gyrus (STG) (left: 57% of studies, right: 50% of studies), left medial temporal lobe (69% of studies), and left medial and inferior frontal gyrus (50% of studies) as the most consistent regional findings in schizophrenia (Honea et al., 2005). Other studies have shown that some of these morphological abnormalities are already observable at disease onset and are therefore unlikely to be solely attributable to the effects of illness chronicity or medication (Gur et al., 1998; Keshavan et al., 1998; Molina et al., 2006; Szeszko et al., 2003). Similarly, metaanalysis has shown that frontotemporal structural alterations determined through VBM are already present in presymptomatic individuals who are at high risk of developing schizophrenia (Chan et al., 2011). Longitudinal studies performed in first-episode (Cahn et al., 2002; DeLisi et al., 1997; Gur et al., 1998; Lieberman et al., 2001), chronic (Davis et al., 1998; Mathalon et al., 2001), and childhood-onset (Rapoport et al., 1999) patients suggest a progressive development of GM volumetric reduction over the course of the illness. Longitudinal VBM analysis of first-episode antipsychotic-naïve schizophrenia patients followed-up for 4 years also found progressive GM changes, some of which were related to functional outcome (Mané et al., 2009). In view of the postmortem data reviewed previously, the neurobiological significance of the observed magnetic resonance signal changes remains to be elucidated (Weinberger and McClure, 2002). Although most likely of neurodevelopmental origin, the implications of the evidenced volumetric brain changes are still a matter of controversy, especially regarding the question of whether the evidenced alterations in the GM and cerebrospinal fluid compartments reflect an actual loss of neurons or, instead, reductions in neuropil and neuronal size (Harrison, 1999). Importantly also, most patient samples vary not only by disease status from healthy individuals, but also by a variety of other confounds that are linked to brain volume changes, such as exposure to alcohol (Taki et al., 2006), tobacco (Brody et al., 2004), social environmental risk factors (Gianaros et al., 2007), and medication. The last aspect is of particular importance, as both acute (Tost et al., 2010) and chronic exposure to antipsychotics (Ho et al., 2011) appear to have profound effects on structural neuroimaging measures, complicating the interpretation of existing data.

While many of these questions remain unresolved, the increasing availability of high-quality, large-scale data on

brain structure also provides the basis for application of advanced computational tools to evaluate the joint relationship of different brain regions with illness. Such integrative analyses are fundamental for gaining deeper insight into illness biology but can also provide the basis for novel clinical tools for diagnosis and prediction. This is particularly relevant in psychiatry, in which alterations in individual regions show effect sizes that are too small to be clinically useful but that can be integrated in more accurate algorithms by using machine learning. Several studies have showed that such algorithms can discriminate accurately between schizophrenia patients and controls (Nieuwenhuis et al., 2012; Schnack et al., 2014; Takayanagi et al., 2011). In another study, performed as part of the FP7 EU project IMAGEMEND (“IMAgEing GEetics for MENTal Disorders”) (Frangou et al., 2016), machine learning was used to identify and validate a signature of brain-structural differences in 2668 individuals with schizophrenia, bipolar disorder, or attention-deficit hyperactivity disorder and healthy controls (Schwarz et al., 2019). This confirmed that GM changes were widely distributed throughout the brains of patients with schizophrenia and highlighted the importance of such global GM changes compared to alterations in individual structures. While this study demonstrated limitations regarding the specificity of the GM signature with respect to bipolar disorder, other studies have provided evidence that brain-structural signatures specific for schizophrenia can be derived from brain-structural data (Schnack et al., 2014). Such machine learning-derived signatures are especially interesting in the context of high-risk prediction, in which it has been shown that frontal cortex volume reductions can predict conversion to full-blown psychosis in ultra-high-risk individuals (Dazzan et al., 2012; McIntosh et al., 2011).

Functional imaging markers in schizophrenia

Since the early 1990s, functional brain anomalies in mental disease states have been examined predominantly by means of one neuroimaging technique: functional magnetic resonance imaging (fMRI). The popularity of the method can be explained by numerous favorable attributes, especially the broad availability of clinical scanners, the noninvasiveness of the technique, and the broad spectrum of complementary MRI applications that can offer additional insights into the structure and biochemistry of the living brain. Concurrent with the growing popularity of this technique, the development of fMRI experiments has been refined from simple paradigms with blockwise stimulation to rapid, event-related task designs. On the data analysis side, sophisticated methods for connectivity analyses of neural network interactions have been developed (Meyer-Lindenberg et al., 2005a; Stein et al., 2007). Advancements in processing technology and speed also paved the way for

real-time fMRI applications that allow providing patients with neurofeedback and the possibility of modulating their own functional brain activity, a promising approach to a new treatment modality for patients with mental illness (Weiskopf, 2012). In the meantime the success of fMRI has given rise to an enormous amount of published fMRI studies in schizophrenia research. The following section reviews major findings in the neurocognitive domains that are most critically affected by the disorder.

Auditory and language processing

Abnormalities of the STG and associated language areas of the temporal and frontal lobe have emerged as some of the most prominent biomarkers in schizophrenia research. In the last two decades, structural and functional deficits of these regions have been extensively examined in the context of auditory hallucinations, a cardinal positive symptom of schizophrenia that has been conceptualized as dysfunctional processing of silent inner articulations (David and Cutting, 1994). Early milestone work in schizophrenia research demonstrated the spontaneous activity of speech areas during hallucinatory experiences, a fact that explains why these internal voices are accepted as real, despite arising in the absence of any external sensory stimulation (Dierks et al., 1999; Ffytche et al., 1998; McGuire et al., 1993; Silbersweig et al., 1995). Subsequently, several fMRI studies have provided evidence of a functional deficit that affects multiple levels of auditory perception and language processing. On a lower level, a diminished response of the primary auditory cortex to external speech has been observed during hallucinatory experiences, a fact that is best explained by the competition of physiological and pathological processes for limited neural processing resources (David et al., 1996; Woodruff et al., 1997). It should be noted that the extent of STG dysfunction during speech processing seems to predict the severity of the patient's thought disorder, a clinical symptom that manifests as irregularities in speech (Weinstein et al., 2006). On the neural network level, an impaired functional coupling of the STG and anterior cingulate gyrus (ACG) seems to be at the core of the misattribution of the patient's own voice to alien voices that frequently characterizes patients with auditory hallucinations (Mechelli et al., 2007).

In terms of brain structure, scientific evidence points to an anatomical correlate of the acoustic hallucinations in schizophrenia. Early morphometric studies repeatedly reported a decrease in the physiological leftward asymmetry of the planum temporale, a higher-order auditory processing area that overlaps with Wernicke's area (Kwon et al., 1999; Shapleske et al., 1999; Sumich et al., 2005). Voxel-based morphometric studies have confirmed a significant GM decrease in the STG that predicted the

severity of experienced auditory hallucinations (Aguilar et al., 2008) as well as several other symptom dimensions that have been quantified using the Positive and Negative Syndrome Scale (Lui et al., 2009). Also, GM reductions of the STG have been shown to be progressive during the transition period from an ultra-high-risk state to first-episode schizophrenia with acute psychosis (Takahashi et al., 2009), an effect that could not be observed in longitudinal assessment of chronic schizophrenia patients (Yoshida et al., 2009). Temporal volumetric alterations have also been identified by a metaanalysis of antipsychotic-naïve VBM studies as possibly underlying the clinical onset of psychotic symptoms (Fusar-Poli et al., 2012). Moreover, folding abnormalities of the STG and Broca's area, as well as microstructural changes of the main connecting the white matter tract to the frontal lobe (arcuate fasciculus), seem to promote the emergence of hallucinatory symptoms (Catani et al., 2011; Steinmann et al., 2014; Cachia et al., 2008; Gaser et al., 2004; Hubl et al., 2004; Neckelmann et al., 2006).

Motor functioning

Disturbances of psychomotor functions are well-known features of schizophrenia and range from subtle deficits such as neurological soft signs to extensive behavioral abnormalities such as stereotypia and catatonia (Schroeder et al., 1991; Vrtunski et al., 1986). In the early years of schizophrenia imaging research, the examination of motor dysfunctions was very popular, usually involving a block-design approach in which simple repetitive motor activities alternated with rest conditions (e.g., finger tapping, pronation-supination). In addition to other findings, hypoactivations of the primary and higher-order supplementary motor and premotor cortices have been reported in schizophrenia patients (Braus et al., 1999; Buckley et al., 1997; Mattay et al., 1997; Schröder et al., 1999; Schröder et al., 1995; Wenz et al., 1994). Moreover, within the highly lateralized motor system, a physiologically abnormal symmetry (i.e., reduced laterality) of recruited neural resources has emerged as a cardinal fMRI biomarker of motor system dysfunctions in schizophrenia (Bertolino et al., 2004a; Mattay et al., 1997; Rogowska et al., 2004). On the neural network level, a functional deficiency of transcallosal glutameric projections has been suggested (Mattay et al., 1997); this assumption is in line with current neurodevelopmental disease models and has been supported by corresponding electrophysiological findings (Hoppner et al., 2001).

In recent years, fMRI studies in the motor domain have been scarce. The pronounced impact of antipsychotic agents on the examined circuitry and the lack of studies in unmedicated patient samples offer two potential explanations for this (Tost et al., 2006). There is evidence suggesting that both schizophrenia patients and their

unaffected relatives are characterized by a lack of striatal activation increase during movement anticipation (Vink et al., 2006). Although this finding is suggestive of an endophenotypic trait marker, it still awaits replication. On the structural level, the functional findings are complemented by reports of GM volume decreases and impaired structural integrity of the connecting white matter tracts of the higher-order processing areas of the motor system (Hirjak et al., 2014; Walther et al., 2011; Exner et al., 2006; Goghari et al., 2005; Matsui et al., 2002).

Working memory

Convergent evidence from several neuroscience disciplines [(epi)genetic, molecular, cellular, physiological, and neuroimaging] suggests that schizophrenia is a complex disorder of brain development that promotes downstream disturbances in dopaminergic neurotransmission and subsequent impairments in prefrontal cortical efficiency. As a result, schizophrenia patients suffer from a variety of higher-order cognitive functions that are known to depend on the integrity of the dorsolateral and medial prefrontal lobe, especially cognitive flexibility, selective attention, response inhibition, and working memory (WM). Previous experimental work in animals and humans has shown that mesocortical DA, especially the stimulation of the dopamine D₁ receptor subtype, plays a crucial role in the modulation of WM (dys)functions (Fuster, 1990; Goldman-Rakic, 1995; Williams and Goldman-Rakic, 1995). According to this evidence, an inverted U-shaped relationship exists between the amount of D₁ receptor stimulation, WM-related activation of PFC neurons, and prefrontal cognitive efficacy (Callicott et al., 1999; Williams and Goldman-Rakic, 1995). Although balanced D₁ dopaminergic tone seems to augment the robustness of PFC network representations by making them less susceptible to background neural “noise,” states of excessive or lacking dopaminergic drive seem to weaken the system’s robustness to interfering stimuli and, as a result, promote the development of cognitive deficits and psychotic symptoms (Durstewitz et al., 2000; Mattay et al., 2003).

In the past decade the neurobiological basis of WM dysfunctions in schizophrenia has been extensively examined with fMRI. On a general level, this cognitive asset can be divided into maintenance and manipulation subprocesses. Classical WM paradigms challenge the active storage and online manipulation of information, often assessed in the form of so-called *n*-back tasks, in which patients are asked to monitor an ongoing sequence of stimulus presentations and respond to items that match the one presented *n* stimuli previously. The majority of studies reported disorder-related dysfunctions of the dorsolateral prefrontal cortex (DLPFC) (Brodmann areas 46

and 9) as well as anomalies in the functional coupling of this area to the medial temporal lobe (Meyer-Lindenberg et al., 2005a; Meyer-Lindenberg et al., 2001). However, the precise pathophysiological background of these findings is still a matter of debate, as diverse anomalies in the form of hypofunctions (Andreasen et al., 1997; Barch et al., 2001; Paulman et al., 1990; Schneider et al., 2007; Weinberger et al., 1986), increased activations (Callicott et al., 2000; Potkin et al., 2009a,b), and combined hyperactive and hypoactive states (Callicott et al., 2003; Karlsgodt et al., 2007; Dae et al., 2009; Lee et al., 2008) have been reported. This inconsistency has raised questions about the traditional theory of pure functional hypofrontality in schizophrenia and has stimulated the formulation of more complex models of PFC dysfunction (Callicott et al., 2003; Callicott et al., 2000; Manoach, 2003; Potkin et al., 2009a,b). In another study reporting combined hyperactive and hypoactive states, increased and decreased DLPFC and parietal activity was associated with higher and lower performance during the fMRI task, respectively, and this has been suggested to support the theory that a combined hyperactivation and hypoactivation may reflect a continuum of behavioral performance in schizophrenia (Karlsgodt et al., 2007). fMRI work in first-episode schizophrenia patients confirmed a preferential impairment of the manipulation component of WM and reported a disproportional engagement of the ventrolateral PFC (VLPFC, Brodmann areas 44, 45, and 47) during task performance (Tan et al., 2005). This finding has been interpreted as a deficiency in the functional specialization and hierarchical organization of the PFC. The deficit is thought to manifest itself in terms of reduced efficiency in DLPFC functioning, which in turn triggers the compensatory recruitment of hierarchically inferior and less specialized areas in the VLPFC (Tan et al., 2006). There is evidence suggesting a relationship between WM capacity at the begin of the illness and the extent of the subsequent prefrontal cortical thinning during course of the illness (Gutiérrez-Galve et al., 2014). Furthermore, WM-related activation and connectivity features have been found in unaffected relatives, suggesting their usefulness as intermediate phenotypes for genetic investigations.

It is notable that interindividual differences in WM can be predicted by DNA methylation differences in DA receptor D4, pointing toward a modulating effect of dopaminergic methylation on cognitive control. Similarly, methylation of the membrane-bound catechol-O-methyltransferase (COMT) promotor is associated with DLPFC activity during WM and methylation of the COMT Val(158) allele with WM performance. These findings are intriguing, since exposure to environmental risk factors strongly influences susceptibility to schizophrenia, and this effect may be mediated by epigenetic modifications, such as changes in DNA methylation.

Accordingly, numerous changes in DNA methylation have been identified in schizophrenia (Aberg et al., 2014; Hannon et al., 2016; Jaffe et al., 2016; Pidsley et al., 2014), but, similar to illness-associated common genetic variants, such changes show comparatively limited effect sizes. To address this, a recent study employed a biologically informed machine learning approach to integrate such changes and identified a blood DNA-methylation signature that could reproducibly differentiate patients with schizophrenia from controls (Chen et al., 2020). When applied to data from the dorsolateral PFC, the signature also significantly differentiated sample donors with schizophrenia from controls, showing that the identified methylation differences were represented in peripheral as well as central nervous system samples. Notably, predictions from the methylation signature were reproducibly associated with brain-functional connectivity between the dorsolateral PFC and the hippocampus in two independent cohorts of healthy participants during two different WM tasks (*n*-back and Sternberg). These associations were not mediated by polygenic risk for schizophrenia, and the methylation signatures showed no increases in unaffected first-degree relatives of patients or in patients with relevant differential diagnoses. These results suggest that environmental risk exposure manifests in methylation differences that are associated with schizophrenia-relevant differences in brain function during WM.

Selective attention

On a general level, selective attention describes the mental capacity to maintain a behavioral or cognitive set in the face of distracting or competing stimuli. The term covers several cognitive subprocesses, especially top-down sensitivity control, competitive selection, and automatic bottom-up filtering for salient stimuli (Knudsen, 2007). Since Bleuler's first clinical descriptions of schizophrenia (Bleuler, 1950), attentional deficits have been considered core symptoms of the condition that eventually leads to overt psychopathological symptoms such as thought disorder, incoherence of speech, and disorganized behavior. Previous neuroimaging research in psychiatry has adopted different experimental techniques to examine the neurobiological correlates of these deficits, the most popular being the so-called continuous performance test (CPT). The term describes a heterogeneous array of non-standardized paradigms in which participants are meant to selectively respond to target presentations while inhibiting responses to nontargets. Most CPTs additionally challenge other cognitive subprocesses such as visual attention (e.g., degraded CPTs), cognitive interference monitoring, or WM (e.g., contingent CPTs like CPT-AX, CPT-IP, and CPT-double-T). The heterogeneity of CPT (and other) paradigms must be taken into account in interpreting

available fMRI data on attentional dysfunctions in schizophrenia.

Many studies in the field have used variations of a contingent CPT to examine the neural correlates of attentional dysfunctions in schizophrenia. Most of these studies reported functional anomalies of the DLPFC (Lesh et al., 2013; MacDonald and Carter, 2003; Perlstein et al., 2003; Volz et al., 1999) and VLPFC (Eyler et al., 2004) in schizophrenia patients and their unaffected first-grade relatives (Diwadkar et al., 2011), findings that are most likely related to the moderate WM demands of this particular task type. Similar findings have been observed in first-episode neuroleptic-naïve patients during CPT task performance, suggesting that this functional biomarker is at the core of the disorder and not merely a medication-induced phenomenon (Barch et al., 2001). In line with this assumption, Aguilar et al. (2008) observed task-related hyperactivation of DLPFC in unaffected siblings of schizophrenia patients, suggesting that this deficit resembles an intermediate phenotype of the genetic susceptibility to the illness. In contrast, most of the studies that used paradigms challenging interference monitoring observed dysfunctions of the ACG. Reported anomalies in schizophrenia include regional ACG hypoperfusion (Carter et al., 1997), disturbed interregional connectivity to the PFC (Eyler et al., 2004; Honey et al., 2005), and the absence or dislocation of activation foci within the ACG (Heckers et al., 2004). Evidence from structural MRI studies suggests that the observed functional and behavioral deficits relate to a morphological and microstructural impairment (Nazeri et al., 2013), in particular the ACG and its main white matter fiber tract, the cingulum bundle (Spalletta et al., 2014; Artiges et al., 2006; Kubicki et al., 2003; Salgado-Pineda et al., 2004; Sun et al., 2003; Yücel et al., 2002).

Imaging of genetic susceptibility factors

Schizophrenia is a highly heritable mental disorder with a complex genetic architecture. Current evidence suggests that multiple genetic risk variants, each accounting for only a small increment of the risk of the development of the disorder, interact both with one another and with the environment. On the neural systems level, this interaction interferes with the functional properties of multiple brain circuits that, in turn, shape a variety of different cognitive, emotional, and behavioral functions and dysfunctions (see Fig. 16.1 for an illustration of these concepts) (Meyer-Lindenberg and Weinberger, 2006).

Multiple genetic risk variants, through interactions with one another and with the environment, affect multiple neural systems linked to several neuropsychological and behavioral domains that are impaired in differing proportions in psychiatric diseases. As examples, the

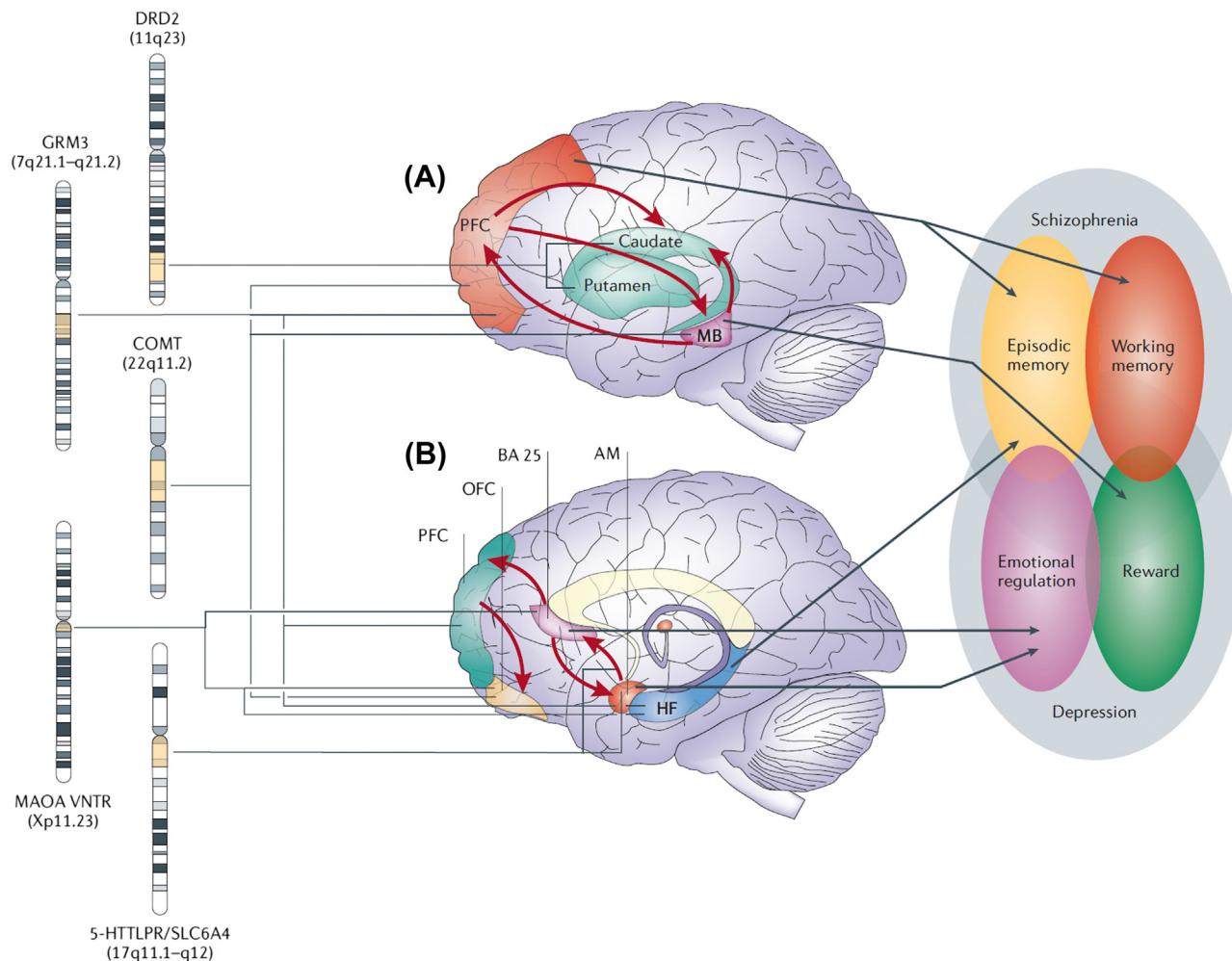


FIGURE 16.1 Depiction of the complex path from genes to behavioral and disease phenotype. From Meyer-Lindenberg, A., Weinberger, D.R., 2006. Intermediate phenotypes and genetic mechanisms of psychiatric disorders. *Nat. Rev. Neurosci.* 7, 818–827., reprinted by permission from Nature Publishing Group, © 2006.

following genetic variants are depicted (chromosomal variation in parentheses): GRM3 single nucleotide polymorphism 4 (7q21.1–q21.2) (Egan et al., 2004), DA receptor D2 (DRD2) Taq 1a (11q23) (Cohen et al., 2005), COMT Val66Met (22q11.2) (Egan et al., 2001; Meyer-Lindenberg et al., 2006b), serotonin transporter length polymorphism (5-HTTLPR/SLC6A4) (17q11.1–q12) (Hariri et al., 2002; Pezawas et al., 2005), and monoamine oxidase A variable number tandem repeat (MAOA VNTR) (Xp11.23) (Meyer-Lindenberg et al., 2006a). These variants are shown to affect a circuit that links the PFC with the MB and striatum (caudate and putamen); this circuit is (1) relevant to schizophrenia, and a circuit that connects the amygdala (AM) with regulatory cortical and limbic areas, and (2) implicated in depression and anxiety. These circuits, in turn, are shown to mediate risk for schizophrenia, depression, and various neuropsychological functions: Brodmann's area 25 (BA 25), hippocampal formation (HF), and orbitofrontal cortex (OFC).

Clearly, there is no one-to-one mapping between risk gene variants and neural system mechanisms or between neural mechanisms and psychopathology, a fact that renders the identification of valid biomarkers on the basis of genetics alone difficult. Imaging genetics, the characterization of susceptibility gene mechanisms on the neural systems level using multimodal neuroimaging, has proven to be a successful research strategy for overcoming this obstacle. Many gene variants associated with an enhanced risk of the development of schizophrenia are frequent in healthy individuals. The imaging genetics approach assumes that the penetrance of gene effects is greater at the neurobiological level than at the level of complex behavior and that these gene effects are traceable at the neural systems level in carriers of risk alleles even when no overt clinical signs of the disease phenotype are expressed. In recent years the attempt to genetically dissect schizophrenia with neuroimaging methods has led to the characterization of several intermediate phenotypes

(i.e., core pathophysiological characteristics observable at the level of the neural substrate that bridge the gap between genetic variation and psychiatric symptoms).

The strongest evidence for the efficacy of this approach arises from intermediate phenotype studies on COMT, a major enzyme for the degradation of catecholamines in the central nervous system. The COMT gene is located in a chromosomal region that has been implicated in schizophrenia linkage studies (Owen et al., 2004; Talkowski et al., 2008); in the 22q11 deletion syndrome (22q11DS) (Karayiorgou and Gogos, 2004), which is a hemideletion syndrome with a 30-fold increased risk of developing a schizophrenia-like illness (Murphy, 2002); and, more recently, in copy number variation in sporadic schizophrenia (Stark et al., 2008). Owing to the lack of DA transporters in the PFC, the regulation of extracellular DA levels in the PFC is critically dependent on COMT functioning (Lewis et al., 2001). As has been shown previously, a common val^{108/158}met substitution in the COMT gene interferes with the thermostability of the transcribed protein, leading to significantly decreased enzyme efficacy (Chen et al., 2004). Studies from our laboratory have demonstrated that the val^{108/158}met coding variant in the COMT gene affects PFC functional measures during WM performance (Egan et al., 2001), modulates subjects' performance in neuropsychological tests challenging executive functioning (Goldberg et al., 2003), and influences the cortical response to amphetamine in healthy subjects (Mattay et al., 2003). These data extend basic evidence for an inverted-U functional response curve to increasing DA signaling in the PFC, and they validate the concept of prefrontal cortical inefficiency as the key endomechanism promoting the risk of schizophrenia. According to this model, the COMT genotype places individuals at predictable points along the inverted-U-shaped curve that links prefrontal dopaminergic stimulation, neuronal activity, and PFC efficiency. Homozygotes for the val-encoding allele are thought to be positioned to the left of met allele carriers at a point of decreased PFC efficiency, whereas the met allele carriers seem to be optimally located near the peak of that curve (val/val: COMT efficacy ↑, synaptic DA ↓; met/met: COMT efficacy ↓, synaptic DA ↑). The genetic risk associated with the COMT val^{108/158}met coding variant is thought to be mediated by a reduced signal-to-noise ratio in the PFC, an idea that is supported by the finding that WM-related and WM-unrelated PFC activity are inversely related to neuroimaging markers of MB DA synthesis, which in turn is directionally dependent on the COMT val^{108/158}met genotype (Meyer-Lindenberg et al., 2005b).

Subsequent work from our laboratory (Meyer-Lindenberg et al., 2007) indicates that a frequent haplotype of the gene encoding of the DA- and cAMP-regulated phosphoprotein DARPP-32 (PPP1R1B) is associated with the risk of

schizophrenia and that it affects measures of frontostriatal structure, function, and cognition. DARPP-32 is a major target for DA-activated adenylyl cyclase and serves as an important functional switch integrating the multiple information streams that converge onto dopaminoceptive neurons in the striatum (i.e., striatal neurotransmitters, neuropeptides, and neurosteroids) (Svenningsson et al., 2004). It has been shown that DARPP-32 is a key node in the final common pathway of psychotomimetic action in the PFC and striatum (Svenningsson et al., 2003), making it an attractive candidate gene for schizophrenia. Our imaging genetics study showed a pronounced and convergent effect of genetic variation in PPP1R1B on the function and volume of the striatum and related measures of frontal-striatal connectivity. Moreover, a pronounced impact of PPP1R1B variation on a wide range of prefrontal cognitive measures was observed. These findings might suggest that PPP1R1B contributes to the risk of schizophrenia by disturbed gating (Swerdlow et al., 2001) of frontostriatal information flow. Interestingly, AKT1, another key molecule in a noncanonical signal transduction pathway for dopaminergic neurons, showed an impact on frontostriatal circuitry as well (Tan et al., 2008), establishing a convergent neural signature for postsynaptic dopaminergic neurotransmission.

In addition to associations between candidate genetic schizophrenia risk variants and brain activity, we have demonstrated that a common genetic variant in ZNF804A that has been linked to both schizophrenia and bipolar disorder through GWAS shows gene-dose-dependent associations with functional coupling (correlated activity) of the DLPFC with hippocampus, mirroring findings in patients (Meyer-Lindenberg et al., 2001), their unaffected first-grade relatives (Rasetti et al., 2011), and genetic mouse model of schizophrenia (Sigurdsson et al., 2010). These results indicated that even in the absence of changes in regional activity, imaging genetics approaches have utility in uncovering disturbed connectivity patterns as neurogenetic risk mechanisms for psychiatric illnesses (Esslinger et al., 2009). The hypothesis that ZNF804A modulates schizophrenia risk through impaired brain connectivity but not structure is consistent with recent studies showing that variation in this gene does not affect total or regional brain volumes in healthy young adults (Cousijn et al., 2012) as well as schizophrenia (Donohoe et al., 2011).

Finally, an interesting avenue for discovering genetic risk variants for mental illnesses within an imaging genetics framework is the direct use of neuroimaging readouts as quantitative traits for GWAS. On the basis of this strategy, Potkin et al., showed that by using the mean activation in the dorsolateral PFC in response to the Sternberg WM task as a quantitative trait for GWAS analysis, genetic variants with significant genotype-diagnosis interactions could be identified (Potkin et al., 2009a,b).

Characterization of antipsychotic drug effects

The standard medical treatment for schizophrenia is antipsychotic medication. First-generation antipsychotics such as haloperidol or fluphenazine (also referred to as conventional antipsychotics or major tranquilizers) belong to a class of antipsychotic drugs that were developed in the 1950s. Owing primarily to their D₂ receptor-blocking properties, this substance class is very efficient at relieving positive symptoms but also has the potential for unwanted side effects, such as extrapyramidal side effects (EPS) or tardive dyskinesia. In the recent years, available treatment options for schizophrenia patients were extended substantially by the development of second-generation, or “atypical,” antipsychotic drugs (e.g., clozapine, olanzapine). The term *atypical antipsychotics* refers to a biochemically heterogeneous group of drugs characterized by the absence of EPS and increased efficacy compared to conventional neuroleptics for the treatment of negative symptoms and cognitive deficits (see Miyamoto et al., 2005 for a comprehensive review). Previous evidence has suggested that the degree and timing of the clinical response to atypical antipsychotics is modulated by the patient’s genetic profile for DA catabolic enzymes. As was discussed earlier, the met variant of the val¹⁵⁸met COMT gene polymorphism inactivates prefrontal DA at a slower rate (Lachman et al., 1996), a mechanism that is associated with a greater improvement of negative symptoms (Bertolino et al., 2004b). Bertolino and colleagues (Bertolino et al., 2007) replicated this finding and provided additional evidence of a faster response to atypical antipsychotics in the met allele carriers. Although the precise mechanism of these effects is not yet fully understood, this finding suggests that met allele–carrying patients may have greater benefit from olanzapine treatment because of their relative excess of prefrontal cortical dopamine. On the neural systems level, several fMRI studies have provided evidence suggesting a favorable impact of atypical antipsychotics on previously “disturbed” functional brain patterns. Bertolino et al. (2004a) examined the impact of olanzapine on motor loop functioning and observed an alleviation of the sensorimotor hypoactivations in the unmedicated state. Similarly, Stephan et al. (2001) reported a restoration of functional cerebellar connectivity during motor task performance under olanzapine. A more recent fMRI study on the effects of antipsychotic treatment on motor task-related activation differences in schizophrenia reported a drug dose–dependent diminishing effect of antipsychotics on activity in cortical and subcortical motor and default mode networks (Abbott et al., 2011). Other relevant performance domains that have been associated with favorable functional effects of atypical antipsychotics include WM (Meisenzahl et al., 2006),

verbal fluency (Jones et al., 2004), and conflict detection (Snitz et al., 2005). Another interesting avenue of investigation has been the assessment of antipsychotic effects on resting state neural activity using fMRI. In this context it has been shown that 6 weeks of treatment with a second-generation antipsychotic resulted in widespread increased regional synchronous neural activity, especially in the bilateral prefrontal and parietal cortex, left superior temporal cortex, and right caudate nucleus, and that this was correlated with a reduction of clinical symptoms and a widespread attenuation in functional connectivity (Lui et al., 2010). Similar restorative effects of antipsychotics on brain function in the visual attention network were reported in a recent longitudinal study in first-episode schizophrenia patients (Keedy et al., 2014).

The differential effect of atypical versus typical antipsychotic drugs has been examined mainly in cross-sectional designs. Several studies examining fMRI task performance related to motor functioning (Braus et al., 1999), WM (Honey et al., 2005), and prepulse inhibition (Kumari et al., 2007) have suggested a superior effect of second-generation antipsychotics over classical neuroleptics on functional brain measures in schizophrenia. On the structural brain level, evidence derived with different MRI techniques supports this notion. In a multicenter, longitudinal study in 161 patients with first-episode psychosis, Lieberman et al. (2005b) observed a reduction of global brain GM volume over time in patients treated with haloperidol but not in patients treated with olanzapine or healthy control subjects. Similarly, previous MRI spectroscopy studies focusing on N-acetylaspartate and phospholipid metabolism have suggested a favorable impact of atypical antipsychotics in the PFC that is superior to the effect of traditional neuroleptics (Smesny et al., 2012; Bertolino et al., 2001; Braus et al., 2001; Ende et al., 2000; Szule et al., 2005). However, the neurobiological basis for this differential drug effect remains unknown. Possible explanations include a greater therapeutic effect of atypical antipsychotics on disease-inherent brain morphological changes as well as the possibility of a neurotoxic effect associated with the application of conventional neuroleptics.

Only a few neuroimaging studies have examined the impact of antipsychotic drugs on functional brain measures in healthy volunteers. In a double-blind crossover drug challenge, Abler et al. (2007) examined the influence of 5 mg of olanzapine on reward-related brain activations and observed a drug-related activation decrease in the ventral striatum, ACG, and inferior frontal cortex. Our own prior work in the visual (Brassen et al., 2003) and motor systems (Tost et al., 2006) on the effect of a single dose of haloperidol (5 mg/kg) confirmed that antipsychotic drugs have a primarily dampening effect on functional brain measures. During motor task

performance, for example, a significant drug-related activation decrease in the dorsal striatum and an increased lateralization of motor cortex activations was observed. In line with previous work in the field, a preferential interference of haloperidol with cognitive measures challenging mental and behavioral flexibility (related to dopamine D₂ receptor functioning) was evidenced, while WM performance (related to dopamine D₁ receptor functioning) remained unaffected (Tost et al., 2006). These results emphasize the need to control for medication effects in fMRI studies examining psychiatric populations.

Multomics and transdiagnostic biomarker discovery

As biological changes that have been identified by large-scale studies on schizophrenia and other mental disorders are widely spread across different data modalities and typically show small effect sizes, there is an increasing push toward the simultaneous analysis of such modalities to identify biomarker signatures with stronger illness association. Such multomics approaches offer the possibility of more comprehensively characterizing illness hallmarks at a biological systems level and provide insight into affected mechanisms as a basis for the development of novel interventions. At the same time, as psychiatric illnesses show a substantially shared genetic architecture (Gallagher et al., 2013) and identified biological changes regularly cross diagnostic boundaries, the necessity for disease-overarching investigations has been widely realized. The increasing availability of transdiagnostic, multomics data repositories offers numerous advantages beyond testing diagnostic specificity of biological findings. They allow the exploration of functional and hierarchical psychopathological constructs with cross-disorder relevance, such as those defined by the RDoC (Insel, 2014) and HiTOP (Kotov et al., 2017) initiatives, and facilitate the application of advanced computational tools that exploit the dependency of different clinical conditions to maximize statistical power. An interesting example for this is the joint genetic analysis of related complex traits, which improves the genetic prediction of conditions such as type 2 diabetes and schizophrenia, albeit with significant heterogeneity across cohorts (Maier et al., 2018). Another interesting example in which the dependency structure of outcome measures is utilized for improving predictive performance is the recently developed “MOSTest” algorithm (van der Meer et al., 2020). Here, the dependency between brain-structural readouts is exploited to increase the statistical power for the detection of associations with common genetic variants. This approach has been found to substantially outperform

conventional genome-wide association analysis. Notably, transdiagnostic data repositories are also fundamental for investigating the factors driving clinically relevant comorbidity. For example, the e:Med systems-medicine alliance COMMITMENT (“COMorbidity Modeling via Integrative Transfer machine-learning in MENTal illness”) utilizes advanced machine learning to stratify multimics data from patients with psychosis in order to identify the biology underlying the somatic comorbidity with cardiovascular disease or type 2 diabetes (Schwarz et al., 2020). The resulting signatures are characterized across the human lifespan in order to define risk periods that are of particular relevance for the occurrence of comorbidity and that can inform intervention approaches. An interesting aspect of the COMMITMENT project is the development of an information technology framework that allows the geographically distributed application of machine learning tools, such that the multimodal data repositories do not have to be combined in a single storage solution but can be analyzed at their source location. This simplifies the logistics of analyzing large data repositories and allows for a maximization of sample size and statistical power.

Conclusions and future directions

Among research disciplines, neuroscience has introduced the most fundamental changes in the way in which mental disease states are conceptualized and pursued today. In terms of funding, modern-day psychiatric research increasingly faces the challenge of bridging the gap between theoretical concepts and practical solutions while focusing available resources on questions that will likely lead to future therapeutic applications. Nowhere is this more obvious than in translational medicine. The slow, arduous empirical process of finding new treatments must be properly incentivized for this work to be done by the current and next generations of psychiatric neuroscientists. This will also require thinking outside of the box regarding the interactions of clinical and industrial research. Neither of the two can effectively go the entire way to a mechanistically new drug alone, and instead of focusing on ethical questions regarding payments to physician-researchers in late or promotional phases of psychiatric drugs, it is urgently necessary to create an ethically secure environment in which academia and industry can interact more closely. Two exemplary initiative in this regard is the Innovative Medicines Initiative of the European Union, which brings together European Federation of Pharmaceutical Industries and Associations pharmaceutical firms with consortia formed from academia and small and medium-sized enterprises to tackle drug discovery, and the ECNP Medicines Chest Initiative, which gives academic researchers access to compounds that are

currently not or no longer pursued by the companies that originally developed them. The practical need for this development is based not only on the tremendous emotional burden that psychiatric illness causes for the affected individuals and their families, but also on the enormous treatment expenses that mental disorders impose on our society in general, an amount that exceeds an estimated \$70 billion per year in the United States alone (Office of the Surgeon General, 1999). As we have seen, in the last decades, neuroimaging methods have provided unique insights into the core neuropathophysiological processes associated with the development and treatment of schizophrenia. As a crucial part of most multimodal research approaches, these techniques have helped to characterize the mechanisms that translate disease vulnerability from the genetic level to the molecular, cellular, and neural systems levels and to the level of overt behavioral disturbances. Within this translational framework the development and application of neuroimaging methods are expected to lead to future improvements in disorder prevention, diagnosis, and treatment.

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Chapter 17

Methodological studies

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Abstract

Early clinical trials are pivotal steps in the humanization of a drug development project. Their design has to accomplish both regulatory aspects and scientific gain regarding the drug target, human mechanism of action, and viable biomarkers; they should ultimately support proof-of-concept (PoC) as early as possible. This chapter details the basic characteristics of such trials and shows the opportunities elicited by smart designs of exploratory trials, exploratory investigational new drug filing, microdosing studies, early decision making, adaptive trial design, and the combination of regulatory and exploratory trials. All this aims at accelerating PoC as a major value inflection point of drug development.

Keywords: Exploratory clinical trial; adaptive trial design; smart clinical design; exploratory investigational new drug filing; microdosing

Conventional phase I trial methodology

Initially, new agents are given to human beings in a single dose to assess the tolerability, safety, pharmacokinetics (PK), and pharmacodynamics (PD) effects and to compare these results with those gleaned in the preclinical setting. The dose is escalated with concurrent observation of the aforementioned parameters in order to define a dose and schedule that can be recommended for phase II efficacy studies.

It is extremely important for phase I trials to be designed in such a way as to include the appropriate number and types of patients in order to minimize the risk of making inaccurate dose recommendations. Otherwise, considerable additional time and effort will need to be invested when the drug is undergoing phase II investigations to further refine dosing.

Aims

The primary aim of a first-in-human phase I trial is to define the maximum tolerated dose (MTD) and to

recommend the correct dose for further studies. Other aims are to describe the pattern of toxicity, PK profile, antitumor effect, and PD endpoints and to describe any relationship between dose and effects on toxicity.

Design

Because development of a new cancer drug is dependent on the phase I trial, it is important that the design, conduct, and analysis of phase I trials are rigorous. If phase I trials are not properly conducted—for example, if the trial includes patients who differ significantly in characteristics compared to those who will be treated in later studies or if the trial schedule is created to prioritize convenience over data—the overall time taken for drug development may increase and/or the future of the new drug may be threatened. Gemcitabine is a case in point (Eisenhauer et al., 2006, example cited with permission from Oxford University Press). In a phase I trial of gemcitabine, it was recognized early on that the schedule played an important role in the pattern of the side effects of the drug. The daily schedule, repeated five times, caused severe flu-like symptoms, so only small doses could be administered (O'Rourke et al., 1994). When doses were given in a 30-minute infusion for three out of every four weeks, more of the drug could be administered per cycle, and thrombocytopenia was the dose-limiting toxicity (DLT). The latter schedule was therefore selected for further development, and the recommended dose for phase II was 790 mg/m² (Abbruzzese et al., 1991). The population enrolled in the study was dominated by heavily pretreated patients, and dose escalation was very conservative, resulting in a large number of patients being treated at lower doses, which led to significant toxic effects. Only a few patients were treated with the recommended doses. When the drug was evaluated at the recommended dose in fitter patients, very few side effects were seen. Doses were escalated first to 1000 mg/m² and then to 1250 mg/m² (Anderson et al.,

1994; Cormier et al., 1994; Mertens et al., 1993). Finally, the phase I trial was repeated in non–small cell lung cancer (NSCLC) patients at a recommended dose of 2200 mg/m² (Fosella et al., 1997). At the same time, the dose was increased by administering the drug at a fixed-dose-rate infusion for increasingly longer periods of time (Brand et al., 1997). This approach was based on information gained earlier that indicated that there was a fixed rate at which gemcitabine could be converted into its active form. Therefore a phase II trial in pancreatic cancer compared the maximal dose of gemcitabine delivered as a 1500 mg/m² dose at a 10 mg/m²/min infusion and 2200 mg/m² at a 30-minute bolus (Temporo et al., 2003). In this trial, there was no observed impact on the primary endpoint of time to treatment failure, but there was a survival advantage (5 months versus 8 months) for the fixed-dose-rate arm of the trial, suggesting that the drug delivery approach needed further evaluation. In this example the starting dose of the phase I weekly schedule was too low because the patients enrolled were not representative of those enrolled in the phase II trial. This added time to the development process.

Let us now consider some important aspects of developing a phase I trial.

Patient entry criteria

Patient entry criteria should be carefully elucidated in the protocol. Some of the criteria that are used to select entry of patients into phase I trials are described in the following sections.

Performance status

Patients who are selected for phase I trials should have a good to moderate performance status. This is critical; otherwise, it might not be possible to distinguish the drug effects from disease-related symptoms if the patient is terminally ill. The tools developed by the World Health Organization (WHO) (Miller et al., 1981), the Eastern Cooperative Oncology Group (ECOG) (Oken et al., 1982), and D.A. Karnofsky (Karnofsky and Burchenal, 1949) are some of the performance status assessment tools that are widely used to assess patients' performance status.

Cancer type

Most phase I trials are conducted on a mixture of solid tumors. Some trials are based on specific antibodies. Some agents are antibodies or small-molecular-weight inhibitors against receptors that are present in specific cancers; trials of these agents should be restricted to patients with cancers that are likely to express these receptors. One example of this is cetuximab, which acts on epidermal growth factor receptors (EGFRs). Table 17.1 gives some other examples of targets.

TABLE 17.1 Examples of novel agents and their targets.

Target receptors	Drugs
EGFR	Cetuximab, gefitinib, erlotinib, sorafenib
HER 2	Trastuzumab
Angiogenesis: VEGF	Bevacizumab
Angiogenesis: VEGFR	Sunitinib, vatalanib
mTOR	Temsirolimus
ABL/c-kit	Imatinib mesylate

EGFR, Epidermal growth factor receptor; VEGF, vascular endothelial growth factor.

Generally, agents that are being tested in phase I trials should initially be tried in patients with a case of recurrent, advanced, or metastatic cancer that has been treated previously with the standard treatments and for whom no further standard treatment is available. In addition, it is common practice to include only patients whose disease can be clinically or radiologically documented, although it can be argued that this is less relevant in phase I trials, in which the primary endpoint is dose finding based on toxicity.

Laboratory investigations within appropriate limits as entry criteria

Phase I trials often restrict entry of patients to those with adequate liver, kidney, and hematological parameters. These parameters can vary from trial to trial and may vary according to whether the patient has a known liver metastatic disease. For example, the inclusion criteria for liver enzymes might be an increase up to twofold in the upper normal limit or an increase up to fivefold in the presence of hepatic metastatic disease. Although these limits are set to maintain patient safety, one could argue that studies should include some patients with at least moderate hepatic or renal impairment in order to find out whether these patients can be included in future development of the agent. It is often patients with the worst liver function tests who have the most to gain from novel drugs and agents, especially in the cancer setting.

Most commonly, pregnant and breastfeeding women are also excluded from phase I trials. This is done to protect the unborn or newborn child.

Special drug administration or procedures

Some trials require measurement of the PD effect of the drug on a tumor, necessitating a fresh tumor biopsy. In this case

the patient clearly must have a tumor that is accessible to biopsy, the doctor must have the appropriate expertise to perform the biopsy safely, and the patient must consent to the procedure. Alternatively, if a drug has to be administered intravenously, the patient will require intravenous access. This access can be obtained through a PICC line if repeat sampling is necessary and if the patient gives appropriate consent.

Patient consent

Any patient entering a trial must be given a careful explanation of the aims of the trial, the drugs involved, any known or expected side effects, and expected investigations and visits to the hospital that may be required. The patient should read the language-appropriate patient information sheet and should be given a period of time to digest this information and the opportunity to ask questions before signing the consent form.

Calculation of the starting dose

A wealth of preclinical information is utilized to inform the phase I trial starting dose and design. Statistical and other research has been undertaken to improve the safety, ethics, and efficiency of trial conduct and dose escalation methodologies. Usually, the murine LD₁₀ is converted from mg/kg to mg/m² using standard conversion factors. The human starting dose is generally one-tenth the mouse LD₁₀ equivalent, unless testing in a nonrodent species shows that this dose is excessively toxic. In this case, one-third to one-sixth the lowest toxic dose equivalent in the more sensitive species becomes the starting dose of human trials. Other factors such as clinical toxicity data from analogs of experimental agent, data from in vitro studies comparing the sensitivity of human and nonhuman cell lines, and data on drug protein binding in human and rodent plasma also help in determining the starting dose.

The US Food and Drug Administration (2005) suggests a method for calculating the safe starting dose in humans. According to this method, to convert the no observed adverse effect level (NOAEL) dose from the toxicology studies to the human equivalent dose (HED) according to body surface area, researchers must select the HED from the most appropriate species. Then a 10-fold safety factor is applied to give a maximum recommended starting dose, which is subsequently adjusted on the basis of the predicted pharmacological action of the investigational medicinal product (IMP).

Another method of calculating the safe starting dose of a new drug is based on the minimal anticipated biological effect level (Early Stage Clinical Trial Taskforce, 2006), which takes into account the potency, mechanism of actions, dose-response data from animals and humans in vitro, concentration response data from animals in vivo, species specificity, PK, PD, calculated target occupancy versus concentration, and concentration of the target and target cells in humans in vivo. If

those methods give different estimates, the lowest value is selected, and a margin of safety is built into the actual starting dose.

Dose escalation

Initially, the dose is usually doubled for each increment if, after the data have been carefully assessed, the previous dose has been found to be safe. Alternatively, serial measurements of the concentration of the new drug in the bloodstream during the trial can allow increases in dose to be guided by exposure to the new drug. It is the responsibility of the investigators, sponsor's physician, and sponsor's expert in PK to review all the data, including the preclinical data, before increasing the dose. If there is any concern about tolerability, an intermediate dose can be administered in order to avoid exceeding the NOAEL.

Dose escalation proceeds until a prespecified endpoint is reached. If toxicity is the primary endpoint, it continues until a minimum number of patients experience a DLT. When DLT happens, the next lower dose is studied to get more information; this will be the recommended dose for further studies. If the lower dose still reveals excessive DLT events, then the dose is further decreased.

If the primary endpoint is a pharmacological measure, escalation will continue until a minimum level, as described in the protocol, is reached. Results of PK analyses and, if possible, PD analyses must be available after each dose level to make accurate decisions about further escalation.

Number of patients required for dose administration

The number of patients dosed on one occasion and the interval between dosing each patient or cohort of patients depend on the route of administration and the type of trial. Usually, a minimum of three patients per dose level is enrolled.

For high-risk agents, the agent is administered to only one subject in the first instance. Often, intravenous doses are given slowly over several hours. For low-risk agents or oral administration, cohorts of up to three patients are dosed over short intervals rather than a single individual patient with a prolonged clinical review.

If the trial is starting at a dose at which no toxicity is observed, then it may be appropriate to enter just one patient per dose level for the first few dose levels (Eisenhauer et al., 2000). As escalation proceeds and the risk of toxicity increases, the number of patients per level is increased to three; this certainly applies once a toxicity of at least grade 2 has been observed in at least one patient. The number of patients per dose level increases to six if one of the three patients has DLT in a protocol-prescribed observation period.

However, if the drug is expected to have large interpatient variability—for example, if there is likely to be large

differences in oral bioavailability—then three patients per dose level is recommended from the first dose level onward. Furthermore, in the later expanded dose levels, patients who are as representative as possible of those likely to be tested in the phase II trial should be enrolled.

Stopping rules

Phase I trials are stopped if significant DLT is observed. The following are a few examples of the stopping rules employed ([Eisenhauer et al., 2006](#), cited with permission from Oxford University Press):

- *Rule of 3:* Stop if one DLT is observed among the three patients. There is a high probability of stopping before a target dose is reached.
- *Rule of 3 + 3:* Stop if at least two out of six patients experience a DLT. The probability of stopping below the target dose is 34%, and the probability of stopping above the target dose is 29%. This can be improved by adding a third cohort of three patients if two out of six patients experience a DLT at a certain dose level. The expected sample size is 11.7 patients.
- *Rule of 3 + 3 + 3:* Stop as soon as at least three out of nine patients experience a DLT. This rule has a higher probability of stopping at a higher dose level than the target. The expected sample size is 16.1 patients.
- *Rule of 2 + 4:* If one DLT is observed in the first cohort of two patients, then an additional cohort of four patients is added. A high probability of stopping at a higher dose level is expected, but the sample size required is 12.6 patients.
- *Rule of 3 + 1 + 1:* If one DLT is observed among three patients, one more patient is added to the next cohort. This rule has a tendency of stopping at higher doses.

The Rule of 3 + 3 is the most widely used, but more aggressive rules may be more efficient if the drug is not expected to be very toxic.

Measuring endpoints

The standard primary endpoint of a phase I trial is to find a recommended dose for future trials. Other endpoints can be employed, such as those that assess dose-related effects of the drug on the body. These are known as pharmacodynamic endpoints and can include measures such as molecular changes, antitumor effects, and toxicity.

Toxicity

Most anticancer drugs target the DNA, so doses that result in antitumor effects are also likely to be toxic for normal cells. The highest tolerable dose is usually the effective dose in clinical studies. In phase I trials the dose of the

TABLE 17.2 Dose-limiting toxicities used in phase I trials.

Event	Dose-limiting toxicities
Neutrophil	Grade 4 ($<0.5 \times 10^9/L$)
Platelets	Grade 4 ($<25 \times 10^9/L$)
Liver function tests	AST or ALT grade 3
Myelosuppression	Febrile neutropenia or grade 3 infection with grade 3 or 4 neutropenia, thrombocytopenic bleeding Grade 3 or greater nausea and/or vomiting despite antiemetic therapy Other CTC: grade 3 or higher toxicities

CTC, Common toxicity criteria.

IMP is usually escalated in cohorts of patients until predefined criteria are met. These are called dose-limiting toxic effects. DLT is a severe but reversible organ toxicity.

In 1981 the WHO published the first toxicity criteria assessment tool ([Miller et al., 1981](#)) to assess drug toxicities. Grades were used, ranging from grade 0 (normal with no adverse effect) to grade 5 (fatal). Similarly, in 1982 the US National Cancer Institute (NCI) developed the common toxicity criteria (CTC), which are now commonly used to record adverse events in trials.

The phase I trial protocol must define the DLT with precise laboratory levels or grades for hematological, renal, and hepatic events or should refer to a standard tool, such as the NCI's CTC. If the patient misses more than a prespecified number of doses because of toxicity, this toxicity is considered to be a DLT. [Table 17.2](#) lists some of the DLTs that are used in phase I trials. The trial design should explain the number of patients who must experience DLT before further dose escalations or dose stops. Usually, two patients out of three or six must experience a DLT for the escalation to stop.

The dose level at which a DLT is seen in the number of patients required to prevent further dose escalations is called the MTD; it is also referred as the maximum administered dose. The recommended phase II dose is usually at a dose level below that at which escalation has stopped. Toxic effects at this dose may still be severe, but their frequency is expected to be lower than that seen at the MTD.

Pharmacodynamic endpoints

Many novel anticancer agents target the intracellular and extracellular pathways and differ from the cytotoxics because of their preclinical dose-effect relationship ([Eisenhauer, 1998](#)). In these cases, once the target is saturated by the drug, the antitumor effect can plateau, but

toxicity may not if the target level is higher in normal tissues. Clearly, then, maximal toxicity may not have been reached at a dose level necessary for maximum efficacy (Parulekar and Eisenhauer, 2002). So the endpoint in these cases would often be pharmacodynamic—for example, molecular biomarkers measured in repeated tissue biopsies, peripheral blood mononuclear cells, buccal mucosa, or skin.

Mechanism-oriented trial design

In the last decade, an extraordinary increase in the number of druggable targets have been discovered by molecular and cell biologists. The role of the enzymes and signal transduction pathways involved in the control of the cell cycle, proliferation, invasion, metastasis, and angiogenesis has been clarified. Not surprisingly, given their centrality to control of cellular homeostasis, many of these new targets have been kinases. This has yielded a new approach to drug development in which attempts are made to titrate drug dose to inhibition of the mechanistic target and to separate the biologically effective and MTD doses. This leads us to consider three related principles of phase I clinical trial design: proof-of-mechanism (PoM), proof-of-principle (PoP), and proof-of-concept (PoC).

Proof-of-mechanism

If a novel anticancer agent has been developed as a specific inhibitor of an individual kinase, then it is possible to construct an assay that reflects the degree of target inhibition, thus providing PoM. We have developed a potent, specific inhibitor of PIM-1 kinase, an enzyme that is widely expressed in a range of adenocarcinomas, the overexpression of which has been shown to be a poor prognostic factor. BCL2 associated agonist of cell death (BAD), one of the proteins involved in induction of apoptosis, is a downstream target of PIM-1, and inhibition of the enzyme leads to a significant and dose-dependent reduction in phosphorylation of BAD. This could therefore be developed as a PD biomarker for the drug. Cell culture studies showed that reduction in phosphorylation of BAD to 90% of baseline levels was associated with induction of apoptotic cell death, suggesting a target that might be adopted as a mechanistic surrogate in a clinical trial. Although in an ideal world we would attempt to take tumor biopsies (at the baseline and at some period after drug dosing) to assess the biomarker, in practice this is difficult, and in most cases, samples are taken of peripheral tissues that express the target (e.g., circulating lymphocytes, skin biopsies, hair follicles, buccal mucosal scrapes). There are a few studies in which research groups have attempted to correlate parallel expression of biomarkers on tumors and surrogate tissues, but these are usually small, underpowered, and rather inconclusive. However, this has not diminished the frequency with which surrogate tissues are used to provide

PoM. PIM-1 kinase is expressed on lymphocytes, and it has been possible to develop a Western blot assay that detects phospho-BAD in the buffy coat of lymphocytes. This means that blood samples taken at intervals after drug dosing can be split into serum for drug concentration estimates and PK modeling and the buffy coat for measuring the decrease in the phosphorylation of BAD. Mathematical models can be constructed relating drug concentration to effect in the clinical setting and reflecting back to the preclinical in vitro cell systems. PoM can now be taken to sophisticated levels; however, the wide interindividual variation in PK and PD variables can make interpretation difficult when only three patients are entered at each dose level.

Proof-of-principle

PoP extends the mechanistic observations to include evidence of consequent phenotypic change. For example, inhibition of a kinase that is essential for survival could lead to induction of apoptosis. There are several markers of apoptosis that could be used in the clinics to correlate the degree of inhibition of the drug target with the extent of cell death—for example, tumor biopsy and microscopic assessment of the fraction of apoptotic cells, disaggregation of the tumor biopsy and fluorescent cell sorting for the TUNEL assay, induction of caspases, and fragmentation of DNA characteristic of apoptosis. Other assays are being developed as serum markers of apoptosis and positron emission tomography (PET)—based imaging using novel tracers that rely on the increased cellular permeability associated with apoptosis, but they have not yet been validated.

Another example of PoP is the examination of the vascular axis following inhibition of the vascular endothelial growth factor (VEGF). Antibodies that bind to and biologically neutralize VEGF (such as Avastin) and small-molecular-weight inhibitors that inhibit the VEGF receptor have been developed as inhibitors of angiogenesis, one of the crucial processes involved in tumor progression and metastases. The impact of VEGF inhibition on angiogenesis can be assessed by tumor biopsy and fluorescent microscopic staining of the microvasculature. The sophisticated imaging techniques depend on the capacity of VEGF inhibitors to reduce tumor vascular permeability. This is determined from complex mathematical models derived from dynamic contrast-enhanced magnetic resonance imaging (MRI) scans that have been correlated with the degree of VEGF inhibition.

Proof-of-concept

The ultimate, of course, is whether the novel agent induces a worthwhile tumor response according to general agreed-upon rules (e.g., RECIST criteria) in which tumor volume is assessed by using conventional scanning methods [computed tomography (CT) scan or MRI] or more developmental techniques such as PET scanning. By WHO criteria, tumor responses are

categorized as complete (disappearance of all measurable disease), partial (a greater than 50% reduction in the bidimensional product of the tumor masses), or stable disease (in which the measurable disease remains the same, within the margins of $\pm 25\%$). Radiological responses themselves are relatively poor surrogates of survival but would be accepted as a PoC.

Can we make go-or-no-go decisions at the end of phase I?

It has been estimated that there are 1000 novel chemical entities in oncology waiting to enter the clinic. This means that there is increasing pressure to make early go-or-no-go decisions on further drug development. Classically, this decision would be made at the end of phase II using the Gehan two-stage trial. For example, if no tumor responses were seen in the first 14 patients treated, the drug would be deemed inactive (assuming that an active drug would have a tumor response rate of 20% or better). The problem with this approach is that

multiple phase II trials, each of a separate tumor type, would be required before a go-or-no-go decision could be made. An alternative model would be to use the PK and PD data gathered during phase I trials. For example, if the maximum plasma concentration of the drug was found to be in the nanomolar range in the clinic setting and if all the preclinical data indicated that micromolar-range concentrations were required for sufficient induction of the appropriate phenotypic changes, then it would be reasonable to conclude that the experimental drug was unlikely ever to be effective. Similarly, if experimental evidence strongly suggested that 90% of enzyme activity had to be inhibited to induce a significant degree of apoptosis and if clinical PoM data showed that only 5% target inhibition was achieved, then, again, it would be reasonable to halt drug development. So it is distinctly possible that these elegant mechanistic endpoints can play an important role in preventing further development of “lame-duck” drugs. These algorithms are presented in Fig. 17.1.

Phase II trials

The industry standard for phase II trial design in oncology has been predicated on demonstrating a tumor response rate of 20% (number of patients with complete remission plus partial remission as 20% of the total patient population treated) and selecting the minimum number of patients with a specified tumor type, stage, and prior number of treatments to be recruited to the trial. Gehan has shown that the probability of rejecting a drug with this level of activity is less than 0.05 if no responses are seen in the first 14 patients treated.

Personalized medicine

Individualizing therapy through understanding which patients will respond to particular drugs will allow doctors to stop ineffective therapies in unresponsive patients during the crucial therapeutic window when alternative anticancer treatment should be initiated, while obviously eliminating associated side effects, expense, and waste of time for terminally ill patients. Omission of patients who are unlikely to benefit from specific therapies will enhance the cost-benefit analysis and make it more likely that the bodies governing health care finance will approve expensive drugs for use. The KRAS gene is one example of a situation in which individualized therapy is likely to improve clinical practice in the very near future.

The KRAS gene has a function in the intracellular signal transduction along the EGFR axis. It mediates proliferation signaling intracellularly downstream from the EGFR. Patients whose tumors harbor the wild-type KRAS gene demonstrate effective disruption of cellular growth signaling through the EGFR pathway when they receive EGFR-targeted therapies, such as cetuximab. As a result, these patients achieve a greater response rate when the EGFR antibody is added to

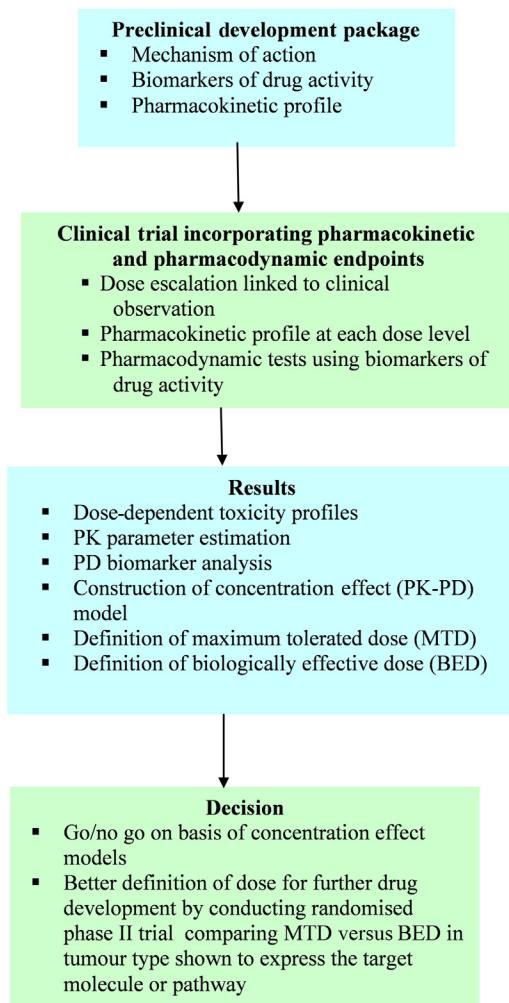


FIGURE 17.1 An early-phase drug development paradigm.

standard chemotherapy compared to standard chemotherapy alone. On the other hand, mutated KRAS genes demonstrate the ability to continue signaling to the proliferation cascade within the EGFR axis even when EGFR inhibitors such as cetuximab have been administered. Consequently, patients with tumors that harbor such mutant RAS do not gain any extra benefit from the additional administration of cetuximab compared to receiving chemotherapy alone ([Genetics Home Reference, 2008](#)). Around 30% of colorectal cancer patients have tumors with the KRAS mutation, which makes them ineligible for EGFR-targeted therapies such as cetuximab and panitumumab ([Amado et al., 2008](#)). Removing these patients from the trial reduces financial cost overall and reduces the number of patients exposed to the side effects of likely useless therapy. Recent data suggest that extension of RAS mutational screening to include additional variants further improves the therapeutic ratio for these EGFR-targeted therapies ([Douillard et al., 2013](#)). Although there has been a significant focus on markers to select patients who are more likely to respond to treatment, there is growing interest in identifying patients who are most at risk of serious toxicity and therefore improve the risk-benefit ratios by reducing the incidence of life-threatening side effects. Consequently, germline markers of toxicity are appearing in the literature ([Church et al., 2014](#)).

The contribution of modern imaging to early-phase trials

Accurate methods to assess treatment response are pivotal in the development of new cancer therapies. While conventional forms of cross-sectional imaging have been of great benefit over many years to assess anatomical response, there is now increasing understanding of the importance and the role of imaging techniques in evaluating metabolic response and the value of incorporating techniques to assess cellular and subcellular response in many tumor types.

This section will address the role of imaging techniques in assessing treatment response in early-phase trials and will highlight emerging techniques to evaluate biomarkers and molecular targets.

To date, clinically relevant data in the evaluation of novel therapies have utilized proven and appropriate accurate quantitative assessment of early clinical trials in their application and subsequent relevance and employment in clinical practice. Current and future drug trial design in oncology needs to reflect the increase in individualized cancer therapies and the expansion of personalized medicine ([Yankeelov et al., 2016](#)).

The introduction and utilization of the newest techniques are not without many challenges, and there have been considerable barriers to overcome to enable their implementation.

In light of the progress made with the appropriate imaging strategies, novel effective targeted therapies can now be assessed and added to the current armamentarium for oncology patients.

Anatomical and molecular imaging evaluation

Traditional cytotoxic chemotherapies have often been nontargeted and have frequently been assessed by using anatomical techniques. Specific assessment of the effect of treatments in specific molecular pathways requires, by necessity, advanced imaging techniques that can evaluate tumor response at the molecular level.

While conventional imaging has enabled assessment of tumor response invariably by evaluation of interval change in size, this may not necessarily correlate with treatment efficacy or predict outcome for novel techniques. Novel therapies require appropriate selection of the relevant imaging modality in order to assess the response of specific targeted treatments to ensure appropriate consideration of new therapies in oncological practice.

The current use of RECIST criteria and the use of size-based criteria at baseline and at follow-up depend on an evaluation of percentage change in the sum of five target lesions to determine treatment efficacy ([Eisenhauer et al., 2009](#)). However, for some therapies, including cytostatic, antiangiogenic, and immune-based therapies, there may be an increase in tumor size or little change in size even in effective treatment, and stabilization of size may be present despite good treatment response.

Fluorodeoxyglucose (FDG)-PET imaging depends on metabolic activity and has a proven benefit in assessing interval molecular change in tumor metabolic activity. FDG-PET specifically has application in the assessment of treatment response to tyrosine kinase inhibitor drugs in assessing progression-free and overall survival, including in those tumors without apparent interval size response ([Mileshkin et al., 2011; Zhao et al., 2006; Sohn et al., 2008](#)).

Objective measurement of metabolic tumor response is pivotal to providing reproducible assessment of novel techniques. Several quantitative imaging methods are available and have further potential in current practice:

1. Perfusion measurements using MRI or CT that assess vascularity, vessel permeability, blood flow, and tissue volume ([Yankeelov and Gore, 2009](#))
2. Cellularity measurements using diffusion-weighted MR techniques to assess rates of diffusion of water molecules in abnormal tissues
3. Assessment of the apparent diffusion coefficient that correlates with tissue cellularity and can be used to determine tumor treatment response

Metabolic measurements using positron emission tomography ligands

Malignant cancer cells upregulate glucose metabolism by upregulating glucose transport into cells by increasing phosphorylation and increasing the activity of specific glucose transporter proteins. Assessing changes in the rate of glucose metabolism provides a predictive indicator of tumor response either by utilizing changes in standard uptake value or by using kinetic modeling.

The range of available PET ligands can provide an alternative assessment of therapeutic response. For example, 18-FLT acts as a marker for cellular proliferation (Shields et al., 1998) and has a potential role in quantifying rapidly proliferating tumor cells.

Choice of imaging modality

The imaging modalities utilized varies among clinical departments, and baseline and follow-up endpoints vary between tumor sites and between individual trials. The correct choice of imaging modality is pivotal, not only to ensure that validated measurements and assessment are used but also to enable relevant validated assessment for specific patient groups, various tumor sites, and specific types of treatment.

Functional and molecular imaging methods have evolved to assess endpoints (e.g., FDG-PET in lymphoma) and can be highly predictive in terms of outcome and survival.

Radiomics

Radiomics is a method that extracts a large amount of data from medical images using data characterization algorithms.

Tumor heterogeneity can be quantified by using radiomic measurements that reflect underlying tumor pathophysiology and variable treatment response in specific tumor types.

Changes of radiomic features over time in longitudinal images (delta-radiomic features, or DRFs) can potentially be used as a biomarker to predict metabolic treatment response to novel therapies, for example, in pancreatic cancer (Nasief et al., 2019). DRFs are an independent predictor of survival and, if combined with the clinical biomarker CA19-9, can improve treatment response prediction and increase the possibility for response-based treatment adaptation (Nasief et al., 2020).

Several studies have recently shown that radiomic features are better at predicting treatment response than are conventional measures, such as tumor volume and diameter or the maximum radiotracer uptake on PET imaging (Tixier et al., 2011; Hatt et al., 2015; van Rossum et al., 2016; Yip et al., 2016; Zhang et al., 2014; Cheng et al., 2015; Cook et al., 2013).

Radiomic algorithms can be developed to predict the probability of tumor response to novel immunotherapy,

providing a demonstration of the clinical potential of radiomics as a powerful tool for personalized therapy in the emerging field of immunooncology (Sun et al., 2018).

Owing to the marked variability in patient outcomes even with the same stage of cancer, accurately determining the aggressiveness of tumors is critical for determining options between curative and palliative treatments. Radiomics studies have shown that image-based markers have the potential to provide information about stage and provide biomarkers and improve prognostication (Aerts et al., 2014; Tunali et al., 2017; Huang et al., 2018).

Quantification imaging methods

Novel imaging methods in early-phase trials require quantification to validate methodology and ensure a reproducible and accurate means of assessment. The applicability of emerging quantitative imaging methods continues to increase in the evaluation of novel therapies.

To assess novel imaging biomarkers and maintain comparable methodologies, standardization is required and involves optimization of imaging protocols, image analysis, and reference to validated methods in order to provide quantitative imaging. For comparable data to be meaningful, it is essential that all sites involved in an early-phase trial use specified clinical protocols and have comparable expertise and validation of the equipment used. Central collection and analysis of imaging data can then provide meaningful assessment.

Anatomical image-based assessment cannot provide adequate data in the assessment of molecular-targeted drugs, and there is now a clear requirement for advancement in evaluation techniques.

The challenges for imaging are primarily the assessment of tumor phenotype, which may not be apparent on conventional imaging, and the assessment of treatment response, particularly at the earliest stages of treatment. RECIST 1.1 assessment is limited in many instances, including where there are poorly defined tumor margins, internal change in morphology, and no interval change in size. Subsequent analyses include techniques to assess and incorporate changes in internal morphology, including internal cavitation (e.g., in NSCLC; Crabb et al., 2009), changes in enhancement pattern (e.g., in GIST; Choi et al., 2007), and assessment of size and metabolic activity in all tumor types, and there is therefore an evolving consideration for quantitative PET response criteria in solid tumor response (PERCIST; Young et al., 1999).

The role of imaging in phase I trials

Exploratory imaging is a biomarker of drug efficacy. The choice of imaging modality depends on the action of the drug, the endpoint, and the appropriateness of the imaging

method in terms of technical issues, reproducibility, local expertise, and cost.

1. Cellular metabolism: measured by FDG
2. Tumor vascularization, including perfusion, angiogenesis, and hypoxia: measured by dynamic contrast enhanced (DCE)-CT and MR
3. Cellular proliferation, differentiation, survival, and apoptosis: measured by diffusion weighted (DW)-MRI and MR spectroscopy (MRS)
4. Proliferation and apoptosis: measured by diffusion-weighted MRI
5. Assessment of water diffusion and informs on cell density, extracellular space tortuosity, and the integrity of cellular membranes
6. MR spectroscopy: assessment of cell density and cellular membrane turnover

MRS uses choline as the metabolite to assess cell membrane synthesis and degradation or uses free lipids to assess necrosis and apoptosis.

Imaging response using a combination of techniques can predict overall good outcome in terms of overall response and assessment of the following parameters: tumor size, vascular response, and cellular response; conversely, a lack of response in these parameters is predictive of a poor outcome.

Challenges for novel imaging methodologies and clinical trials

An increasing number of novel imaging biomarkers are available. However, there are ongoing challenges for translation that require technical and biological validation. Validation as a trial endpoint criterion is necessary prior to definitive evaluation studies. The requirements include technology that is stable and broadly available, specifiable acquisition parameters with defined normal ranges, standardized image interpretation, and documented reproducibility.

If the imaging endpoint is to serve as an early accurate indicator of promising treatment effect, it needs to correlate with progression-free survival and the overall survival.

Conclusion

Imaging has an important role in clinical trials in terms of objective response assessment and trial endpoint. RECIST 1.1 remains the international standard for response assessment. However, other response criteria may be appropriate but require further validation.

Imaging biomarkers may have a role in early-phase clinical trials. However, considerable challenges remain in the implementation of novel imaging markers, although significant progress continues to be made in the fields of molecular imaging and radiomics.

Open access clinical trials

Drug discovery has been steadily declining in productivity over the past 30 years. Currently, more than 80% of clinical trials fail for pioneer drug targets. There are many reasons for this, the dominant reason likely being poor target validation. A new public-private initiative led by the Structural Genomics Consortium (SGC) of the Universities of Toronto and Oxford aims to provide structural models of proteins of interest to the pharmaceutical and biotechnology industries as potential drug targets and make this information, along with chemical inhibitors and monoclonal antibodies to these proteins, freely available to the oncology community (academia and industry). Authors of this open access partnership have hypothesized that this approach will greatly improve the quality of target validation and improve the odds of a novel drug making it into the clinic ([Edwards et al., 2009](#)).

Is it possible to conceive of open access phase I clinical trials of these new agents, overcoming the prohibitive secrecy that most pharmaceutical companies employ in drug development? This would be the clinical equivalent of the experiment being performed by the SGC and would make clinical data publicly available in real time so that information on the risk-benefit ratio of the novel target could be shared with others in the field. Increasing access to clinical data brings obvious concerns about confidentiality and poses outstanding questions over how far the reporting of data will go. Will it be necessary to document every blood test, every scan? If data are shared and to be used by others, this puts huge pressure on the accuracy of the data.

Performing a phase I trial with a clinical probe—akin to the chemical probes released by the SGC—is predicated on the likelihood that the probe, which will not be patent protected, allows clinical validation of the target but is unlikely to be the final, best-in-class agent that gets market approval. Financing for such trials could come from a consortium of academia, charities, and industry, which share the goal of getting a greater number of effective medicines into wider use. Obviously, numerous issues still need to be addressed with regard to the wider sharing of data, but this may be one means of modifying early-phase trial design to better improve the efficiency of cancer drug development.

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Chapter 18

The pharmaceutical research and development productivity crisis: can exploratory clinical studies be of any help?

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Abstract

The development of a new medical intervention is rarely a fixed linear process; rather, it is a highly complex, dynamic process with multiple options and a wide range of possible development routes. Critical development questions and decisions vary for individual products. Although development typically starts with the expected label claim (or target product profile) in mind, the route to getting there depends on the product, indication, and target group. Development plans can change significantly during the course of development, sometimes because of unexpected results or novel insights, requiring an iterative process rather than a linear, phase-oriented approach. Obviously, the incorporation of flexibility (or having different options) in a project has its price, but it also has value. The higher the uncertainty, the higher the value of flexibility incorporated in the project. Exploratory studies address the critical, potential show-stopping development questions and support decision making. Details of their contribution to drug development will be discussed in this chapter.

Keywords: Exploratory studies; high attrition rates; development routes; value perspective; decision processes; development questions

Traditional drug development

In a recent publication in *Nature Reviews on Drug Discovery*, Michael Ringel and colleagues provide evidence that after decades of declining pharma productivity in the pharmaceuticals industry, this trend came to a halt around 2010 with the average cost per market entry of a new molecular entity (NME) of US \$1.4 billion. One of the reasons the authors ascribe to this change in productivity decline is an increased focus on better decision making along the stage-gate process of drug research and development (R&D) (Ringel et al., 2020).

This R&D process is traditionally structured into a preclinical portion and a clinical portion that “meet in the valley of

death” (VoD) (Fig. 18.1) (Dessain and Fishman, 2017). After an elaborate preclinical research phase in which optimized compounds (e.g., with respect to their physicochemical pattern, target activity, safety profile, and pharmacokinetic properties) are selected in a series of validated cellular and animal tests, a patent is filed, and eventually the clinical stage of development can start (ICH E8, 2020). The clinical stage ends with larger phase IIb (dose-finding or dose-ranging) and phase III studies (confirmatory) studies, which are also called label relevant or label enabling.

Definition of exploratory clinical studies

In 2006 the U.S. Food and Drug Administration (2006) referred to “exploratory IND studies” as clinical trials that

- are conducted early in phase I before a full pharmacokinetics (PK) characterization has been acquired
- have no therapeutic or diagnostic intend,
- expose individuals only to a limited degree (e.g., between one-fiftieth and one-fourth of rat NOAEL dose), and
- are conducted over a limited time (≤ 7 days).

In a guiding document by the European Medicines Agency (2019) this scope was broadened to later studies in which more PK and efficacy information was available and where an exploratory approach could also be used in nonconfirmatory Phase IIa trials to assess specific subgroups re. safety and efficacy, for example, prior to conducting larger trials in a Phase IIb or III.

In his textbook *Clinical Trials: A Methodologic Perspective*, Steve Piantadosi lays out an even larger frame that applies the term *translational studies* to a “vitally important, widely used, but poorly described type of clinical trial” (Piantadosi, 2005). In a similar way, Schwarz (2011) in a

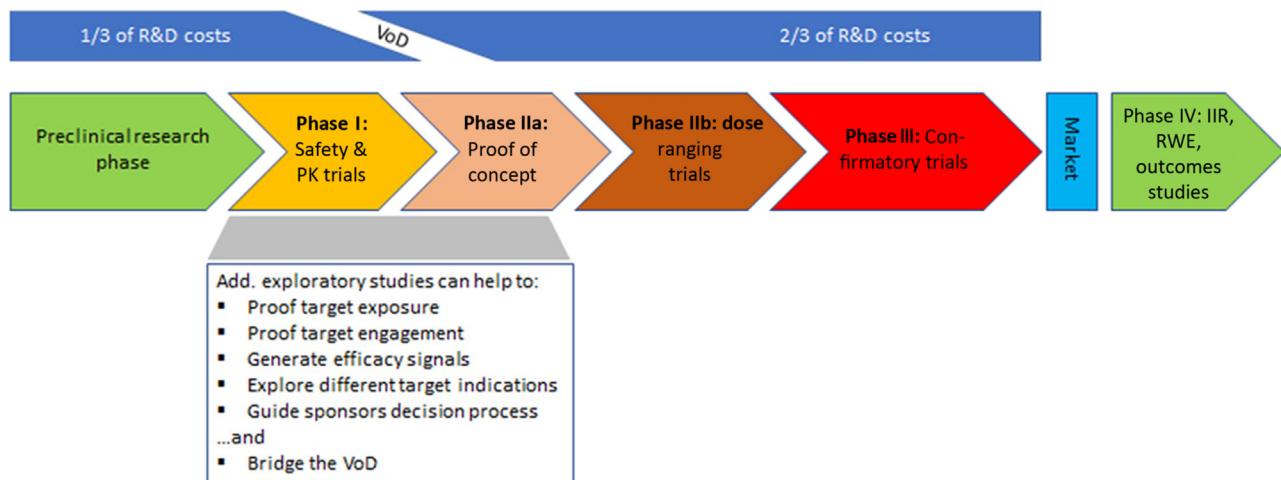


FIGURE 18.1 A simplified depiction of the pharmaceutical development stages along a stage-gate process in which project uncertainty is reduced at subsequent steps.

textbook on clinical trial conduct, applies a broader scope that lists exploratory trials as those that could be microdosing as well as phase IIa proof-of-concept trials.

Decision making: regulatory perspective versus company internal perspective in phase I and phase IIa

When a new molecule is monitored by its progression through the traditional phases, little is learned about what questions are actually being answered. Managers have to assume that this is being done adequately. The first time the answers are sometimes critically examined is by the regulatory authority that has to give approval for marketing. A common misconception is that the European Medicines Agency or the U.S. Food and Drug Administration requires stringent, phase-oriented drug development before approval is granted. Early translational clinical development should therefore focus on addressing the critical development questions early in order to derisk the product as efficiently as possible. After all, there is substantial value in early discontinuation of a failing product (Zurdo, 2013) as well as reducing uncertainty in the VoD of drug development (Truebel and Thurston, 2020).

The problem at hand: attrition versus opportunity focus

The notoriously high attrition rates in developing novel, innovative medical interventions fuel discussions about optimizing the costs involved. A 2019 Gupta Strategists analysis (Gupta Strategists, 2019) suggests that the frequently mentioned \$2.5 billion 2017 average R&D costs of a new chemical entity are composed of a mere 7% out-of-pocket costs, 40%

out-of-pocket failure costs, and a significant 53% cost of capital. This clearly shows the value of efforts to reduce the development costs of products that fail. Out-of-pocket failure costs form a substantial part of total R&D costs. Therefore reducing these costs has a considerable impact on overall R&D costs, and with the goal of increasing the efficiency of investment decisions, more efforts have been directed toward addressing critical uncertainties in translational development.

From phase thinking to question-based development

All of these descriptions have in common that for an exploratory study, a focus is placed on early derisking of a mode of action without specifying to what extent. It is important to realize that the process description of (clinical) development phases does not dictate the content of these phases. During the classical phases I to III (and IV), a number of generic questions need to be answered. The detailed questions have to be determined on a case-by-case basis, but these question groups provide some structure to the list.

Does the biologically active compound or active metabolites get to the site of action?

This main generic question contains several issues that need to be determined, such as absorption, distribution, metabolism, and excretion of the drug. Not only the parent compound but also any possible active metabolites should be included in answering this question. Additional items can be relevant for certain drugs, such as the ability to penetrate the blood-brain barrier for central nervous system-active drugs. Unexpected biologically active metabolites can be formed in vivo, or unexpected sites of

action can be discovered, which should be incorporated in this main question as soon as they are observed.

Does the compound cause its intended pharmacological/functional effect(s)?

Answering this question includes the demonstration of the mechanism of action of the investigational drug. For example, a new drug for hyperlipidemia will at least have to reduce the plasma cholesterol in a dose-dependent or plasma concentration-dependent manner.

Does the compound have beneficial effects on the disease or its pathophysiology?

This question reflects the question traditionally answered in the classical phase III studies to establish the effects on the disease but also the alteration of other physiological systems that result in clinical side effects.

What is the therapeutic window of the new drug?

The therapeutic window of each investigational drug needs to be established in order to identify the optimal dose that is clinically efficacious at tolerated levels. This question includes important subquestions: Which dose regimen will keep the drug's concentration within the therapeutic window? What is the optimal dosing interval relative to the intended indication (chronicity of intended drug exposure)? Can controlled drug delivery improve the product's action? What is the forgiveness of the product (i.e., the difference between the product's post-dose duration of effective therapeutic action and the recommended interval between doses)?

How do the sources of variability in drug response in the target population affect the development of the product?

The sources of variability in drug response can be defined as dose (formulations and compliance), PK (absorption, distribution, metabolism, and elimination), pharmacodynamics (sensitivity and maximum response), and other (disease, other drugs, and circadian rhythms). The main question should include "Are there any specific factors in the target population that may affect dosage?" A general subquestion can be: "Is there any food interaction with this compound?" But more drug- or population-specific questions can also arise. The regular use of comedication in the target population may require extra drug interaction studies. Ethnopharmacological issues and pharmacogenomics

can play key roles in some development programs (e.g., for the introduction of a "Western" drug in Japan).

One question can be concealed in several studies, and one study can provide partial answers to multiple questions.

High risk and variable costs: how to address the risk-cost combination in development?

Important lessons can be learned from other high-risk, high-cost industries, such as the oil and gas industry, which have some features in common with drug companies (Fig. 18.2). The discovery and development of a new oil field takes several years, the costs of exploring different potential fields are high, and the price of oil per barrel in the future (and thus future revenues) is hard to predict. Still, investment decisions have to be made even with the inherent uncertainties. The classical way is to base the investment decision on net present value (NPV) calculations for the project. In a NPV calculation the future cash flow (i.e., investments and revenues) of a project is translated to the value of today. The NPV method does not fully take into account the risks and flexibility during the course of the project.

Obviously, the incorporation of flexibility (or having different options) in a project has its price, but it also has value. The higher the uncertainty, the higher the value of flexibility incorporated in the project. This may seem logical, but in the NPV methodology, only time, costs, and revenues determine value. To describe the impact of flexibility on investment decisions, Steward Myer was the first to suggest in 1977 that the theory of financial options also be applied to nonfinance options (or "real options") that can be incorporated in investment decisions. In financial options an investor can take an option to buy a stock at a fixed time and price. The investor has the right, but not the obligation, to buy the stock. The obtained option has a value, and the option itself can be sold at a certain price to other investors. Similarly, nonfinancial options in a project will give a company's management the flexibility to terminate the project prematurely with no obligation to complete the project according to a predetermined fixed plan.

The possibility (or option) to terminate the project prematurely or modify it has a certain value. Myer's aim was to incorporate this value in the analyses to support the investment decisions. Of course, the flexibility that is incorporated in a project cannot be traded, as financial options can, but the essence is the same. With real options analysis (ROA) one can determine the value of this flexibility and address the associated risks. Simply correcting NPVs for historical risks does not provide insight into the real options in a project (Paul Janssen Futurelab, 2020a).

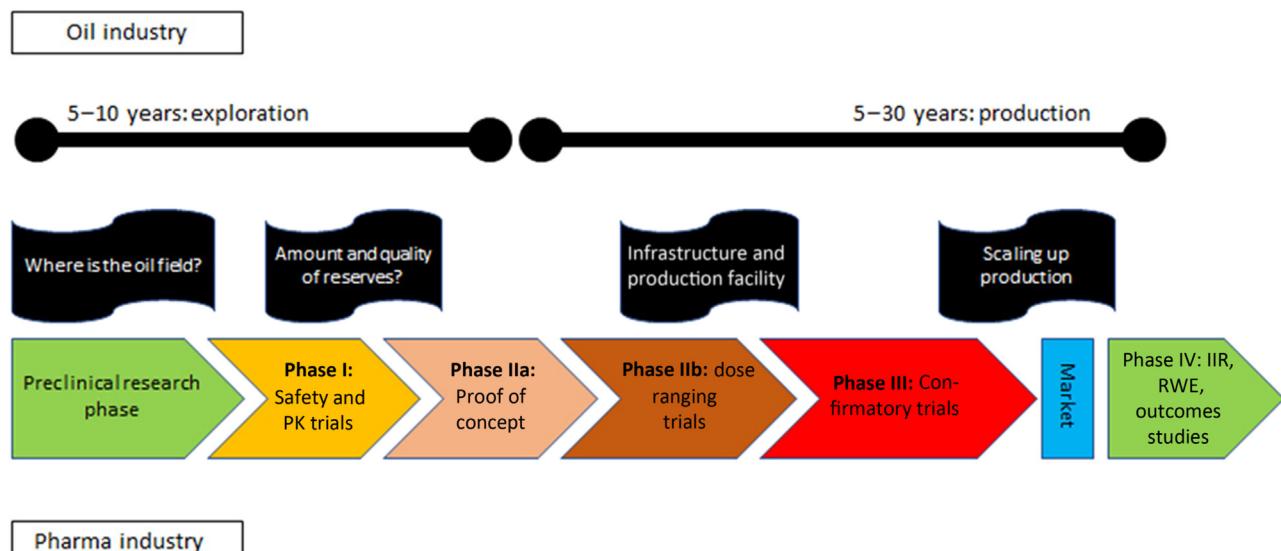


FIGURE 18.2 The oil and pharmaceuticals industries have some common features, such as long setup and exploration times with substantial up-front investments and associated risks.

Real options in translational development

The development of novel medical interventions typically starts with defining a target product profile (the expected label claim). The route to get there varies enormously depending on the product, indication, and target treatment population. The process is rarely a fixed, linear process; rather, it is a complex, highly dynamic process with several options and possible routes. This is reflected in the fact that development plans are frequently altered and adjusted because of unexpected results or novel insights. Therefore the development process requires an iterative process rather than a fixed, linear, phase-oriented approach. ROA is a much more flexible approach to R&D project valuation in this nonlinear process.

Entrepreneurial biomedical researchers who are involved in developing new medical interventions are ideally suited to integrate financial and scientific considerations in order to calculate the value of an R&D project. A 2020 *Nature Biotech* paper (de Visser et al., 2020) explains classic financial valuation by the measures of NPV and risk-adjusted NPV. Moreover, an alternative method based on ROA is presented that integrates financial project valuation with scientific considerations for critical path finding in product development. By defining scientific questions, such as those presented previously, as real options, a question-based priority list can be analyzed. This list will be generated on a case-by-case basis and can be monitored and adjusted as new insights and results become available during development.

Let's assume that you are considering the development of a novel medicinal product. You assume (based on literature data) that the average success rate for the type of product you are developing is around 15%. Furthermore,

you apply the overall development out-of-pocket costs of 7% of \$2.5 billion (= \$175 million) quoted earlier (de Visser et al., 2020). The success rates and costs (or really the efforts needed to successfully address the question and get an answer, either positive or negative) are distributed evenly over the questions as described above except the success rate for the “functional effect” question. The latter is deemed to have a lower than average success rate of only 50%.

A free-to-use tool to analyze question-based development using this methodology and these settings is available online (Paul Janssen Futurelab, 2020b). This can clearly demonstrate the value of addressing critical questions early. The tool (Table 18.1) calculates the estimated project values for the optimal order of questions, the second best order of questions, and a user-defined order of questions addressing the functional effect question last. The overall success rates, overall development costs, and estimated revenues are identical for these three routes. In this example you would obviously consider addressing the critical functional effect question first. This yields a risk-adjusted project value of approximately \$38 million, while the effect of addressing this key question last would drop the project value by more than half compared to the optimal route: to approximately \$15 million! The second-best order returns an expected intermediate value of approximately \$29 million (Fig. 18.3).

Exploratory studies: the scorn perspective

Ideally, exploratory studies address the critical, potentially show-stopping development questions. However, owing

TABLE 18.1 Example of the question-based evaluation approach.

Questions
Question 1: Does the intervention get to the site of action? Key words: Site of action
Question 2: Does the intervention cause its intended functional effect? Key words: Functional effect
Question 3: Does the intervention have beneficial effects on the disease or its clinical pathophysiology? Key words: Clinical effects
Question 4: What is the therapeutic window of the new intervention? Key words: Therapeutic window
Question 5: How does the variability in the target population's response affect the development? Key words: Target population
Valuta \$M
Development limits 175 \$M, overall costs 15%, overall probability of success 800 \$M estimated revenues
Input
Question 1: Site of action Succes rate: 74% Cost: 35 \$M
Question 2: Functional effect Succes rate: 50% Cost: 35 \$M
Question 3: Clinical effects Succes rate: 74% Cost: 35 \$M
Question 4: Therapeutic window Succes rate: 74% Cost: 35 \$M
Question 5: Target population Succes rate: 74% Cost: 35 \$M

to the increased pressure on the high-cost, high-risk valley-of-death analogy mentioned in our introduction (Dessain and Fishman, 2017), exploratory studies are perceived differently by small biotech companies than by “Big Pharma.” Small clinical studies are sometimes aimed at a fast exit for small companies. The exploratory aspect is disregarded; rather, the studies are used to tick the box of clinical development. This actually fuels a risk-averse approach in exploratory studies.

Alternatively, increased pressure (related to both time and investments) might drive small companies toward unwanted attempts to leap over the VoD by taking too much risk. Ignoring multifactorial risks can introduce unacceptable risk taking. This can have dramatic results,

as in recent history has shown (Kaur et al., 2016; Chaikin, 2017; Hünig, 2012; Attarwala, 2010), and clearly shows boundaries overstepped by translational scientists. Larger companies with an extensive pipeline of products view exploratory studies rather as a stepwise means of derisking the portfolio. This can lead to a linear, steady procedural progression rather than rigorous decision making. If the value of addressing the key issues is not appropriately valued, larger companies can generate a perception that exploratory studies add to overhead by introducing added costs and precious development time. Therefore Big Pharma might be geared to preserve a portfolio that misses out on opportunities to fully capture an NME’s value, which deflates overall productivity.



FIGURE 18.3 Depiction of different development routes.

Exploratory studies: the value perspective

It is important to realize that exploratory studies can be used to address the critical development questions safely and early, but they do not necessarily relate to the questions one on one. In other words, one question can be (partially) addressed by multiple studies at different points in time, and one study can (partially) address multiple questions. Also, questions can be customized, added, and deleted. The questions presented here may give some structure to the list but are obviously not set in stone. An optimal order of development questions can help to direct resources to derisk the product in an optimal way. Furthermore, discussing the success rates and efforts needed to answer the questions stimulates open, transparent interdisciplinary communication about the risks and optimal development route for each individual novel medical intervention.

In a recent analysis one of the authors looked at the valuation increase for projects that make it across the VoD, that is, projects for which a first exploratory clinical study was at least started. There appears to be a larger than ever before increase in value when this bridge can be provided (Truebel and Thurston, 2020). This holds true not only for single (biotech) projects but also for portfolios of large pharmaceutical companies, in which, according to Paul et al. (2010), productivity $P = (WIP \times pTS \times V)/(CT \times C)$, where C represents costs, CT represents cycle time, pTS represents probability to success, V represents value, and WIP (i.e., work in progress) reflects the number of projects in a pipeline.

One can argue that exploratory studies can help to maximize a portfolio through increasing the pTS (e.g.,

through learnings in an exploratory study) while increasing the value of V (e.g., different indications appear feasible). Furthermore, terminating a project early might decrease the overall project costs C , further positively affecting the overall equation. Both perspectives represent in essence company internal views. When another market external dimension is added (i.e., value for society), the V in the equation can in essence also be related to future unmet medical needs for patients and society at large (Vennemann et al., 2019).

Summary: challenges and outlook

Targeted exploration focuses on the true unmet medical need for patients. As society drives development toward a clear added value rather than solely toward positive safety and efficacy, the balance of optimal development strategies changes. After optimizing the procedural path to registration and the financial apparent shareholder value creation by licensing and takeover strategies, modern drug development fully integrates the value of optimally addressing the key issues. Obviously, this requires an early definition of the value for society and subsequent early evaluation of this potential in development. This is in turn reflected by the increasing efforts to incorporate health technology assessment in development, making a strong case for further integrating flexible, translational approaches in development of innovative medical interventions and incorporating appropriate valuation tools that reflect these efforts.

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Chapter 19

Adaptive trial design

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Abstract

At earlier stages of clinical development, a static type of trial design to cover phases I–III may seem to be suboptimal. New findings from the running trial, from another trial involving the same compound, or even from unrelated trials testing novel biomarkers may render the original trial design either outdated or insufficient even while the trial is still ongoing. As a common example, one could envision the impact of dose-finding studies on running clinical trials in which the dose estimate from previous experiences, including animal results, may have been used in a prospective larger trial but was found to be insufficient in those newer trials or even in the earlier subjects of the same trial. Thus the ability to adapt a given trial design during the course of its execution seemed highly desirable, and a clinical trial design that allows for prospectively planned modifications to one or more aspects of the design based on accumulating data from subjects in the trial seems to be most appropriate. This adaptive trial design will be discussed in this chapter.

Keywords: Clinical trials; phases I–III; adaptive trial design; flexibility

The scientific value of a clinical trial critically depends on the exclusion of bias that would render the results invalid. For example, if 100 readouts of clinical trials were available and the choice of readouts was unregulated, one would always find five significant results, on average, if the alpha error was set to 5%. This is the minimal hit rate for positive readouts if there is absolutely no interventional effect in these trials, thus representing chance findings. To avoid this, a prospective trial will be designed in a very specific manner in that, for example, the main readouts (endpoints) are prespecified and classified as primary and secondary endpoints. The statistical construct will assume that there is only one primary endpoint (or maybe two coprincipals), and truly positive finding will be accepted only if the preset chance of 5% chance finding level is surmounted. The same need to prespecify trial details to avoid bias holds true for a long

list of study parameters, such as inclusion/exclusion criteria, number of patients or subjects to be included, duration of the intervention, blinding, statistical analysis, and interpretation of readouts.

This strict interpretation of prospective planning yields the highest possible relevance and reliability of conclusions drawn from a clinical trial. It is still the leading design for late-phase trials, such as phase IIb or phase III trials. In these trials the main properties of new drugs or devices, that is, efficacy and safety, need to be proven beyond a reasonable doubt. This is best achieved through the fixed design described above, prespecified at all levels of sophistication, and of course other major features of gold standard trials (controlled, double-blind, randomized, prospective trials).

At earlier stages of clinical development this static type of trial design may seem to be suboptimal because new findings from the running trial, from another trial involving the same compound, or even from unrelated trials testing novel biomarkers may render the original trial design either outdated or insufficient even while the trial is still ongoing. As a common example, one could envision the impact of dose-finding studies on running clinical trials in which the dose estimate from previous experiences, including animal results, may have been used in a prospective larger trial but found insufficient in those newer trials or even in the earlier subjects of the same trial. Continuing the trial according to its original design would be a waste of time and money but, above all, would represent an ethical problem because it is quite foreseeable that no results of clinical importance are likely to emerge. This implies that the subjects taking the test drug would have been exposed to avoidable risk that is clearly unacceptable under ethical standards.

Thus the adaptation of a given trial design during the course of its execution seemed highly desirable, and in 2006 the U.S. Food and Drug Administration (FDA) ([U.S. Department of Health and Human Services, Food and Drug Administration, 2006](#)) and the European Medicines Agency ([European Medicines Agency, 2006](#)) published

the first guidelines on the adaptation of clinical trial designs. In 2019 the FDA published a more extensive and elaborate document on this topic ([U.S. Food and Drug Administration, 2019](#)).

In this document, “an *adaptive design*” is defined as a clinical trial design that allows for prospectively planned modifications to one or more aspects of the design based on accumulating data from subjects in the trial.”

This definition restricts the use of the term to adaptations derived only from data from the same study. However, by a broader definition, the term is also used for such modifications resulting from data of other studies or sources, as mentioned previously. Invariably, it is important that the adaptations must be planned prospectively (and should be discussed with the authorities beforehand); if it is not planned as an adaptive trial, modifications of the design during a running trial are very difficult and hard to analyze statistically. It is often unpredictable whether authorities accept modifications of a trial design as an amendment allowing for continuation of the original study or require a new study to be planned and executed from scratch.

Two levels of data impact need to be separated: design modification in response to blinded and unblinded data. Responses to unblinded data (interim analyses of clinical endpoints with intervention/control comparisons) may essentially weaken the value of results or render the entire study invalid. Responses to blinded data, such as the increase in

subjects if aggregated endpoints (sum of endpoints in all study arms with blinding maintained) are below expectation, are much less troublesome and may even be imposed in an acceptable amendment if not prospectively planned.

Examples for modifiable parameters proposed in the recent FDA document are given in [Table 19.1](#). The document further details statistical approaches comprising appropriate modeling, for example, by Bayesian approaches (for details, see also [Stallard et al., 2020](#)). There is an increasing number of software tools to support adaptive trials ([Grayling and Wheeler, 2020](#)).

Depending on the main areas of modification, different types of adaptive trials may be separated. [Chow \(2014\)](#) classifies adaptive trials into the following categories:

- An adaptive randomization design
- An adaptive group sequential design
- A flexible sample size reestimation design
- A drop-the-losers design
- An adaptive dose-finding design
- A biomarker-adaptive design
- An adaptive treatment-switching design
- An adaptive-hypothesis design
- A phase I/II (or II/III) adaptive seamless trial design
- A multiple adaptive design

Most classifiers are self-explanatory. Adaptive randomization means adjustment of randomization schemes,

TABLE 19.1 Possible study design modifications in adaptive design clinical studies.

- **Group Sequential Designs:** allow for one or more prospectively planned interim analyses of comparative data with prespecified criteria for stopping the trial
- **Adaptations to the Sample Size:** to prospectively plan modifications to the sample size based on interim estimates of nuisance parameters from analyses that utilize treatment assignment information
- **Adaptations to the Patient Population (e.g., Adaptive Enrichment):** consideration could be given to a design that allows adaptive modifications to the patient population based on comparative interim results
- **Adaptations to Treatment Arm Selection:** to prospectively plan modifications to the treatment arms included in the clinical trial based on comparative interim results
- **Adaptations to Patient Allocation:** adaptations based on comparative baseline characteristic data and adaptations based on comparative outcome data
- **Adaptations to Endpoint Selection:** a design that allows adaptive modification to the choice of primary endpoint based on comparative interim results
- **Adaptations to Multiple Design Features:** It is possible for a clinical trial to be more complex by combining two or more of the adaptive design features discussed in this guidance.
- **Adaptations in Time-to-Event Settings:** additional considerations specific to adaptive trials in which the primary endpoint is the time to occurrence of a certain event, such as time to death or time to tumor response.
- **Adaptations Based on a Potential Surrogate or Intermediate Endpoint:** an adaptive design can be based on the potential surrogate or intermediate endpoint.
- **Secondary Endpoints:** adaptive designs can have consequences for the analysis of these secondary endpoints
- **Safety Considerations:** adaptive design clinical trial planning often focuses on outcomes intended to demonstrate effectiveness, however, safety objectives may also play a critical role
- **Design Changes Based on Information From a Source External to the Trial:** Unpredictable events that occur outside of an ongoing trial may provide important new information relevant to the ongoing trial and may motivate revisions to the trial design

especially in studies with small sample sizes. In adaptive group sequential design studies, a variety of parameters may be varied (sample size, treatment arms, study endpoints, dose, and/or treatment duration). In the drop-the-loser design, study arms without expected results may be closed and replaced by new study arms, for example, at higher doses. In the adaptive treatment-switching design, a patient may be switched from the unsuccessful study arm to a successful arm; this approach is mainly used in cancer studies. In the phase I/II (or II/III) adaptive seamless trial design, two phases of clinical development are combined in one study. In the phase I/II design, the maximal tolerable dose may be defined first and then applied to test efficacy and safety in patients in the same trial.

Adaptive design clinical studies are increasingly used in drug development. In a recent survey on 142 adaptive design trials (Bothwell et al., 2018), prevalent types of adaptations were seamless phase II/III (57%), group sequential (21%), biomarker adaptive (20%), and adaptive dose-finding designs (16%).

The increasing use of the adaptive trial design is the response to its obvious benefits (Chow and Corey, 2011):

1. The investigator may correct wrong assumptions made at or even before the beginning of the trial.

2. The investigator may choose the most promising option early.
3. The design allows for using emerging external information to improve the trial.
4. The design opens the opportunity to cope with unexpected events.
5. The design helps to speed up development process.

Conversely, there are major concerns about disadvantages because every adaptation, even if prespecified, may carry the risk of bias (selecting the good, avoiding the bad).

Three adaptive design types have been compared for benefits and challenges or obstacles (Chow and Corey, 2011).

As can be seen in Table 19.2, the downsides are related mainly to statistical problems but also to loss of information. A major concern of authorities is also that statistical power is lost and biases could be introduced by adaptive trial design, in particular if unblinded data are used for modification. Conversely, the use of blinded data is seen as much less critical.

The statistical background of adaptive design clinical trials is given in Chapter 26, Orphan drugs: why is translation so successful?.

TABLE 19.2 Comparison of benefits and risks for three adaptive trial design types.

Design	Flexibility/benefits	Challenges/obstacles
Adaptive randomization design	<ul style="list-style-type: none"> • Unequal probability of treatment assignment • Assign subjects to more promising treatment arm 	<ul style="list-style-type: none"> • Randomization schedule not available prior to the conduct of the trial • Not feasible for large trials or trials with long treatment duration • Statistical inference is often difficult, if not impossible, to obtain
Adaptive dose-finding design ^a	<ul style="list-style-type: none"> • Drop inferior dose group early • Modify/add additional dose groups • Increase the probability of correctly identifying the maximum tolerated dose with limited number of subjects 	<ul style="list-style-type: none"> • Selection of initial dose • Selection of dose range under study • Selection criteria and decision rule • Risk of dropping promising dose groups
Two-stage seamless adaptive design (either phase I/II or phase II/III)	<ul style="list-style-type: none"> • Combine two studies into a single study • Fully utilize data collected from both stages • Reduce lead time between studies • Shorten the development time • Additional adaptations such as drop-the-loser, adaptive randomization, and adaptive hypothesis may be applied at the end of the first stage 	<ul style="list-style-type: none"> • The control of the overall type I error rate • Sample size calculation/allocation • How to perform analysis based on combined data collected from both stages? • Is the O'Brien-Fleming type of boundaries feasible?

^aFor example, adaptive dose escalation designs for cancer trials.

From Chow S.C., Corey R., 2011. Benefits, challenges and obstacles of adaptive clinical trial designs. *Orphanet J. Rare Dis.* 6, 79, Open Access.

To illustrate the utility of this type of design, a simple example is given here for illustration, as published by [Veal et al. \(2013\)](#).

The compound 13-cis-retinoic acid is used as treatment for high-risk neuroblastoma patients. It is sensitive to a pharmacogenetic variation (cytochrome P450 polymorphisms in variants 2C8, 3A5, and 3A7). Thus fixed dosing in patients under 12 kg of body weight resulted in plasma levels below 2 µmol/L in 8 of 11 patients. The dose regimen had to be adjusted to reach sufficient exposure. This dosing by plasma levels is one of the most basic and valuable examples for an adaptive design study.

Another example for an adaptive trial design demonstrates that accumulating data will govern patient allocation to different drug doses to improve the gain in knowledge that would not be possible if the number of subjects in the study arms were invariably predefined ([Geiger et al., 2012](#)).

AWARD-5 was a double-blind randomized, placebo-controlled, adaptive, dose-finding, inferentially seamless phase II/III study with individualized dosing. In the original plot of the study protocol ([Geiger et al., 2012](#)), two randomization schemes were proposed and divide the study into two stages: adaptive randomization (stage 1) and fixed randomization (stage 2). Seven doses of dulaglutide, a glucagon-like-peptide 1 analog, should have been evaluated in stage 1 along with comparators. Patients were adaptively allocated across these doses on the basis of accumulating data. The decision point was defined as the time when sufficient information has accrued to either select dulaglutide doses or stop the trial. When dose selection was done, stage 2 should began. Only patients who were assigned the selected doses and comparators continued to be followed; all others were anticipated to stop further participation in the trial. Additional patients were enrolled and were assigned to the comparators or dulaglutide. In the final trial, two out of the seven tested doses of dulaglutide were chosen at the decision point, namely, 0.75 and 1.5 mg/day, for the long-term intervention. Thus this seamless trial was both a dose-finding trial (at the beginning) and a phase III trial to test efficacy and safety of the selected doses in a long-term setting. For both doses of dulaglutide, superiority over sitagliptin was demonstrated at week 52 ([Nauck et al., 2014](#)).

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Chapter 20

Combining regulatory and exploratory trials

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Abstract

Regulatory authorities have issued clear guidelines about the clinical studies that are necessary to support market approval of a new drug. Early human experience with new compounds appeared as one of main strategies to cope with the challenge of attrition for drug development at all stages, but this aspect is not fully covered by the regulatory set of mandatory studies. This was the background for so-called phase 0 studies, in which early orientation about human pharmacokinetics could be found, for example, by microdosing or by understanding the mode of action, the quality of novel or traditional biomarkers, or the pathophysiological processes that seem to be essential for proper profiling of a new compound. In this chapter, trial designs are discussed that combine regulatory and nonregulatory aspects of drug development.

Keywords: Regulatory clinical trials; nonregulatory aspects of clinical trials; combined trial designs; reduction of attrition

Regulatory authorities have issued clear guidelines about the clinical studies that are necessary to support market approval of a new drug. Typically, such regulatory trials (meaning that they are required by authorities) are allocated to three or four phases of clinical development. Phase I mainly looks at pharmacokinetics (PK) in healthy volunteers if possible (with exceptions for toxic drugs, e.g., in oncology, which have to be explored exclusively in patients for ethical reasons) to determine the fate of the drug in the human body. This is normally done by using single ascending dose (SAD) and multiple ascending dose (MAD) studies with major interest in plasma levels, route of excretion, metabolites, and parameters of distribution. In phase II, efficacy and safety are tested in a smaller group of patients (up to 400). In phase IIa the very important dose finding for clinically relevant effects is mandatory. In phase IIb, doses are applied to patients to prove efficacy and safety at a basic level (so-called proof of concept). In phase III (so-called pivotal trial), all previous

data are corroborated, and thereby, the safety and efficacy of a new drug in the given patient population are finally established; this is the ultimate prerequisite for market approval. In general, the emphasis at this stage is on safety, as this aspect of drug development usually requires a larger patient number than proof of efficacy. Phase IV describes data from studies performed after market approval, which may be mandated by authorities to extend approval beyond preset time frames.

This somewhat idealistic linear sequence of clinical trials has grave limitations in drug development; it would be fine if the success of a novel compound were granted in all cases, which, however, is not true for 99% of all projects on new compounds (see Chapter 2: Problems, challenges, and initiatives in translation). Late attrition of projects is costly and time-consuming; the need to reduce it has been the major driver behind the hype about translational medicine in industry.

Therefore early human experience with new compounds appeared as one of main strategies to cope with that challenge, but that aspect is not fully covered by the regulatory set of mandatory studies. This was the background for so-called phase 0 studies in which early orientation about human PK could be found, for example, by microdosing. In addition to early pharmacokinetic data, understanding the mode of action, the quality of novel or traditional biomarkers, or pathophysiological processes in conjunction with a new compound seems to be essential for the proper profiling of a new compound. This may even include the identification of a disease treatable by this compound.

Upon reflection of this demand, the term *exploratory clinical trial* (see Chapter 10: Human studies as a source of target information) was coined, with a variety of synonyms such as *physiological study* and *nonregulatory trial*. The latter synonym should not be confused with the term *nonregulated*, which is absolutely not true for these studies; they need to be performed under the same auspices of

subject protection, proper statistical planning, and good clinical practice-conforming conduct as do regulatory studies, including the requirement of informed consent and Institutional Review Board consultation.

According to the main related U.S. Food and Drug Administration (FDA) document ([U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research CDER, 2006](#)), an exploratory investigational new drug (IND) study can help sponsors to do the following:

- Determine whether a mechanism of action defined in experimental systems can also be observed in humans (e.g., a binding property or inhibition of an enzyme)
- Provide important information on PK
- Select the most promising lead product from a group of candidates designed to interact with a particular therapeutic target in humans, based on PK or pharmacodynamic properties
- Explore a product's biodistribution characteristics using various imaging technologies

On the FDA's current website ([US Food and Drug Administration, 2020](#)), "the phrase exploratory IND study is intended to describe a clinical trial that

- is conducted early in phase 1,
- involves very limited human exposure, and
- has no therapeutic or diagnostic intent (e.g., screening studies, microdose studies)."

This definition also includes the term *IND*, which is used to describe a precursor or low-dose application of a drug candidate. Thus the term *exploratory clinical trial* is used more broadly than just to describe a trial on an IND. While early documents on exploratory studies and phase 0 studies emphasized pharmacokinetic profiling (e.g., by microdosing), other aspects have gained more interest recently, especially those relating to biomarker development and validation, mode of action, and disease subclassification (personalization). An exploratory clinical trial may

be combined with regulatory trials in that the drug candidate is studied in the regulatory set of studies (SAD or MAD studies), but nonregulatory aspects may be added to the design to add valuable information on top of data demanded by the regulatory process. A given study may measure the established outcome biomarker for proof of concept in phase IIb but at the same time generate valuable data on other biomarkers to be established and developed. This may be exemplified by studies on cancer treatment. In studies on solid tumors, response to treatment may be measured by the RECIST criteria ([Eisenhauer et al., 2009; Gomez-Roca et al., 2011](#)), which are mainly based on tumor diameters and new lesions. However, the rapid progress of modern imaging techniques has given rise to more sophisticated and stratifying biomarkers that may be used after further exploration and refinement in the future. **Table 20.1** shows how novel cancer biomarkers may evolve from regulatory trials to which exploratory components are added ([Cousin et al., 2012](#)).

Fig. 20.1 demonstrates how an imaging procedure may affect the future readouts of related studies. While RECIST criteria would indicate stable disease, imaging clearly shows massive regression/necrosis but also a relapse of tumor growth over time ([Cousin et al., 2012](#)).

Meanwhile, such strategies have led to more refined RECIST criteria, such as iRECIST reflecting pseudoprogression in immunotherapeutic situations ([Seymour et al., 2017; Dromain et al., 2020](#)). An imaging example for the application of iRECIST is shown in **Fig. 20.2**.

With this approach representing the profiling of novel imaging techniques as one example for exploratory aspects of regulatory studies, obviously the same approach may be utilized for serum biomarkers. In a regulatory study on different anti-HIV treatments a substudy was embedded in which inflammatory biomarkers were profiled as predictors of disease response or progression. As can be seen in **Fig. 20.3** ([McComsey et al., 2014](#)), commonly used biomarkers of inflammation (high-sensitivity C-reactive protein, interleukin 6, and the soluble receptors for tumor

TABLE 20.1 Stepwise validation of exploratory data generated by modern imaging as potential classifier biomarker.

Step 1	Carry out a large phase II trial or several parallel phase II trials (100–200 patients). Collect the data provided by the new imaging tool and the data provided by the gold standard method (e.g., RECIST ^a). Collect the response rate according to the gold standard.
Step 2	Compare in responders and nonresponders the distribution of the potential classifier biomarkers generated with the different methods of analysis. Determine the optimal thresholds (receiver-operator curves).
Step 3	Determine the performance of the potential classifier biomarkers: the discrimination (sensitivity, specificity, positive predictive value, negative predictive value, accuracy) and the calibration (e.g., Brier score).
Step 4	External validation with the use of a large multicenter prospective trial (clinical usefulness).

^aRECIST = Response Evaluation Criteria in Solid Tumors.

Source: From Cousin, S., Taieb, S., Penel, N., 2012. A paradigm shift in tumour response evaluation of targeted therapy: the assessment of novel drugs in exploratory clinical trials. *Curr. Opin. Oncol.* 24, 338–344, by permission of Wolters Kluwer Health, Inc.

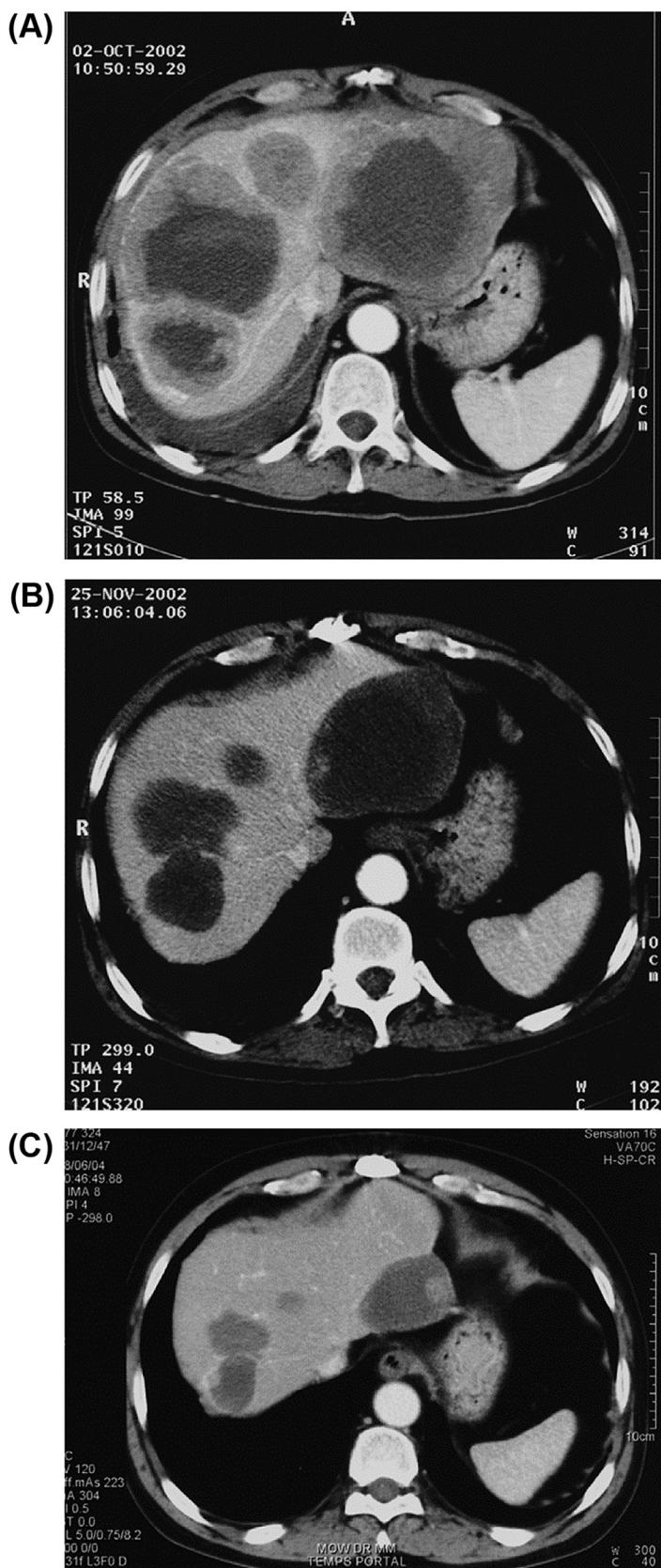


FIGURE 20.1 Assessment of liver metastasis from gastrointestinal tumor treated by imatinib mesylate. (A) Baseline assessment. (B) Assessment after 6 weeks, showing massive necrosis of all target lesions (stable disease according to RECIST, partial response according to Choi criteria). (C) Assessment 2 years later, showing nodules in the mass and an increase in tumor density (stable disease according to RECIST, progressive disease according to Choi criteria). From Cousin, S., Taieb, S., Penel, N., 2012. A paradigm shift in tumour response evaluation of targeted therapy: the assessment of novel drugs in exploratory clinical trials. *Curr. Opin. Oncol.* 24, 338–344, by permission of Wolters Kluwer Health, Inc.

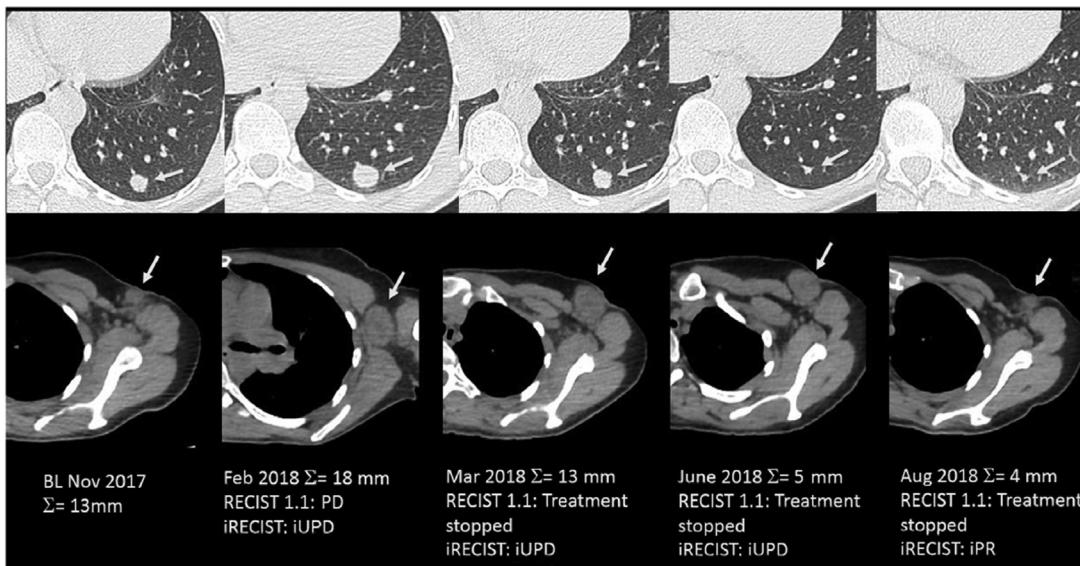
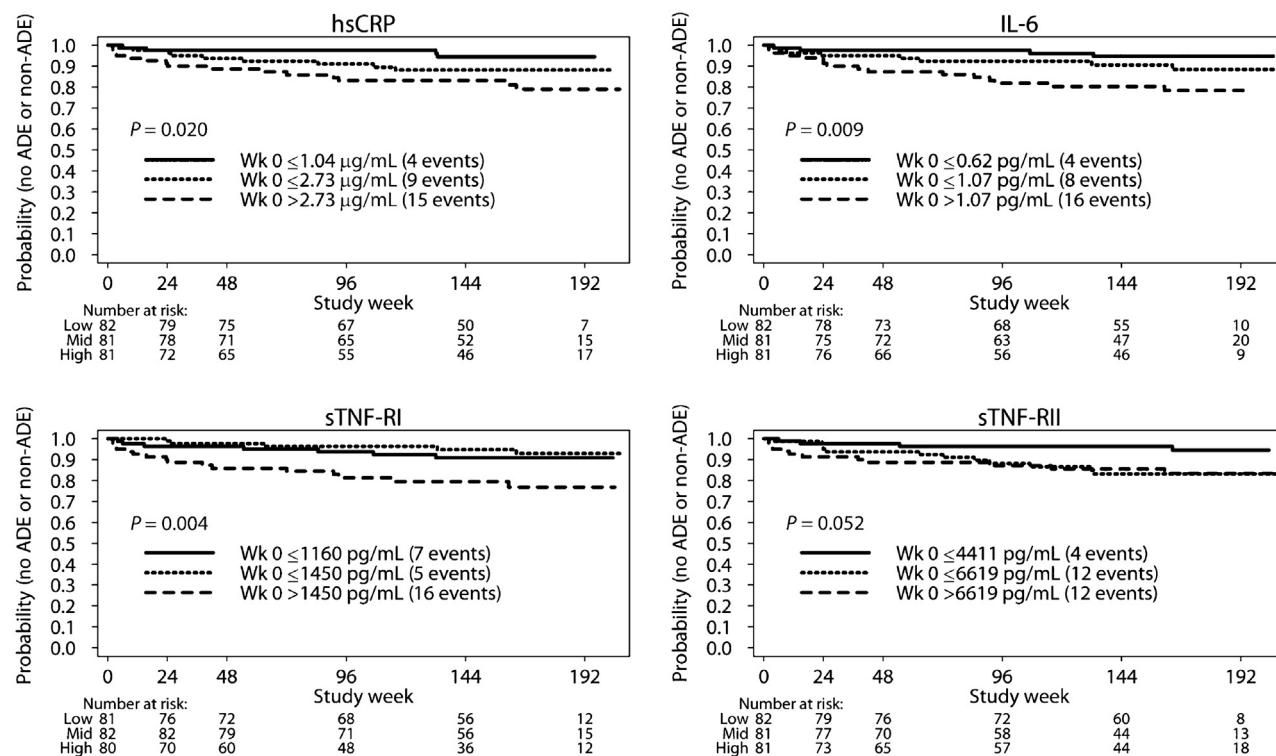


FIGURE 20.2 Comparison of RECIST 1.1 and iRECIST criteria for evaluation of a 45-year-old woman with metastatic melanoma treated with ipilimumab (antiCTLA-4) and nivolumab (anti-PD-1). The baseline CT image (November 2017) shows a 13-mm lung metastasis (target lesion, *upper panel*, arrow) and a 10-mm short-axis axillary lymph node (non-target lesion, *lower panel*, arrow). On a 3-month follow-up, both lesions were enlarged, with an increase of 38% of the target lesion leading to a progressive disease (PD) and a stop of treatment according to RECIST 1.1 and an unconfirmed PD with treatment maintained according to iRECIST criteria. On the two following CT examinations (March and June 2018) the lung metastasis had decreased in size, but the axillary lymph node was stable (unconfirmed PD) but still significantly enlarged compared to the baseline (still unconfirmed PD according to iRECIST criteria). Finally, on August 2018, CT images showed a decrease in size of both lesions, confirming the pseudoprogression with a response assessed to be -70% , leading to a partial response according to iRECIST criteria. From Dromain, C., Beigelman, C., Pozzessere, C., Duran, R., Digkia, A., 2020. Imaging of tumour response to immunotherapy. Eur. Radiol. Exp. 4 (1), 2.



ADE = AIDS-defining event

FIGURE 20.3 Kaplan-Meier plots stratified by tertiles for high-sensitivity C-reactive protein (hsCRP), interleukin 6 (IL-6), and the soluble receptors for tumor necrosis factor alpha, type I (sTNFR-I) and II (sTNF-RII). Samples were from a substudy (A5202) of AIDS Clinical Trials Group A5224s in which different antiviral regimens were tested in HIV patients. AIDS-defining events (ADE) and non-AIDS-defining events were measured. From McComsey, G.A., Kitch, D., Sax, P.E. et al., 2014. Associations of inflammatory markers with AIDS and non-AIDS clinical events after initiation of antiretroviral therapy: AIDS Clinical Trials Group A5224s, a substudy of ACTG A5202. J. Acquir. Immune. Defic. Syndr. 65, 167–174, by permission of Wolters Kluwer Health, Inc.

necrosis factor alpha, types I and II) correlated well with AIDS-defining events (and non-AIDS-defining events). Thus patients who are at high risk of developing AIDS as defined by those novel biomarkers may be stratified into more intense treatment schemes or may receive second-line treatments earlier.

Another very important area of exploratory clinical trials is the understanding of the disease to be exploited as a source of druggable targets. For example, exploratory aspects were added to a weight-loss intervention, and several adipokines and other metabolically relevant parameters were measured. A correlation between quality-of-life measures and leptin was found, which should help to further understand its role in weight regulation; ultimately, the understanding of this role could guide new drug development and finally support weight loss and weight-associated diseases such as cancer (Linkov et al., 2014). The search for a therapeutic application of leptin itself is still ongoing. Meanwhile, an analog, metreleptin, is being applied to patients with lipodystrophy (Grewal et al., 2019). The search for a widely applicable antiobesity drug has been disappointing so far. New routes of leptin application are being discussed (Khafagy et al., 2020).

In the trial mentioned above, the exploratory aspects have not been combined with a regulatory trial demonstrating that exploratory aspects may be implemented in almost all interventional studies. The given example is typical for academic research, which is still the main source of disease understanding as a prerequisite for successful drug development. Thus the term *exploratory trial* has a much wider use than only describing drug-related trials; in general it describes any human experiment that cannot directly be used in regulatory processes. Such trials may guide drug development with a great impact on speed and quality and need to be considered especially at the earliest human experiences with new drug candidates. As there is tremendous pressure to streamline clinical development and avoid costly late attrition, the intertwining of regulatory (“must do”) and exploratory trials is pivotal for success, and their integration into trials fulfilling

both goals (“must do,” “need to know”) represents a valid and successful strategy.

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Chapter 21

Accelerating proof of concept by smart early clinical trials

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Abstract

The time required to develop a new drug takes over 12 years and often much longer, and its development is very expensive. This dilemma was the main reason for launching translational medicine as a potential remedy. From the beginning, it was clear that a pivotal early step in clinical development is the proof-of-concept (PoC) study, typically obtained in the phase IIb clinical trial. It is only a natural consequence to adapt the development strategy for a new drug to obtain a positive PoC as early as possible. This goal was one of the major objectives of the Critical Path Initiative in the United States and similar constructs elsewhere. In this chapter, methods and pathways to accelerate PoC are discussed and examples given.

Keywords: Early proof of concept; accelerating drug development; phase IIb trials; Critical Path Initiative

Newer estimates about the costs of a new drug have been claimed to range from U.S. \$2.6 billion (DiMasi et al., 2016) to \$5 billion (Herper, 2013), reflecting the costs not only of successful but also of all failed drugs. A current, more specific cost estimate reports an average of U.S. \$985 million per product, ranging from \$323 million for a less costly therapeutic area (lower limit of the 95% confidence interval for nervous system agents) to \$5366 million for the most expensive therapeutic area (upper limit of the 95% confidence interval for antineoplastic and immunomodulating agents) (Wouters et al., 2020). The divergent numbers reflect the problem of accurately assessing the cost of drug development, as investments may not be fully reported, especially for failed drugs, or may be shared among several projects.

Developing a new drug takes over 12 years and often much longer (DiMasi et al., 2010). The rapid increase in both costs and time has many causes, including highly sophisticated regulation of clinical trials to protect participants by

complex safety surveillance and reporting, strict and extensive rules regarding preclinical toxicology data, and reduced margins of success in the light of competing drugs—to name just a few culprits. It is obvious that the ever-shrinking remaining patent life of 5–15 years, which guarantees a high return on investment after market approval, cannot generate enough funds to maintain this model of exorbitant costs and excessive development times.

This dilemma was the main reason for launching translational medicine. From the beginning, it was clear that a pivotal early step in clinical development is the proof-of-concept (PoC) study, typically obtained in the phase IIb clinical trial. As can be seen in Figure 10.2 (Chapter 10: Human studies as a source of target information), this is the time at which the highest (relative) value increment occurs because the translational risk drops dramatically if a positive (clinical) PoC is reached. This still does not guarantee market success because safety or patient stratification issues may be detected only in the very large phase III trial(s), but it reduces the risk of failure considerably.

It is only a natural consequence to adapt the development strategy for a new drug to obtain a positive PoC as early as possible. This goal was one of the major objectives of the Critical Path Initiative in the United States (U.S. Food and Drug Administration. Critical Path Initiative, 2020) and the Innovative Medicines Initiative in Europe (Goldman, 2011). This initiative was originally derived from the New Safe Medicines Faster initiative (European Federation for Pharmaceutical Sciences, 2014) by the European Federation for Pharmaceutical Sciences. The wording already implied one of main objectives: to speed up drug development.

As these compelling forces were behind the new movement called *translational medicine*, almost all efforts in the context were aimed at an accelerated PoC as a prerequisite

of early, less-costly attrition and shorter timelines in development. Thus this book detailing the manifold aspects of translational science in medicine could also be seen as supporting accelerated PoC in its entirety.

As was shown in Chapter 10, Human studies as a source of target information, the translational risk critically depends not only on the quality of biomarkers, among other factors, but also on the early program of clinical studies. This chapter focuses on the translational power and its improvement in the early studies of clinical development. Clinical studies that are optimized under these auspices (gaining a maximum of translational power out of the smallest number of subjects studied for the shortest possible duration of an intervention) are called *smart early clinical trials* here. This term is not in common use, nor has it been defined elsewhere; it should just underline the potential of shaping the early trial program in a translationally relevant way to reach the objectives mentioned previously.

The following short list presents the major goals of smart early human trials:

- To render proof of mechanism (PoM), proof of principle (PoP), and proof of concept (PoC)
- To validate biomarkers with particular emphasis on patient stratification and responder concentration
- To find effective and safe dosing, a key issue in designing successful phase II and III trials
- To gather as much information as possible in the least feasible number of subjects
- To profile competitive developments early on

The terms *PoM*, *PoP*, and *PoC* are defined in Chapter 16, Translational medicine in psychiatry: challenges and imaging biomarkers. Clearly, PoM is often achieved in pharmacokinetic regulatory phase I trials. Measurements of serum biomarkers may be added, which are proximal effectors of the receptor addressed by the drug. Thus adding a little complexity by drawing few additional milliliters of blood may demonstrate that the drug not only is seen at appreciable levels in the blood, but also reaches to the target in sufficient amounts. It only then may trigger the cascade of events that may eventually lead to clinical effects.

More sophisticated biomarkers could be added, such as imaging of radiotracers to visualize the distribution of a drug in the body, in particular its entry into the central nervous system. These are just a few examples for combining regulatory trials with exploratory aspects, thereby gaining much more information in much shorter times.

Phase 0 studies (microdosing), adaptive trial design, combining phase I and II, and exploratory trials are key components of smart designs, which are essential for successful early human trials.

The main avenues to achieve acceleration of PoC are convincingly summarized in the session topics of a

scientific meeting organized by Cambridge Healthtech Institute (<http://www.healthtech.com/Proof-Of-Concept/>, accessed March 6, 2020):

- Precision & Personalized Medicine Initiatives and The Effects on PoC Decision Making
- Accelerating Clinical Decision Making: Patient Stratification Using Pharmacogenomics and Protein Biomarkers
- Novel Indications Development—New Strategies for Old Drugs
- Improving PoC Study Designs & Methods
- Novel Alternate Endpoints for Clinical PoC

The latter two points are directly related to the topic of this chapter (smart early clinical trials) and should be exemplified by a virtual case study on a new antiatherosclerotic drug. The unmet medical need is to reduce major cardiovascular events—mainly myocardial infarction and stroke—and related morbidity and mortality by altering the biology of atherosclerotic plaques in a manner that should reduce plaque progression and render lesions more stable, less inflammatory, and less thrombogenic. Thus stabilization of the vulnerable plaque is the goal (Hafiane, 2019), and so far the most effective principles are statins (cholesterol-lowering drugs), which were proven to reduce plaque inflammation and clinical endpoints (major cardiovascular events, death).

In a traditional model (Fig. 21.1), a two-stage set of early human trials would try to prove PoP in a 3-month trial demonstrating PoM markers (direct products of the target, e.g., in the lipoxygenase pathway), inflammatory serum markers (e.g., high sensitivity C-reactive protein), and measurements of vascular function (e.g., nitric oxide-mediated vasodilation) to change as expected. However, this might indicate but would not prove that the vulnerable plaque is stabilized, that its inflammatory activity is reduced, which would be required as PoC (this still does not prove that major clinical endpoints, including deaths, are reduced; this needs to be proven in the large phase III trial). PoC would require a second study over 1–1.5 years in which plaque size and morphology would be measured by intravascular ultrasound and magnetic resonance tomography (MRT). The entire time to PoC starting from phase IIa (proper dose finding) to phase IIb (PoC) would be 2 years. In a smart

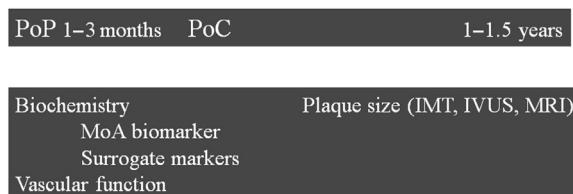


FIGURE 21.1 Phase II traditional scenario (antiatherosclerotic drug): low risk—slow pace, two studies, PoP and PoC biomarkers separated.

study design, modern biomarkers for plaque imaging would be employed (Fig. 21.2). In particular, MRT with or without molecular markers or gadolinium- or iron-containing contrast media positron emission tomography with or without computed tomography (CT) are promising readouts to rapidly demonstrate plaque stabilization (Fleg et al., 2012). Ultrasound and histology methods (e.g., in atherectomy samples from carotid surgery) could be used as well. Because atherosclerosis is a generalized disease, easily accessible plaques, for example, in the carotid artery, may serve as indicator plaques for the disease in smaller, hidden arteries, such as the coronaries. Meanwhile, the spectrum of potential biomarkers has become even wider and now includes CT, spectroscopy, palpography, virtual histology, optical coherence tomography, and high-frequency intravascular ultrasound, which are discussed as biomarkers for plaque instability (Hafiane, 2019).

The combination of such biomarkers directly visualizing drug effects on the plaque morphology and stability with PoM markers in one trial would cut the time to PoC down to 3–6 months. This means a gain of 1–1.5 years in the development of such a drug.

A similar example is given in Chapter 20, Combining regulatory and exploratory trials, for cancer (Figures 20.1 and 20.2), in which more powerful imaging biomarkers would detect effects more rapidly and sensitively if used in future studies. An adaptive trial design would allow for using such novel biomarkers even if they are identified as being reliable, sensitive, and accessible just in the early segments of the same trial.

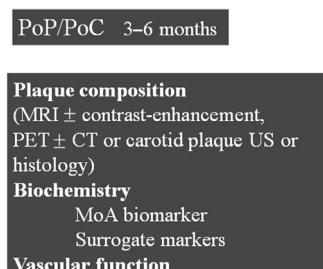


FIGURE 21.2 Phase II innovative scenario (antiatherosclerotic drug): higher risk—high speed, one study, PoP and PoC biomarkers combined.

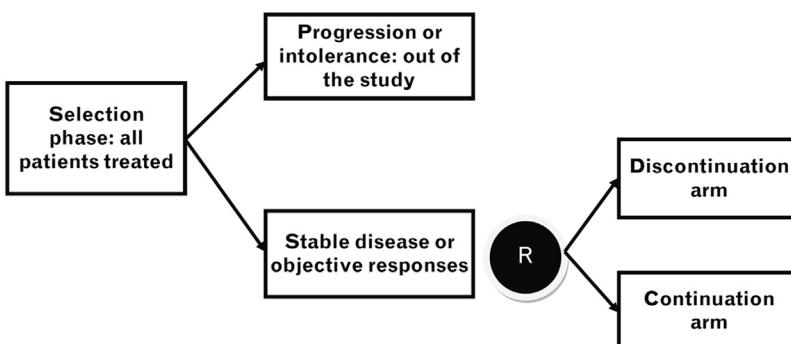


FIGURE 21.3 Discontinuation scheme for an adaptive design clinical trial exploring (early segment) and utilizing (later segments after interim analyses) a novel biomarker for stratification. *From Cousin, S., Taieb, S., Penel, N., 2012. A paradigm shift in tumour response evaluation of targeted therapy: the assessment of novel drugs in exploratory clinical trials. Curr. Opin. Oncol. 24, 338–344, with permission from Wolters Kluwer Health, Inc.*

Another important area for the acceleration of PoP relates to patient stratification and responder concentration (the first two and—in part—third points in the short list shown previously). In Chapter 10, Human studies as a source of target information, a famous (or, more accurately, infamous) example of the beneficial impact of responder concentration is given for gefitinib (Table 10.4). Patients with activating mutations in the *EGFR* gene were much more likely to respond to therapy for lung cancer than were those without this mutation. Obviously, there were indications for this association even before the unstratified trial was initiated, but they were not implemented in this study. Only after its failure a new trial was designed that included this stratification; this trial was successful enough to allow for market approval. At the time those studies were designed, the principles of translational medicine were just in their infancy, and the potential of adaptive trial design was not yet fully appreciated. Today, a smart early human trial combining phase II regulatory aspects and the need to find stratifying markers very likely would be addressed by an adaptive design. It would allow for interim analyses to detect the most useful markers to predict a positive response to be used in the later stages of the same trial, eventually leading to clear-cut, clinically relevant effects. As shown in Fig. 21.3, the novel markers will lead to discontinuation of treatment in the putative nonresponders and to responder concentration in the remaining sample (Cousin et al., 2012).

Of course, if a novel drug has already been specifically designed for a mutated receptor structure that has been identified beforehand, responder concentration will take place at the level of patient inclusion already in that only patients whose tumors expose the relevant mutation will be included. This applies to the following example for the development of successful treatments for malignant melanoma. B-RAF mutations are present in 50% of all melanoma tumors, and specific inhibitors (e.g., vemurafenib or, more recently, encorafenib) are being used successfully, sometimes in combination with MEK inhibitors also interfering with the MAP kinase pathway. Alongside this, novel immunotherapeutics (ipilimumab) are used.

TABLE 21.1 Stepwise model to characterize the mutational status of melanomas.

- (1) Screen for V600E *BRAF*^a mutation in melanoma patients with advanced disease (i.e., unresectable stages III and IV) as well as those at high risk of disease progression (stages IIIB and IIIC).
- (2) In case of negative-V600E *BRAF* mutation, look for other non-V600E *BRAF* mutations (i.e., K, M, R, D).
- (3) Melanomas not showing *BRAF* mutations should be investigated for *N-RAS* mutations.
- (4) Double-negative *BRAF* and *N-RAS* melanomas should be further explored for *KIT* mutations or amplifications. This is even more relevant for acral and mucosal melanomas that should be investigated for both *BRAF* and *KIT* mutations at the first step.
- (5) Triple-negative melanomas may benefit from *GNAQ* mutation evaluation, especially for uveal melanoma.

^aFor abbreviations, see the Human Genome Organization (<http://www.genenames.org/>).

Source: From Giovanni, P., Giovanni, P., Aldo, T., Pietro, L., Gabriele, L., Fabio, G., et al., 2014. Molecular targeted approaches for advanced BRAF V600, N-RAS, c-KIT, and GNAQ melanomas. *Dis. Markers* 2014, 671283, Open Access.

However, not all patients respond to B-RAF inhibitors, and they may have other relevant mutations (Giovanni et al., 2014). In a so-called targeted approach, a finer sub-classification of the individual set of relevant mutations should be utilized to target treatments to those mutations and use related inhibitors (for a review, see Cheng et al., 2018). Giovanni et al. (2014) propose a scheme of genetic analyses for currently known mutations (Table 21.1) as a sequential stratification approach. This scheme could be used in early clinical trials with adaptive design, which would not test a single intervention but would find the optimal markers and clinical effects of related inhibitors. After approval, the same approach would have to be used in individual patients as a mandatory combination of diagnostics (companion diagnostics) and treatment. Meanwhile, the refinement of various target mutants in the treatment of melanoma has become more complex with a multitude of pathways shown to contribute to growth, including the K-RAS (Dietrich et al., 2018) or the TERT promoter (Shaughnessy et al., 2020). With the larger variation in individual and combined strategies to inhibit tumor growth, an individualized cocktail of agents may be designed that is tailored to an individual patient's situation. This would result in different treatments for almost all individual patients. Whether this is called precision medicine or the ultimate form of individualized medicine is just a matter of wording. In any case, these very differentiated approaches aim at maximal responder concentration by the sophisticated molecular characterization of tumor cells.

Similar examples of molecular subclassification may now be given for almost all malignancies. In most cases such concentration strategies are key to clinical success, often resulting in considerable effects on tumor progression and even mortality.

If taken together, the proper stratification of patients and related responder concentration is one of the most promising approaches not only for clinical success, but also for the reduction of development times, in particular those up to clinical PoC.

As a commentary, it may be noted that this approach, however, is all but new; in the past, it was simply called *disease classification*. The main differences between disease classification 50 years ago and today are the range and power of biomarkers applied, mainly reflecting the breakthrough discoveries in genetics, known as “omics,” but also and maybe even more important, imaging and serum biomarkers.

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Chapter 22

Pharmaceutical toxicology

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Abstract

This chapter aims to highlight the core principles of pharmaceutical toxicology. It is an interrelated discipline that needs to be applied at all stages of the drug development process to appropriately characterise the safety profile of a drug compound and acknowledge the uncertainties associated with models available. With a strategic mindset, the preclinical safety activities aim to build a comprehensive profile of the drug so that potential hazards can be identified and the risks for healthy trial subjects or patients quantified, and, if necessary, suitable means for eliminating or reducing unacceptable risks can be put in place. We focus on the 2 distinct phases of pharmaceutical toxicology: Discovery toxicology and regulatory toxicology, to explain how the thinking is built upon at each stage and how the mindset shifts from enabling the selection of an optimally derisked clinical candidate through to thorough risk characterisation and management of those risks for clinical development. Considering attrition due to safety reasons, whether clinical or preclinical, is one of the main reasons for drug project failure, safety assessments should be viewed with equal importance as drug efficacy assessments.

Keywords: Regulatory toxicology; discovery toxicology; dose-response relationship; pharmaceutical safety; drug development; risk assessment

Introduction

All novel therapeutics have the potential to cause harm, so the quest for new medicines must include a comprehensive evaluation of the potential for adverse effects. Pharmaceutical toxicology is one of the few disciplines covering the entire span of drug discovery and development. It can be divided into two distinct phases: discovery toxicology, which seeks to identify an optimally derisked clinical candidate from amongst the thousands or tens of thousands of potential chemical leads, and regulatory toxicology, in which the objective is to characterize in detail, the toxicity of a single compound, in order to ensure

patient safety. Both disciplines require a clear understanding of the translational uncertainty of the data generated in preclinical models, a subject that will be addressed in more detail in the following chapter 23.

An important point to make at the outset is that the aim of the nonclinical safety testing of new drugs is not to demonstrate that a pharmaceutical is safe or to predict human toxicities. It is to characterize the properties of a molecule as it relates to potential human risks. This information will then be included as part of the clinical trial design. The emergence of a compound's safety profile is an incremental process that builds on preliminary data from the literature, *in vitro*, and *in vivo* preclinical models. It then builds through early clinical trials in relatively small controlled subject groups before broader, more diverse patient populations are ultimately exposed to the compound. Additional nonclinical safety data supplements the existing profile at each clinical stage and ensure that the correct monitoring procedures are included for the trial population. In many cases a drug's true safety profile will not be understood until after marketing approval, once use of the drug is widespread in real-world settings.

Safety assessment is not a single isolated discipline. It is important to understand the interrelationships among the different properties of a molecule and to adequately characterize its inherent risks for the populations exposed to the drug. Therefore it is necessary to build a strategy from early discovery to identify likely issues to be encountered as the project progresses. We will look at the drug development process and outline some of the common considerations and pitfalls at each stage. For each drug modality, whether small molecules, biologics, or any of a wide variety of novel approaches, it is useful to think about safety risk in the framework of the target, the chemistry, and the patient. This thinking can be applied to each type of therapeutic intervention to provide a bespoke set of key questions for the toxicologist. Whether these

questions warrant further investigation will depend on the level of risk suspected. These questions may be developed further as the project continues, may be discharged with mitigating data, or may simply be carried forward and become part of the project's risk log. Table 22.1 shows how early thinking about safety can be built upon at each stage of development.

Discovery toxicology

Drug development remains an expensive and high-risk endeavor. The high rate of project attrition throughout drug development is a major contributor to the increasingly unsustainable costs of a successful drug reaching the market (DiMasi et al., 2016). A comprehensive analysis of over 7000 drug projects from more than 1000 companies showed a low likelihood of approval for drugs in clinical trials between 2005 and 2016. For example, in oncology, only around 5% of compounds in phase I clinical trials ultimately made it to the market. Other therapy areas do not fare much better (Wong et al., 2019). Attrition due to safety reasons, whether clinical or pre-clinical, is one of the main reasons for drug project failure. In recent years, considerable investment has been made in the discovery phase to identify molecules carrying potentially unacceptable safety concerns and eliminate those from consideration much earlier. Therefore the toxicologist's aim within a research team environment is to work with colleagues to identify higher-quality clinical candidates with fewer potential safety liabilities and a better chance of long-term success. To achieve this, they must ask the right questions regarding the target, the chemistry, and the patient context. This is the discipline of discovery toxicology.

In this early phase, the aims of the discovery toxicologist contrast markedly with those of the regulatory toxicologist in a clinical development team. There are no prescribed study designs, no regulatory guidelines, and few expectations of what types of data should be generated. In that sense, the scope of the discovery toxicologist to define the relevant questions and the most appropriate experimental models with which to answer those questions is entirely open (Blomme and Will, 2016). Which activities to undertake as part of discovery toxicology depends entirely on defining the right critical questions. Relevant questions may relate to an understanding of the target's role in normal tissue or from the knowledge of compounds affecting the same target or pathway. There may be cause for concern about properties within the chemical series or about metabolites that themselves have the potential to cause adverse effects. If toxicities arise from modulating the therapeutic target itself, then fundamental changes to the project may be required to achieve a sufficient therapeutic window. For toxicities that are not related to the primary target but due to

properties within the chemical series, discovery toxicology provides a potential opportunity to develop structure-activity relationships (SARs) and a chance to evaluate alternative chemistries with such liabilities designed out. Those considerations can be included as compound nomination criteria.

Consequently, the discovery toxicologist's experimental approach depends on the questions to be addressed and the models to be employed to answer these questions. Within the research project environment the toxicologist will work hand in hand with other disciplines to assess the balance of risks and whether concerns identified require further experimental investigation. At this point, the variety of experimental options available to the toxicologist are as broad as the questions themselves. These may include investigations using knock-out or knock-in transgenic animals, imaging techniques, *in vitro* and *in vivo* models, gene manipulation using CRISPR or short interfering RNA (siRNA), genomics and proteomics screening, computational modeling, three-dimensional organoids, or models using human-derived tissues. These represent only a small fraction of the techniques available. It is the toxicologist's skill to provide clarity on the questions being asked and identify the most appropriate models to answer them (Hornberg et al., 2014). Discovery toxicology is not a fishing expedition; it is scientifically driven and focused on delivering informative, decision-making data.

We will continue this theme of target, chemistry, and patient throughout the chapter.

Target

The starting point for identifying potential safety concerns for any new project is with an understanding of target biology. The primary pharmacological target (e.g., a receptor, enzyme, protein, or nucleic acid) has usually been chosen for its purported role in a disease process, and that modulation of this target will provide some therapeutic benefit. Occasionally targets are chosen to direct an active molecule to the site of disease [e.g., antibody-drug conjugates (ADCs)]. However, once administered, the drug will interact with the target wherever it is found in the body, and disruption of its physiological role in healthy tissue(s) may then be a cause of toxicity. Indeed, in a recent analysis, it was estimated that of the compounds stopped in development for safety reasons, approximately 50% were due to adverse effects from the primary target (Cook et al., 2014; Morgan et al., 2018).

There is often an enormous amount of information about a target that can be gleaned from the scientific literature and other publicly available data sources to better understand the target's role in biological processes and the potential for adverse effects. Databases of gene expression profiles, pathway analysis data, and genetically modified

TABLE 22.1 Early thinking about safety can be built upon at each stage of development from discovery to market, ordered by relevance for chemistry, target, and the patient.

	Discovery	Development	Phase I	Phase II	Phase III	Market
Patient	Target ID Identify, validate targets, ID broad liabilities	Lead selection “Best of the bunch” Optimized efficacy vs liability profile	Characterization of risk profile for phase I	Select dose for tolerability & target cover. Human PK/PD effects. PK-driven &/or high frequency safety	Early efficacy signals. Safety/tolerability	Benefit-risk quantification for submission
Target	Start strategy documents: What's the opportunity? What do people need? Start development plan: data needed, how/when to collect it? Example: use RWE to support population choice + characteristics (comorbidities, etc). Study safety profiles of similar compounds. ID potential biomarkers: safety/efficacy.	Decisions phase I: requirements, population (volunteer/patient), single/repeat dose. Risk mitigation, monitoring, management. Relevance of data: mechanisms, differences between species. Refine biomarker strategy. Define safety margins: safety profile vs exposure and how this is likely to relate to dose in humans.	ID: Key clinical safety issues, PK-driven effects, tolerability that affects most on patients. ID novel management guidelines. Define PRO strategy. ID population with best benefit-risk profile and biomarkers to select them.	PRO selection/creation. Fix monitoring strategies for phase III.	PRO delivery. health economics. Write labeling. Monitoring.	Additional populations, indications (pediatrics)
Chemistry	Hypothesis generation: Understand target and predict safety profile resulting from binding to target + off-target effects. Example: use of literature + in silico, in vitro, in vivo studies.	Understand hypotheses: Measure potential efficacy – in vitro and in vivo models. Characterize on-target and off-target effects in vivo to understand liabilities. Modeling.	Identify and characterize human on-target/off-target effects vs dose. Exposure profiles + early proof of mechanism. Assess clinical use of biomarker for safety monitoring; diagnostics	Efficacy and longer-term/lower frequency safety profile characterisation. Additional studies to understand effects in special populations: e.g., impact of drug on cardiac QT length; use in renal or hepatically impaired people. Validate any diagnostics or bespoke monitoring requirements	Chronic toxicity assessment in phase III. Understand chemistry-driven effects of large batch of drug that's to be scaled for launch. Undertake reproductive toxicity and carcinogenicity nonclinical studies to inform label and appropriate use in humans.	RWE-observed safety effects. May need in vitro/in vivo studies to ID mechanism + back translate.
	Hypothesis generation: Understand liabilities because of chemistry: safety, pharmacokinetics, manufacture, formulation. Example: Research the chemical series, early assessments to derisk molecule (in silico, in vitro, in vivo). In vivo formulation development (tolerable, stable, manages DMPK).	Characterize drug toxicity profile: in vivo repeat dosing in line with predicted dosing in phase I. Understand exposure profile and how this drives safety observations. Define formulation requirements for phase I: use safety profile and maximum allowable impurity levels. Any metabolite issues?	Characterize clinical suitability of formulation. Select final formulation at the end of phase I. Impurity level check: Larger batches vs earlier batch levels. ID + characterize chemistry-driven tolerability/safety effects in humans (HV vs patient).	Manufacture and batch-to- batch variability. Drug/device complaints.		

mice can provide useful information on a new target. It is also vital to understand whether the target is conserved and similarly distributed across species and whether variation in genetic or protein sequence may affect the drug's activity. This is particularly true for monoclonal antibodies, affibodies, siRNAs, and antisense oligonucleotides that are so exquisitely selective for their intended target that on-target pharmacology may be the only activity it has. In these circumstances, all the potential safety considerations will come from an understanding of where the target is expressed and its physiological role in those tissues. Furthermore, knowledge of target distribution and biological function becomes critical to identify potential pharmacologically mediated toxicities and confirm the appropriate choice of species for toxicity testing.

In an ideal situation a target would be expressed only in the cell or tissue of interest, such as a mutated protein in a tumor or a component of a bacteria's wall. In these cases, selective inhibition of the target may not be a significant cause for concern for the toxicologist. Nevertheless, it is important to recognize that for some molecules, as doses and tissue concentrations rise, selectivity may well be lost, and activity at closely related structures become apparent. For monoclonal antibodies, ADCs, and RNA-based therapies, the antibody's specificity for the intended target is such that on-target activity is truly reflected only in humans or other primate species, severely limiting the choice of relevant preclinical models. In some cases, to understand the pharmacological consequences *in vivo* better, this may mean codevelopment of a surrogate that recognizes the mouse or rat target.

Epidermal growth factor receptor (EGFR) remains a target of huge interest in new lung cancer treatments and other epithelial tumors. This was initially investigated with small molecule inhibitors, then with monoclonal antibodies and ADCs, and now by a variety of additional approaches. However, EGFR is present in the skin, particularly the epidermis' basal cells and the GI tract's mucosal cells. Consequently, blocking EGFR signaling leads to recognized on-target effects, including a characteristic papulopustular skin rash and diarrhea. These adverse events can markedly affect a patient's quality of life and can be severe enough to cause dose reduction or cessation of treatment. Today, newer agents have been developed that are able to selectively target mutant forms of the EGFR that are present only in the tumor, thereby sparing most patients the effects on the skin and GI tract. However, at higher dose levels, the selectivity for these mutant forms diminishes, and the adverse effects reappear.

Another example to consider is that of cyclooxygenase 2 (Cox-2). Cox-2 inhibitors are widely used nonsteroidal antiinflammatory agents that effectively treat pain, fever, and inflammation by reducing local prostaglandin production at sites of injury. However, they show very little

tissue selectivity in their distribution and thus would be expected to affect essentially all Cox-2 wherever it is present. The pharmacological mechanism outlined for the primary beneficial therapeutic effect of Cox-2 inhibitors is complex and is reviewed elsewhere (Warner and Mitchell, 2004). There are a vast array of associations between Cox-2 and other proteins, small molecules, and physiological processes. As a result, unwanted effects of Cox-2 inhibition can occur, including coagulation disturbances, gastrointestinal effects, and a higher risk of heart attacks and stroke.

There is a long-term benefit to a clear understanding of on-target toxicity. For most drugs, demonstration of a toxicological profile, including dose-limiting toxicities, is a fundamental requirement prior to regulatory submission. Consequently, all drugs at that stage are expected to have a toxicity profile, which forms the basis for patient safety and risk management plans. An important question that is often asked is whether the dose-limiting toxicities that have been observed might be avoided with another molecule, thereby allowing a higher exposure to be reached. Such backup strategies are common, as the first molecule selected for clinical testing may not necessarily be optimal in all respects. Resources will be allocated for a new chemistry effort to identify further potential candidates that perhaps do not show the same toxicity. However, if the dose-limiting toxicity is due to on-target effects, a second compound with the same pharmacology is unlikely to be toxicologically distinct.

An example is ALK5, a tyrosine kinase that is overexpressed in human tumors and is a promising anticancer target. Compounds blocking the TGF- β –ALK5 pathway have been shown to be effective at slowing tumor growth in rodent xenograft models. However, ALK5 is also expressed in cardiac valve leaflets and has an essential role in maintaining the valve's structural integrity under high pressure within cardiac ventricles. Would blocking ALK5 signaling *in vivo* be detrimental to cardiac valve integrity and present a safety risk to patients? It is now known that ALK5 inhibition can cause the degeneration of cardiac valves in multiple species (Anderton et al., 2011). If such a toxicity were to occur in patients even in clinical trials, it could be difficult to monitor and potentially life-threatening. Therefore this knowledge presents a significant issue to be overcome if the promise of this target is to be realized.

It seems appropriate that in embarking on long and expensive research programs, a thorough understanding of potential safety issues arising from the pharmacological modulation of a target modulation is an important first step. Safety issues related to the intended pharmacological activity of a drug remain with the program regardless of which chemical series or compounds are selected, and that knowledge can help to shape the project strategy.

Chemistry

When a compound is nominated for clinical development, its inherent properties and characteristics are mostly fixed and waiting to be revealed. It is in the discovery phase of a project when medicinal chemists have the greatest scope to adapt the chemical design and choose compounds with fewer potential liabilities. In the same way in which compounds are optimized iteratively for desirable features such as potency or clearance in design-make-test cycles, the same thinking should also apply to safety. Adverse features may not be eliminated entirely, but this process allows the opportunity to minimize those adverse activities that could ultimately hinder or stop the compound's progression. The toxicologist's goal is not to deny progression but to help find the optimal balance of properties that contribute to a higher-quality candidate drug (CD). Each scientific discipline contributes information in support of a CD's nomination. It is useful at the outset for the team to work toward a common goal, which may best be captured in a Candidate Drug Target Profile (CDTP). This sets out quantitative criteria, across all relevant disciplines, on the features required of a high-quality clinical candidate. In early research, when compounds are still a long way from CD quality and multiple chemistries may be in play, it is impossible to conduct thorough risk assessments. Therefore the early focus is aimed primarily at hazard identification and comparison across molecules to identify and deselect those with the least attractive properties. This iterative process is familiar to most research teams as they progress through lead optimization.

In considering the kinds of safety-related investigations that should be conducted by the research team, the initial focus is usually on those areas with the highest potential to cause profound safety issues. There will likely be a spectrum from potential "showstoppers" to dose-limiting adverse effects that could constrain the ability to dose escalate, through to effects that simply would preferably be avoided. It is important to consider what aspects of concern are worth spending the time and resources on during this period. At this stage, available compound is likely to be limited, so needs to be used wisely. There are several areas where certain properties might well signal a death knell for the compound, or at least a difficult path to progression. These may include the potential for DNA mutation or chromosome damage, secondary pharmacology activity with recognized physiological effects, for example, blood pressure, central nervous system (CNS) effects, arrhythmia, and attributes associated with low incidence clinical problems, such as reactive metabolite formation, transporter effects, or hERG inhibition.

The ability to cause DNA mutations is considered an early surrogate for potential carcinogenicity in humans. Therefore it is important to know at an early stage whether the lead chemical series can cause mutational change.

Medicinal chemists are well versed in avoiding many structural motifs that suggest that a structure (or metabolite) would have inherent DNA reactivity (such as aromatic amines). Perhaps with one notable exception, compounds that cause mutations are almost certain not to be developable in anything other than an advanced cancer indication. When the patients already have advanced cancer, the risk-benefit skews heavily toward benefit even for mutagenic compounds. Bear in mind, though, that progressing with a mutagenic compound into an advanced cancer indication could still mean that the opportunity to move into an earlier line of cancer therapy later in clinical development or postmarket authorization could be affected.

Additionally, when one considers the number of people who may come into contact with the drug during manufacture, shipping, preparation, and so on, a nonmutagen is always preferable. Simple assays exist for detecting mutagenicity and representative compounds can be checked periodically to ensure that the pharmacophore is not mutagenic. The most common screens for detecting genetic mutagenicity or clastogenicity (chromosome damage) are the *Salmonella typhimurium* reverse mutation (Ames test), the mouse lymphoma assay, the chromosomal aberration assay, and the micronucleus assay. All have been adapted for early screening with low compound requirements. Ultimately, genetic toxicity testing is part of the mandated set of safety assays that must be performed before humans are exposed to a new drug. Therefore it is a critical area to evaluate early on to avoid a significant issue later in the process.

Many compounds have the potential to interact with a broad array of molecular structures far beyond the intended pharmacological target(s). This off-target or secondary pharmacological activity provides no benefit to the patient but can cause adverse effects (Bowes et al., 2012). As with mutagenicity, simple early profiling of representative compounds against a standard panel of receptors, enzymes, and transporters can provide early information on selectivity and whether there are concerns about target activity with known physiological consequences. Broad promiscuity at multiple targets is unlikely to stop a compound on its own, but the compound can suffer from poor tolerability that may hinder the exploration of efficacious dose levels. For potent hits within 100-fold of the primary target, it is important to establish whether that binding has function consequences and, if so, whether they behave as agonists or antagonists. This functional information can give an early indication of potential adverse effects based on existing clinical knowledge, or it may prompt further experimental investigation. For example, if a compound series binds the alpha-1 adrenergic receptor within two orders of magnitude of the primary target, an early exploration of the compound's effects on cardiovascular parameters might be considered.

Such questions may prompt the team to develop some SARs around this liability, which may then feed into compound selection decisions.

An area of secondary pharmacological activity that has been of considerable focus has been the ability of many molecules to bind to an ion channel known as hERG. This ion channel is present in cardiomyocytes and allows potassium ions to leave the cell following each heartbeat, restoring the resting membrane potential. Compounds that bind to the hERG channel block the potassium current delay repolarization, which is shown as an increase in the QT interval on the electrocardiogram. This change has been associated with a very low incidence but potentially fatal arrhythmia known as *torsades de pointes* (TdP). A number of drugs in different indications have been removed from the market after case reports of TdP, including the nonsedating antihistamine terfenadine, the antifungal astemizole, and the prokinetic cisapride. Other drugs have been halted late in clinical development after showing increases in the QT interval. There have been many advances in our understanding of the physicochemical properties that drive hERG blockade, and protein models of the channel are now available (Kalyaanamoorthy and Barakat, 2018). Scores of papers have been published on hERG SARs, although designing away from hERG activity while retaining other desirable properties can be challenging. From the tens of thousands of compounds that have been characterized for this liability, chemists can now use a few relatively simple rules to try to avoid, if not eliminate, blockade at the hERG channel:

- Avoid extended cylinder-shaped molecules with hydrophobic features at the ends.
- Avoid aliphatic amines with a pK_a above 7.
- Avoid compounds with $\log P$ or $\log D$ values over 2.

There is difficulty in confidently establishing what level of hERG inhibition might be considered acceptable, and this is likely to be different for each project in considering the intended population and current standard of care for the indication. We can also ask whether modest inhibition of the current at, say, IC_{20} concentrations is sufficient to increase the proarrhythmic risk. Given the potential for expensive late-stage failures, companies have tended to err on the side of caution and set at least a 100-fold window between hERG activity and the primary target. Today, it is recognized that effects on other ion channel currents can also contribute to proarrhythmic risk, and therefore the propensity to block sodium and calcium channels should be avoided if possible. The fact that in the last decade, no drugs have been removed from the market due to TdP is remarkable and definitely an instructive case of how medicinal chemists, armed with the right tools and information, can substantially improve product safety.

There are a huge number of in vitro assays that can be deployed as part of the compound selection cascade

during the discovery phase. Each will provide information on endpoints that are considered relevant in terms of potential safety liabilities. The wide range of assays includes in vitro cytotoxicity in a favored cell type, assays to assess mitochondrial toxicity, transporter inhibition, phototoxicity, phospholipidosis, hepatotoxicity and steatosis, and neurotoxicity. Which assays are useful is a topic of ongoing debate and mostly a matter of personal or institutional choice. Huge strides have been made in making such in vitro assays more physiologically relevant, moving from two-dimensional to three-dimensional formats and the development of microfluidic technology that has allowed organ-on-a-chip technology (Cong et al., 2020). However, whether these relatively expensive, lower-throughput assays make significant inroads into safety screening will be interesting to observe.

The propensity of a drug to form reactive metabolites has been a significant problem for drug development. Reactive metabolites are believed to be a primary cause of idiosyncratic adverse drug reactions, which can occur in skin, liver, and hematopoietic tissues. Several drugs have been withdrawn from the market or late development because of low-incidence severe toxicities. Metabolism of some drugs can lead to the release of electrophilic radicals, which bind covalently to cellular components such as enzymes, intracellular proteins, and DNA, potentially leading to toxicity. Screening for reactive metabolite formation can be conducted at an early stage using chemical trapping agents, incubated with liver microsomes, to identify the potential for reactive metabolite generation. The formation of reactive metabolites can then overwhelm the body's natural detoxification systems. Reactive metabolites do not generally cause overt toxicity in preclinical and early clinical studies and may become apparent only after years of use in many thousands of patients. These toxicities are sometimes labeled idiosyncratic, in that they are not predictable from preclinical studies and are manifested only in susceptible patients, perhaps with a genetic or environmental predisposition (Thompson et al., 2016).

A catalog of in vivo pathologies that have been associated with reactive intermediates is a useful tool and can be used in combination with a comprehensive library of the functional groups and substructures that can give rise to reactive intermediates.

The following are a few simple rules for avoiding reactive intermediates:

- Keep the dose low. The higher the dose, the greater the reactive metabolite burden.
- Actively use a “warning” substructure list.
- Characterize biotransformation as early as possible.
- Warning signs: overt cell toxicity in genetic toxicity assays using metabolic activation, time-dependent inhibition of P450 activity, cytotoxicity in P450-expressing cell lines.

A multitude of in silico models have been developed to assist the toxicologist in identifying potentially toxic compounds even before they have been synthesized. This area has been given significant impetus in recent years following the EU regulation titled Registration, Evaluation, Authorisation and Restriction of Chemicals, which aims to protect humans and the environment from the adverse effects of the use of chemicals and from the ban on animal testing of cosmetics. Consequently, the use of in silico prediction models (e.g., QSAR and read-across methods) as alternatives to animal testing are now integral to both industries (Hasselgren and Myatt, 2018).

The promise of in silico predictive models is in predicting the potential adverse effects of a chemical based on its chemical structure alone. These SARs are based on the proposition that structurally similar chemicals are anticipated to show similar effects and that knowledge of one set of chemicals can be used to predict the behavior of others. There have been some notable successes in this area, particularly in mutagenicity models arising from the wealth of Ames data available from the pharmaceutical, chemical, food additive, and cosmetics industries. Consequently, many QSAR models have been developed for assessing potential mutagenicity, including DEREK, Leadscope, and MultiCase, among many other (for a review, see Honma, 2020). However, there are currently many limitations to the broadening of this approach into areas beyond mutagenicity, owing to our relatively poor understanding of which characteristics are important with respect to toxicity. Seemingly minor tweaks in chemical structure can lead to large shifts in many activities. Although such shifts in behavior open up areas of opportunity for medicinal chemists seeking to optimize leads, they are unhelpful in the programming of computational models. The next incremental step in the development of computational tools in drug discovery will be the introduction of applications using artificial intelligence and machine learning. Such tools are already being introduced in areas such as predictive pharmacokinetics and the detection of histopathological changes. In theory, millions of possible molecular structures can be evaluated and rejected prior to synthesis. Time will tell whether these applications can replace or more likely, complement the medicinal chemist or pathologist's skills and judgments.

Despite the ever-increasing data generated by in silico and in vitro models, it remains the case that only when a compound is administered in vivo does the true safety profile begin to emerge. Important indications about tolerability can be obtained early on from in vivo pharmacokinetic studies and efficacy models that allow comparisons to be made across different molecules. As dose and duration increase, dose-limiting effects become more apparent, prompting further rounds of SARs and chemical optimization in the search for a well-tolerated compound at efficacious exposure levels.

Patient

Ultimately, the hazards that are identified in early toxicity testing need to be given patient context. The farther away from a clinical candidate, the more difficult the judgment. However, a broad idea of the clinical trial and patient population is key to understanding the potential impact of the emerging toxicity profile. Important considerations include whether the patient population is relatively young or elderly, the prevalence of comorbidities, and whether dosing in combination is anticipated. The duration of treatment, the route of administration, and whether dosing will be at home or in a hospital setting all potentially influence the desirable toxicity profile in the CDTP.

Long-term risk-benefit assessments are often focused on the patient, but for most indications the clinical trial program will begin with short-term dosing in healthy volunteers. Therefore the toxicology profile should be acceptable for dosing to clinical trial subjects who receive no benefit.

Regulatory toxicology

When a compound is finally selected to be the clinical candidate, it enters the regulatory phase of toxicology testing (often described as IND-enabling). Here, there are very definite expectations on the toxicologist regarding the data that should be provided to national authorities in support of any new compound entering clinical trials. The objective is to characterize the compound's safety profile in preclinical models to influence the design of clinical trials design and to ensure patient safety. These regulatory toxicity studies must be conducted to conform to an internationally recognized standard known as good laboratory practice (GLP).

In designing a nonclinical safety package of scientifically driven studies, we again return to the framework of target, chemistry, and patient. The target's specific nature, including its function and tissue distribution, will influence the selection of appropriate species for toxicity testing. The chemistry (or drug modality) and its mode of action, distribution, and metabolism will influence the design of the safety studies to be conducted, for example, whether genotoxicity, the potential for off-target effects, effects of metabolites, and phototoxic potential are relevant concerns. The patient context is important in considering the appropriate dosing frequency and duration, whether healthy volunteers will be involved in the program, and whether combination toxicity studies may be needed. In this way, the toxicologist builds a scientifically credible dataset that recognizes the specific risks of a new therapeutic for the subjects of the upcoming clinical trials.

Regulatory toxicity testing can be broadly categorized into three phases:

1. Preclinical testing supporting the first administration of a new compound to human subjects in phase I trials generally to assess safety, tolerability, and pharmacokinetics.
2. Toxicity testing that allows for a longer duration of dosing or broadening of the patient population to include women of child-bearing potential or children.
3. Testing to support a marketing application. Data from these toxicity studies are intended to support widespread clinical use after reaching the market and include long-term studies in rodents to assess carcinogenic potential as well as effects on fertility and perinatal and postnatal development.

International guidelines from regulatory agencies such as the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use, the World Health Organization, and the Organisation for Economic Co-operation and Development provide useful frameworks for understanding what data are necessary to provide in support of clinical populations. These guidelines were initially developed with small molecules in mind; they have been extended to include biologically derived therapeutics. With the enormous variety of therapeutic approaches and modalities in clinical development today, many do not easily fit the prescribed guidelines. The guidance reflects the regulatory agencies' thinking on certain topics but should not be considered a rule book. In every case it is essential for the toxicologist to consider the scientific questions relevant to each individual project and design a regulatory toxicity package of safety studies that address the relevant issues. In doing this, it is again useful to consider safety risks arising from the target, the chemical modality itself, and the patient context. In special cases it is always a good idea to discuss safety and testing strategies proactively with regulatory agencies.

Regulatory toxicology involves the identification of hazards (potential adverse effect) and then the assessment of risk (making a judgment about the likelihood and tolerability of that risk under the conditions of use). This risk assessment is used as part of regulatory agencies' and industry's decision making as to whether to allow the use of potential new medicines whose benefits outweigh the risks associated with their use.

As described by the National Research Council of the National Academy of Sciences ([Lehman and Laug, 1949](#)), risk assessment involves four components:

1. Hazard identification: An evaluation of the adverse health effects the agent is capable of causing. Examples might include the capacity of an agent to cause liver or nervous system damage or to cause cancer.

2. Dose-response assessment: A determination of how much of an agent is required to cause a pharmacological versus toxicological effect and prediction of the exposure levels at which risk is likely to be negligible or nonexistent.
3. Exposure assessment: A determination of how much of an agent people could be exposed to under various conditions, such as the use of a drug or a consumer product over a long period of time or perhaps environmental exposure at a hazardous waste site.
4. Risk characterization: An integration of the pertinent information from the preceding steps to characterize the exposed population's risks. For example, what is the likelihood of liver toxicity if an individual uses a particular drug? The risk characterization also includes an explicit description of the assumptions and uncertainties that go into the risk assessment and the overall confidence in the results of the analysis.

Although the principles of safety evaluation delineated by Lehman and colleagues in 1949 still describe the current approach to risk characterization relatively accurately, toxicology is a dynamic discipline that must evolve to continually improve the value of the data generated and to improve the ultimate risk assessment calculation. There is significant momentum in the pharmaceutical industry and regulatory agencies' combined effort to evaluate opportunities for improved approaches to toxicological assessment. The Critical Path Institute and the Innovative Medicines Initiative are at the forefront of these efforts.

Historical context

In the first half of the 20th century there were no recognized independent standards for evaluating medicinal products before they were allowed on the market. In the United States the role of the U.S. Food and Drug Administration (FDA) in regulating novel medicines began following the Elixir of Sulfanilamide tragedy of 1937, when 71 adults and 34 children died after taking a purported remedy for a wide variety of ailments. This event spurred Congress to empower the FDA to monitor drug safety and impose strict regulations. In Europe the thalidomide tragedy in the 1960s drove a similar desire for stricter regulation. Thalidomide had been very well tolerated in animals and was licensed over the counter as a sedative and treatment for colds, nausea, and morning sickness in pregnant women. It is estimated that in the short time that thalidomide was available, over 10,000 babies were affected by the drug. Around half died shortly after birth, and others were born with limb deformities such as phocomelia.

For most countries the 1960s and 1970s saw a rapid increase in laws, regulations, and guidelines for reporting

and evaluating the data on the safety, quality, and efficacy of new medicinal products. The different regulatory systems were based on the same fundamental obligations to evaluate quality, safety, and efficacy, but until recently, the detailed technical requirements had diverged over time to such an extent that the industry found it necessary to duplicate many time-consuming and expensive test procedures in order to market new products internationally. Harmonization of regulatory requirements was pioneered by the European Community in the 1980s, as the EC (now the European Union) moved toward the development of a single market for pharmaceuticals. At the same time, there were discussions between Europe, Japan, and the United States on possibilities for harmonization. This finally resulted in the birth of the International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) in 1990, a joint regulatory and industry project to improve, through harmonization, the efficiency of the process for developing and registering new medicinal products in Europe, Japan, and the United States. ICH topics are divided into four categories: S (Safety), Q (Quality), E (Efficacy), and M (Multidisciplinary).

ICH M3 (R2) is the key guideline that describes the non-clinical safety studies required to support a new pharmaceutical's clinical development and marketing approval as well as appropriate scientific and ethical considerations. It was developed primarily for small molecules and has been supplemented with further guidelines in specific areas of interest. For example, ICH S6 provides relevant considerations in the

nonclinical safety assessment of biotechnology-derived products and ICH S12 for gene therapy products. ICH S9 describes the nonclinical data that are important in the development of therapies in advanced cancer. In each case the guidance reflects regulatory agencies' thinking in an attempt to orientate the developer rather than providing a roadmap to be followed. For some newer drug modalities, including RNA-based therapeutics or therapeutic vaccines, no specific ICH guidance exists, and one must consider whether particular studies are needed on a case-by-case basis. There are often helpful publications from national agencies or from working groups such as the Oligonucleotide Safety Working Group ([Marlowe et al., 2017](#)).

A selection of ICH guidelines relevant to drug safety are listed in [Table 22.2](#).

Good laboratory practice

To ensure the quality, consistency, and reliability of data submitted to support clinical testing, the system of GLP was introduced in the 1970s. This global experimental standard was introduced following cases of fraud in drug safety data submitted to the FDA. GLP standards are now required of all pivotal nonclinical data and include toxicology (in vitro and in vivo), safety pharmacology, pathology, and pharmacokinetics or toxicokinetics, including bioanalytics.

As defined by the Organization for Economic Cooperation and Development, the primary goal of GLP is "to ensure the generation of high quality and reliable test data related to the

TABLE 22.2 Key ICH guidelines relevant to nonclinical safety.

ICH guideline	Topic
M3 (R2)	Nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals
S1A-C	Need for carcinogenicity studies of pharmaceuticals
S2 (R1)	Genotoxicity studies and data interpretation for pharmaceuticals intended for human use
S3	Toxicokinetics and pharmacokinetics
S4	Duration of chronic toxicity testing in animals
S5 (R3, R4)	Guideline on reproductive toxicity
S6	Preclinical safety evaluation of biotechnology-derived pharmaceuticals
S7A and S7B	Safety pharmacology studies
S8	Immunotoxicology studies for human pharmaceuticals
S9	Nonclinical evaluation for anticancer pharmaceuticals
S10	Photosafety evaluation of pharmaceuticals
S11	Nonclinical safety testing in support of development of pediatric medicines
S12	Nonclinical biodistribution studies for gene therapy products
Q3A-E	Impurities in pharmaceuticals

safety of industrial chemical substances and preparations in the framework of harmonizing testing procedures for the Mutual Acceptance of Data.” The detailed rules for GLP in the United States are described in the code of federal regulations 21CFR58 by the FDA, which addresses GLP for non-clinical laboratory studies. Those of the European Union are described in the two basic directives 2004/10/EC and 2004/9/EC (on the harmonization of laws, regulations, and administrative provisions relating to the application of the principles of GLP and the verification of their applications for tests on chemical substances) of the European Parliament and Council. As a consequence, any preclinical research that is not conducted according to these guidelines might not support an IND in the United States or a clinical trial application in Europe.

In general, GLP embodies a set of principles that provide a framework within which laboratory studies are planned, performed, monitored, recorded, reported, and archived. It assures regulatory authorities that the data submitted are a true reflection of the results obtained during the study and can therefore be relied on in making risk or safety assessments. GLP monitoring units are part of regulatory authorities and are involved in the inspection of GLP compliance.

The goals of regulatory toxicology

Regulatory agencies rely heavily on nonclinical data before giving consent to begin clinical trials. Before initiating first-in-human studies, the toxicologist and reviewer play similar roles in identifying safety signals in the data that inform critical aspects of the clinical protocol to minimize risk to human subjects. This is accomplished by thorough data review and translational evaluation, identification of no-observed-adverse-effect levels (NOAELs) and a safe starting dose, appropriate representation of nonclinical findings in informed consent documents, and risk mitigation strategies, such as additional clinical monitoring, clinical trial participant exclusion criteria, and exposure limitations.

The nonclinical safety data that are submitted should identify key target organs of toxicity, the relationship to dose, exposure, time, and potential for reversibility. Not all therapeutic approaches carry the same risks; therefore each safety program should always be customized to the type of compound being investigated and its intended clinical use. There is no point in generating data that are not scientifically valid or do not contribute to clinical risk assessment. Again, we can return to the framework of target, chemistry, and patient.

A target’s distribution, function, and homology across species is an important consideration in selecting species for toxicity testing and in the interpretation of data. Monoclonal antibodies represent a significant proportion of investigative agents in clinical development, but as large proteins, they do not cross cell membranes, so they have no potential to

interact with DNA. Consequently, genetic toxicity testing for mutagenicity or clastogenicity is not relevant for this type of molecule and should not be conducted. On the other hand, small molecules are generally broken down into a range of metabolites, all of which may have their own toxicological profile to be considered.

The biological responses to any given molecule can vary depending on the species, gender, ethnicity, age, disease, and so on. Furthermore, toxicological effects depend not only on the substance in question, but also on the dose, the dosing regimen, the resulting concentration to the parent substance or metabolite in different organs and tissues, the nature of the exposure profile (e.g., sharp, short plasma peaks vs long stable exposure profiles), and the exposure duration. These differ from organism to organism, from species to species, and from individual to individual as well as over time. For example, even if the molecular interaction at a particular receptor in a highly conserved pathway was exactly recapitulated in different models or individuals, other aspects, such as the pharmacokinetic variation, the metabolism of the compound, and the underlying activity of the pathway, may be different enough to elicit significantly different pharmacodynamic responses.

It is critical to be aware of the limitations of toxicological testing and respective risk mitigation measures. Such measures can be, for example, safety margins, which try not only to mitigate limited human translatability of toxicological data raised in animals but also to take individual, variable susceptibility into account. It is important to recognize the limitations of the toxicology data that are generated and to be aware that they are valid only in the context in which they were created and may not translate to other species or even other strains of the same species.

A key element of the characterization is establishing the dose-exposure-toxicity relationship. In animals we measure test substance concentrations in blood (plasma or serum) *in vivo* and occasionally in relevant organs and tissues. Methods of choice for bioanalysis in blood or plasma are high-performance liquid chromatography and/or mass spectrometry. Assessment of tissue distribution and retention can be made with whole-body radiography using a radioactive marker molecule attached to the test molecule. Radiolabeling a test compound is not always possible and poses the risk that the modification may change the test substance’s behavior. Postmortem methods have the disadvantage of measuring test substance concentrations in organs and tissue at a single time point only. Establishing time courses and dose-response relationships is more informative and can be achieved by using more dynamic imaging techniques, such as positron emission tomography or matrix-assisted laser desorption ionization-time-of-flight mass spectrometry. In humans, apart from direct sampling, such as cerebrospinal fluid to estimate CNS exposure, the assessment of organ and tissue test substance concentration is confined to sophisticated imaging methods

and is not an option for most test substances. That leaves classical blood-based pharmacokinetic assessment in both animals and humans as a universally available tool to assess tissue exposure.

By assessing the dose-exposure-toxicity relationship in nonclinical studies, toxicologists can begin to establish a picture of which tissues are most sensitive to the effects of a novel compound, the severity of change that occurs, and the steepness of the dose response. These data allow identification of a no-observed-effect level, a NOAEL, and a maximum tolerated dose. Ideally, the exposure range at which toxicities begin to appear occurs at a concentration above those required for efficacy, providing a therapeutic window or therapeutic index.

Dose-response relationships

The relationship of dose or exposure and frequency or severity of a toxicological response is one of the most fundamental concepts in toxicology. Such dose-response relationships can be looked at from two principal angles. One describes the gradually increasing severity of a particular toxicological response relative to the dose in one individual or a cohort of animals. The other describes the variability of such an effect by the frequency of its occurrence.

For example, variable doses of a sedative may be plotted against the depth of sedation achieved in an individual or a fixed dose against variable, individual levels of sedation in a group of animals. Such dose-response relationships may illustrate minimal and maximal toxicological responses relative to variables doses, often plotted on a semilogarithmic scale, resulting in a sigmoidal curve with a linear portion. The linear part's steepness is a measure of the extent of change of a toxicological response relative to dose. A steep line indicates large changes in the toxicological response relative to small changes in dose; a shallow angled line indicates the opposite; relatively small changes in the toxicological response relative to the same change in dose.

The minimal dose at which a certain toxicological effect is observable but is not considered detrimental is referred to as the NOAEL. The NOAEL is frequently used as an anchor point for comparison with other parameters. The safety margin (Fig. 22.1) is the difference between the exposure required for therapeutic effects versus that of the NOAEL. A large margin or difference would indicate a relatively safe compound, and a narrow margin would suggest the opposite. However, the NOAEL that is generated in young, healthy animals of one species may not be the same as that in other species, including humans. One must also consider the steepness of the dose-response curve above the NOAEL and how further increments in dose may quickly lead to severe toxicity.

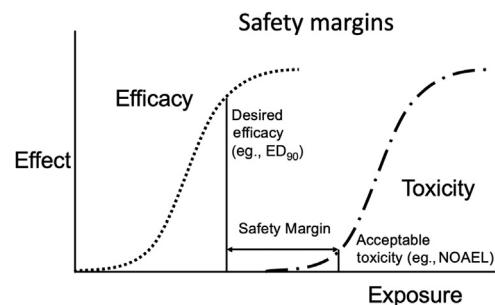


FIGURE 22.1 Illustration of the safety margin concept. Note that the efficacy and toxicity curves are not necessarily parallel. The two curves may even overlap.

Fig. 22.2 illustrates the most common dose-response curve—a sigmoid-shaped curve—but others are possible as well. For example, vitamins have a U-shaped graded dose-response curve in which doses below the daily requirements and above a toxicity threshold are associated with side effects.

Translational considerations and context

In designing toxicological studies and interpreting results, it is critically important to take the context into account. Several angles can be considered:

- *Time:* To predict the relative risk of a given compound, time plays a critical factor. For example, side effects that occur after several weeks of dosing may be somewhat irrelevant for a medicinal product that is to be given only once to an individual.
- *Effect:* Not every effect is of equal importance for all patient segments. Organ and tissue damage is of relevance for all humans, but, for example, teratogenicity may be of limited relevance if the substance is intended to be used in postmenopausal women only.
- *Target organ or tissue:* Pulmonary toxicity found with a compound that is intended to treat asthma by the inhalation route may be more problematic than oral use in other indications.
- *Mechanism:* As was outlined previously, mechanistic knowledge may be important for translating toxicological information. For example, knowledge of some types of toxicity can clearly indicate that the toxicity in question is relevant only to the animal species in which it was detected and therefore is irrelevant in humans because of the lack of similar mechanisms.
- *Medical control:* This is important in terms of the intended size of the patient population and medical control. It is evident that a drug that is intended to be given to millions of outpatients must be looked at differently than one that is to be given to a few individuals under close clinical surveillance.

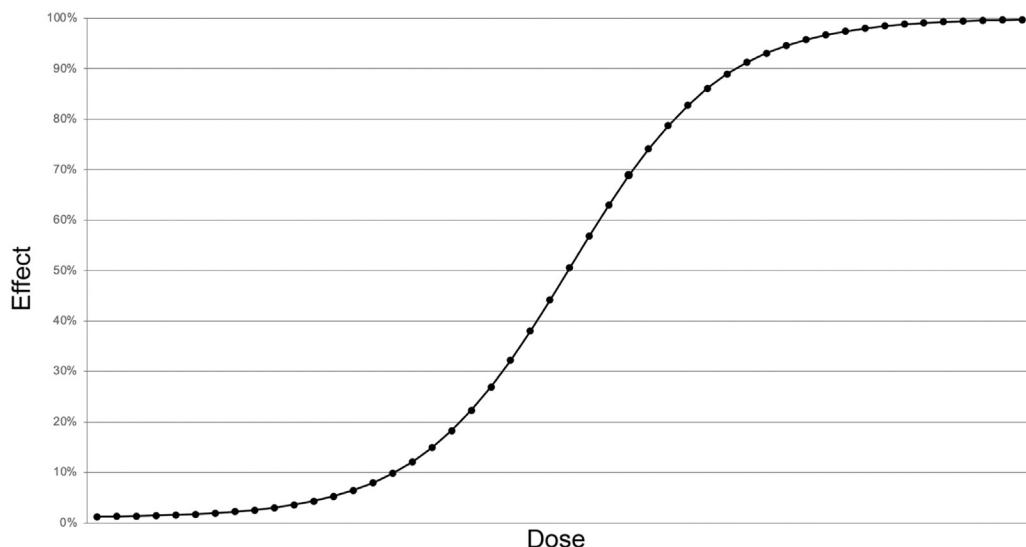


FIGURE 22.2 Classical sigmoidal dose-response curve.

- *Route of administration:* Because the route of administration has a significant impact on the exposure, the exposure duration, and even the biological and toxicological profile of a substance, toxicological studies have to use the same route of administration as will be used in future clinical applications.
- *Reversible effects versus irreversible effects:* It is obvious that irreversible effects carry significantly more weight than those that heal and resolve over time. However, this argument is of limited use for chronic treatments.

Animal models and future perspectives

Repeat-dose toxicity studies as described by the ICH include testing in two mammalian species—usually a rodent and a nonrodent—in which the duration of treatment should equal or exceed the duration of the human clinical trial. Laboratory-bred animals such as rats, mice, minipigs, and beagles are preferred because of the wealth of available background data.

The careful choice of species for toxicity testing is a key early decision. Several factors will affect that decision, including pharmacological relevance, metabolite profiles, tolerability, the extent of available background data, practical considerations around the route of administration, and precedent with certain drug classes. It is also important to consider ethical as well as scientific justification in the choice of species. Selecting a species simply to minimize drug quantity requirements is not a valid consideration.

Rats and mice are the typical rodent species of choice because of their extensive background data and well-characterized biology. For nonrodents, beagle dogs have traditionally been the default species, although alternatives

such as minipigs are becoming more common. For highly targeted agent, when the pharmacological similarity to humans is critical to understanding safety risks, nonhuman primates may be the only species suitable for this purpose. However, the use of nonhuman primates should always be seen as an exception when no other alternative is available. For all species the toxicologist must always be aware to apply the strictest principles of the 3Rs (reduction, refinement, and replacement) wherever possible. The welfare of the animals is also of paramount importance and essential to obtaining high-quality data. These high standards of husbandry, animal welfare, and environmental enrichment are described in various FDA, European Council, and national directives.

In certain circumstances the pharmacological activity of the therapeutic agent may be such that it cannot be studied in normal, healthy animals. For example, an anti-diabetic medicine that lowers blood glucose would be very poorly tolerated in an animal with normal blood glucose levels and is unlikely to provide useful data within a tolerated dose range. In such cases, alternatives should be considered that allow full exploration of clinically relevant plasma concentrations, even if that involves a non-standard model such as the hyperglycemic ob/ob mice.

In a concordance analysis of the toxicity of pharmaceuticals in humans and animals, the results showed a positive human concordance rate of 71% for rodent and nonrodent species combined, with nonrodents alone at 63% and rodents alone at 43% (Olson et al., 2000). The highest incidence of overall concordance was seen in hematological, gastrointestinal, and cardiovascular human toxicities, the lowest incidence was seen in cutaneous toxicities. It is also recognized that many toxicities that are commonly described in humans, such as headaches,

dizziness, or nausea cannot be adequately assessed in animal models. Therefore it is clear that the aim of the regulatory toxicologist is not to predict human toxicities but to explore the potential for toxicity based on preclinical models and to use that information to design clinical studies that adequately protect the subjects.

The researcher's confidence derived from an extensive background dataset in standard species means that the number of animals needed to identify a compound-related change can be relatively small. In a typical study, equal numbers of male and female animals are tested in three doses with an administration route identical to that intended for humans, and there is one control group. The number of animals used in any particular study is generally linked directly to the number indicated in the published regulatory guidelines. It is appropriate for specific endpoints that the number of animals is set after consultation with professional statisticians.

By definition a repeat-dose toxicity study aims to identify dose-limiting and target organ toxicities and an understanding of the dose-response. A large amount of historical data is available for laboratory-bred animals and is of great benefit in the identification of toxicity. However, owing to the relatively homogenous genotype and phenotype of these animals and the fact that only small groups of young, healthy animals are generally used, low incidence effects or effects related to certain diseases or ages will rarely be identified. At first glance, this provides an opportunity for broader use of disease models or genetically modified animals and may be warranted if, for example, the pharmacodynamic effect by itself will cause toxicity. In general, however, the lack of extensive control data and the fact that even the best models do not accurately reflect human disease pose a challenge for the process of risk assessment that needs to be considered prior to testing.

Alternative approaches in regulatory toxicology: the exploratory investigational new drug approach

The preclinical testing strategy described in the section on regulatory toxicity studies was first enshrined in the ICHM3 guideline and is the most common approach currently used in the pharmaceutical industry. It describes the preclinical data that are typically required for sponsors to explore a clinical exposure range for a specified period of time. Additional ICH guidelines address safety issues in certain circumstances, such as those presented by biologically derived therapies ([ICH Harmonised Tripartite Guideline, 1997](#)) and drugs intended to treat advanced cancer (ICH S9). Further, in an attempt to reduce the time and resources necessary to bring a CD into phase I, a

revised ICH M3 (R2) document (International Conference on Harmonization, 2008) as well as the FDA guidance titled “Guidance for Industry, Investigators, and Reviewers—Exploratory IND Studies,” published in January 2006 (U.S. Food and Drug Administration, 2006), offer alternative approaches to testing drug candidates prior to phase I clinical trials in humans and to accelerating drug development. Because of the limited preclinical safety data supporting the clinical trial, human exposure is limited and has no therapeutic or diagnostic intent. The value of these early clinical trials is related to exploring the pharmacokinetic profile—for example, microdosing—and the readout of specific safety or pharmacodynamic biomarkers. The preclinical safety package very much depends on the duration and extent of human exposure.

The microdose approach is as follows:

- ≤ 100 µg: An extended single-dose toxicity study in one species, usually rodents
- Five or fewer administrations of a maximum of 100 µg: A 7-day toxicity study in one species with SAR assessment of the genotoxic potential

The approach for single-dose studies up to the subtherapeutic or intended therapeutic range is as follows:

- Extended single-dose toxicity studies in rodent and nonrodent species
- Assessment of genotoxic potential (Ames mutagenicity test)
- Safety pharmacology core batteries

The approach for multiple-dose studies that last up to 2 weeks and do not test the maximum tolerated dose is as follows:

- Approach 1: Two-week repeated-dose toxicity studies in rodent and nonrodent species with exposure multiples of anticipated human area under the curve (AUC), safety pharmacology, and genotoxicity
- Approach 2: Two-week repeated-dose toxicity studies in rodents testing up to a maximum tolerated dose and confirmatory studies in nonrodent species for a minimum of 3 days at the exposure intended to be the NOAEL in the rodent, safety pharmacology, and genotoxicity

However, since publication of the first edition of this book, it has become apparent that only a few pharmaceutical companies are actively using alternative regulatory approaches to any appreciable degree. This is believed to be due to the delay required to backfill the data and restart the program if the early clinical data look positive. In contrast, smaller companies and research institutes often use such approaches to save on costs prior to applying for additional funding or outlicensing of the drug asset.

Biomarkers

As the limitations of currently available safety biomarkers become increasingly evident—for example, the lack of sensitivity of common renal biomarkers—joint efforts are taking place between agencies and the pharmaceutical industry, resulting in the establishment of collaborations such as the Predictive Safety Testing Consortium (PSTC), an organization formed under the umbrella of the C-Path Institute with the goal of improving the biological evaluation of preclinical and clinical bridging biomarkers of drug safety.

One success has been the approval by the FDA and the European Medicines Agency (EMA) of seven proteins or biomarkers of drug-induced kidney injury in animal studies that were present in urine and can provide additional information about drug-induced damage to kidney cells (Kim-1, albumin, total protein, B2-microglobulin, cystatin C, clusterin, and trefoil factor-3). Previously, the FDA and EMA had required drug companies to submit data from blood tests to demonstrate that potential therapies will not cause kidney damage. Although the blood tests accurately measure toxicity, they do so in a delayed fashion. By the time the blood tests indicate toxicity—after a week of treatment—the kidneys may have sustained severe damage. The new tests for kidney biomarkers offer more immediate results and greater sensitivity. Instead of yielding results after days of treatment, the biomarker tests will indicate whether kidney cells are being damaged only a few hours after treatment begins, and the tests will pinpoint precisely which kidney cells a drug is affecting. Other groups of the PSTC are working on novel biomarkers for liver, muscle, and blood vessel toxicity and carcinogenicity. Novel insights can also be gained from older, established biomarkers, for example, cardiac troponin, for which new, ultrasensitive detection methods can reliably measure baseline values in healthy animals and humans.

In some cases there can be value in conducting bespoke preclinical (e.g., using clinically relevant biomarkers) studies or including additional endpoints in ongoing preclinical studies to build confidence that a particular patient safety-monitoring strategy would be useful to manage a potential liability. For example, a specific study looking at the time course for the onset of a particular biomarker change as an early indicator of injury in relation to the presence of histopathological changes could provide valuable information for implementing a risk management strategy to monitor for a potential toxicity. A biomarker change ahead of the occurrence of histopathology could provide a means to enable early intervention to keep clinical trial patients safe. Provided that the study in question will address a specific question and be of value for the clinical study, these can be considered on a case-by-case basis.

Preclinical safety from a translational perspective

As outlined at the beginning of this chapter, pharmaceutical toxicology or safety assessment is a translational discipline through and through. All aspects of preclinical safety activities aim to identify potential hazards, identify and quantify risks for healthy trial subjects or patients, and, if necessary, assess suitable means for eliminating or reducing unacceptable risks. However, there are many uncertainties in this approach. For practical reasons, only a limited number of aspects can be addressed in preclinical animal and *in vitro* studies. Furthermore, considerable species differences leading to changes in biological activity or pharmacokinetic properties and individual variations in susceptibility to certain effects and compounds add to uncertainties in the risk assessment process. Therefore any toxicological risk assessment process for a pharmaceutical is always incomplete and associated with uncertainties. Such uncertainties are compensated for by empirically derived safety factors. It is evident from the overall incidence of pharmaceutical treatment-related side effects that such factors are occasionally insufficient to completely rule out unexpected side effects.

Despite the many developments in the regulatory and investigative toxicology area, one aspect remains underaddressed: There is no element in the preclinical safety package focusing directly on patients. All studies are conducted in healthy young animals and are therefore primarily risk assessment tools for healthy human subjects. However, many diseases are known to increase patients' susceptibility to side effects. This is certainly an area in pharmaceutical toxicology that requires further development.

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Chapter 23

Translational safety medicine

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Abstract

This chapter looks at the entire safety value chain of pharmaceuticals, from the start of a drug project in early discovery to the product's withdrawal, brand, or generic, from the last market. Many different functions, disciplines, and experts are involved in the safe exploration and development of pharmaceuticals. In the absence of a discipline or even a common name for this activity, we called it *translational safety medicine*. This intends to emphasize the practical and applied nature of the work and stress the importance of clinical and postlaunch safety development. This chapter takes a critical look at current practices, analyses and highlights areas of improvement, and suggests best practice approaches wherever possible. The critical reader may be surprised to see that many opportunities do not relate to cutting-edge science and predictive methods but rather down-to-earth organizational and strategic aspects.

Keywords: Translational Safety Medicine; safety value chain; pharmaceutical safety development; pharmacovigilance

Introduction

The previous (Chapter 22, Pharmaceutical Toxicology), focused on the thinking, strategy, and practical delivery of preclinical safety studies in preparation for clinical development. In this third edition of *Principles of Translational Science in Medicine*, a separate chapter on translational safety medicine (TSM) has been added for the first time. This builds on the previous (Chapter 22, Pharmaceutical Toxicology), and describes how this preclinical knowledge is supplemented and interwoven with other information sources to form a robust and continuously evolving development strategy throughout the value chain.

All activities to improve medicinal product safety, from early drug discovery to market withdrawal, have been grouped under one heading we call TSM. In this context, TSM may be understood as a subdiscipline of translational medicine. However, as translational medicine traditionally

focuses primarily on therapeutic efficacy, TSM may also be understood as its safety-focused sister discipline.

We are defining TSM in the broadest possible terms. TSM comprises all thought models, strategies, and activities and the organizational setup that are intended to actively minimize risks and increase the risk-benefit profile of emerging and marketed pharmaceuticals at all stages. In addition, the definition of safety that is used throughout this chapter is a broad one and covers all traditionally accepted areas of safety recorded in clinic as well as the tolerability, the burdens, and ultimately the unwanted impacts that the drug may have on the lives of people who take it.

In this context, TSM comprises all aspects and activities intended to improve the safety profile, safe exploration, and safe use of medicinal products in every imaginable context. This includes but is not limited to target selection, design of a pharmacological modulator, choice of formulation, preclinical and clinical exploration and testing, market release, medical and geographical expansion (life cycle management), change of legal status (prescription to over-the-counter), and market withdrawal. All aspects supporting the safety value chain at various stages are also well within the scope of TSM. Examples are quality control and quality management of preclinical studies and clinical trials, manufacturing, packaging, distribution, storage, and medical use—of course, always relative to safety. Of particular importance are the patient context and medical practice characteristics.

The differences between TSM and classical translation of preclinical findings are in the work's scope and purpose. Whereas classical translation focuses on using the preclinical results to set the starting dose and risk minimization and monitoring in phase I trials, TSM applies a more strategic and broader data-driven and scientific approach. In this, translation of preclinical phase I is only one small part of the thinking. Throughout the safety science approach, a range of data sources gives the context and a thorough understanding of all

significant translational safety work elements. These include (but are not limited to) the following:

1. Population identification and characteristics: Who is likely to have the best risk-benefit balance and why? How prevalent is the condition? What is the current standard of care for this condition? How are the right patients identified? What is the best time to treat to maximize benefit/risk? How long should the treatment last to maximize benefit compared to risk? Which common comorbidities and comedication should be taken into account? What characteristics are required to deliver the product to the patient optimally? What are the specific needs of this patient population?
2. Target: Deep understanding of on- and off-target effects is important. When may they occur? Why do they occur? What are the side effect profiles associated with each of the off-target effects, and are there ways of optimizing benefit while minimizing adverse effects? Can we back-translate the human data in phase I to readjust our understanding from the pre-clinical space to improve models and make better predictions for both subsequent stages of clinical development and backup research programs?
3. Chemistry: Are there any safety-related consequences due to the molecular structure? What is known about other, similar molecules? What does the chemistry mean for manufacturing? Consider chemical stability, degradation products, and impurities and their consequences. What type of formulation will be needed? Are there any patient-specific requirements that need to be taken into account (e.g., drug delivery device, tablet vs liquid, size and number of tablets)?

These are just some of the elements that need to be built into a long-term strategy. Constant back-translation of learning throughout all phases of development means that the team does not forget about the work that has already been done but rather looks at the previous information with fresh eyes, adjusts models, then keeps the key learnings relevant and useful for interpreting future data. Together, these build a deep understanding of safety, one that is predicted, measured, and understood and one that is developed to a long-term strategy to meet the needs of the patients who are waiting for the treatment.

With this broad and holistic approach to safety, we are entering relatively uncharted territory. While safety activities are well established in early drug discovery, the situation is somewhat unclear in clinical development and even on the market.

On the basis of personal experiences gathered in long careers in the pharmaceutical industry, we present the current state of affairs, the main pitfalls to avoid, some

best-practice advice on effective safety work, and some glimpses into the future.

We cannot stress enough that TSM is a holistic concept that must be practiced continuously and thus at all stages of a product's life cycle. A broken safety value chain is almost impossible to repair, and an approach that uses only some of the relevant datasets or only applies part of the thinking is unlikely to deliver the desired results.

Conceptually, the description in this chapter starts from the point at which a candidate drug has been identified. In the world of small molecules, this is at the end of lead optimization with pivotal (conforming to good laboratory practice, or GLP) toxicology studies as the next major step toward clinical studies.

We address the specific aspects and challenges of translational safety work at crucial inflection points along the value chain in pharmaceutical development: investigational new drug/clinical trial application—enabling preclinical safety studies, preclinical to clinical transition, clinical development (phases 0–III), filing for market authorization, product launch, and postmarket product development (phase IV) and surveillance. We specifically demonstrate the purpose and value-adding nature of TSM and investigate the challenges, blockers, and organizational discrepancies that are keeping TSM principles from being developed to their full potential in most organizations.

This chapter will not explicitly address the so-called predictive sciences. There are high expectations regarding predictive safety tools, models, simulations, and biomarkers. All are certainly integral parts of TSM. Who would not like to have a crystal ball predicting all future safety issues of a given candidate drug or product? However, on the basis of personal experience, we believe that such tools and models have only limited value and impact in isolation. Extracting the value of such predictive tools requires well-established and effective TSM working principles and supporting organizational structures. For these reasons, we concentrate on addressing the working principles of TSM rather than on an outline of currently available predictive tools and safety biomarkers.

Before moving to specific sections of the chapter, we propose a four-dimensional framework of TSM goals and objectives (see [Table 23.1](#)). Ideally, every activity and organizational feature of TSM should relate to at least one of the four dimensions. We hope that every safety-accountable function, body, or person can identify with these objectives, regardless of their focus along the safety value chain.

We would like to stress that TSM is not a defined scientific or pharmaceutical industry discipline. Feedback on

TABLE 23.1 The four-dimensional framework of *Translational Safety Medicine* goals and objectives.

1. *Patient/healthy trial volunteer dimension*
 - a. Minimizing risks for healthy trial subjects
 - b. Maximizing the benefits compared to risks for patients
2. *Clinical trial dimension*
 - a. Safety-optimized trial design
 - i. Right trial population
 - ii. Right placebo/verum cohort ratio
 - iii. Right starting and end dose
 - iv. Right dose intervals/dose escalation steps
 - v. Right exposure limits
 - vi. Right safety endpoints
 - b. Optimal safety surveillance and oversight
 - c. Safety-focused stop and intervention criteria and mechanisms
 - d. Safety concerns and hypotheses testing implemented
3. *Development project or marketed drug product dimension*
 - a. Concise and unbiased development and documentation of safety profile and risk-benefit balance according to pre-defined goals
 - b. Effective and unbiased safety surveillance
 - c. Proactive exploration of safety uncertainties and testing of safety hypotheses
 - d. Clear safety stop criteria
 - e. Proactive, informed, safety-related attrition at earliest possible point in time
 - f. Fast and effective intervention strategy on basis of reasonable level of concern
4. *Company dimension*
 - a. Protection of company moral and ethical standards, reputation and license to operate
 - b. Safeguarding company from safety-related litigation

the concepts, thoughts, and strategies presented here is most welcome and encouraged.

State of affairs of translational safety medicine

Is TSM essential? Put another way, when is the safety of a pharmaceutical product good enough? One could say—and some people do—that research and development (R&D) of new pharmaceuticals are already complex and expensive enough, and additional safety activities not required by the regulatory authorities are not warranted. Safety is acceptable when it does not compromise the delivery of the desired therapeutic effect to most patients.

We believe that this is not good enough. None of us would set foot in an airplane or even an automobile that had been designed and built according to minimum regulatory requirements only. The aviation and automotive industries discovered some time ago that safety is a rather compelling, competitive selling argument—an insight that has yet to penetrate the world of pharmaceuticals. In the airplane and automobile industries, security has long become a critical competitive edge that is value-driven by ever-improving technical possibilities and cutting-edge science rather than basic regulatory requirements. In our opinion the same thinking should

be applied to pharmaceuticals. Ethical but also competitive aspects warrant that medicines and the ways in which they are used should be continuously safety-optimized to the extent possible according to technical and scientific possibilities. This is what everyone expects when entering an airplane, a car, a ship, or even a high-rising building, and it should be the same when they take any pharmaceutical product.

Let us have a look at how the safety optimization of pharmaceuticals works today. In practical terms, we must distinguish between two different workstreams ([Fig. 23.1](#)): one comprising all activities leading to a new pharmaceutical modulator and respective formulation (together called drug product or candidate drug), the other comprising the continued exploration, testing, development, and medical use of the drug product. The latter may address diverse topics such as how the emergent product is best explored and characterized in animals and humans, how theoretical safety concerns are being handled, how comparatively unclear safety concerns are addressed as early as possible, the selection of patients who are likely to benefit the most and suffer the least from the product, identification and exclusion of patients who are at high risk, or the definition of safe use of the product in every possible medical context.

In the previous (Chapter 22, Pharmaceutical Toxicology), we described how a growing number of screening batteries are used to optimize emergent drug substances, for example,

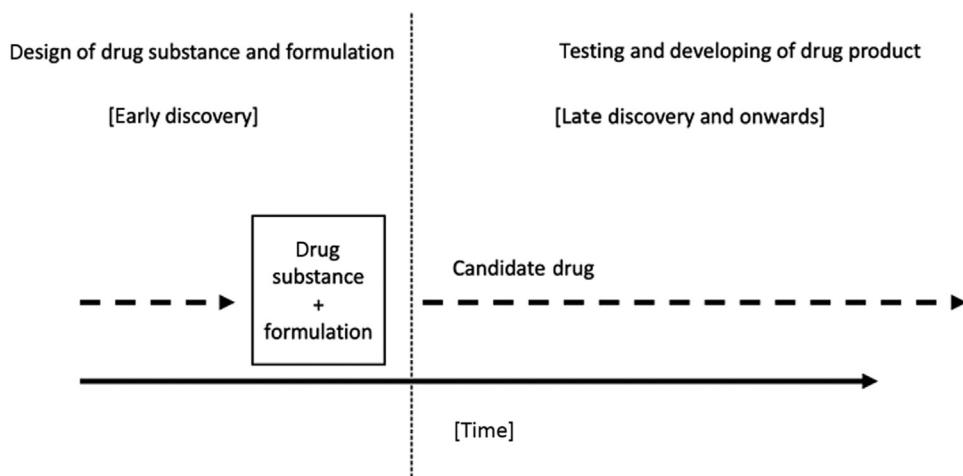


FIGURE 23.1 We are distinguishing between two distinct phases in translational safety medicine. The first included all safety activities leading to a candidate drug (CD; drug substance + formulation), also called drug product. The second includes all activities for the continued exploration, testing, and development of the drug product. This phase ends only with withdrawal of the drug product from the market.

activity on the hERG channel, genotoxicity, mutagenicity, reactive metabolite formation, phospholipidosis, or safety pharmacology liabilities, not to mention pharmacokinetic, metabolism, and pharmacological receptor interactions. In our opinion this part of drug discovery is relatively well established and tends to work well unless there are limited resources, such as insufficient funds or expertise, in which case the opportunity to optimize emergent candidate drugs for safety and realize potential patient benefits in an ongoing manner may be missed.

When one looks beyond the drug substance, the design and choice of the right formulation often lack safety considerations. Most formulations are chosen for convenience, cost savings, and manufacturability, not proactively for safety. Of course, there are exceptions, an example of which is developing an extended-release formulation intended to cap high maximal plasma concentrations (C_{max}) associated with an adverse effect while maintaining overall exposure on an area-under-the-curve (AUC) basis. However, formulation development for optimization of safety risks reflects unrealized opportunities in many pharmaceutical companies. A poor choice of formulation can negatively affect the safety profile further. Occasionally, a smart, safety-focused formulation can enable an otherwise impossible product to be developed.

Once a candidate drug has been selected, some simple questions need to be answered: What is being done to optimize the drug product's safety profile further and maximize the risk-benefit ratio? Who is doing this? However, this process can quickly become confused and muddled without clarity in structure and approach. There is often no consistent approach to developing safety strategies or objective, quantified optimization, and balancing the product's risk-benefit profile. The underlying assumption often is that all efforts and hopes leading to the candidate drug are locked in the construct. What the regulators expect regarding safety at subsequent development stages is taken care of by regulations and guidelines. Should the product unexpectedly

experience safety issues or even be terminated for safety reasons, it is considered nothing but bad luck and part of the game, similar to drawing a blank in the lottery. Of course, we are exaggerating, but not much. It is undoubtedly true that guideline-conforming preclinical and clinical activities generate a great deal of safety and derisking for trial subjects and patients. However, there is a lot of room for improvement.

To understand the meaning behind the current guidelines, we must briefly look at our legislation's roots. Two main lines of argument must be considered. One regulates the way in which we are conducting clinical trials, the other regulates how safety is meant to be established for pharmaceutical products.

The current legislation ensuring appropriate moral and ethical standards and the safety, integrity, and dignity of humans in clinical trials reaches back to the despicable human experiments in Nazi Germany before and during World War II. This triggered first the Nuremberg Code (1947), followed by the Declaration of Geneva (1948), and then the all-important Declaration of Helsinki in 1964, in which the World Medical Association defined the ethical principles for medical research involving human subjects, including research on identifiable human material and data. It followed the implementation of Good Clinical Practice in 1996, which was refined with Directive 2001/20/EC in the European Union (2001) and the 2014 Regulation 536/2014. In simplified terms, this regulatory framework is intended to ensure the safety, dignity, and integrity of trial subjects by specifying quality, safety, and efficacy criteria in clinical trials from a sponsor obligation perspective.

The other regulatory framework also has its roots in extraordinary adverse events (AE) in the past. One was the sulfanilamide incident, in which a toxic elixir killed over 100 people in the United States. This led to passage of the Federal Food, Drug, and Cosmetics Act in 1938 in

the United States, thereby setting the foundation for quality standards for foods, pharmaceuticals, cosmetics, and medical devices as well as government oversight and enforcement. The other incident was the infamous thalidomide disaster in the late 1950s and early 1960s. The catastrophic side effects were not recognized for many years and ultimately led to the death of or severe congenital malformations in thousands of children. The resulting EEC Directive 65/65 eventually triggered two major inflection points in the pharmaceutical industry. One was implementing mandatory and standardized preclinical testing of emergent pharmaceutical products. The other was the introduction of pharmacovigilance to monitor marketed pharmaceutical products' fate and behavior.

Both regulatory frameworks have a common theme: They are reactive to severe safety incidences in the past. The pharmaceutical industry has generally been noncommittal and passive in forwarding new safety standards for emergent and marketed medicines. Thus the reactive nature of safety-related legislation remains unbroken. Until this changes, we are likely to see more unpleasant safety issues involving new and old drugs as the driving force in the evolution of new safety legislation.

From this perspective the above-stated assumption that compliance with existing guidelines is enough to establish acceptable safety in clinical trials and marketed products is unsustainable. The regulatory framework could not prevent disastrous clinical trial sponsor obligation violations in the theralizumab (TeGenero) and BIA 10-2474 (Bial-Portela) phase I trials in 2006 and 2016, respectively, leading to the death or severe, lasting injuries of healthy young trial subjects. Nor could they anticipate the safety-related market withdrawals of, cerivastatin (Baycol, Lipobay) or Vioxx (rofecoxib), for example, which brought even mighty pharmaceutical companies to the brink of collapse.

Through these examples we see that existing regulations are there to prevent a repeat of known disasters. This is very different from producing and establishing competitive, optimized safety profiles of pharmaceuticals. And we have only spoken about major safety incidences, not the ocean of near misses and unrealized opportunities to improve the safety profile of medicines and to optimize the risk-benefit ratio that a person is likely to experience. Significantly more creativity and initiative are required in the pharmaceutical industry to tap into this wealth of opportunities. We recognize that it is not easy and may take some time, but it is definitely achievable.

On a more positive note, we recognize that preclinical and nonclinical toxicology has experienced a dramatic development since structured, quality-controlled studies became mandatory following the thalidomide disaster. The area has expanded in the form of, for example, discovery safety and safety pharmacology, test assay and test battery development, assay refinement, and predictive sciences,

such as computational toxicology and predictive safety modeling. In a way, this is not surprising. The demands on preclinical safety that were brought to the fore in the 1970s were met by existing structures within pharmaceutical companies and the well-established scientific discipline of toxicology, of which pharmaceutical safety is only a sub-discipline. Today, robust and lively expert societies form global networks and ensure close communication, proximity, and collaboration among industry, academia, and government. This is reflected in many joined working groups, which have resulted in the generation, refinement, and harmonization of some of the most prominent preclinical and clinical safety guidelines in existence today.

We wish the same could be uniformly stated for the discipline of pharmacovigilance. Following the thalidomide disaster, regulatory demands for the surveillance of marketed products met the industry somewhat unprepared. The principles of monitoring the safety of medicines and medical devices on the market had to be developed literally from scratch. There were no organizational structures, working principles, processes, or dedicated prior expertise to build on. Pharmacovigilance departments had to be established from nothing. Required expertise was, and is still, somewhat unclear. Although there are early glimmers of hope that this is about to change with the European programme in Pharmacovigilance and Pharmacoepidemiology ([IMI Innovative Medicines Initiative et al., n.d.](#)), it is surprising that even 50 years later, we have no explicit role descriptors or standardized, accredited educational programs for pharmacovigilance and clinical Patient Safety professionals.

Nevertheless, considering the limitations of information technologies in the 1970s and 1980s, it is still quite impressive how quickly some working structures and pharmacovigilance departments were set up. Establishing the principles of adverse event (AE) reporting, case handling, case reports, safety databases, signal detection, signal evaluation, and risk management was undoubtedly no small feat.

Nevertheless, pharmacovigilance was—and is—not always seen as a value-adding discipline. Successes in surveillance, signal detection, and risk management often mean limitations in a drug product's commercial business case. This may be one reason why many pharmacovigilance and patient safety departments are frequently isolated and operate from a comparatively peripheral position in many pharmaceutical companies.

Furthermore, past successes may be why the discipline today appears to be stuck in the past—at least in our opinion. Many rate-limiting pharmacovigilance features still reflect the technology standards of the times when electronic typewriters, floppy disks, and fax machines were the norm. For example, AEs are still reported in the form of (tedious!) individual case reports based on the old paper CIOMS II form. Electronic versions, such as the

British Yellow Card Scheme, provide only marginal improvements as a computer can be used to fill in the form. Even so, front-line physicians in the health services are in time-pressured environments, and typing all the information into the form is both repetitive and laborious. Theoretically, almost all information could be taken from other sources (e.g., the patient journal or health records). Physicians should need to use the time only to add their perspective and insight. Even though this should be possible, cross-referencing to existing data and information is currently quite limited.

The discipline of pharmacovigilance is still weak or even blind to medical realities such as generics, drug combinations, polypharmacy, and the higher incidence of AEs in multimorbid patients who are in critical condition or at the end of their lifespan. Scientific aspects such as pharmacological and toxicological mechanisms, epidemiological or genetic data, or even direct competitor information are still challenging to implement in the existing pharmacovigilance framework. There is also a strong element of individual bias built into the system's spontaneous reporting of AEs. Today, pharmacovigilance acts in many aspects as the administrator of the AE-reporting machinery. Again, the main objective is compliance with the strict reporting timelines in the pharmacovigilance guidelines and legislation. Recent attempts to introduce more scientific content into patient safety functions, sometimes called safety sciences, are still far from being the industry's norm, despite some encouraging success stories in recent years.

Pharmacovigilance was initially meant to monitor marketed drug products only. It is no wonder the discipline is still struggling to adjust to the demands of managing safety in clinical trials. In many cases, pharmacovigilance is not involved in clinical trial safety or is simply copying principles from marketed product surveillance and AE reporting without taking the risks, dynamics, data richness, and demanding safety oversight obligations of clinical trials into account.

It is not surprising that pharmacovigilance departments have not picked up the baton from preclinical safety as the ultimately accountable function to drive safety in clinical development and even for marketed products. Addressing this void is essential for practicing TSM.

Constraints to effective translational safety medicine

This section investigates four major, closely interrelated pitfalls that teams need to avoid in order to have the seamless, value-adding safety optimization of pharmaceuticals throughout the value chain:

1. the broken safety value chain;
2. the missing conceptual approach to safety;

3. the nature of safety work;
4. organizational constraints.

The broken safety value chain

As alluded to in the previous (Chapter 22, Pharmaceutical Toxicology), many companies invest heavily in predictive safety in the early stages of drug discovery. Safety criteria drive, at least in part, the thorough assessment of potential drug targets, target selection, and objective-driven design of new chemical entities or biological modulators. This often happens at the very edge of technological and scientific frontiers. In many cases the level of integrated biological, chemical/structural, and pharmaceutical knowledge created is genuinely impressive. Every resulting candidate drug carries a considerable amount of safety-relevant information and as-yet-untested safety hypotheses and concerns. The problem starts here.

With the selection of a new candidate drug, a change of guard to a different, development-focused team is in order in most companies. This transition is almost always incomplete. A significant amount of the data, information, and experience accumulated up to this point does not undergo a formal and structured handover to this next team or is stored for future teams studying the drug later in the value chain. This results in vast amounts of valuable information being forgotten. Furthermore, many safety-relevant aspects, mostly hypothetical or speculative, are never recorded in early discovery.

We call this effect “the discovery-development dip” ([Fig. 23.2](#)), and it is present to some extent in all organizations. Occasionally, the loss of safety knowledge can be near-absolute, for example, when a candidate drug is externalized or transferred to another location. It is important to emphasize that we are talking about hard data and facts getting lost *and* about the plethora of open and hidden safety hypotheses and concerns. Lack of concise safety records and the absence of safety strategies inevitably lead to a break in the safety value chain at every transfer or change of staff. Who is remembering a theoretical safety concern that was raised in the process of target selection when the resulting candidate drug shows some unexpected features in clinical trials 5 years later on?

Breaks in the safety value chain due to lack of records and communication can happen at any stage, for example, at the preclinical-clinical transition, in some companies at the handover of a late-stage clinical development Team, at market launch, and during postmarket product development and surveillance.

The conceptual approach to safety

Another critical aspect is the relatively immature, inconsistent, and unstructured approach to safety in the pharmaceutical

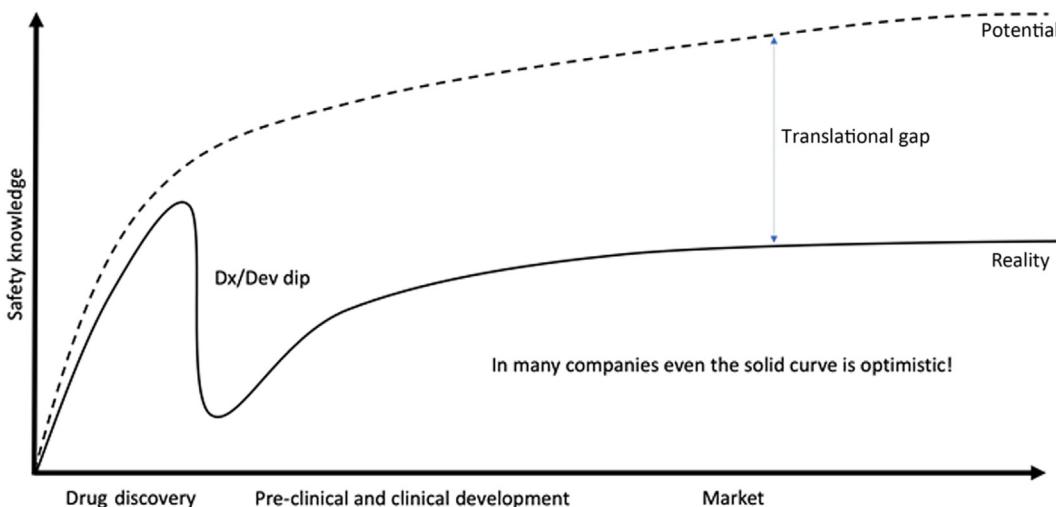


FIGURE 23.2 Dx/Dev dip, discovery-development dip. The graph illustrates the loss of early safety information in the transition to a later-stage team. In many cases the loss in translational safety knowledge remains evident at all later stages of the product. It must be realized that additional data losses happen at all later team transitions as well (not shown).

industry. Many companies still appear to entertain the philosophy “No news from safety is good news.” In a way, this is understandable. Bad safety news can limit or destroy the efficacy-focused commercial business case of a drug product, especially when safety is seen only as an add-on quality. However, suppressing important safety aspects is certainly not a viable response to safety concerns. If the drug is successful, then any unstudied but logical safety concerns will be unveiled either later in development (when it is very difficult to manage them) or when the drug is on the market (which may result in product withdrawals). Neither option is worth not looking at early in clinical development as it is feasible to do so. And who says that news from the safety experts are always bad? In any case, the net effects are that many companies do not have a structured approach to safety and safety strategy development.

Drug discovery’s early stages seem to have overcome such reservations and discovery safety is seen as a constructive value-adding discipline. However, this changes with the first clinical trial—enabling preclinical or non-clinical toxicology studies in many companies. In the worst case, such studies are designed to demonstrate a favorable safety profile rather than to characterize the candidate drug objectively. Another poor practice is to conduct preclinical studies in a self-contained manner relative to generic regulatory compliance criteria rather than aligned to the clinical trials the studies are supposed to support. This apparent uneasiness about objective and aligned safety work is compounded at subsequent, more costly stages of clinical development.

All too often, the focus in clinical development of an emergent drug product is on developing the efficacy claims. There is no real or consistent parallel development of a differentiating and optimal safety—at least not at the

same level of detail and granularity. As long as no significant safety incidents impair or stop the emergent product, safety is generally considered acceptable. The objective of such a nonverbalized safety strategy is entirely on avoiding show-stopping safety issues, not on developing safety knowledge and relieving the tolerability burdens on the patient. In such environments, safety plays a secondary, sometimes passive role through clinical development and beyond, receiving attention only when the marketing authorization application documents are being filled in.

This attitude is also reflected in the fact that safety in clinical development and even for marketed products does not really have an accountable owner. There is no doubt that the toxicology or preclinical and nonclinical drug safety function is responsible for the preclinical space. However, this is far less clear in clinical development and beyond. True, every company has a pharmacovigilance or patient safety department. However, as was noted above, these departments are all too often engaging only in procedural pharmacovigilance and respective compliance activities. The pharmacovigilance department is frequently not involved before the first AE reports from clinical trials trickle in. Operating from a passive, peripheral position, the patient safety experts may not be part of cross-functional, product development teams. The concept of proactively developing a drug product’s safety profile is not yet built into pharmacovigilance’s DNA. Even for marketed products, patient safety departments are frequently engaging only in safety topics resulting from traditional pharmacovigilance activities or health authority inquiries.

Who, then, is formally taking care of maximizing safety in clinical development and beyond? The sad truth is that nobody is! Often, clinical functions have overall accountability for the risk-benefit balance of a product and oversight

of clinical trials. If this does not involve joint accountability between the clinical and safety functions, it creates a purely efficacy-focused environment. This can often lead to safety development being the follow-on activity, playing second fiddle, as discussed above. It is no wonder that safety development is hardly ever conducted in a structured, coordinated, and integrated manner.

Another critical aspect is the limited, strategic approach of preclinical and clinical drug product development per se. Most companies are still following a mindset of “milestone achievements” (Fig. 23.3). They follow the generic stages and associated regulatory hurdles of drug discovery and development (drug discovery, phases I–III). The perception is that once a milestone has been reached, a new game begins for meeting the criteria for the next milestone. The fixation is entirely on the next milestone. It is not unusual for staff performance objectives to hinge on starting and completing tasks rather than on the optimal exploration of critical scientific issues. The aim then becomes pushing the product to the next step and addressing the known hypotheses quickly, addressing new hypotheses only when they cannot be avoided. The accumulated knowledge from the past is mostly ignored, and long-term concepts such as safety development spanning many (or all?) milestones are not reflected in structured strategies. The opportunities of a hypothesis-driven, iterative, and value-focused strategy are entirely missed.

Closely related to the lack of safety ownership and strategy is the apparent lack of safety expertise and experienced staff availability. It is undoubtedly true that some operational aspects of pharmacovigilance are relatively simple and repetitive. However, nothing could be farther from the truth regarding the demands on an effective safety professional and expert development of safety profiles for emerging and marketed medicines. Pharmaceutical product safety optimization requires the acquisition and processing of information from a broad spectrum of company internal and external information sources. Excellent command of industrial processes and a solid understanding of other functions’ datasets are an absolute must. Indeed, people joining patient safety departments from other functions often develop into the best safety experts available, but there are still far too few of them. This situation is unlikely to change as long as safety expertise is considered to be synonymous with mastering pharmacovigilance processes

and until educational and training contents and professional accreditations for clinical safety experts are agreed upon and implemented. Lack of expertise and talent is likely one of the most potent barriers toward more effective safety work in the industry.

The nature of safety work

The nature of safety work is another critically important barrier to effective safety development. This is a topic very few companies reflect upon. It is fundamentally different from developing a pharmaceutical product’s efficacy claims.

Efficacy must be demonstrated in an objective, robust, and statistically verified manner in the population selected for the study. The data alone make the case. There is little room for personal interpretation. Working with safety is fundamentally different. The selection of information sources, data interpretation, risk assessment, and risk management are highly subjective activities. Even two individuals rarely agree 100% on their interpretation and implications of the same safety dataset. It may be compared to riding motorbikes: The same information exists for all to see, yet some people conclude that the risks are acceptable, while many others think they are not. There is no right or wrong.

It would be highly unethical to demonstrate some specific safety endpoints in an objective, statistically verified manner. For example, the oncology product lenalidomide, which can be understood as a potent version of the infamous thalidomide, carries a warning about teratogenicity. Teratogenicity of lenalidomide is assumed in analogy to thalidomide but, strictly speaking, has not been proven. Such conclusions by analogy would be unacceptable for efficacy claims. However, it is easy to understand that a clinical trial to demonstrate lenalidomide’s teratogenic effects in pregnant women is ethically immoral. This shows that in contrast to efficacy claims, safety operates on “the principle of reasonable levels of concern.” However, what constitutes a “reasonable level of concern” is highly subjective and likely to be different for every individual involved.

This implies that safety work requires a plethora of subjective assessments and decisions. Provided that the processes, decisions, and actions that are taken are plausible and unbiased, every outcome is acceptable. However, nothing could be less ill-tuned to the organizational setup and line managerial authority in pharmaceutical companies.

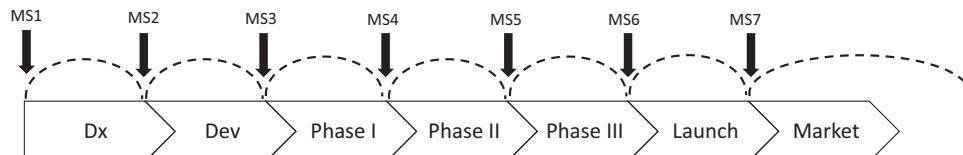


FIGURE 23.3 The prevailing milestone-focused strategy of product development in the pharmaceutical industry. The attention is only on the next milestone and regulatory deliverables. Once a milestone has been achieved, the past is forgotten, and the focus is on the next milestone. This philosophy is a potent blocker to a concise, iterative, value-adding product strategy.

Who is making such decisions? Who is ultimately accountable? How are experts protected when a subjective decision turns out to be wrong? How are subjective safety decisions made in the tension field of other potent interests within a pharmaceutical company? How do subjective safety decisions hold against objective, data-driven efficacy claims? In most companies there is no easy answer to any of these questions. Very few companies have a culture in which safety can be addressed and practiced freely in an unbiased fashion.

We can illustrate this situation through a generic example ([Box 23.1](#)).

Organizational structure

Taking the initiative to drive safety exploration and decisions proactively is not a trivial feat, especially when it is perceived to be contrary to corporate interests. This is difficult enough at the stage of clinical trial—enabling toxicology studies and even more so during clinical development or for marketed products. Apart from the inherent constraints outlined above, a significant issue is the relative lack of corporate structures to support effective safety work during clinical development and beyond. Going back to our example, we can ask ourselves which ethical standards, mindsets, capabilities, roles, responsibilities, cross-functional platforms, and governance structures are necessary to process such a safety concern appropriately?

Practicing translational safety medicine

Organizational setup

The organizational setup and definition of roles and responsibilities necessary for practical safety work must serve several purposes:

1. Seamless and continuous safety-focused working principles from target identification onwards.
2. A protected environment supporting independent and unbiased safety development and separated from efficacy work and other corporate objectives.
3. Structures supporting and protecting safety experts relative to the subjective nature of safety.
4. Operational structures reflecting the cross-functional nature of safety work.
5. Implementation of safety as an indispensable and interwoven value-driver in the mainstream corporate decision and governance processes.

On the basis of initial successes in some organizations, we propose to meet these objectives by doing the following:

1. Implementing concise program-specific or product-specific safety strategies.
2. Implementing cross-functional safety working groups.
3. Establishing supporting governance and conflict resolution structures.

We would like to emphasize that the proposed changes do not require additional resources or significant investments in most organizations. Well-organized and aligned safety work often saves resources and adds to the overall value of the company's products and reputation. Proposed changes are possible by reconfiguring existing structures and working principles. However, we must stress that these intuitively trivial and simple concepts require profound, substantial changes in both people's mindsets and operating principles. They do not come easily to any organization. Harsh, multi-layered, cross-functional resistance is quite common.

An organization should not attempt to implement TSM principles without support and commitment from senior management and the availability of well-established change management capabilities. Is it essential to highlight that

BOX 23.1 A typical safety situation in the clinical development setting

Assume that a company is developing a pharmaceutical product that is currently in highly promising phase II clinical trials. The company has spent significant resources on the project, and many individuals involved are linking their career prospects to the program's success. A competitor product is at the same stage in clinical development. A hypothetical concern derived from the initial target safety assessment years ago suggests that the target's pharmacological modulation may impair blood coagulation and thus might lead to thromboembolic events. The concern was never addressed. Three cases of serious thromboembolic events were noted in a phase II clinical trial involving more than 100 patients. The relationship of these events to the study treatment is not clear. Alternative explanations, although weak, do exist in all three cases.

Imagine yourself in the role of a safety-accountable person. Speak up or stay quiet? Terminate the project, continue, or investigate and potentially fall behind? How to defend whichever decision is taken? What if a decision turns out to be wrong? How to face your colleagues, your line manager, or senior management in the company? Where does it leave the company and patients if staying quiet backfires in the results of more extensive phase III trials or in further (and potentially preventable) poor outcomes for the patients in these future studies? Even if you bring up the issue in a product team meeting, you are up against six or seven functions with different objectives than yours. Such situations are the daily bread-and-butter of safety professionals in all companies.

the proposed changes are minimum, closely interrelated requirements of a basic TSM system. Leaving out critical elements is unlikely to deliver good results and may even lead to friction and dissent.

The safety management team

We have previously mentioned the relative absence of an accountable, operational body for safety and product safety development in many organizations. It does not mean that safety-focused teams do not exist. In discovery the preclinical/nonclinical safety department is usually responsible for safety. However, these groups may have ended up isolated at the start of the value chain, and their work is focused on broad exploration rather than the strategic, forward-looking cross-functional integration required elsewhere.

In the clinical development space, some companies established safety-focused teams. However, these are often formed too late, have unclear objectives, lack defined working principles, are too large, are poorly structured, and sometimes even lack formal safety expertise. Operational and governance aspects are frequently confused, and conflict resolution mechanisms are absent.

A simple, but effective, solution is the set-up of safety management teams (SMTs) as described below. Please note that some companies even have teams with a similar name. However, they often resemble the inadequate setup described above and are not what we are proposing.

The key feature of an SMT is the purposefully assigned ultimate authority for all program-level safety aspects, including clinical trials. *Program-level* means a drug project or marketed product in contrast to, for example, the individual clinical trial level. By *ultimate authority*, we mean exactly that: the authority to make all day-to-day safety decisions in a drug project or for a marketed product without line-managerial or hierarchical interference of any kind. It is this level of authority that creates the space for unbiased, independent safety work. The high level of authority levels must be supported and contained by effective corporate governance and conflict resolution structures and principles.

Assigning ultimate authority to any given group is the hardest part for most organizations. It is in direct conflict with the hierarchical setup and line-managerial control principles found in most companies. There may be the fear that such powerful SMTs become “gray eminences” and influence program- and corporate-level processes and decisions beyond their remit and purpose. However, nothing could be farther from the truth. The subjective nature of safety simply requires that subjective safety positions be developed independently before the results are subjected to higher-level corporate decision-making processes. Otherwise, safety initiatives can easily be suppressed or biased at an immature stage as a result of the subjective nature of safety. It is a matter of

governance and conflict resolution principles to support the SMTs’ high level of authority and independence.

The purpose of the SMT is strictly operational. The day-to-day safety work is managed and done by this team and nowhere else. The team must be small enough to facilitate the operational nature but large enough to integrate the cross-functional expertise necessary for effective safety work. Experience suggests that a good starting point is having a core team that includes the following lead representatives:

1. preclinical/nonclinical drug safety;
2. R&D clinical organization;
3. patient safety/pharmacovigilance.

In other words, the core team consists of the toxicologist, the medical director (clinical physician), the patient safety physician, and the patient safety scientist (safety scientist). Other functions should be considered on an as-needed basis, although of course there are times in the value chain where other representatives end up being present as the norm (e.g., pharmacokineticist through the early stages of drug development). However, the need for additional members should be assessed carefully to maintain the team’s operational nature. Often, this can be managed on a consultation basis ([Fig. 23.4](#)).

Ideally, the patient safety scientist and the physician are coresponsible for the scientific strategies and data interpretation. They are also accountable for the SMT process, team composition beyond the core team, and assignment of permanent or *ad hoc* voting rights to non-core members. In this capacity the patient safety scientist is chairing the SMT and contributing their scientific discipline to the data collection and interpretation throughout by all experts. In our opinion this setup supports the operational nature of SMTs. However, we realize that it may require adjustments in physician-centric organizations.

The patient safety physician works with their safety scientist counterpart and is responsible for the safety strategy

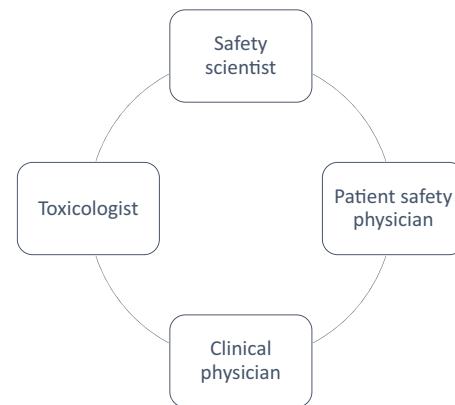


FIGURE 23.4 Safety management core team. It can be extended by adding other functions as needed.

and overall safety profile of the SMT's project or product. The clinical R&D physician provides the clinical context and efficacy information of the drug project or product to the SMT to ensure that the balance of benefits and risks can be discussed in an ongoing manner and undergo a thorough reevaluation as needed when new information emerges.

The toxicologist is providing preclinical/nonclinical drug safety and safety science content to the team. We cannot question enough the common fallacy of assuming the toxicologist is redundant once a candidate drug has entered clinical development.

The SMT generally works on a strict consensus basis. In most companies, drug-projects and marketed products are managed by cross-functional teams, often called global product teams (GPTs) or the like. In larger clinical development projects or for major drug products, clinical subteams are specifically tasked with developing the therapeutic efficacy claims. It makes sense to establish SMTs that are responsible for safety and safety development in a similar manner. Work and value streams (safety and efficacy) should be equally represented and organized. Just as clinical R&D is represented in the SMT, the patient safety or pharmacovigilance lead safety physician and their scientific counterpart (where scientific elements are discussed) should be members of the efficacy-focused clinical subteam along with the clinical physician and scientist working on the product. Both the clinical R&D and patient safety lead physicians should be core members of the GPT. This setup keeps both efficacy and safety aspects close to the attention of the GPT and maximally supports the integrated, qualitative, and quantitative risk-benefit assessment process. It must be emphasized that the SMT is accountable for safety only. The GPT owns the risk-benefit integration and assessment (Fig. 23.5).

To overcome the above-mentioned “discovery-develop gap” and information losses at subsequent stages, project-specific SMTs should be formed at the time of candidate drug nomination. They remain in place until the project's termination or withdrawal of the resulting product from the market. The first task of an SMT is to take over the safety responsibility from the discovery safety team, which exists in various formats in most companies. The SMTs are responsible for capturing all relevant safety information, hypotheses, and concerns that have been generated so far in a strategic safety record system. This system is developed continuously and remains with the SMT as long as the SMT exists. We expand on this topic later in this chapter.

Some companies maintain specific subteams for large clinical trials under the level of the clinical subteam. It is prudent to organize such clinical trial teams under the authority of both the clinical subteam and the SMT. The SMT is responsible for ensuring that the declared project safety strategy is reflected in the clinical trial design, and the trial's sponsor safety oversight is conducted appropriately.

Safety governance

The SMT's autonomous and highly exposed position must be supported, balanced, and contained by practical corporate safety governance principles. Unfortunately, few companies have robust processes in place. All too often, the interpretation of immature safety-relevant information is subject to governance intervention and is evaluated by objectives other than safety—sometimes by functional representatives who lack specific safety training or expertise.

Strictly speaking, the interpretation of safety data and the basis of a safety concern should always stay among the experts in the SMT. Only the resulting decisions, activities, and strategies should be subject to corporate governance. In this sense, SMTs must present concisely worked-out cases and plausible positions. Decisions by the governance body are confined to “approval,” “rejection,” and “imposition.” The latter two outcomes are related. Proposals cannot be rejected indefinitely without halting the development program. A rejected proposal may eventually lead to an imposition. Impositions transfer the corporate safety accountability for the task at hand from the SMT to the governance body and its chair and must be documented accordingly. The governance body may also provide counsel, but this aspect is outside the formal decision-making process.

It must be recognized that pharmaceutical companies' safety governance demands may emerge from entirely different contexts and topic areas. One governance body is usually not adequate to address all such needs appropriately. Therefore we propose three separate, strictly

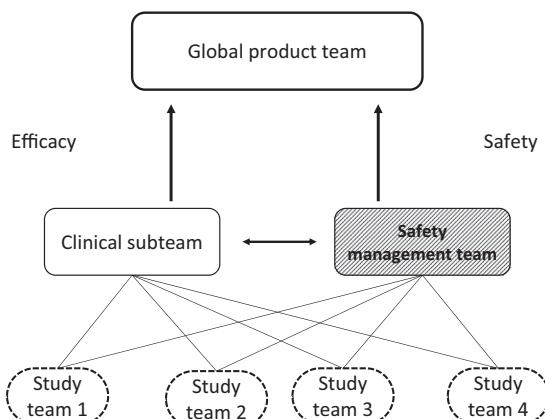


FIGURE 23.5 The position of safety management teams in the organizational context. The duality of concomitant efficacy and safety development is ingrained in this structure. Clinical study teams are applicable only for large, parallel clinical trials.

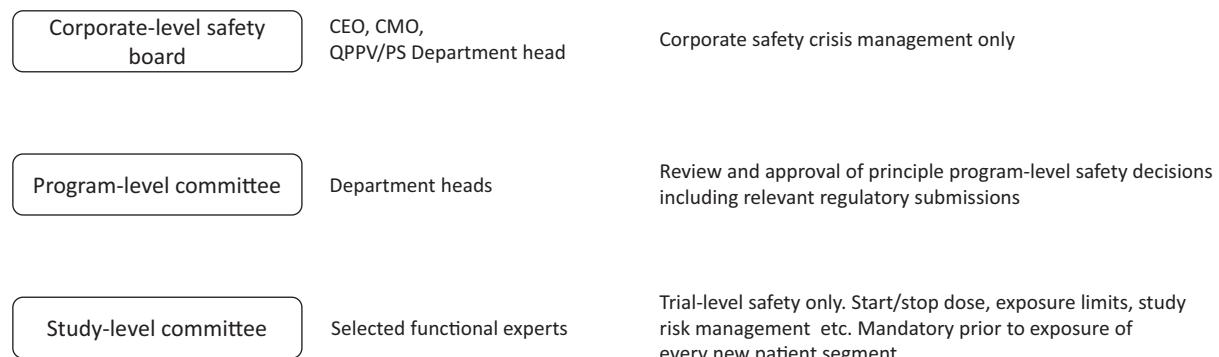


FIGURE 23.6 Three-tiered, nonhierarchical safety governance bodies.

nonhierarchical governance bodies: one covering clinical trial safety, another focusing on program-level safety (projects and products), and a third dealing with corporate-level safety crisis management (Fig. 23.6).

Every clinical trial is a human experiment and demands particular care and caution. All clinical trial-specific safety aspects should be evaluated and approved by a formal governance body of experienced and explicitly trained cross-functional safety experts. The chair can be a senior member of the patient safety or pharmacovigilance department. This team should have the highest level of specific safety expertise within the company.

Interaction with this governance body is mandatory every time a new, relevant patient segment is exposed to a candidate drug for the first time (e.g., healthy volunteers, patients, women of childbearing potential, elderly, children). It is common practice to review aspects such as cohort size, dosing regimen (including, e.g., sentinel dosing), trial subject exclusion/inclusion criteria, starting dose, dose intervals, stopping criteria, exposure limits, safety screens, use of biomarkers, and management of potential and identified safety risks. There is a European Medicines Agency guideline ([Guideline on strategies to identify and mitigate risks for first-in-human and early clinical trials with investigational medicinal products, n.d.](#)) that provides excellent guidance in the topic areas to be covered in this respect. Any relevant changes in the approved plan and scenario require a new governance review and approval. Unexpected, severe safety issues in ongoing trials that affect program-level safety should also be presented to this body before submission to the program-level governance body. However, this does not imply a hierarchical nature of the trial and program-level safety governance. The SMT is the accountable body for interactions with clinical trial-level governance and presentation of respective clinical study plans.

Program-level safety topics should be reviewed by a specific program-level governance body, which often comprises R&D functional department heads and is chaired by the chief medical officer or the patient safety or pharmacovigilance

department head. This body reviews significant changes in a drug project or drug product safety and risk-benefit profile, including safety-related program terminations, significant safety-relevant strategic changes, and notable regulatory submissions. The SMT is accountable for presenting program-level safety positions. The GPT is responsible for delivering an integrated risk-benefit position, even if it has been provided by the same clinical R&D and patient safety lead physicians on the SMT. It is critical to separate safety and efficacy input from the risk-benefit integration process. It is all too tempting to tweak the risk-benefit assessment by downplaying the safety part and overemphasizing efficacy aspects.

Furthermore, the governance body must be careful to remain within the constraints of safety and risk-benefit assessment. It takes only a short step to make the mistake of evaluating the corporate business case instead of safety and risk-benefit topics. Representatives of commercial functions are generally excluded from safety governance processes.

The response to corporate safety crises requires yet another type of safety governance and management. Here, fast, empowered leadership is needed to prevent harm to patients, the company, employees, stakeholders, or the environment. A small team typically comprising the chief executive officer, the chief medical officer, and the patient safety or pharmacovigilance head of function is appropriate. It is sometimes called the executive safety committee or the like. Strictly speaking, this is not a pure safety governance body; it is more a crisis management team. For drug projects or product-specific safety crises the respective SMT is accountable for providing input to the crisis management and decision process.

Conflict resolution

As was mentioned above, the SMT is operating on a strict consensus basis. The demand for consensus is a crucial element in ensuring sound and thorough operational principles. However, on rare occasions, an SMT may be unable to achieve consensus on a particular topic. Well-defined

conflict resolution principles are asked for. However, most companies do not have any such structures, processes, or principles in place. Conflicts are resolved - or shall we say, suppressed - by line managerial authority or, worse, are entered into corporate governance processes. Of course, line managerial conflict suppression is in direct conflict with the SMT's safety accountability. The governance track is a paradox loop as enforced conflict resolutions result in positions that must be reviewed and approved by the same body. It would be nothing else than an imposition on the SMT's accountability. We see many benefits in establishing clear conflict resolution principles. It creates not only transparency and clarity but also discourages deadlock situations by their mere existence.

We are proposing a tiered approach to conflict resolution in an SMT (Fig. 23.7). It should be emphasized that all conflict resolution steps are strictly advisory and supportive in nature and do not usurp the SMT's authority. In case of significant dissent in an SMT, the first step should be to seek joined counsel by the heads of functions represented in a respective SMT. This is already a significant escalation step and is unlikely to be taken lightly by any SMT. Almost all conflicts should be resolved at this level. However, we stress again that the heads of function provide counsel only. They have no executive power over the SMT and its members to force them into a particular position. A line manager certainly has the authority to exchange a functional SMT member, but this should be considered poor practice in a conflict situation.

If a conflict cannot be resolved at this level, it is possible to delegate the controversial task to the clinical trial

governance body members. In turn, they present a position supported by a simple majority vote to the program-level governance body. Any result of this (ultrarare) process is generally considered an imposition on the SMT. The accountability lies with the program-level governance body.

Safety strategy

Safety development of a candidate drug follows an incremental, stepwise principle. Animal studies are undertaken to characterize and manage the risk for humans. Subjects in human trials are often young, healthy male volunteers (phase I). The healthy volunteers in turn are derisking the next step to patients. The implied assumption is that healthy volunteers are potentially less at risk than health-compromised patients are. The first patient segment is usually defined in comparatively narrow terms (phase II) and derisks the next step, in which broadly defined patient segments (phase III) are exposed. This principle is not broken even after successful phase III trials. Phase III in broadly defined patients is simply meant to derisk the step to market exposure with potentially even broader characteristics and considerably less means of medical control and supervision compared to clinical trials.

It is a not uncommon misconception to see this process as a simple, linear process represented by the mandatory clinical development phases. Preclinical testing continues in parallel to clinical trials and contributes to derisking of new patient segments (e.g., women of childbearing potential, children, adolescents) or longer exposure durations or

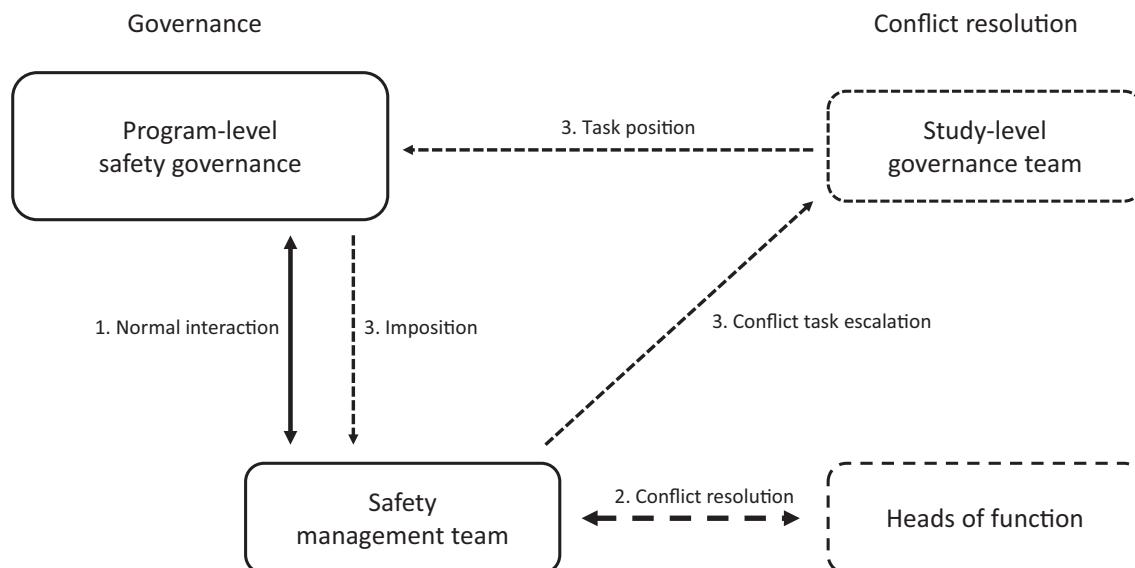


FIGURE 23.7 Conflict resolution in case of safety management teams (SMT) disagreement. 1: The normal interaction between SMT and the program-level safety governance body. 2: Conflict resolution by the heads of functions represented in the SMT. 3: Escalation of conflict task from SMT to the study-level governance body (not acting as a governance body) that presents the case to program-level safety governance. Any decision is an imposition on the SMT.

to evaluate possible long-term toxicities such as cancer or postnatal development over several generations.

Special situations apply for oncological and some rare disease and emergency medication indications for which initial clinical trials in healthy volunteers are considered inappropriate. The inherent risks of candidate drugs in such areas are justifiable only relative to a potential benefit in respective patients. In oncology, first-time-in-man studies are often conducted in so-called end-stage patients who have no other treatment options. Caution is warranted, as these patients do not represent the same predictivity as healthy volunteers do in nononcology settings. Such patients have often suffered considerable organ and tissue damage as a result of prior therapies. This may alter the response to a new candidate drug in many ways. This should be consciously taken into account at all times to protect this trial population. Consequently, the predictivity toward the intended, less affected patient segments is limited—for better or worse!

The development of a coherent, value-adding safety strategy for each project or product may be seen as one of the lowest-hanging fruits in the implementation of TSM. As a matter of principle, safety strategy development must start with the activities leading to selecting a pharmaceutical target. Just as safety aspects cannot be introduced into an airplane's construction when the blueprints are mostly done, safety planning and strategy cannot be left to a late stage in pharmaceutical R&D. Ideally, safety consideration should already be part of the target selection process. However, there is a fundamental difference from safety management in engineering. Our understanding of biology, pathophysiology, chemistry, and human behavior—related safety concerns lags far behind the level of control of mechanical engineering risks.

For this reason, any safety strategy in a pharmaceutical context is far more uncertain and speculative. A safety strategy must rely on continuous exploration and learning and therefore must be continuously adjusted. Whereas safety strategies in mechanical engineering may be linear (goal setting → strategy development → execution), safety strategies in a pharmaceutical context are far more iterative. Losing control over the iterative cycling of explorative, investigative safety development means losing control over the safety value chain. This is the reason why “milestone achievement” approaches and mindsets do not work in safety development in the pharmaceutical context. The iterative mindset for safety development must be initiated in early drug discovery and be maintained throughout all subsequent stages. The emerging pattern is one of small, rapid integrative learning cycles in drug discovery with larger, slower, more powerful, and more costly learning cycles as the candidate drug evolves.

Target safety assessment demonstrates that no pharmacological drug target is free of potential safety risks. Concerns

may arise from the target's specific biological action, related upstream or downstream pathway modulation, target specificity, or expression variability, among other issues. In many instances, concerns that are raised at this early stage are hypothetical and cannot be investigated experimentally due to a specific target modulator's unavailability. It is subject to a well-developed safety strategy to capture such safety concerns, rank them by perceived relevance, develop suitable hypothesis-testing strategies, and ensure execution at the earliest point in time. A well-developed safety strategy also includes quantitative safety risk evaluation relative to the development timelines. For example, a potential show-stopping safety concern that can be investigated and verified at the preclinical stage would carry significantly less weight than one that may reveal only itself in late-stage clinical development or on the market.

Developing and implementing a proactive, strategic approach to safety development comes fairly naturally to most scientists. The challenge is to maintain the momentum and to keep the baton in the game at the various subsequent handover processes and when other objectives gain weight and influence. Naturally, the safety strategy will continuously evolve and be adjusted as more information becomes available, sometimes at a dramatic pace. To stay focused, it is highly advisable to develop a safety-focused target product profile (TPP). The fact that the safety TPP will change frequently is no reason not to compile one. Such a document should clearly state the optimal and minimal realistic safety requirements quantitatively and qualitatively at significant development milestones and on the market.

Almost all companies compile TPPs for their emergent candidate drugs. However, such documents are frequently biased toward efficacy criteria, sometimes to the extreme. Safety targets are either absent or expressed in nondescript terms such as “acceptable.” Such target criteria lack the purpose and direction to guide the development of a concise safety profile.

To illustrate the value and purpose of a well-thought-through safety strategy and defined safety targets, we can look at the spectacular safety development of lenalidomide (e.g., Revlimid, developed for the treatment of multiple myeloma) ([Galustian and Dalgleish, 2009](#); [Zeldis et al., 2011](#)). The product is closely related to the infamous thalidomide, which caused death and severe congenital malformations in thousands of children. The invention and development of a product that shares the mode of action and the safety liabilities of thalidomide truly require very conscious safety-focused development capabilities and strategic planning. We are not aware that any attempts were made to change the toxicological profile from that seen with thalidomide in the design of lenalidomide. The known adverse profile is related to the pharmacology of the molecule, but by applying this knowledge to a careful and focused selection of the right patient segments and

implementation of appropriate risk mitigation measures, a favorable risk-benefit profile was created for an indication different from that of thalidomide. At the time of writing this chapter, lenalidomide was the third best-selling pharmaceutical product globally, with sales of U.S. \$9.7 billion per year (Urquhart, 2020).

Recording safety information and strategy

This is not an easy task. Appropriate safety records must serve several purposes:

1. state the short-term, intermediate, and long-term safety goals (TPP);
2. reflect the current safety profile;
3. state identified risks, safety hypothesis, and concerns;
4. inform about risk mitigation and investigative activities;
5. state summaries and references to the relevant data sources.

In contrast to the comparatively linear, accumulative nature of developing efficacy claims, safety development is far more iterative, dynamic, and occasionally disruptive. New data, information, and insights or changed program strategies may change previous safety assumptions almost daily. For this reason, safety records must be built cumulatively and support the recreation of the state of safety knowledge and strategy for any point in time in the past. A sound system also supports the automated creation of safety summary reports for senior management. Such a system may be operated by preclinical or nonclinical drug safety up to candidate drug identification and then by the patient safety or pharmacovigilance department or a cross-functional SMT.

Table 23.2 shows a simplified but typical information base shortly before candidate drug nomination. It is evident

that safety decisions must be taken and that no possible option is unambiguously supported by the data available. It is also important to highlight that many potential risks may be addressable only in the future. Decisions must be taken on a subjective basis. There is not enough information to support data-driven decisions. For example, are the potential receptor selectivity issues relevant in patients and healthy volunteers? How should the datasets for lead 1 and lead 2 be evaluated? Which is the better compound? What do the discovery toxicology results tell us? Is lead 1 better, or should lead 1 and lead 2 be further developed in the same manner? If not, how should GLP toxicology studies be adjusted? Depending on the lead chosen, how should phase I clinical trials be designed? Are studies in typical male, healthy volunteers justified and meaningful? What are the limitations? What could phase II and III studies look like? What are the safety requirements for possible market authorization in this indication?

There are clearly no right or wrong answers to such questions. Many different permutations, scenarios, and decisions are possible. The only evil would be not to ask the questions, not to take safety decisions, and not to process the given information from a safety perspective. Right is every plausible and meaningful, agreed decision and strategy. However, decisions and strategy can be revisited, evaluated, and refined only if the thinking, assumptions, hypotheses, and information base have been recorded in a meaningful way.

In this context it is worth mentioning the concept of informed attrition. This is simply the proactive attempt to terminate a candidate drug with unacceptable safety features as early as possible. It implies that key safety concerns are translated into a well-formulated hypothesis and are tested at the earliest possible opportunity. This approach is often

TABLE 23.2 Example of a selected information basis shortly before candidate drug identification.

Target safety assessment	Chemical leads	Discovery safety studies	Patient info
Mutant constitutively active receptor responsible for disease. Human specific. No validated animal model. 4 structurally related receptors in 2 different regulatory pathways present in all mammals: Likely effect of inhibiting pathway 1 → sedation, cognitive impairment. Likely effect of inhibiting pathway 2 → tachycardia	Lead 1: a. Excellent suppression of mutant receptor in vitro b. Not crossing blood-brain barrier in rat and dog c. Not active on pathway 2 receptors, active on 1 d. Active on hERG and alpha adrenoceptors at high concentrations e. Short elimination half-life in rats Lead 2: a. Medium target inhibition b. Crossing blood-brain-barrier in dog c. Low level inhibition of pathway 1 and 2 receptors d. Very long elimination half life in dogs	Lead 1: a. No organ/tissue toxicity rat and dog b. Short-duration tachycardia in rats c. Negative genotoxicity screen Lead 2: a. Mild liver histopathology in dogs b. Mild subdued behavior in dogs after 5 days doing c. No adverse effects in rats d. Negative genotoxicity screen but test substances precipitates noted in assay	Median age at fully developed disease: 70 years Age-typical comorbidities and medications Onset of symptoms: >60 years Suggested start of preventive treatment: 40 years Carrier of mutated receptor can be identified via genetic screening.

perceived as intuitively counterproductive. However, isolated and exploratory safety issues that can be tested consciously in early stages are bound to reveal themselves at later, much more expensive and sensitive stages anyway. Aiming for informed safety attrition opportunities at pre-clinical or early clinical development stages should be an integral part of all product safety strategies. Open stop-go safety topics that cannot be addressed in these early stages of product development pose an almost unacceptable risk in most instances. Ethical considerations as well as the need for preserving a company's scarce resources demand the implementation of informed safety attrition concepts.

Safety strategy document

An essential task of every SMT is to create, maintain, and develop a safety strategy for its project or marketed product. This should contain the cumulative and always up-to-date summary of safety information, hypothesis, concerns and speculations, ongoing safety workstreams, and, most important, a forward-looking safety strategy according to defined criteria (e.g., a safety TPP). Because safety changes continuously, the system should be set up to allow easy entry of new information. The system should support the recreation of the records for any point in time in the past. For example, it must be possible to extract the safety information basis, the thinking, and the strategy before a phase I trial, during the trial, and after the trial.

This safety strategy serves as the blueprint for all program- and study-level safety activities and high-level safety documents (e.g., clinical study protocols and reports, investigator brochures, risk management plans, periodic safety update report, /periodic risk-benefit evaluation reports).

It is evident that we are not talking about a conventional paper or paper-like electronic system. That would be far too tedious to maintain and develop. This task requires a digital database setup that can be continuously updated by relevant new information while retaining all previous records. A smartly set up system should support the automatic generation of a readable document whenever it is needed. In our experience, senior management frequently requests an up-to-date excerpt. This sounds demanding from an IT perspective but can be realized relatively quickly by, for example, an experienced Microsoft SharePoint programmer.

In the introduction to this chapter we said that TSM is about developing and optimizing emerging and marketed pharmaceutical products' safety profile. However, a concise outline would fill a rather voluminous textbook of its own. Here, we can only sketch out some basic principles.

In a way, the demands on safety experts in the pharmaceutical industry are substantial. Meeting the above-mentioned safety objectives for healthy trial subjects, patients, clinical trials, the development program or

product, the company, regulators, and the law is no trivial matter. It requires the following:

1. a continuously developed, objective, proactive safety strategy right from the start in early discovery;
2. a solid record of cumulative safety data, information, hypothesis, and concerns;
3. constant 360-degree, three-dimensional vigilance;
4. a curious and critical investigative (forensic) mindset;
5. preparation for the unexpected.

Constant 360-degree vigilance

The emerging safety profile and potential safety risks may change literally every single day in a drug project or for a marketed product. A good safety expert is continually scanning the environment for new information and, especially, the earliest signs of an emerging safety issue or problem.

There are no excuses if animals and humans come to harm because a relevant piece of information was not processed adequately and promptly. Maintaining a constant finger on the pulse of the data and information flow from all directions is quite challenging. A significant obstacle lies in the fact that most potentially safety-relevant data and information is not generated and owned by the safety experts themselves. Data may come from other functions or subdisciplines within their own function, from outside sources such as scientific literature or competitor companies, from distant external service providers, or from complicated processes, for example, within pharmacovigilance. This is also the case in clinical trials, in which the flow of information to the sponsor can be languid, tedious, and unstructured, despite the legal requirement for oversight.

The selection of relevant information sources is a critically important part of an asset's safety profile and respective safety strategy. However, it is not always clear what kind of information or data are relevant. It is never enough to consider only toxicological data from the pre-clinical or nonclinical drug safety department and safety signals derived from pharmacovigilance AE reporting. Nor is it acceptable to derive alerts only from clearly spelled out safety concerns. A fair safety assessment takes into account the biological mode of action in health and disease, animal and human pharmacokinetics (PK) and metabolism data, competitor and related product information, patient descriptors (including comorbidities and comedications), and current as well as emerging country- and region-specific medical practice. A relevant safety concern may well be derived from connecting information and data from many different sources. All information sources feeding into these topics are relevant. In many instances, datasets serve more purposes than safety development. For example, PK and metabolism datasets are not necessarily constructed with a focus on safety. However,

they make up one of the essential aspects of quantitative safety and risk assessment.

Extreme vigilance is particularly required in clinical trials, especially when new cohorts are exposed to a candidate drug for the first time, such as the first time in humans, the first time in women of childbearing potential, the first time in patients, the first time in children, and so on. This sounds trivial and self-explanatory. However, the definition of a new cohort is not always easy. For example, we assume a candidate drug with fast metabolism-based elimination in all animal species, healthy volunteers, and most patients. In this scenario the small subset of patients who metabolize the drug very slowly can well be understood as a new cohort requiring specific focus and care. It is of critical importance to identify as early as possible any individuals, groups, and populations with particular safety concerns and risks.

It cannot be stressed enough that the strengths, weaknesses, and gaps of a clinical trial must be understood and risk mitigation measures must be put in place. This includes fast and adequate communication of all relevant data to the sponsor and processing the information competently and comprehensively. Sadly, there are many instances in which the sponsor treats clinical trials like mail-order items, especially when the trials are conducted through a clinical research organization (CRO). However, no external party, such as CROs or data-monitoring committees, release the sponsor from the strict legal oversight obligations. Lack of oversight and conducting clinical trials generically can have catastrophic consequences. Especially phase I trials are occasionally seen as a generic but necessary burden on the road toward more exciting and higher-profile phase II and phase III trials. However, every clinical trial is nothing more or less than an experiment on humans subjects. Lack of care and oversight may lead to catastrophic outcomes, especially in phase I trials, in which things can happen fast and little is known yet about the candidate drug's action in humans.

The following example demonstrates the possible consequences of ignoring the fundamental principles of safety vigilance in clinical trials. The Portuguese company Bial-Portela & Ca. SA conducted a phase I clinical trial with a novel, centrally active fatty acid hydrolase inhibitor (BIA 10-247) through a CRO (Biotrial) in Rennes, France (Randerson, 2016). The company assumed a favorable safety profile, as toxicology studies and competitor trials had not revealed any significant concerns. Much can be said about the trial design, conductance, and oversight. However, here we highlight only that neither the sponsor nor the CRO reacted to mild and unspecific central nervous system (CNS)-related AEs at lower doses in the multiple-ascending-dose part of the trial (Eddleston et al., 2016). The dose level was further increased and resulted in the death of one healthy volunteer and severe

neurological injuries in another four. The CNS symptoms that had been seen at lower doses were not related to the test substance; no suspicions were raised, and both CRO and sponsor were poorly prepared for the unexpected.

This example shows how important it is to think through what is actually happening in a clinical trial or market exposure scenario and be aware of sensitive inflection points, especially in apparently nondescript phase I trials. In the BIA 10-247 trial it would probably have been a good idea to model and simulate very carefully when appropriate CNS exposure levels are expected to be reached and intensify surveillance and vigilance accordingly at that point.

Preparing for the unexpected

Constructive suspicion is one of the most valuable virtues of practicing TSM. Never trust that one knows everything about a candidate drug or drug product, no matter how well it has been studied in the past. Biological variability alone is simply too great. Besides, in any given exposure scenario to a drug candidate or drug product, relevant variables are never really under control. Most dangerous scenarios are those in which one believes that one is controlling the situation—often on the basis of inadequately scrutinized information. For example, in the BIA 10-247 trial the investigators assumed that fatty acid hydrolase inhibitors generally have a benign safety profile, as competitor products did not show any severe side effects in clinical trials. However, to the best of our knowledge, these candidate drugs did not show relevant efficacy either. Thus accountable safety staff in the BIA 10-247 trial should have questioned the competitor trials' predictive value. Possibly, those compounds did not penetrate the CNS to a relevant degree, as is suggested by the lack of efficacy. Consequently, they were not predictive of high levels of central fatty acid hydrolase inhibition.

Well-applied suspicion is often evident by raising the “what if” question over and over again. This is an integral part of good scenario planning in a clinical trial or for a product on the market. For example, had the investigators of the infamous TeGenero (TGN1412) trial asked themselves, “What if … T-cell activation via CD28 is spinning out of control in humans?” it is possible that four to six healthy volunteers would have been spared the horrors of a phase I trial that had such a catastrophic outcome (Attarwala, 2010).

Practical aspects

It may be useful to provide a mental framework of points to consider in the safety development of a candidate drug or drug product. In the previous (Chapter 22, Pharmaceutical Toxicology), we highlighted that every biological response to a xenobiotic is a function of the test substance, the dose, the exposure, and the biological mechanism:

$$\text{test substance} \rightarrow \text{dose} \rightarrow \text{exposure} \rightarrow \text{effect}$$

The relationship between these variables can effectively drive translation and translational safety thinking and hypothesis development. The key to this approach is to critically assess each of the variables in terms of their identity, quality, quantity, and relevance when, for example, comparing species differences, building safety hypotheses, or developing risk mitigation measures.

Test substance

The test substance must not be taken for granted. The culprit behind an AE may be a degradation product, an impurity, a metabolite, or a formulation excipient. An AE may not have anything to do with the test substance in humans and could result from a comedication or even a food ingredient. It is always a good idea to verify that the actual test substance is plausibly driving desired and undesired effects, especially when extrapolating between species (e.g., rats or dogs and humans) or between different patient segments or even other medical practice cultures. For example, consider reports of new AEs after a drug product is launched in a tropical country. Taking the test substance for granted may keep one from considering inadequate storage conditions leading to degradation of the test substance or even product counterfeits with no or a different active ingredient. The appropriate action would be to consider climate effects before launching the product, of course. These principles should be considered proactively by raising the question how well the test substance is controlled in different scenarios.

Dose

The dose must be subject to consistent scrutiny as well. The amount of active ingredient in a drug product may have decreased due to chemical instability. Dosing errors may occur in animal studies, in clinical trials, and in medical practice. The product may have been taken incorrectly (e.g., with or without food) or at the wrong time.

Exposure

The dose-to-exposure relationship is one of the most important anchors in translational work. The exposure resulting from a given dose or dosing regimen can be highly variable between different species and individuals. The dose may be differently taken up (absorption) due to, for example, solubility, particle size, ionization (pH), transporter activity, or presence or absence of food. The distribution to various organs and tissues may be fundamentally different in different species and different individuals for many reasons. Residence time in organs, tissues, and blood may differ owing to transporter activities, protein binding, or metabolism. Significant differences in exposure, for example, accessed by blood plasma concentration, should also be a reason to pause and reflect, as

the previous studies may not necessarily derisk the new situation. For example, a new candidate drug shows much higher exposure levels (blood plasma concentrations) in a first-time-in-man study than are expected from animal studies. This should be reason enough to pause and consider the situation. For example, the dose-to-exposure relationship may be much steeper in humans than in animals. Maintaining the planned dose escalation steps may lead to excessive exposure and toxicity even at the next escalation step. Changes of the formulation in clinical development are of particular concern in this respect. Even small changes may disrupt the established dose-exposure profile significantly.

Effect

On the basis of past studies and experiences, the safety expert should have a reasonably good understanding of the spectrum and quantitative expression of effects relative to the candidate drug or drug product, dose, exposure, and time. Deviations from well-formulated expectations are always a reason for concern and should be investigated. Especially in clinical trials and marketed-product safety surveillance, the focus is often on detecting new signals. However, an emergent safety issue may well be hidden in unexpected variability of a known effect, a steeper dose-exposer-effect relationship, or exaggeration of an effect that had not been considered to be of relevance for safety up to now. Low-incidence but potentially significant safety effects are of particular concern, as they may not reveal themselves until late in clinical development or after product launch. Particularly challenging are drug combinations. Even with good simulations and scientific predictions, the effects of combinations are not established until they have been tested and demonstrated.

On the basis of the test substance, dose, exposure, effect relationship, further variables such as the species, differences in health and disease, and medical practice may be taken into account.

In summary

The application of TSM principles in practice requires ongoing cross-functional collaboration by a team of experts with the responsibility and autonomy to make decisions. A proven successful approach for achieving this is through the delivery of structured and data-driven SMT and the integration of their outputs into the clinical program. These teams are supported by clear structure and conflict resolution principles. As the oversight of these SMTs covers all relevant safety data, both within the program and outside it, they are empowered to achieve their aims: strategic drug development, data-rich risk characterization, predictive risk

profile management, and optimized risk-benefit balance for the target populations.

Translational safety future

Similar to the future perspectives for toxicology (Chapter 22, Pharmaceutical Toxicology), early-phase clinical trial design and delivery approaches are changing at an unprecedented pace. This brings with it a huge opportunity for the future of the translational safety science discipline, with both improved forwards and backwards translation and the continuous buildup of knowledge. The fundamentals underpinning these advances remain the same: Teams need to use systems to provide digital records of information and decisions made so that knowledge can build, and they need fast access to emerging data, biomarkers to detect safety effects quickly, a way for healthcare practitioners to rapidly interpret and action key information being sent, and, finally, a way to integrate the new knowledge with existing data to understand the impact on any previous assumptions and models.

The widespread acceptance of the internet and mobile technologies provides the opportunity to push safety monitoring and mitigation methods closer to the patient and strengthen the information exchange between patients and their healthcare team. Traditional clinical trial approaches are starting to be adapted, using innovative data science and technology to improve the timeliness, accuracy, and personalization of decisions in trials as well as to provide data to improve back-translation of knowledge and the improved prediction of safety profiles. There are also new organizations to help and support teams with innovative new ways of predicting, translating, and mitigating risks. An example is the not-for-profit organization TransCelerate BioPharma ([TransCelerate - Pharmaceutical Research and Development \[WWW Document\]](#), n.d.), whose mission is one of collaboration, working across the whole global biopharmaceutical research community to identify, prioritize, design, and facilitate the implementation of novel solutions that are designed to drive the efficient, effective, and high-quality delivery of new medicines. It is hoped that such collaborative approaches will speed the pace of advance, and project teams will need to rapidly adopt new methods into their thinking when trying to translate signals of their own.

Several areas of advancement are discussed in more detail below.

Creating a digital memory

Safety translation units and pharmacovigilance departments should have an appropriate auditable filing system to demonstrate that they have conducted their experiments and trials to appropriate standards and have shared (expedited) information according to pharmacovigilance

legislation. However, documenting action is not enough for creating a digital memory of a team's current understanding of a product. For this there needs to be a record of how the information was evaluated, what decisions were taken, and why they were made. This is not about writing huge masterpieces; in fact, when it is done well, being able to extract the key points and present them simply makes all the difference. This information should be stored in a such a way that it can be easily found and built upon in the future. The world of safety is rarely black and white; therefore giving future teams an easy way to find and quickly understand the rationale behind the current understanding (with supporting evidence a click away if needed) and build upon this with new data is essential for the evolution of TSM. Many systems already exist that can provide such digital support, and most of the influential contract research organizations or pharmacovigilance service providers will have one. In the future it is likely to get even easier to keep track of the many moving parts in data collection and interpretation with the application of blockchain technologies. Although in its infancy currently, the potential for having an end-to-end evolving system that maintains complete access to data, federated visualization, and a complete audit trail is temptingly close ([Beninger and Ibara, 2016](#); [Chen et al., 2019](#); [Hasselgren et al., 2020](#); [Lewis and McCallum, 2020](#); [Vazirani et al., 2020](#)).

Changes in sampling

Clinical trials have routinely required patients to visit their hospital or trial site on a regular basis for routine sample collection. A wide variety of novel microsampling approaches are finding their way into mainstream medicine, along with trials that offer three main advantages to the traditional approach: (1) Sampling can be done by the patient or care person at home, (2) there is the potential to engage geographically or culturally hard-to-reach populations, (3) there is the potential to massively increase the data density and coverage of PK, safety, and efficacy biomarker monitoring. It is this third benefit that is likely to be so important for translational medicine. Having a clearer understanding of predicted versus actual after a drug goes into clinical use is likely to help enormously with translational models in the future.

Pharmacokinetics sampling

An example of how home sampling has changed just a single parameter in a hospital is the recent improvement in the monitoring of the immunosuppressant levels in renal transplant patients ([Gustavsen et al., 2020](#); [Vethe et al., 2019](#)). Although the most accurate monitoring approach for the drug tacrolimus is to use the AUC, for practical reasons most clinics have abandoned this and instead use the trough

measure. Visits to outpatient clinics for blood sample collection are frequent and time consuming. In addition, the time point needed to accurately identify the trough level rarely links up with availability of appointments and consultant clinics for interpretation. Using home capillary microsampling techniques, one hospital has replaced this approach with patient home-based collection of dried blood and in a recent study has demonstrated that this is an achievable, practical, and reliable alternative. With home sampling, more frequent data collection can occur, enabling exposure profiling for the full dose interval in renal transplant recipients. With this slight change in approach, outpatient clinics are not needed, patients can be monitored over time and at more regular intervals than is possible with a clinic appointment-based approach, and the physician has an accurate reflection of the AUC with which to personalize the patient's treatment regimen.

It is only a matter of time before such PK sampling becomes commonplace in early trials. This innovation is likely to collect far more insightful PK data than the traditional visit schedule, which is usually limited to sampling over the first 12–24 hours followed by individual timepoints. The timepoints that are selected are entirely based on nonclinical-to-clinical translation work; if this translation is perfect, then safety surveillance can be optimal. However, the exposure profile in humans turns out to be different, and these points are suboptimal (e.g., completely missing the C_{max} measure), so the traditional approach is quite brittle. New timepoints need to be decided on the basis of these incomplete human data, and a protocol amendment needs to be put in place before any more learning can be gathered. This process can add months to a timeline. Novel sampling approaches mean that the PK-based translational approaches can be maximized, even if the exposure profile is different from that in preclinical studies. The availability of data ensures that research teams can back-translate these findings, amend their safety predictions, and be aware of any gaps in knowledge as they arise. This means better ongoing correlation between the predicted and emerging safety profiles and improved understanding. This context provides clinicians with greater understanding in making cohort dose selection decisions and for the next phase of clinical development as treatment durations get longer and drug combinations may be included. It also paves the way for having exposure, rather than dose, cohorts in the early studies and improved translation of from one phase of development to the next.

Safety and efficacy biomarker monitoring

Remote and digitally enhanced monitoring of actionable efficacy and safety biomarkers is likely to increase our understanding of the events themselves as well as

enabling earlier intervention and improved care when things go wrong (Coravos et al., 2019a, 2019b). This practice is not new. Many people have thermometers and blood pressure monitors in their own homes. The change relates to the use of information as a data source in clinical trials. The increased reliability and consistency of measures that come with improved technology and the novel application of measures that can provide more useful endpoints have increased their value for monitoring patient's health.

Monitoring disease state

An example of improve efficacy biomarker monitoring can be seen in Parkinson's disease. The currently accepted measure of disease severity requires subjective data collection backed up by in-clinic assessments. This process is highly influenced by rater-dependent variation. Novel approaches to the same issue use smartphone sensor data and machine learning to generate an objective disease severity score that has been validated with traditional methods. This ensures that a more robust measure of between-visit health states can be not only detected, but also transmitted to the patient's care team and trusted enough to trigger robust and timely action (Arora et al., 2015; Zhan et al., 2018).

Improved monitoring of known safety biomarkers

In clinical trials, electrocardiograms (ECGs) are usually undertaken only during visits. This usually means that 3-minute ECG recordings may be gathered from patients weekly or monthly. Atrial fibrillation is an important cardiac event that, in addition to the event itself, presents a significant risk factor for stroke and thromboembolism. With the current methodology the background incidence of this effect cannot be quantified within the study, which means that if it is recorded in clinic, it is interpreted as a single event—the first and only. This clinic-only view cannot optimally capture whether the patient had arrhythmia in between visits, how often it occurred, and whether there was a pattern to the profile of what the patient was doing when arrhythmia occurred. With the emergence of m-Health devices, cardiac data are starting to be gathered 24/7. This means that if people are monitored from their consent and eligibility screen and then throughout the study, not only will a full characterization be achievable within the study population, but earlier intervention can be undertaken when required. In addition, bimodal classification algorithms are starting to emerge to objectively and reliably record ECGs, measure effects, and trigger patient management, which means that their use is not only helpful, but also achievable within the trial setting (Kruger et al., 2019).

Detecting previously nonquantifiable tolerability

Many tolerability effects that affect a patient's life are subjectively reported and thereby vulnerable to recall bias and individual variability. Itchiness is a classic example. It is very hard to measure the level of itchiness someone is experiencing, but for anyone who has experienced intense itch, it can be distracting at best, and it can also be annoying, frustrating, embarrassing, and isolating. The potential burden of persistent and intense itching should not be underestimated, especially if the patient starts to believe that the developmental drug is causing it. Recent advances in technology have used high-resolution wrist actigraphy (three-way accelerometers) to record nighttime movement (Smith et al., 2019). The sensor data that are received are then integrated and algorithmically processed to separate scratching motions from others. This gives an objective measurement of the amount of time a patient unconsciously scratches and whether the patient used one hand or two. Comparison between such automatic detection and manual scoring from infrared video data demonstrated the approach's reliability. Use of techniques such as these is likely to transform the level of insight and objectivity that can be used in early trials to predict tolerability and burden on patients and guide management approaches.

Identifying the right population

One of the key aspects of risk-benefit analysis is the ability to identify the population that is likely to derive the most benefit with the least safety risk. As described throughout this and the previous (Chapter 22, Pharmaceutical Toxicology), this balance is applied through translational safety thinking from the very beginning. As access is improved to more integrated real-world data and health records and analysis techniques for deriving meaning from large datasets are improved, this means that initial identification of potential indicated populations can be better defined. In addition, during nonclinical and clinical programs, the more integrated use of the safety knowledge plus the improved monitoring capabilities that home sampling, data algorithms, and wearable and /sensors bring allow for better ongoing management of risks in trials. This improved management opens the possibility of expanding study eligibility criteria and allowing a more representative population into studies. In turn, the researchers' understanding of the drug is enriched, the risk of moving from a rarefied clinical trial population into the real-world setting becomes better characterized, and the generalizability of the trial results is increased.

Embracing data

Virtual clinical trials are becoming a reality and are starting to embrace technology like never before. The use of

real-world evidence to give context, predict outcomes, and support safety modeling is becoming more and more prevalent (FDA, 2020). Direct use of electronic health record data in particular is likely to provide greater insight into what's happening in the real world than ever before. Combine this with the wearables, sensors, and other remote data collection methods, and we are starting to get a glimpse into the impacts of developmental drugs across a patient's whole experience rather than just what's happening (or remembered) during a clinic visit (Izmailova et al., 2018). Although many technologies are not advanced enough for routine use, it is only a matter of time before the accuracy of outputs is improved to a sufficient level for broad acceptability. It remains to be seen whether such improvements will come via the technology itself, as a result of improved analytical approaches to remove noise from the data, or through combining endpoints to form novel early-detection biomarkers in which the accuracy and specificity come from triangulation of data rather than from a single input.

This means that improvements in the monitoring of existing biomarkers, novel early-detection safety and efficacy biomarkers, and novel study endpoints are all on the horizon. All of this has the potential for similarly enhancing preclinical data-gathering methods and improved translation for the next stage of clinical development (Table 23.3).

Understanding tolerability

A huge amount of effort has been expended on researching the translation of clinically significant safety risks, yet a key risk to patients is their own compliance. Many decisions that patients make in the areas of adherence to treatment requirements involve other factors, such as emotional status, formulation, schedule requirements, and potentially low-grade burdensome tolerability effects (Bender et al., 2014; Mazzocco et al., 2019). Low-grade tolerability effects are typically the type of events that can be characterized only in the clinic (e.g., impact of nausea, itch, sleep disturbance, fatigue, depression), and it is quite often not the effect itself that causes the issue, but the impact it has on people's lives and how they feel. This is very difficult to assess in non-clinical studies, but as methods improve for capturing this information in early trials using ePROs (electronic patient reported outcomes) to understand quality-of-life measures, digital ethnography-like practices, and digital phenotyping, the daily impact can begin to be understood. This ensures that targeted, detailed information can be gathered to characterize the most impactful measures in phase II. Patient-driven self-management approaches can also be identified, woven into the clinical support pathways, and also can be tested for risk minimization effectiveness in later studies. Through back-translation of these qualitative and quantitative impact data, the preclinical data, which are currently

TABLE 23.3 Examples of novel technologies and digital approaches that are changing monitoring and outcomes.

Opportunity	Example
Potential for improved data coverage and density of existing biomarker	Vital signs such as heart rate, heart rate variability, blood pressure, or respiratory rate (Breteler et al., 2020; Liu et al., 2020; Miao et al., 2020; Mutlu et al., 2018; Schuurmans et al., 2020). Improved timing of ECG when a symptom is experienced: with smartphone single-lead ECG traces, it's possible for patients to take an ECG while a symptom is occurring at home. Although the data quality is lower, the timeliness is improved. In addition, recent work by Muhlestein et al. (2020) found that multiple serial single-lead ECGs from a smartphone can identify ST-elevation myocardial infarction with good correlation to a standard 12-lead ECG.
Novel biomarkers	Parkinson's disease: The use of step angle to provide improved functional status and effects of the treatment on gait (Wang et al., 2020). The use of a wearable inertial device on the upper limb to monitor motion data during six tasks to support and objective diagnosis of Parkinson's disease (Cavallo et al., 2019).
Novel patient selection and prediction approaches	Development and validation of a novel MR imaging predictor of response to induction chemotherapy in locoregionally advanced nasopharyngeal cancer (Dong et al., 2019).
Novel endpoints	Accurate read of functional status is usually relevant in studies involving elderly patients. For those where activity and mobility are primary endpoints the approaches normally used are self-reported approaches combined with clinic-based assessments such as the 6-min walk test, both of which have limitations. Using wearable devices researchers has shown that real-world gait patterns differ significantly in real life when compared with a treadmill and also that digital gain analysis can reliably predict survival (Bohannon and Glenney, 2014; Godfrey et al., 2015; Hardy et al., 2007; Rispens et al., 2016; Studenski et al., 2011).

poor at predicting patient impact from such tolerability events, can be given context, and a database of impact outcomes can start to be created. This ensures that over time, preclinical signals for such events can start to be identified so that tolerability management and potential compliance issues can be considered even earlier in the value chain.

Summary: looking to the future

Soon, the best practices of safety translation are likely to gain more and better data to enhance forward predictions and more seamless back-translation of context and learning. This may result in swapping subjective, remembered feedback for passive and objective continuous data collection. Computers will be used to integrate large real-world evidence datasets in decisional balance and predicted future outcomes. The application of artificial intelligence will help to combine parameters into more complex biomarkers that can detect pathological changes earlier in their clinical course. Simply having the opportunity of hearing patients' voices and descriptions of what it is like to take their drug can provide important information. Our clinical insight is on the tipping point, moving from periodic and/or remembered data to more comprehensive data from across the whole of the patient's experience with the disease and treatment. The parameters themselves can start lining up between disciplines more reliably than ever before too, such as the potential for using exposure groups for both preclinical and early clinical studies. When combined with the predicted safety profile derived from predicted on- and

off-target effects, this provides huge additional insight as well as a chance to understand how well the human data are reflecting previously known information, and it gives the researcher a more reliable read on how much to trust predictions for each situation. In short, the improvements in data collection, application of strategy, and routine forward and backward translation of learning ensure that the future is bright for the science of TSM.

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Chapter 24

Cancer vaccines: translational strategies

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Abstract

Therapeutic cancer vaccines have been pursued for several decades to harness the immune system to treat cancer without cytotoxic or destructive measures. Different from the expectation and to the disappointment of many translational researchers, there has not been much success of a cancer vaccine as a single agent once translated into clinical trials. However, new understanding of the immunoregulatory mechanisms has allowed the development of approaches to potentiate the effect of vaccines by removing the obstructions presented by tumor. Thus vaccines that previously showed strong T cell responses in the preclinical model or in vitro assays without clear clinical benefit may now have a potential for demonstrating full efficiency. This chapter will review a variety of cancer vaccine platforms in the journey of historic achievement in immunology and identify a role of cancer vaccine as an important element of the fourth pillar of cancer therapy, cancer immunotherapy, as it joins the three pillars of surgery, chemotherapy, and radiation.

Keywords: Cancer vaccine; immunotherapy; lymphocytes; antigen presentation; tumor immunology

Introduction

The current field of vaccines can be divided in two distinctive categories: infectious disease and cancer. The general public often thinks of the vaccines in the light of prophylactic vaccines against infectious organisms from their personal experience in daily life, but even in those vaccines to prevent infection, many of the concepts regarding the mechanism of action or vaccine technologies are not different from the ones in cancer vaccines. On the other hand, some of the vaccines for infection prevention also play a great role in cancer prevention as well, as is exemplified by vaccines for hepatitis B virus or human papillomavirus. In this chapter, cancer vaccines will be highlighted with a focus on therapeutic vaccines along with historic landmarks in immunology (Fig. 24.1) (André et al., 2020; Burton

et al., 1994; Cheever et al., 2009; Eberlein et al., 1982; Fontenot et al., 2003; Harrington et al., 2005; Hermans et al., 2003; Hori et al., 2003; Ishida et al., 1992; Jones, 1988; Kaufmann, 2008; Kobayashi et al., 1989; Krammer, 2020; Krummel and Allison, 1995; Matzinger, 1994; Naito et al., 1998; Nishimura et al., 1999; Park et al., 2005; Ráki et al., 2007; Rosenberg et al., 1988; Sakaguchi et al., 1995; Sievers et al., 2001; Speiser et al., 2005; Yang et al., 2007).

Historic perspective of immune system involvement in the biology of cancer

Spontaneous regression

A search of the keywords “spontaneous regression (resolution or remission)” and “cancer” in journal collection at US National Institutes of Health’s National Library of Medicine will return a few hundred results. Along with the mention of “a disappearing tumor” in ancient Egypt, spontaneous regression of tumors is one of the initial events that led to the concept of tumor immunology. In the early 20th century, researchers debated the credibility of presented cases of spontaneous regression, but at least they came up with a partial agreement that spontaneous regression is a true phenomenon despite its suspected rarity, occurring in an estimated 1 out of 100,000 cases (Challis and Stam, 1990; Rohdenburg, 1918). Some of the events suggested a sequential relationship of spontaneous regression with certain viral or bacterial infection. As the technology advanced, the importance of pathological confirmation of the tumor negated the suspicion of any diagnostic error as an alternative explanation, but the mechanism is not fully understood even though there is no doubt that immune reaction is the key player in this phenomenon. The cancer types that were reported to show spontaneous regression included melanoma, renal cell cancer, lymphoma, and lung cancer more frequently than other types of cancer (Challis and Stam, 1990). These cancers overlap with the disease indications for many of immune checkpoint inhibitors, providing additional clues of mechanism in

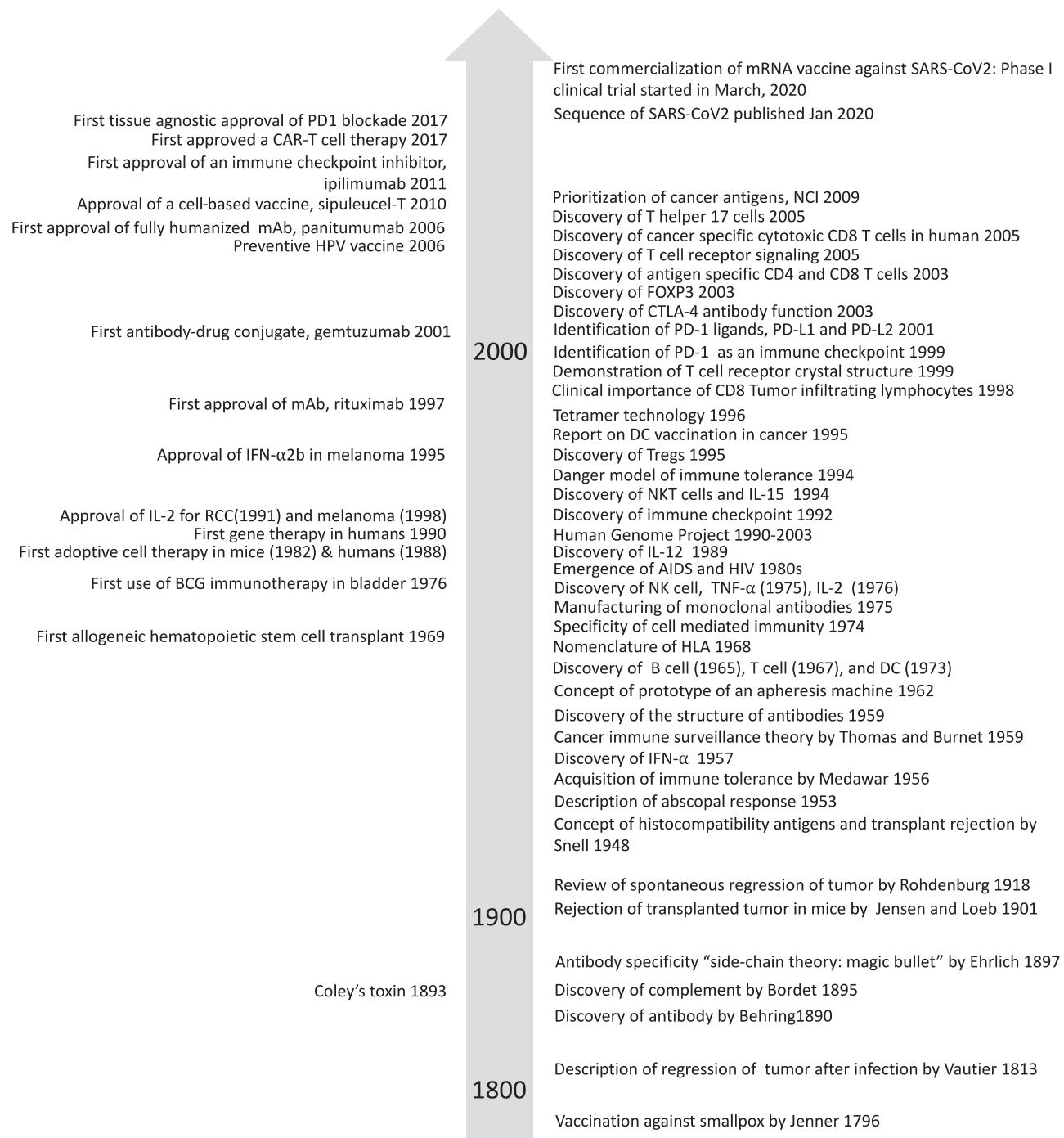


FIGURE 24.1 Timeline of cancer immunology, immunotherapy, and vaccines; major discoveries and events in immunology (*right*) and clinical achievements in cancer immunotherapy (*left*).

spontaneous regression and further suggesting the role of immune reaction as accountable for the finding.

Abscopal response

Abscopal response or effect is one of the earliest descriptions that suggested there could be a factor in one's body

with remote effect on a tumor when the treatment targeted a separate tumor at a site. In 1953, Mole published a report that radiation to a tumor on one side of a mouse led to a shrinkage of a tumor on the other side of the animal and termed the phenomenon "abscopal" which means "away from the target" in its Latin root (Mole, 1953). This concept was debated for the authenticity of the

findings, but growing evidence of the same type of observation eventually led to acceptance over time. The abscopal response has been documented in multiple types of cancer, including melanoma, breast, and lung cancer, even though it is extremely rare. The current concept of abscopal response is that it is a result of induction by dying tumor cells of immune effector cells that can travel to and work in remote sites after a local cytotoxic therapy, most often radiation. The reason for the rarity is thought to be a combination of suppressive immune modulation from the tumor and inefficient immune function in recognizing and killing tumor cells. Recently, the abscopal response has gained increasing attention as a model for amplification of immunity by combination therapy with immune checkpoint inhibitors (Buchwald et al., 2018).

Microbes: the first cancer vaccine platform in the history of modern medicine

Microbes have the longest and most diverse track record in the study of immune responses in humans. They can be functional even when the tissue is necrotic, unlike most of normal human physiological mechanisms, and they impose an immune modulatory effect independently that can favor cancer cell killing (Forbes, 2010). The first record of an observation of a bacterial infection that led to tumor shrinkage is thought to be the observation in 1813 by the French physician Dr. Arsène-Hippolyte Vautier, who described the disappearance of a tumor after a clostridial infection that caused severe gas gangrene (Barbé et al., 2006). Attempts to use bacteria for the treatment of cancer date back to late 1800s. Drs. Fehlensein and Busch reported tumor regression after bacterial skin infection but could not reproduce the finding with an intentional inoculation of the bacteria (Oiseth and Aziz, 2017). Soon afterwards, Dr. Coley, the father of cancer immunotherapy, observed a patient with sarcoma that improved with erysipelas. Inspired by the observation, he infected the patients with inoperable sarcoma by inoculating “Coley’s toxin,” which comprised *Streptococcus* and *Bacillus prodigiosus*, now known as *Serratia marcescens*; he reported a regression of the disease that lasted several years in remission in one patient and more than three years until suspected metastasis in another patient among three patients who had successful induction of erysipelas out of a total of ten patients who participated, illustrating the challenges of effective ways of truly “infecting” the patients while he was convinced of the efficacy (Coley, 1910). Several other organisms, such as *Clostridium*, *Listeria*, *Salmonella*, *Shigella*, and *Mycobacterium*, have been tested with the intention of treating cancer either as a vector to deliver tumor antigens or as an immune-activating element to induce immunological synergy (Kocijancic et al., 2017;

Le et al., 2012; Patel et al., 2008; Roberts et al., 2014). As the importance of microbiomes, including normal flora in specific organs, is being unveiled, the concept is gaining credence that the interaction between the microbiome and the host immune system may hold the key to success, as has been shown in the study of checkpoint inhibitor response in relation to the microbiome (Pinato et al., 2019). Considering the hypoxic tumor microenvironment (TME) in which regulatory immune responses predominate, favoring protection of cancer cells, the capacity of facultative anaerobes that can boost tumor antigenicity and can overcome the tolerogenic condition strengthens its potential.

Bacteria-based vaccines can decrease checkpoint molecule expression on the tumor immune infiltrates and aid in the induction of central and effector memory T cells. Tolerogenic dendritic cells (DCs) possess an immune suppressive nature, and these in addition to myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages, and tumor-trained neutrophils are found in the tumor throughout many stages of cancer. Monocytes that migrate to a tumor differentiate into M2 macrophages, which carry the opposite function of M1 macrophages, contributing to immune evasion. In studies in mouse models, injection of attenuated *L. monocytogenes* (*Lm*) or *Salmonella typhimurium* showed a shift of the macrophages to a mature phenotype in the tumor or spleen and increased macrophage infiltrate of predominantly the M1 profile, suggesting the role of the microbiome in reprogramming the immune cells favoring proinflammatory condition and preferentially differentiating the DCs (Bronchalo-Vicente et al., 2015; Lizotte et al., 2014). Tumor-associated neutrophils (TAN) can alter the TME by secreting proinflammatory cytokines to promote immune infiltrates in the tumor and trigger systemic responses that can affect the local milieu. Injection of microbials can increase the recruitment of TANs that can be potentiated further when engineered to express cytokines for enhanced antitumor activity. Such an effect was shown in the administration of *Salmonella* sp. or *Lm*. Virulence factor listeriolysin O protein of *Lm*, which is known to function as an adjuvant once administered in the tissue, can induce interleukin (IL)-12, IL-18, and interferon gamma (IFN- γ) and thereby improve the ratio of effector to regulatory T cells (Grille et al., 2014; Vendrell et al., 2011). The clinical use has been studied in both hematological and solid cancers, including lymphoma, HPV-associated cancer, melanoma, and breast cancer (Chen et al., 2014).

Bacillus Calmette–Guérin (BCG) vaccine, a live attenuated strain of *Mycobacterium bovis*, is the only bacterial vaccine that is used in the field of standard cancer treatment so far, mainly in carcinoma in situ of the bladder and high-grade non-muscle-invasive bladder cancer (Sylvester et al., 2002). Since the beginning of its routine

use in the 1980s, BCG has replaced a significant portion of destructive treatments that can diminish the quality of life, sometimes even to an irreversible extent. Since the late 2010s, as the global BCG shortage became a practical threat in providing what is already known to be beneficial, several academic societies and treatment guideline-making agencies have provided guidance to prioritize the use of BCG in induction and maintenance therapy (Babjuk et al., 2019; Chang et al., 2016; Thomas et al., 2020). Despite the challenges in supply, robust innate and potentially tumor-specific immune responses mediated by BCG should be remembered as an important milestone in cancer immunotherapy. Intravesical instillation of BCG in the clinic was first described in 1976 (Morales et al., 1976). BCG is believed to attach to fibronectin of disrupted urothelial basement membrane via fibronectin attachment protein on BCG (Ratliff et al., 1988). Then BCG is rapidly cleared from the urine, but the persistence of increased inflammatory cytokines in the urine and increased neutrophils, natural killer (NK) cells, macrophage, and activated antigen-presenting cells (APCs) after the treatment suggest immune activation in the bladder tissue. Also, adaptive immune response via urothelial cells and APCs presenting BCG antigens is suggested to be responsible for robust infiltration of CD4 and CD8 T cells after BCG instillation (Brandau et al., 2001; Luo and Knudson, 2010; Pettenati and Ingersoll, 2018; Pryor et al., 1995; Ratliff et al., 1993). To improve the efficacy, combination therapy and molecular subtype–specified treatment are being actively pursued, as several checkpoint inhibitors have showed significant benefit in urothelial cancer (NCT02792192, NCT02324582, NCT02808143) (Hedegaard et al., 2016; Inman et al., 2007).

Discovery of cytokines and the first usage in cancer treatment

The first cytokine to be discovered was interferon alpha (IFN- α) in 1957, followed by the discovery of IL-2 by Ruscetti and Gallo in 1976 (Isaacs and Lindenmann, 1957; Morgan et al., 1976). IL-2 allowed in vitro culture of T lymphocytes and soon entered the clinical realm. It was manufactured by using recombinant technology as the first successful immunotherapy agent for cancer, approved for metastatic kidney cancer (1991) and metastatic melanoma (1998) (Fyfe et al., 1995; Rosenberg, 2014; Rosenberg et al., 1988; Taniguchi et al., 1983). Cytokines became a popular adjuvant of many vaccine platforms and other cellular therapeutic studies along with other immune stimulants manufactured with newer technologies in the 1980s and afterwards. One of the most outstanding landmarks of such a usage is tumor-infiltrating lymphocyte (TIL) therapy, which used T

lymphocytes expanded in vitro using IL-2. IL-2 is the most extensively studied cytokine, and it has shown quite broad impact on various immune cells, especially T cells (Fehniger et al., 2002; Rosenberg, 2014). IL-2 was first used as a vaccine adjuvant in 1985 (Kawamura et al., 1985), and other cytokines emulsified with antigens were also compared as vaccine adjuvants (Ahlers et al., 1997). Tumor antigens in all available forms from DNAs to tumor cells or the lysates of tumor cells have been frequently used with one or more cytokines, including granulocyte-macrophage-colony-stimulating factor (GM-CSF) or IL-2, which seems to be a reasonable concept (Kawamura et al., 1985; Rosenberg et al., 1999). However, several studies showed that cytokines such as IL-2 can increase toxicities or unwanted immune cell subsets, such as regulatory T cells (Tregs), rather the effector T cells, which can limit the usefulness of a vaccine. Because of such effects, these cytokines did not demonstrate superiority in clinical efficacy when they were added in many studies. In those cases it was hard to justify the added cost and complexity of clinical care, which are important factors for further clinical development (Hersey et al., 2008; Powell et al., 2006; Redman et al., 2008; Smith et al., 2008; Sosman et al., 2008; Zhang et al., 2005). More recently, IL-7, IL-15, and IL-21 have become of interest on the basis of preclinical data showing properties similar to those of IL-2 without an increase in regulatory T cells (Brilot et al., 2007; Frederiksen et al., 2008; Iuchi et al., 2008; Kasaian et al., 2002; Kim-Schulze et al., 2009; Oh et al., 2004; Pellegrini et al., 2009; Rosenberg et al., 2006; Thompson et al., 2008; Wurster et al., 2002). In particular, IL-15 not only demonstrates the improved T cell response but also can enhance NK cell activity, which can synergize with other immunotherapeutics to turn an immunotherapy-unresponsive tumor into a responsive tumor by diversifying the mechanism of tumor cell killing (Capitini et al., 2009; Huntington et al., 2009; King et al., 2009; Teague et al., 2006; Waldmann, 2006).

Discovery of white blood cell subtypes and translation from bench to bed

Identification of new white blood cell types intercalated with the discovery of cytokines, and they became partners for each other in immunotherapy for both cancer and non-cancer indications. Identifying B cells and their function to make antibodies by Cooper et al. in 1965 was critical for our understanding of the immune system and established an early stepping stone in its application (Cooper et al., 1965). Soon, technologies to produce pure forms of monoclonal antibodies (mAbs) used in the lab or for therapeutic indications, from the Nobel Prize-winning

discoveries of Kohler and Milstein in 1975 (Kohler and Milstein, 1975), added practical applications to the earlier concept of a “magic bullet” for infection such as syphilis by Nobel Prize winner Paul Ehrlich in early 1900s (Kohler and Milstein, 1975; Piro et al., 2008; Schwaber and Cohen, 1973). Likewise, the T lymphocyte and its critical role in immune function were identified in 1967 by Jacques Miller and coworkers, and Steinman et al. first described the presence of DCs in mice as the primary professional APCs in 1973 (Miller et al., 1967; Steinman and Cohn, 1973).

T cells have diverse functions, and the detailed classification into subsets based on surface markers and associated functions has now been defined. Individual subsets are able to behave in an agonistic, antagonistic, or complimentary manner. So far, CD8⁺ killer T cells have been spotlighted and used for in vitro manipulation with an intention to expand and reinforce the ability to identify and kill the tumor cells. The first applications of T cells in cancer therapy started with nonspecific T cell activation exemplified in lymphokine-activated killer cell and TIL therapy cultured with cytokine supplements and have now progressed to the use of antigen-specific T cells. After more than 10 years of effort since the first application for an investigational new drug to clinically test genetically engineered T cells that are capable of recognizing a specific tumor antigen of interest, the US Food and Drug Administration (FDA) approved the first chimeric antigen receptor (CAR)-T cells targeting CD19 in B cell malignancies (2017). CAR-T cells not only attack the antigen-bearing malignant B cells but also can proliferate in the patient and continue with immune surveillance as well. Additional CAR-T cell approvals have all been in B cell malignancies to date, and the attempts to use CAR-T cell in solid tumors continue (June and Sadelain, 2018). Discovery of additional T cell subsets such as Tregs or helper T cells has added more understanding of diverse outcome after a treatment in individuals and added great value in developing more finely targeted immune modulation. In the field of cancer vaccines, combining CAR-T cells with a vaccine platform is being attempted to continue immune stimulation to prolong CAR-T cell proliferation and survival so that immune surveillance can occur for a longer period and enhance the clinical efficacy (Chan et al., 2020; Ma et al., 2019).

Because DCs can regulate both innate and adaptive immune responses by cross-presentation to induce strong effector T cells against cancer, DCs became very popular as a cancer vaccine platform once ex vivo generation became available (Anguille et al., 2014). The first clinical trial using ex vivo manufactured DCs in patients with lymphoma was first reported in 1996 (Palucka and Banchereau, 2013). Since then, DCs have been used in numerous platforms to introduce the most diverse antigen

types or have been combined with other immune modulators. Some platforms used DCs that are fused with tumor cells or tumor cell lysates; others loaded peptides, messenger RNA (mRNA), or DNA. Electroporation or transduction of the DCs with antigens during culture is another way to introduce the antigen (Maeng et al., 2019; Wood et al., 2016). The advantage of using autologous DC vaccines is that the mode of antigen presentation is the closest to the natural pathway and not limited by specific human leukocyte antigen (HLA) types, since the DCs express the patient’s HLA repertoire unless short peptides that are presented by only some HLA molecules are used to introduce antigens. As we learn more about how immune memory is induced, the importance of effector memory T cell development is considered one of key steps in durable control of the disease. Thus naïve T cells primed by DCs, which can differentiate to be effector memory T cells and circulate in the peripheral blood to rapidly proliferate in case of antigen recall or a booster dose of vaccine, became very attractive (Todryk, 2018). The DC vaccine boom started in 1990s and led to a long list of both preclinical and clinical studies. The clinical studies were mainly in melanoma, renal cell carcinoma, prostate cancer, and malignant glioma in the first decade of intensive research. The adverse events often involved low-grade injection site reactions, whereas systemic reactions were uncommon. Rare systemic reactions were associated mostly with immune adjuvants administered together with DCs. Despite the immunogenicity to induce CD8⁺ T cell responses and even NK cell responses in some platforms, DC vaccines have been viewed with skepticism, owing to suboptimal clinical efficacy in shrinking tumors, which was 5%–15% in most of the clinical studies, and challenges in standardized manufacturing. Even though immunogenicity can provide useful insight into the vaccine’s mechanism of action, clinical activity needs to be supported by survival benefit to gain broad support and consensus for advanced clinical development. The one and only success of a DC vaccine’s achieving regulatory approval that showed survival advantage in the field of advanced cancer is sipuleucel-T. Sipuleucel-T is manufactured by using autologous peripheral blood monocytes that are cultured with a recombinant protein called PA2024, which is prostatic acid phosphatase (PAP), a prostate cancer antigen, fused to GM-CSF. Treatment is given three times every 2 weeks in patients with metastatic castration-resistant prostate cancer who are not symptomatic. The treatment did not decrease prostate-specific antigen (PSA), a well-known tumor marker of prostate cancer, but there was a tendency for patients who received the treatment with lower PSA to survive longer, and the treatment group showed a survival advantage of 4.1 months (Kantoff et al., 2010a). The complexity of manufacturing and the difficulty of developing

standardized release tests or functionality assays in vitro have been challenges to scaling up the manufacturing that are often linked to additional financial burden to the investigators exploring advanced clinical trials. Efforts to improve the shortcomings include batch manufacturing employing automated closed systems and the use of alternatives to autologous DCs such as artificial or allogeneic APCs (Suhoski et al., 2007; Ye et al., 2011). Nanoparticles, which are mentioned in this chapter, can function as alternatives to DCs eliminating the need of processing patient's blood cells to provide a mechanism for an antigen presentation. Regarding the improved clinical efficacy, combination strategies are actively pursued for the vaccines that have shown immunogenicity. The other way of improving the efficacy is engineering of the DC to augment antigen processing and presentation. Most DCs in vaccine platforms are peripheral blood monocyte-derived DCs (MDDCs) that might not be as efficient in antigen presentation to the effector T cells or migration to draining lymph nodes compared to natural tissue-dwelling APCs. Thus the strategy to engineer the DCs to secrete IL-4 or GM-CSF or using stem cells to virally transduce FLT3L expression for self-differentiation into DCs can overcome some of the defects that artificially cultured DC vaccines might have (Perez and De Palma, 2019; Rosa et al., 2018; Sundarasetty et al., 2015). The fate of DCs after the injection has been debated. Investigators expect the cells to migrate to the draining lymph node (DLN), where antigen presentation should occur. Recent in vivo magnetic resonance imaging of DC trafficking after injection in mice showed that DC migration to the DLNs correlated with tumor shrinkage, which supports the original postulation on DC vaccine's mechanism of action (Grippin et al., 2019).

As more became known about the immune cell types and their production of stimulatory or suppressive cytokines, disease classification based on the cell markers or cytokines for control of the disease became popular (Arber et al., 2016). The effort toward skewing a dysfunctional immune system in one direction or the other has led to opposing strategies for rheumatological or other immune-induced illnesses and cancer with inadequate or suppressed immune surveillance. For example, immune suppression in transplant patients that prevents graft rejection is associated with an increased risk of developing certain type of lymphoma or solid cancer in the recipients. Compared to rheumatological indication of immune suppressive measures, the risk of developing posttransplant lymphoproliferative disease in solid organ transplants where broader immune suppressive agents are used is clear. Withdrawal or decrease of immune suppression is one of the standard care options whenever possible, making researchers think that narrowing the target may be beneficial to mitigate the risk of developing cancer

(DeStefano et al., 2018; Tokuhira et al., 2019). In rheumatological indications of immunotherapy, long-term studies assessing the risk of malignancy as an endpoint are not available, and retrospective analysis debates the true increased risk. However, uncontrolled chronic inflammation is believed to increase the risk of other detrimental conditions in the musculoskeletal and cardiovascular systems, justifying the judicious use of immune suppression in weighing the risk and benefit in each individual setting. Also, there is a need to consider the factor of using other types of immune suppressants that can cause cancer, such as methotrexate, or underlying conditions that are known to be associated with increased risk of cancer, such as inflammatory bowel disease, that challenge the ability to accurately assess the true risk of developing cancer while on immunotherapy. Thus in selecting an immune suppressant that will be used in the long term, it is critical to review whether more broad suppression would be more appropriate or whether there could be options to suppress specific pathways or molecules as nonspecific immune suppressants (e.g., glucocorticoid, cyclosporin, azathioprine, or mTOR inhibitors) that may introduce a higher risk of developing cancer compared to more narrowly targeted inhibitors (e.g., TNF- α blockade) (Abou-Raya and Abou-Raya, 2006; Beyaert et al., 2013; Kandiel et al., 2005; Kappelman et al., 2014; Ketelhuth and Hansson, 2015). Chronic viral infections by EBV, HHV-8, or other members of the family of HSV, HIV, or HPV in immunocompromised patients are compounding factors in accurate assessment of cancer risk, and the strategy in surveillance and prevention should be individualized in the clinic (de Fijter, 2017; Dugue et al., 2015). The observation that immune suppression heightened the risk of lymphoproliferative disease led to new insights into the importance of immune surveillance in removal or control of tumor cells.

Nucleic acid–based cancer vaccines

One of the major advances in 1980s was the use of nucleic acids in preventive or therapeutic indications as demonstrated initially in gene therapy. The first attempt at a recombinant DNA transfer was in a mouse by Cline et al. Unfortunately, Cline's clinical work raised ethical concerns and prohibited him from performing further research (Beutler, 2001; Mercola et al., 1982). The first human gene transfer was conducted in a child with adenosine deaminase deficiency type severe combined immunodeficiency by Anderson, Blaese, and Rosenberg (Anderson and Fletcher, 1980; Rosenberg et al., 1990).

DNA has been a popular tool for a vaccine development, starting with influenza vaccines developed by Margaret Liu and Jeffrey Ullmer at Merck in the 1993 (Liu and Ulmer, 2000a; Ulmer et al., 1993). The use of

RNA has caught more attention only in the past decade, in part related to the need for personalized vaccines. DNA vaccines were further improved by Pavlakis and Felber using codon optimization to improve expression (Rosati et al., 2005). Using DNA or RNA instead of any microorganisms offers a chance of inducing both humoral and cellular immunity without causing or transmitting an illness associated with the microorganism that is used as a vector and with potentially fewer side effects. Initial success has been more prominent in veterinary medicine, leading to the approval by the US Centers for Disease Control and Prevention and the US Department of Agriculture of vaccines against West Nile virus in the early 2000s (Chang et al., 2007). DNA vaccines are prepared by cloning the sequence of interest into a plasmid to be expanded and purified before being administered to patients. DNA vaccines are safe, with relatively simple manufacturing, and do not trigger neutralizing antibody, an advantage when repeat doses are required. The route of delivery can be variable, including intradermal, subcutaneous, intramuscular, or intratumoral injections (Liu and Ulmer, 2000b). When the injected plasmid enters APCs in the tissue or surrounding cells, such as myocytes, transcription occurs to express the protein of interest with species-specific posttranslational modifications. If APCs were directly transfected, the cell will process the peptides and present them on MHC class I molecules to CD8 T cells. If some cells other than APCs were transduced with the plasmid, the protein of interest would be presented through cross-presentation after being taken up by APCs (Aptsiouri et al., 2007). DNA vaccines can also serve as a priming vaccine while combined with other types of vaccine, such as viral vaccines, which are used as boosts in heterologous prime-boost strategies as tried against viral infections (Sun et al., 2016; Vaccari et al., 2018).

In the early phase, lesions caused by high-risk HPV were one of the most popular targets of DNA vaccines. Premalignant or *in situ* lesions associated with HPV-16 and HPV-18 were treated with VGX-3100 that encodes HPV-16/18 E6/E7 in clinical trials. Trimble et al. reported that the samples from patients with cervical intraepithelial neoplasia (CIN) grade 2 and 3 demonstrated cellular immune response, and the responders who had regression of the lesions were able to avoid lesion resection with a clinical outcome comparable with that of patients who underwent conventional surgical treatment (Bhuyan et al., 2020; Trimble et al., 2015). GX-188E is another DNA vaccine with E6 and E7 but also with additional *Fms*-like tyrosine kinase-3 ligand (Flt3L) that is known to activate DCs. The study showed antigen-specific cell-mediated responses in both IFN- γ -secreting CD8 and CD4 T cells and HPV-specific polyfunctional T cell responses in patients with CIN. The interim report of a phase 2 study

of GX-188E in combination with pembrolizumab in patients with HPV-16- or HPV-18-positive recurrent or advanced inoperable cervical cancer showed an improved response rate of 42% (11 among 26 patients) compared to 14.3% with pembrolizumab alone at week 24 (Chabeda et al., 2018; Youn et al., 2020).

Human epidermal growth factor receptor 2 (HER2), a receptor tyrosine-protein kinase encoded by the ERBB2 gene, has been studied by many researchers as a target of DNA vaccines. V930 against HER2 with or without V932 against carcinoembryonic antigen (CEA) followed by adenoviral boost was tested in patients with solid tumors, demonstrating a well-tolerated safety profile without autoimmunity, including no cardiotoxicity (Diaz et al., 2013). pNGVL3-hICD, a plasmid-based vaccine encoding HER2 intracellular domain, administered with GM-CSF in stage 3 HER2-positive breast cancer, has shown immunogenicity and safety (Salazar et al., 2009). DNA vaccines against rhesus PSA in prostate cancer (pVAXrcPSAv531), Mam-A, a breast cancer antigen expressed in about 85% of breast cancer (pING-Mam-A), or Wilms tumor antigen with the indication for leukemia (pDOMWT1) are some of additional examples that were tested in clinical trials (Eriksson et al., 2013; Lopes et al., 2019; Ottensmeier et al., 2016; Tiriveedhi et al., 2014). Even though DNA vaccines have shown immunogenicity, the endeavor to improve efficiency should continue. Electroporation, use of a gene gun, and preparation of DNA nanoparticles are examples of such efforts (Garu et al., 2016; Huang et al., 2012) (Buchan et al., 2005; Fynan et al., 1993). The possibility of large-batch manufacturing and long-term stability are practical advantages in clinical translation compared to other types of vaccine that require significant coordination and preparation of resources to manufacture and store, such as cell-based vaccines or relatively easily degradable RNA vaccines. Another advantage of a DNA-based platform is versatility of construction such that developers can easily include multiple epitopes or additional genes that can work synergistically or additively, such as cytokines or chimeric DNAs that encode xenogeneic antigens (Lopes et al., 2019; Riccardo et al., 2017). On the other hand, the ease of construction becomes a double-edged sword when the risk of integration of vaccine DNA into cellular DNA is brought up. Regulatory guidelines from the FDA, the European Medicines Agency (EMA), and the World Health Organization are available to address the safety concerns in developing DNA vaccines, focusing on the preventive vaccines against infectious organisms (Kutzler and Weiner, 2008).

RNA as a tool for cancer therapy has an advantage with a variety of characteristics from specific binding or digestive activities to the ability to express or regulate genes of interest. RNA may not serve as a standalone

tool, but it certainly has value as a combinational partner, especially in the field of personalized treatment. Among different types of RNAs, mRNA has been the most popular as a vaccine platform. Other types of RNA, such as structural RNA (e.g., rRNA or tRNA), interference RNA (including microRNA, small interfering RNA (siRNA), and Piwi-interacting RNA), long noncoding RNA, aptamers, or viral RNAs, have been studied with the intention of tackling cancer from different angles in cancer immunotherapy (Pastor et al., 2018). The attempts to use mRNA as a therapeutic agent started in the 1990s, usually in rare disorders and infectious illness such as influenza, Zika, or rabies as well as in cancer by Eli Gilboa's group (Nair et al., 2002). However, numerous attempts disappointed the researchers until the technology came to prominence owing to the urgent need for a vaccine against the SARS-CoV2 pandemic in 2020 (Abbasi, 2020). Unlike many other conventional vaccines, mRNA does not mandate microorganisms as a vector. There is no risk of integration of vaccine components into host DNA in the absence of reverse transcriptase, as mRNA will enter cytoplasm where the RNA sensor is located and will not go into the nucleus. The possibility of rapid design and manufacturing is an outstanding benefit of mRNA vaccines, as conventional vaccines require a minimum of several months if not years of research just to obtain clinical-grade materials before clinical trials, let alone the time required for clinical trials (McNamara et al., 2015). The mode and site of delivery are important aspects in mRNA vaccines, as in any other vaccine platform. Naked mRNA suspended in buffer is simple, and it was used frequently in the early 2000s (Probst et al., 2007). One way to improve the stability of mRNA is to encapsulate mRNA in lipid nanoparticles. The lipid layer of these nanoparticles can protect mRNA from the natural process of degradation and preferentially select the type of cells to which the mRNA can be delivered (Reichmuth et al., 2016). As is seen in other cancer antigen forms, mRNA can be loaded into DCs in vitro before administration to patients, and this method showed induced immune responses. Unlike single-epitope peptides, mRNA-based DC vaccines are not restricted by HLA, and many epitopes of the same antigen can be loaded. This advantage complements the ease of production and low cost burden of any changes in the sequence as described in DNA vaccines (Benteyn et al., 2015). Among different injection sites, intranodal injection seems to elicit the highest T cell response compared to subcutaneous, intradermal, intravenous, or intramuscular injections (Kreiter et al., 2010). With the concept of personalized vaccine to tackle neoepitopes, RNA-based vaccines are especially advantageous. Personalized RNA mutanome vaccines were tested first-in-human in patients with melanoma after sequencing and neoepitope prioritization based on computational prediction of neoepitopes and MHC

affinity. The patients vaccinated with RNA-based poly-neoepitope vaccines developed T cell responses against the neoepitopes in all recipients and tumor infiltration of vaccine-induced T cells that were able to kill the tumor cells infiltrated the tumor in two biopsied patient samples. Among five patients with metastatic disease, one of two patients who experienced objective response showed complete response when the patient had multiple lesions that were unresponsive to radiation and CTLA-4 blockade prior to the vaccination. Eight patients who received vaccines while there were no radiographically detectable lesions at the time of enrollment developed strong immune responses and remained recurrence-free for up to 23 months the follow-up period (Sahin et al., 2017).

Synthetic peptides as a cancer vaccine

Synthetic peptide vaccines have probably been the most popular cancer vaccine platforms. owing to their simplicity, consistent quality, and tolerability since the early 1990s when the first clinical peptide products were manufactured with synthesized peptides initially in the synthetic peptide hormone field (Pontiroli, 1998). Some of the earliest use in cancer vaccines was in melanoma epitopes (Kawakami et al., 1994; Parkhurst et al., 1996) and in peptides encompassing mutations in the patient's tumor p53 and K-RAS proteins (Carbone et al., 2005, 1994; Smith et al., 1997; Yanuck et al., 1993). These were some of the first personalized neoepitope vaccines. The manufacturing process is not as complicated as that of some other platforms, and stability of the stored product is secure for a relatively long term compared to most of the other platforms. Ease of coordination and safety in drug administration are very attractive in the drug development. If the epitopes can stimulate only T cells, the risk of developing antibodies that can lead to anaphylaxis can be avoided. Changing the peptide composition is done in a simple, straightforward manner. Therefore the addition or modification of a few amino acids upon any changes in the plan or epitope enhancement to change the amphiphilicity and increase the immunogenicity can be done (Berzofsky, 1995; Cornette et al., 1995). The amino acid length for an epitope is a minimum 8–11mer that will bind to HLA-I alleles without any further processing. For HLA-II, longer peptides of 13–18 amino acid residues are needed, and this type of peptide will stimulate CD4 T cells. Synthetic peptide platforms are HLA-restricted, requiring HLA typing of the patients. In the late 2000s the concept of using synthetic long peptides (SLPs) of 15–30 residue length was introduced. Both short and long peptides are proven to elicit T cell immune responses. However, SLPs that are processed by professional APCs have a potential to generate stronger CD8⁺ T cell responses, as was initially shown by Melief et al. in

HPV-associated malignant lesions, because of costimulation by the professional APCs and the opportunity to include helper epitopes in the longer peptides, which expanded a strategy used in DNA-based vaccines (Kenter et al., 2008; Melief and van der Burg, 2008).

In B cell malignancies, such as B cell lymphomas or myelomas, a unique vaccine platform was based on the tumor-unique variable region antigen-binding site (called the idiotype) of the monoclonal antibody on the surface of the monoclonal malignant cells, which serves as a personalized surface antigen that specifically identifies tumor cells, as pioneered by Levy, Kwak, and others (Hsu et al., 1997; Kwak et al., 1992). Tumor antigens in a form of idiotype produced by the hybridoma of tumor cells or myeloma cell lines can be used as a personalized vaccine (Lee et al., 2007; McCann et al., 2015; Schuster et al., 2011).

Vehicle of delivery is another important issue in peptide platforms, as in any other platform. Usually, peptides are designed to be small enough that they can easily penetrate the target cells or bind directly to MHC molecules on the surface. However, if the molecule is too small, the vaccine content might be absorbed into systemic circulation and be degraded by proteases. There are various types of nanoparticles that enable encapsulation of the peptides to enhance the antigen uptake by APCs, prolong the bioavailability until vaccine contents arrive in the target area, and also provide the capacity to conjugate with other molecules, such as immune adjuvants, as needed. Liposomes are the most popular type, and the safety profile of the lipid contents of the nanoparticle and the consistency and reproducibility of the manufactured particle are critical factors for further investigation, along with the clinical outcome. Besides liposomes, several other preparations, such as polymer-lipid hybrid, carbon nanotube, or virus-like particles, which are the basis of the HPV vaccine to prevent cervical and anogenital cancers, are examples of currently explored platforms (Boks et al., 2015; Hubbell et al., 2009; Oyewumi et al., 2010; Varypataki et al., 2017; Wen et al., 2019).

One of the challenges in using a peptide vaccine platform is that there is a gap in knowledge of the trafficking, intracellular processing, and fate of administered peptides in humans, as the vast majority of the data are based on murine models. This information can be used in improving the design of vaccine platforms.

Medical advances with monoclonal antibodies in clinical fields

mAbs entered the realm of therapeutics with the invention of the hybridoma technique that can generate mAbs by Kohler and Milstein in 1975 (Kohler and Milstein, 1975). It took over a decade to translate this preclinical Nobel

Prize-winning discovery into the first antibody therapeutics that received FDA approval, an anti-CD3 antibody for kidney transplant rejection in 1986 (Jaffers et al., 1986). Since then, the indications of several mAb therapeutics have been spread out among autoimmune disease and other allergic or inflammatory conditions, cancer or hematological conditions, infection (*Clostridium difficile*, anthrax), and rarely metabolic or degenerative conditions (bone loss, hypercholesterolemia, and macular degeneration). Since the FDA approval of rituximab in 1997 until 2020, there have been about 35 FDA-approved monoclonal therapeutics for use in cancer, and the rate of development has expanded in the past 10 years. Tumor antigen-targeting mAbs such as rituximab (anti-CD20), multiple HER2-targeting mAbs (or antibody drug conjugates), or inhibitory antibodies, including immune checkpoint inhibitors, are frequent partners of vaccine studies (Lu et al., 2020). The concept of making cold tumors hot by using vaccines to generate tumor-infiltrating effector cells to attack the tumor directly complement checkpoint inhibitors is a well-accepted approach. The duration of vaccination, the advantage or disadvantage of different types of immune checkpoint inhibitors or any other anticancer treatment as combination partners, and the sequence of treatment, that is, whether vaccine should precede or be given concurrently with a checkpoint inhibitor, are all actively pursued topics. Bispecific antibody bintratufusp- α targeting PD-L1 and TGF- β with the intention of controlling the TME while unmasking tumor cells' immune evasion using the PD(L)-1 axis is undergoing a clinical trial in combination with an HPV vaccine (NCT03427411).

Virus-based cancer vaccine platforms

With the development of molecular biology techniques enabled by the invention of the polymerase chain reaction since the early 1980s, viral platforms have been widely investigated (Jackson et al., 1972). Several viruses that causing human illness such as measles, mumps, rubella, smallpox, and poliovirus have been better controlled by attenuated or killed viral preventive vaccines, for the most part by preventing or decreasing the severity of viral illness. Thus these viruses could be safe choices with less concern for causing a clinical infection when an attenuated virus is modified to encode tumor antigens as a vaccine vector. Also, the ability to aid in inducing immune responses even in less immunogenic tumors has additional merit. Viral pathogen-associated molecular patterns act as built-in adjuvants that contribute to increased immunogenicity. Toll-like receptor (TLR) activation by virus-encoded TLR ligands is critical in increased antitumor activity (Lizotte et al., 2016; Rojas et al., 2016). When a viral vector is used in cancer vaccines, it can be designed to deliver a tumor-associated antigen (TAA), with or

without costimulatory molecules or cytokines. Then the proteins produced by the engineered virus will be processed in the MHC class I or class II molecule processing pathway depending on the cell types that are infected. Producing a TAA alone in a nonlytic virus is likely not enough to generate strong immunogenicity for a tumoricidal effect. Thus codelivery of a costimulatory molecule or cytokines is reasonable. Proinflammatory responses at the injection site will induce immune responses against the injected virus in nearby TMEs or even normal tissues that can result in cancer cell death.

Adenoviruses have been popular to deliver TAAs, and many vectors were designed using replication-incompetent types (Lubaroff et al., 2009). Environmental exposure to adenoviruses in the community and development of immunity against adenoviruses that exist in the majority of adults posed a problem in the efficacy of adenoviral vaccines unless rare serotypes or chimp adenoviruses to which humans are generally not immune are used. Similarly, poxvirus has a disadvantage that preexisting immunity is associated with previous vaccination against smallpox over the past two centuries, but this can be circumvented with novel vectors, and on the positive side, the safety and efficacy profile is well established in extensive vaccination track records. The cowpox virus that Edward Jenner used against smallpox was passed down through generations for over 100 years, and it is no longer same virus but is named vaccinia virus, which does not exist in nature (Baxby, 1999). Poxviral tropism that can directly infect epithelial cells and APCs is another benefit (Yu et al., 2009). Modified vaccinia Ankara (MVA) was attenuated by over 500 passages of vaccinia virus in chicken embryo fibroblasts. During the passages, MVA became extremely attenuated, losing many poxviral genes, and became mostly replication incompetent in mammalian cells. Attenuated recombinant vaccinia virus or fowlpox virus engineered to express recombinant TAAs with optional cytokines or costimulatory molecules as in PANVAC against CEA and MUC1 have been tested in several clinical trials (Arriola and Ottensmeier, 2016; Madan et al., 2007). In building a prime-boost strategy, using a different vector for priming and boost may induce stronger immune responses. In PANVAC, priming by vaccinia will not cause immunity against the fowlpox vector that is used for booster doses, which can amplify the immune reaction (Grosenbach et al., 2001). PROSTVAC adopted a similar concept of priming with recombinant vaccinia virus designed to express PSA and a triple costimulatory molecule combination called TRICOM followed by recombinant fowlpox virus constructed to express PSA and TRICOM. PROSTVAC showed promising phase II results with improved superior overall survival of 26.2 months compared to 16.3 months in the control group in patients with asymptomatic or minimally symptomatic metastatic castration-resistant prostate cancer, but the phase III randomized trial as a single agent did not confirm that in a planned

interim analysis, and combination studies are underway (Gulley et al., 2019; Kantoff et al., 2017, 2010b).

Neopeptope vaccines

When the concept of personalized medicine was first brought up in clinical oncology, it was about certain cell surface markers or cell signaling pathways. However, marked improvement in molecular biology techniques that made sequencing faster and cheaper for individual patients coupled with the success of immunotherapy, addressing the mutations that are unique to the individuals, is getting considerable attention. When the prioritization of the antigen as a cancer treatment target was announced in 2009, using criteria of (1) therapeutic function, (2) immunogenicity, (3) role of the antigen in oncogenicity, (4) specificity, (5) expression level and percent of antigen-positive cells, (6) stem cell expression, (7) number of patients with antigen-positive cancers, (8) number of antigenic epitopes, and (9) cellular location of antigen expression, the term *tumor antigen* meant that TAAs as a personal approach using molecular strategy was not available until recently (Cheever et al., 2009). Even though the treatments targeting shared TAAs, such as CD20, HER2, or vascular endothelial growth factor (VEGF), paved the way with significant clinical benefit in cancer patients over the past 20 years, the risk of on-target, off-tumor effects has been inevitable, associated with noncancer cells expressing the same or a cross-reactive target. In contrast, neopeptides that arise from tumor-specific antigens arise from a somatic mutation that is unique to the cancer and therefore is not present in normal cells. They are seen as “foreign” and recognizable by effector cells not subject to negative selection, unlike nonmutated shared TAAs, making them ideal for immunotherapy (Tureci et al., 2016). The immune system is extremely sensitive in recognizing differences between self and non-self even down to the level of a single amino acid change. Also, the immune system can provide systemic effects and elicit long-term memory with a recall response that is ideal in cancer control. However, immunologists have been discouraged for a few decades by the challenges in efficacy once the findings in preclinical model were translated to the clinic. Newly brewing enthusiasm was brought in with checkpoint inhibitors that can work synergistically with vaccines to “turn a cold tumor hot.” One of several proposed mechanisms of primary checkpoint resistance is deficient T cell clones that can identify tumor antigens at baseline, to explain low response rate of 5%–20% across most of the solid tumor types, whereas the analysis of checkpoint inhibitor studies has shown that the response is better in patients with cancers with a high tumor mutational burden. However, if vaccines can generate T cell immune responses to tumor antigens, they can

compensate by providing the needed T cells and synergize with checkpoint inhibitors, turning the cold tumor hot. Effector T cells that can specifically recognize mutated epitopes showed the ability to induce the regression of tumor. Manufacturing of cellular products is challenging to scale up, and the impact of single-agent vaccine therapy is not strong enough to induce clinical responses in most patients. In dealing with neoepitopes, shared neoepitopes that are present in a group of patients, such as mutations in RAS, EGFR, or TP53, were among the first such neoepitope targets (Carbone et al., 2005). Vaccines for TAA can be attractive in that the product can be premade in a large batch for off-the-shelf supplies (Carbone et al., 2005; Sampson et al., 2008). Over time, the interest has expanded to a more individualized approach. Rapid screening using high-throughput next-generation sequencing and prioritization of multiple neoepitopes to deliver vaccines in a more manufacture- and delivery-friendly platform gained momentum, leading to multiple personalized vaccine studies (Castle et al., 2019). Currently, multiple neoepitope vaccines in DNA, RNAs, and peptide platforms are being tested in vaccines alone or in combinatorial settings with other immunological (checkpoint inhibitors, cytokines, mAbs, or immune costimulatory molecules) and nonimmunological (chemotherapy or radiation) agents. At the moment the studies are predominantly focused on using immune checkpoint inhibitors (Hacohen et al., 2013; Lee et al., 2020).

Combination strategies

Despite the paradigm-changing success of several immune checkpoint inhibitors in the past decade, the response rate and long-term remission rate are still far from what we aim to achieve even in the type of cancers, with FDA or EMA approval mostly running below 20% with the exception of melanoma, urothelial tumor, rare Merkel cell carcinoma associated with polyomavirus, and microsatellite instability (MSI)-high tumors (Diaz et al., 2018; Haslam and Prasad, 2019; Heery et al., 2017; Le et al., 2020). To overcome resistant mechanisms that hamper the effectiveness of cancer immunotherapy, regimens that can provide synergism among the reagents with different mechanisms are essential. The partners in combinational regimens can be in the category of biological, immunomodulatory, or cytotoxic agents in conventional usage. Several combinational studies of cancer vaccines and immune checkpoint inhibitors have been launched in the past 5 years with a belief that vaccine can induce an immune response, making a cold tumor hot, to work synergistically with immune checkpoint inhibitors. On the other hand, several conventional cytotoxic agents or targeted agents that are used in the clinic as a part of standard care are reported to have immune stimulatory or inhibitory effects or to affect negative regulators of the immune system as more immune

effects are unveiled. Contrary to theoretic concerns that effector cells may disappear after chemotherapy, a study in a PD-1 blockade-resistant melanoma showed that tumor-reactive CD8⁺ T cell subsets from the patients' blood increased in number and antitumor activity after carboplatin. Also, longer overall survival (3.5 years vs 1.8 years) and higher overall response rate (59% vs 15%) in patients when certain chemotherapies were combined with immunotherapy compared to chemotherapy alone were reported (Vera Aguilera et al., 2020). The studies in small cell lung cancer (IMpower133 and ECOG-ACRIN EA5161) using standard regimen chemotherapy in combination with checkpoint inhibitors suggested improved progression-free survival and overall survival; the final report is pending (Leal et al., 2020; Liu et al., 2020). Another significance of chemotherapy lies in the control of negative immune regulators such as MDSCs. A study in glioblastoma that is known to have higher MDSCs than other tumors showed that the use of metronomic capecitabine reduced the MDSCs and suggested a role of capecitabine as an immune modulator (Peereboom et al., 2019). The impacts of VEGF on the immune system beyond the impact on the vascular system are being investigated in depth. VEGF is reported to negatively affect differentiation of the cell and DC maturation while promoting activities of MDSCs and regulatory T cells (Draghiciu et al., 2015; Gabrilovich et al., 1996; Ko et al., 2009). Thus combining VEGF inhibitors with immunotherapy agents is a reasonable strategy, especially in types of cancer that have shown benefit in using either one alone. The potential of a durable response of vaccines can be achieved when each tumor type's biological findings are considered instead of a fixed uniform approach, to build a synergistic or stepwise strategy. Initial attempts to incorporate other agents involved cytokines such as IL-2 or GM-CSF have been expanded to the administration of genes encoding cytokines with added variety, including IL-12, IL-15, IL-21, and more (Slingluff et al., 2009; Small et al., 2007; Touba et al., 2007; Waldmann, 2018). Numerous vaccines that previously demonstrated tolerable adverse event profile and immunogenicity, such as ISA 101, GVAX (a genetically modified whole tumor cell vaccine), PROSTVAC, DC vaccines, and several peptide and nucleic acid-based vaccines, are being tested in clinical trials in combination with immune modulatory agents or drugs that have been used in the standard care aiming for a synergy (NCT03199040, NCT02451982, NCT02933255) (Le et al., 2013; Massarelli et al., 2019; Wilgenhof et al., 2016).

Cancer vaccines with broader goals

Vaccines targeting the tumor microenvironment

The TME is important in cancer cell proliferation and resistance to treatment. It is well known that cellular components of the TME are polarized to suppressive

phenotypes (Hinshaw and Shevde, 2019). Therefore measures that can reverse the negative regulatory phenotype of cells in the TME to a stimulatory phenotype can amplify the immune mechanism of tumor cell killing. Blocking extracellular protein by antibody treatment or intracellular protein with siRNA has been studied (Sioud, 2019). The other way to boost the immune reaction is by enhancing inflammatory processes by providing additional cytokines by local injection. The purpose of local injection of immunomodulatory agents is to prevent their degradation in the systemic circulation and metabolism while decreasing off-target effects compared to systemic therapy. This approach has been tested in first-in-human trials using mRNAs of proinflammatory molecules or cytokines (Luheshi et al., 2019; Patel et al., 2020).

Vaccines with preventive or adjuvant purposes

Considering the development of immune tolerance after prolonged exposure to overexpressed antigens, preventive vaccines for high-risk groups (primary prevention) or vaccines for secondary prevention as an adjuvant to prevent recurrence or metastasis can be more efficient than usage in advanced cancer (Bol et al., 2016; Mapara and Sykes, 2004; Saeterdal et al., 2001). HPV vaccines for dysplastic *in situ* cancer cells are great examples, as described in the discussion of peptide vaccines earlier in this chapter. Among many vaccine platforms in adenocarcinoma, targeting MUC1 has a longstanding history in vaccine development. Finn et al. have established the link between tumor antigens and disease-associated antigens (DAA) in MUC1, supporting how vaccines can be used for prophylactic indications in a series of research studies. A DAA is defined as a self-antigen expressed during the course of acute or chronic inflammation that is abnormal. MUC1 is a DAA in mumps but also a TAA for several solid tumors, mainly in adenocarcinomas, including cancer of

the pancreas, colon, ovary, breast, and lung. In cancer, MUC1 often presents as aberrant glycosylated forms. A history of mumps in younger ages was shown to be protective against ovarian cancer that correlated with the anti-MUC1 antibody that was capable of rejecting tumor cells in mice (Vella et al., 2009). Using the same strategy, a preventive vaccine in high-risk individuals with colonic adenoma was designed using a 100 amino acid synthetic peptide containing five repetitive 20 amino acid residues of MUC1. In a phase 1 clinical trial the vaccine induced high IgG levels against MUC1 with immune memory, and failures of response correlated with high circulating MDSCs. The vaccine was studied further in a phase 2 trial (NCT02134925) (Kimura et al., 2013; Lohmueller et al., 2016). In using DAAs as a target, it will be critical to ensure the safety profile that includes the risk of developing autoimmunity that can be detrimental to achieving prevention, which should not be at the price of developing an autoimmune condition. Thus the choice of antigens unique to tumors that are not expressed in normal tissue is essential. Translational efforts in preventive vaccines are well illustrated in vaccines for individuals with Lynch syndrome presenting with MSI-high tumors and vaccinations for breast ductal carcinoma *in situ* against HER2 (Gelincik et al., 2019; Sharma et al., 2012).

Challenges and future directions

Cancer vaccines have faced serious challenges in translating the findings from preclinical models to the clinic. In early years the gaps between the expectation and reality led to a negative impression of cancer vaccines' utility. However, as this chapter has shown, each vaccine platform (see Fig. 24.2) has unique potential that can synergize with agents that are already in the clinic or are in the pipeline or in preclinical studies. One major player in the category that needs further exploration is oncolytic

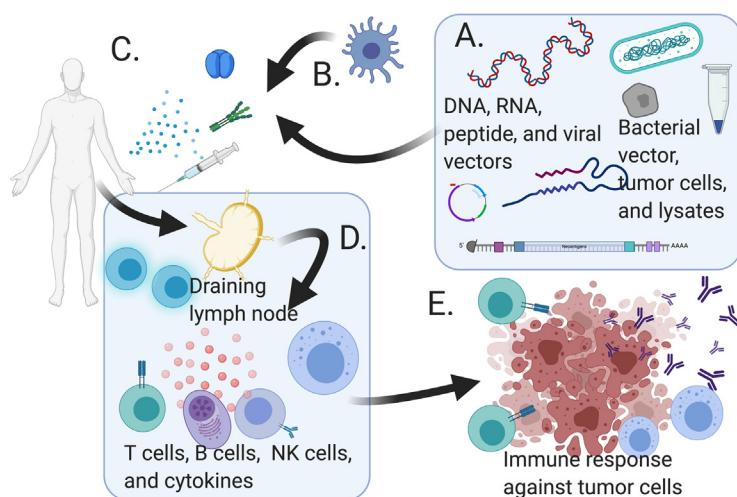


FIGURE 24.2 Simplified cancer vaccine platforms and their action. (A) Selected types of tumor antigen sources and vectors: DNA, RNA, peptides, tumor cells, and their lysates bacteria and virus. (B) Dendritic cells can be manipulated in vitro to enhance the efficacy of antigen presentation and subsequent immune response. (C) Administration of the vaccines is often accompanied by immune adjuvants. (D) After the administration of the vaccines, antigen presentation and other immune stimulation occur to induce innate and adaptive immune response. (E) Innate and immune response results in tumor cell killing. Created with BioRender.com.

viruses that can serve as “an endogenous vaccine” by posing a direct cytopathic effect and releasing other tumor antigens, leading to an immune cascade and so-called epitope spreading. We need to remind ourselves of the immune surveillance hypothesis proposed by Burnet and Thomas in 1957 that the immune system is capable of recognizing and destroying nascent transformed cells, which had not gained consensus for more than 50 years, although it has finally been proven in the work on immunoediting by Schreiber and coworkers (Burnet, 1957; Dunn et al., 2004). Aside from scientific achievements, overcoming regulatory challenges, stringent quality control of the product, and reducing the high manufacturing cost are some of the critical factors in bringing the success of vaccines against infectious organisms to cancer care (Goldman and DeFrancesco, 2009, 2010).

Conclusion

In this chapter the history of immunological discovery and success was intercalated with several different types of cancer vaccines, and the challenges of translating pre-clinical findings to the clinical application were discussed. With the successful start of cancer immunotherapy, opening a new era of cancer treatment with immune checkpoint inhibitors, cancer vaccines have a great potential to become the third leg of the immunotherapy stool, along with immune modulators and adoptive cell therapies, as immunotherapy rapidly becomes the fourth pillar of cancer care in addition to the three pillars of surgery, chemotherapy, and radiation.

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Chapter 25

Translational aspects of biologicals: monoclonal antibodies and antibody-drug conjugates as examples

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Abstract

Monoclonal antibodies are at the forefront of novel therapeutic strategies for cancer and have made a positive impact on the clinical management of several malignancies. Success in the clinic was originally demonstrated in hematological cancers with the regulatory approval of the anti-CD20 monoclonal antibody rituximab for the treatment of non-Hodgkin's lymphoma in 1997 and subsequently with the anti-HER2 trastuzumab for the treatment of breast cancer in 1998. Early research and development efforts concentrated on the design of antibodies that selectively target cancer cells or tumor-associated vasculature. In the last decade, two further antibody classes have emerged: checkpoint inhibitors capable of targeting checkpoint molecules on immune cells to activate immune responses against tumors and antibody-drug conjugates, comprising monoclonal antibodies conjugated to toxic payloads to deliver these specifically to tumor cells. A clearer understanding of an antibody's functional profile may pave the way for identification of the most highly efficacious agents for specific patient groups.

Keywords: Monoclonal antibodies; Fab regions; Fc regions; effector functions; IgG, IgE, antibody-drug conjugates (ADCs); checkpoint inhibitors; cancer immunotherapy

Howard more than a century ago. In the past two decades, monoclonal antibodies have been used in cancer treatments, becoming especially successful in the clinical management of hematological malignancies (Scott et al., 2012), although good progress is now being made with their use to treat solid tumors. Monoclonal antibodies are therapeutically and clinically advantageous, owing to their high selectivity and specificity for their target antigens. Unlike polyclonal antibodies, which recognize several epitopes of an antigen, monoclonal antibodies recognize only a single unique epitope of an antigen structure. This attribute has been exploited to target cancer cells specifically.

Treating cancer with monoclonal antibodies became a clinical reality in 1997, when rituximab (a chimeric anti-CD20 monoclonal antibody) was approved for the treatment of CD20-positive non-Hodgkin's lymphoma, followed by the approval of trastuzumab, a humanized antibody that recognizes the tumor-associated antigen HER2, for the treatment of HER2-positive breast cancer in 1998 (Galluzzi et al., 2014; Carter and Lazar, 2018). Therapeutic antibodies can function through a variety of mechanisms: directly causing alterations in target antigen signaling, blocking receptor-ligand interactions that affect vital cancer survival functions, or triggering effector mechanisms to engage the immune system via the Fc regions. Monoclonal antibodies in clinical use for cancer therapy can selectively target cancer cells, tumor-

Overview of monoclonal antibodies for cancer therapy

The concept of employing antibodies to treat diseases, including cancer, was proposed by Ehrlich, Bolduan, and

associated vasculature, or checkpoint molecules on immune cells (Marin-Acevedo et al., 2018). Furthermore, antibodies linked to cytotoxic drugs, resulting in antibody-drug conjugates (ADCs), use antibodies as vehicles to specifically deliver their payload directly and specifically to tumor cells.

Structure and functions of antibodies

Antibodies, also referred to as immunoglobulins (Igs), are multimeric glycoproteins expressed by B cells and secreted by plasma B cells. Antibodies play vital roles in protecting the host by triggering immune responses against antigens expressed by pathogens (Schroeder and Cavacini, 2010).

Antibodies are heterodimeric glycoproteins composed of two identical heavy chains and two identical light chains linked through covalent disulfide bonds as well as noncovalent interactions (Schroeder and Cavacini, 2010). Together, these four chains make up two regions of the immunoglobulin molecule: the fragment antigen binding (Fab) region and the fragment crystallizable (Fc) region (Fig. 25.1).

The Fab region determines the antigen specificity through a variable region (V) at the N-terminal end of the protein formed by both heavy (V_H) and light chains (V_L). This variability, caused by alterations in the amino acid sequence, gives rise to the recognition of an unlimited spectrum of antigens and antigenic structural epitopes in a lock-and-key fashion. Antibody light chains can be one of two types, kappa or lambda, and these can contribute to antigen epitope recognition, binding, and affinity (Schroeder and Cavacini, 2010). Every Fab region consists of the framework regions and the complementary determining regions (CDRs), representing the antigen-binding sites. The CDRs are directly involved in antigen recognition and binding, whereas the FWRs are

considered a scaffold for the CDRs (Sela-Culang et al., 2013). As B cells are exposed to a specific antigen and influenced by specific immunostimulatory environments, the CDRs go through a series of gene arrangements, culminating in the selection of antibody clones with the highest affinity to a specific antigen. Part of the constant region domain above the hinge region, at which the two heavy chains are covalently bonded through disulfide bridges, also represent part of the Fab region.

The Fc region is composed of the heavy chain constant domains at the C-terminal region of the protein. The number of immunoglobulin domains making up the constant regions can differ between antibody classes (Fig. 25.1). There are five main classes of antibodies: IgA, IgD, IgE, IgG, and IgM. Of these, IgG and IgA can be further classified into four and two subclasses (or isotypes), respectively. These are IgG1, IgG2, IgG3, and IgG4 for IgG and IgA1 and IgA2 for IgA. IgD antibodies contain three C_H domains (C_{H1} – C_{H3}), while IgG and IgM classes comprise four C_H domains (C_{H1} – C_{H4}) (Raghavan and Bjorkman, 1996; Schroeder and Cavacini, 2010). With antigen and cytokine stimulation, immature B cells expressing IgM and IgD antibodies can class switch to express mature antibody classes IgA, IgE, and IgG (Stavnezer and Schrader, 2014). The various antibody classes also show differences in their assembled structures, since IgD, IgE, and IgG immunoglobulins are found only as monomers, while IgA and IgM antibodies also exist as dimers and pentamers, respectively (Fig. 25.1).

The primary roles of antibodies are to recognize antigens by the variable regions on the Fab domains and to engage with Fc receptors expressed on the surface of immune effector cells through their Fc region. Antigen recognition via the Fab region allows the induction of innate and adaptive immune response events via Fc effector cell binding, thereby neutralizing and eliminating pathogens as well as triggering protective memory T cell

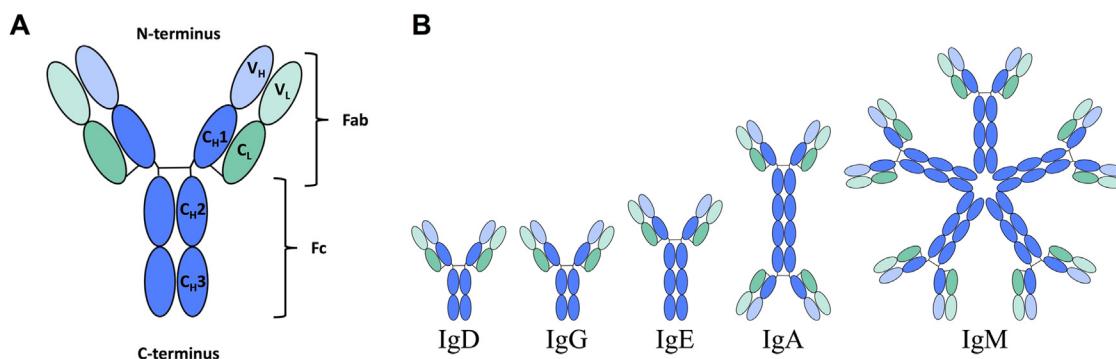


FIGURE 25.1 General structure of immunoglobulins and of the five antibody classes. (A) Schematic diagram showing the general structure of an antibody in a simplified format. The blue regions are the heavy chains, and the green regions are the light chains. Constant (C) domains of the heavy and light chains are indicated by darker shading, and the variable (V) domains by lighter shading. Whereas IgD, IgG, and IgA antibodies follow this structure, IgE and IgM antibodies contain an additional C_H domain (C_{H1} – C_{H4}). (B) Diagram representing the overall structures of the five human antibody classes. IgD, IgG, and IgE are illustrated as monomers, IgA as a dimer, and IgM as a pentamer.

and B cell responses. Through Fc receptor engagement, immunoglobulins can trigger multiple biological responses (Raghavan and Bjorkman, 1996; Schroeder and Cavacini, 2010). These include innate mechanisms of cell killing, such as antibody-dependent cellular phagocytosis (ADCP), antibody-dependent cellular cytotoxicity (ADCC), and complement-dependent cytotoxicity (CDC) (Carter, 2006; Weiner et al., 2010; Zahavi and Weiner, 2020).

Generation of monoclonal antibodies for cancer therapy

Initially, monoclonal antibodies for therapeutic use were derived by using the hybridoma technology (Kohler and Milstein, 1975; Little et al., 2000). This generated monoclonal antibodies from immunized mice by fusing mouse splenic B cells to myeloma cells, thereby creating antibodies of murine origin. In clinical application these antibodies triggered strong immunogenicity in humans (Presta, 2006) and the production of human antimouse antibodies. This adverse immune reaction to monoclonal antibody therapy led to quick neutralization and clearance of the murine antibody from the blood circulation, leading to poor efficacy and systemic toxicity in recipient patients. Moreover, the Fc portions of these antibodies were not effectively recognized by human Fc receptors on immune cells and thus failed to trigger effector functions through recruitment of immune effector cells. To overcome these challenges, new types of antibodies were developed, including chimeric (murine variable regions assembled with human constant regions), humanized (human antibodies with murine CDR structures), and finally fully human monoclonal antibodies (Weiner et al., 2010) (Fig. 25.2). For the production of chimeric antibodies the murine constant regions, based on hybridoma-derived antibodies, are replaced by human constant regions using molecular cloning techniques. For the generation of humanized antibodies, murine framework regions are replaced by homologous regions of human origin, with CDR grafting representing the most popular technique (Dondelinger et al., 2018; Lu et al., 2020). Since CDR and framework

regions influence the three-dimensional structure and binding activity of antibodies, web servers are being developed to provide tools for template selection, grafting, backmutation evaluation, and antibody modeling (Lu et al., 2020).

More recently, antibodies for therapeutic use have been produced mostly as fully human agents by using either phage display (in vitro) or humanized transgenic mice (in vivo) technologies. The most prominent host cell lines for recombinant monoclonal antibody expression are Chinese hamster ovary, murine myeloma derived NS0 and Sp2/0, human embryonic kidney cells (HEK293), or human embryonic retinoblast-derived PER.C6 cells (Kunert and Reinhart, 2016; Dangi et al., 2018).

Mechanisms of action of antibodies for cancer therapy

Currently, there are more than 60 clinically available monoclonal antibodies in different formats approved by the US Food and Drug Administration (FDA) for the treatment of hematopoietic and solid tumors (see Table 25.1 for examples of monoclonal antibodies approved for the treatment of solid tumors), and many more are in clinical development. There are various mechanisms by which engineered monoclonal antibodies can reduce or prevent tumor survival and growth. The antitumor functions of antibodies can be grouped into four major categories: (1) Fab-mediated effects, (2) Fc-mediated effects, (3) engagement of the adaptive immune response, and (4) antitumor immunity by immunomodulation based on immune checkpoint inhibition (Scott et al., 2012; Weiner, 2015; Zahavi and Weiner, 2020). Many clinically approved antibodies work via one or more of these mechanisms.

Fab-mediated mechanisms of antibodies for cancer therapy

Monoclonal antibodies can recognize tumor-associated antigens and bind to those through their Fab regions. This can result in receptor antagonist activity by the antibody binding to a tumor cell surface receptor, in which case it can prevent receptor dimerization and inactivate kinase activity and downstream signaling, all of which can ultimately lead to reduced proliferation, disruption of target tumor-associated vasculature, or apoptosis and result in target cell death (Scott et al., 2012).

Cetuximab, panitumumab, and trastuzumab are examples of this category of monoclonal antibodies used in the clinic, as they interfere with the downstream signaling of epidermal growth factor receptor (EGFR/HER) family molecules. Cetuximab and panitumumab competitively

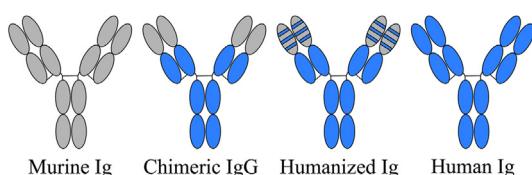


FIGURE 25.2 Schematic representation of murine, chimeric, humanized, and human immunoglobulins. Chimeric antibodies consist of murine variable regions and human constant regions, and humanized antibodies contain murine CDRs only. The gray shading indicates murine regions, while blue shading indicates human regions.

TABLE 25.1 Examples of monoclonal antibodies approved by the FDA for the treatment of solid tumors.

Monoclonal Antibody	Drug Name	Specification	Target	Proposed Mechanism of Action	FDA Approval	Main Therapeutic Indication
Atezolizumab Avelumab Bevacizumab	Tecentriq Bavencio Avastin	Humanized IgG1 Human IgG1 Humanized IgG1	PD-L1 PD-L1 VEGF	CP inhibitor, CTL activation CP, CTL activation, ADCC Inhibition of VEGF signaling	2016 2017 2004	Metastatic NSCLC NSCLC, Merkel cell carcinoma Metastatic colorectal cancer, NSCLC, metastatic breast cancer, ovarian cancer Cutaneous squamous cell carcinoma
Cemiplimab	Libtayo	Human IgG4	PD-1	Inhibition of PD-1/PD-L1/PD-L2 signaling	2019	
Cetuximab	Erbitux	Chimeric mouse/human IgG1	EGFR	Inhibition of EGFR signaling, ADCC	2004	Head and neck cancer, colorectal carcinoma
Daratumumab Denosumab	Darzalex Xgeva	Human IgG1 Human IgG2	CD38 RANKL	ADCC, CDC Inhibition of RANKL signaling, ADCC	2020 2011	Multiple myeloma Breast carcinoma, prostate carcinoma, bone metastases
Dinutuximab	Unituxin	Human/mouse chimeric IgG1	GD2	ADCC, ADCP, CDC	2015	Neuroblastoma
Durvalumab Elotuzumab Ipilimumab	Imfinzi Empliciti Yervoy	Human IgG1 Humanized IgG1 Human IgG1	PD-L1 SLAMF7 CTLA-4	CP, CTL activation ADCC, NK cell activation CTLA-4 blockade, checkpoint inhibition, ADCC, CTL activation	2017 2018 2011	Advanced/metastatic urothelial carcinoma Relapsed multiple myeloma Melanoma
Necitumumab	Portrazza	Human IgG1	EGFR	Inhibition of EGFR signaling, ADCC and CDC	2015	NSCLC
Nivolumab	Opdivo	Human IgG4	PD-1	CP inhibitor, Inhibition of the PD-1/PD-L1 interaction	2015	NSCLC, Melanoma
Olaratumab Panitumumab Pembrolizumab	Lartruvo Vectibix Keytruda	Human IgG1 Human IgG2 Humanized IgG4	PDGFR- α EGFR PD-1	Blockade of PDGFR- α /Ligand binding Inhibition of EGFR signaling Inhibition of the PD-1/PD-L1 interaction; CP inhibitor	2016 2016 2014	Sarcoma Metastatic colorectal carcinoma Melanoma
Pertuzumab Ramucirumab	Perjeta Cyramza	Humanized IgG1 Human IgG1	HER2 VEGFR2	Inhibition of HER2 signaling Inhibition of VEGF signaling	2012 2014	Breast carcinoma Gastric or gastroesophageal junction adenocarcinoma
Trastuzumab	Herceptin	Humanized IgG1	HER2	Inhibition of HER2 signaling, ADCC	1998	Breast cancer, metastatic gastric adenocarcinoma

ADCC, antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; CDC, complement-dependent cellular cytotoxicity; CP, checkpoint; CTL, cytotoxic T lymphocytes; NK cell, natural killer cell; NSCLC, non–small cell lung cancer.

Source: Information adapted and updated from various sources.

bind to EGFR, thereby blocking its epitope recognition site for activating ligands, including epidermal growth factor and transforming growth factor alpha (Purba et al., 2017). This results in inhibition of ligand binding and receptor homodimerization and heterodimerization, thus blocking downstream signaling cascades and ultimately reducing tumor cell survival and proliferation (Moradi-Kalbolandi et al., 2018). Trastuzumab targets HER2, another member of the EGFR protein family, expressed by 25%–30% of breast cancers. HER2 receptors do not have ligands, so trastuzumab binding does not compete with ligand interaction. It can instead prevent HER2 receptor dimerization and downstream signaling, consequently inhibiting aberrant cell proliferation and survival (Masuda et al., 2012; Vu and Claret, 2012). The mechanisms of resistance to trastuzumab are further discussed later in the chapter.

Monoclonal antibodies can also be designed to target a naturally occurring ligand rather than a cell surface receptor. For example, bevacizumab was designed to target the tumor-associated vasculature by recognizing the vascular endothelial growth factor (VEGF). VEGF, a mediator of angiogenesis, supports vasculature formation, which supplies nutrients and oxygen to tumors while removing waste products, and also provides a means for cancer cell metastasis and dissemination to other anatomical sites (Ferrara et al., 2005). Furthermore, VEGF may exert immunosuppressive effects to prevent immune attack against cancer cells in the tumor microenvironment (TME), for example, by suppressing dendritic cell maturation and adaptive immune responses (Gabrilovich et al., 1996). By binding to circulating VEGF, bevacizumab prevents the interaction of VEGF with VEGF receptors on the surface of endothelial cells. This results in the inhibition of angiogenic activity, leading to a potential reduction in blood supply to a tumor, thus leading to tumor growth reduction (Ferrara et al., 2004). Bevacizumab was initially approved for the treatment of colorectal cancer, but it is now widely prescribed to treat various cancers, such as glioblastoma, as a single agent. It is also used in

combination with chemotherapy to treat other disease types such as non–small cell lung cancer, metastatic renal and cervical cancers, and ovarian cancer.

Fc-mediated mechanisms of antibodies for cancer therapy

Antibodies are also capable of inducing tumor cell killing by engaging their Fc regions with Fc receptors present on the surface of immune cells. The main mechanisms of Fc-mediated tumor cell killing are ADCC, ADCP, and CDC (Bakema and van Egmond, 2014; Kellner et al., 2017; Graziano and Engelhardt, 2019) (Fig. 25.3).

For ADCC to be triggered, immune effector cells recognize the Fc region of antibodies bound to the surface of target cells, which could be tumor cells, thereby stimulating the release of cytotoxic (e.g., granzymes, proteases, perforin) and immune (e.g., tumor necrosis factor alpha and interferon gamma) mediators that promote lysis of tumor cells (Iannello and Ahmad, 2005; Rajasekaran et al., 2015; Redman et al., 2015; Sharma et al., 2017). Natural killer (NK) cells are classic effector cells that mediate ADCC; however, monocytes, macrophages, and neutrophils have also been shown to trigger ADCC (Yeap et al., 2016; Li et al., 2017). Efforts to enhance the ADCC effect include the Fc engineering of antibodies to increase the affinity for Fc receptors on effector cells (Herter et al., 2014). NK cells in particular release cytolytic granules containing perforins, which create pores on the surface of tumor target cells, and granzymes, which induce apoptotic cell death (Trapani and Smyth, 2002; Iannello and Ahmad, 2005; Lo Nigro et al., 2019). Monocytes and macrophages can promote cytotoxicity through several mechanisms, such as the release of reactive oxygen species, reactive nitrogen species, and tumor necrosis factor alpha (Yeap et al., 2016).

ADCP is mediated by phagocytic cells recruited to the target cell as part of their innate immune response, where they engage with the Fc region of tumor cell–bound antibodies. Phagocytes such as monocytes, neutrophils, and

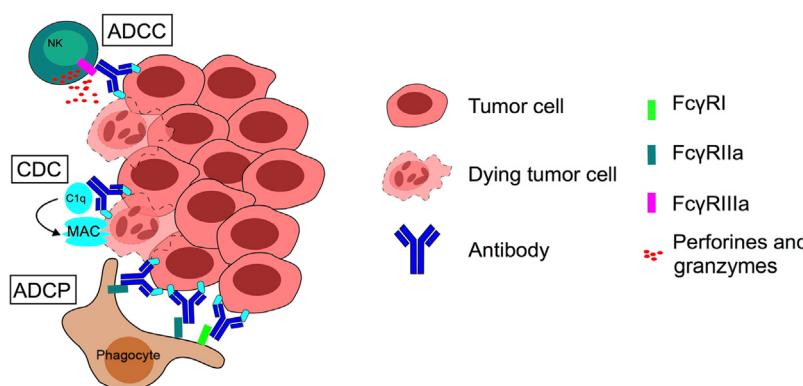


FIGURE 25.3 Schematic diagram depicting the main mechanisms of Fc-mediated tumor cell killing by monoclonal antibodies. Antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity [CDC, e.g., via C1q/membrane attack complex (MAC)] are caused through Fc-mediated engagement of the immune system. Modified from Hoffmann, R.M., Coumbe, B.G.T., Josephs, D.H., Mele, S., Ilieva, K.M., Cheung, A., et al., 2018. Antibody structure and engineering considerations for the design and function of Antibody Drug Conjugates (ADCs). *Oncioimmunology* 7 (3), e1395127.

especially macrophages are capable of engulfing and lysing target cells that are opsonized with antibodies or complement (Gregory and Devitt, 2004; Bakema and van Egmond, 2014; Merle et al., 2015). The level of ADCP carried out by macrophages can be enhanced when stimulated by the presence of tumor antigen-specific monoclonal antibodies on the surface of target cells. For instance, monocyte/macrophage-mediated phagocytosis of HER2-overexpressing cancer cells has been reported with the antibody trastuzumab, which is clinically used to treat HER2-positive breast cancer (Karagiannis et al., 2009).

CDC can be mediated by antibodies bound to the surface of target tumor cells. An activated complement cascade can trigger or aid tumor cell killing by three mechanisms: (1) opsonizing antigens and inducing phagocytosis of the killed cell debris by immune cells, (2) causing inflammation, which recruits effector cells such as macrophages and neutrophils, and (3) formation of a membrane attack complex as part of the complement activation cascade, which can generate pores on target cell surfaces and induce cell lysis (Kolev et al., 2011; Rogers et al., 2014).

Engaging the adaptive immune response

Apart from contributing to antitumor immunity through their Fab and Fc region engagement, monoclonal antibodies can also stimulate an adaptive immune response. This is triggered by phagocytes that have antigen-presenting capacities (APCs), such as monocytes and macrophages and especially dendritic cells. This enables them to process tumor antigens and present their processed antigenic peptides bound to MHC Class I and Class II molecules on the cell surface to cognate T cells. In the presence of costimulatory signals between the APC and cognate T cell, this can elicit the adaptive immune system and trigger subsequent cytotoxic T cell immune effector mechanisms (Weiner et al., 2012). For example, the clinically used antibodies trastuzumab and cetuximab have been shown to facilitate the activation of anti-HER2-specific and anti-EGFR-specific T cell responses, respectively, in patients. Trastuzumab has been shown to enhance both the uptake of HER2 by dendritic cells and the cross-presentation of HER2 peptides, thus resulting in increased priming of naïve CD8⁺ T cells or cytotoxic T cells (CTLs) (Park et al., 2010; Gall et al., 2017). The role of the adaptive immune response upon treatment with cetuximab has been reported in an in vivo study in Rag1 knock-out mice bearing human EGFR-dependent tumors. The study demonstrated tumor regression only upon administration of cetuximab plus transgenic lymph node cells, while neither cetuximab nor lymph node cells individually could significantly inhibit tumor growth (Yang et al., 2013).

In addition to the antagonistic clinically approved antibodies, agonistic antibodies targeting immunostimulatory coreceptors through Fab engagement are being developed. Targets that are being investigated include CD40 expressed on APCs, 4-1BB expressed on NK cells, and CD28 expressed on T cells (White et al., 2015). Interestingly, the IgG2 isotype with the h2 disulfide bond conformation is agonistic for multiple targets, and its activity is FcγR-independent (White et al., 2015; Yu et al., 2018, 2020).

Engaging antitumor immune responses via checkpoint blockade

Developments in our understanding of human immunity, tolerance, and inflammation in the last decade have highlighted the importance of checkpoint molecules that regulate immune responses, especially T cell–driven immunity, and these have given rise to the immune checkpoint inhibitor antibodies. Our immune system employs immune checkpoints to regulate immune responses, maintain homeostasis, and prevent reactivity to self-antigens and the onset of autoimmunity. However, tumor cells often exploit these protective mechanisms to promote immune suppression, immune escape, and, ultimately, cancer cell survival and proliferation. Hence immune checkpoint inhibitors are designed to target these immune checkpoint molecules and to counteract the immune-suppressive mechanisms.

The first anticancer therapy targeting an immune checkpoint was ipilimumab, a cytotoxic T-lymphocyte-associated 4 (CTLA-4) blocking agent that was approved in 2011 for the treatment of melanoma. CTLA-4 is expressed on the surface of naïve activated T cells as a brake to continuous activation but is also constitutively expressed by regulatory T cells (Tregs), which are often found in tumors, where it can turn off T cell receptor signaling and activation (Seidel et al., 2018). Administration of ipilimumab prevents the immunosuppressive functions of CTLA-4, thus restoring or enhancing antitumor T cell functions. Additionally, ipilimumab is thought to deplete CTLA-4 high-expressing tumor-associated Tregs that are likely to be found in the TME. These effects may be achieved by multiple mechanisms, such as expansion of ICOS + CD4⁺ T cells (Liakou et al., 2008; Chen et al., 2009). Another possible mechanism may involve tumor-infiltrating monocytes and macrophages, and NK cells can potentially trigger ADCC against Tregs that highly express CTLA-4 (Romano et al., 2015; Arce Vargas et al., 2018).

The checkpoint axis based on the programmed death 1 receptor (PD-1) and its ligand (PD-L1) is a central mediator of immunosuppression in the TME. In a cancerous

disease state, the interaction of PD-L1 expressed on tumor cells and immune cells in the TME with PD-1 expressed on T cells, B cells, and myeloid cells after activation, reduces immune cell function signaling, and thus prevents tumor immune clearance by T cells through multiple immunosuppressive pathways, including anergy and apoptosis (Seidel et al., 2018). Therefore the use of PD-1 and PD-L1 inhibitors to block the interaction between the two can prevent immune cell suppression and enhance immune responses. Along with the anti-CTLA-4 antibody ipilimumab, monoclonal antibodies targeting PD-1 and its ligand PD-L1 have been approved for clinical use for various types of cancers. PD-1 inhibitors include pembrolizumab (humanized IgG4), nivolumab (human IgG4) and cemiplimab (human IgG4). Atezolizumab (humanized IgG1), avelumab (human IgG1), and durvalumab (human IgG1) are examples of PD-L1 inhibitors.

Since CTLA-4 and PD-1/PD-L1 act at different locations and times in the generation and differentiation of effector T cells, it has been suggested that combination therapies may act in a complementary or even synergistic fashion (Das et al., 2015; Wei et al., 2019). Furthermore, preclinical and clinical studies on novel agents that are specific for other checkpoints, including the adenosine A_{2A} receptor, the lymphocyte activation gene 3, and the indoleamine-pyrrole 2,3-dioxygenase, can potentially combine with anti-CTLA-4 and anti-PD-1 antibodies to offer therapeutic benefits and possibly help to overcome treatment resistance to checkpoint inhibitor therapy (Khair et al., 2019).

The positive impact that immune checkpoint inhibitors have on cancer treatment was further acknowledged with the award of the Nobel Prize in Medicine in 2018 to Tasuku Honjo and James P. Allison, the pioneers of studies on immune checkpoint pathways and of developing the concept of immune checkpoint inhibition.

Harnessing IgE-mediated immune surveillance against cancer cells

All approved monoclonal antibodies for cancer therapy are designed with Fc regions of the IgG class, and the potential use of other antibody classes has not received significant attention. Although IgG monoclonal antibodies have shown excellent antitumor efficacy in many hematological malignancies, they have not always been successful in targeting solid tumors.

Despite the low levels of IgE antibodies in the blood, they are known to be involved in the pathology of allergic diseases. IgE antibodies also participate in immune protection from parasitic infections and can exert effector functions against parasites in tissues under Th2-biased conditions. Furthermore, interesting associations between IgE immunity and cancer have been emerging. For

example, negative correlations between individuals with allergies and their rates of malignancy occurrence have been reported (Helby et al., 2015; Kozlowska et al., 2016; Wulaningsih et al., 2016). Consistent with this finding, very low levels of serum IgE have been associated with a higher incidence of cancer (Ferastraoaru and Rosenstreich, 2018; Ferastraoaru et al., 2020a; Ferastraoaru and Rosenstreich, 2020; Ferastraoaru et al., 2020b). IgE class antibodies have several biological attributes, such as (1) high affinity for cognate IgE Fc receptors (Fc ϵ Rs) (i.e., 2–5 orders of magnitude greater than that of IgG for Fc γ Rs), (2) expression of Fc ϵ Rs on a distinct spectrum of tissue- and tumor-resident immune effector cells (e.g., monocytes, mast cells, macrophages) (Fig. 25.4), and (3) retention of IgE by Fc ϵ R-expressing immune effector cells, which can translate to a long tissue residency time and powerful immunological surveillance in tissues (Sutton et al., 2019). Therefore it has been proposed that antibodies of the IgE class directed against tumor-associated antigens should be able to activate human immunity against cancer cells (Karagiannis et al., 2017).

Several studies have demonstrated the efficacy of IgE antibodies in eliminating cancer cells but also in boosting the immune response against cancer in various models (Karagiannis et al., 2007; Platzer et al., 2015; Nigro et al., 2016; Josephs et al., 2017, 2018; Singer et al., 2019). A first-in-human phase I clinical trial of the first-in-class IgE therapeutic candidate MOv18 IgE, a chimeric antibody targeting folate receptor alpha (FR- α) and designed for the treatment of solid tumors, is ongoing (i.e., NCT02546921). The trial reported promising interim results in 2020 (https://cancerres.aacrjournals.org/content/80/16_Supplement/CT141). Based on these developments, the emerging field of allero-oncology represents an exciting and emerging research area focused on developing a better understanding of the interface between Th2 immunity, allergy responses, and malignancy (Jensen-Jarolim et al., 2017). Ongoing studies of novel IgE-based therapeutic strategies may help to elucidate whether harnessing the unique biological properties of IgE antibodies can help to unleash previously undiscovered immune mechanisms, including effective antitumor surveillance against tumors (Chauhan et al., 2020; Pellizzari et al., 2020).

Antibody-drug conjugates

Despite providing benefits for the clinical management of different tumor types, many monoclonal antibodies targeting tumor-associated antigens suffer from numerous limitations, such as the generation of intrinsic and acquired resistance (McGranahan and Swanton, 2015). To overcome these limitations, ADCs have been developed and

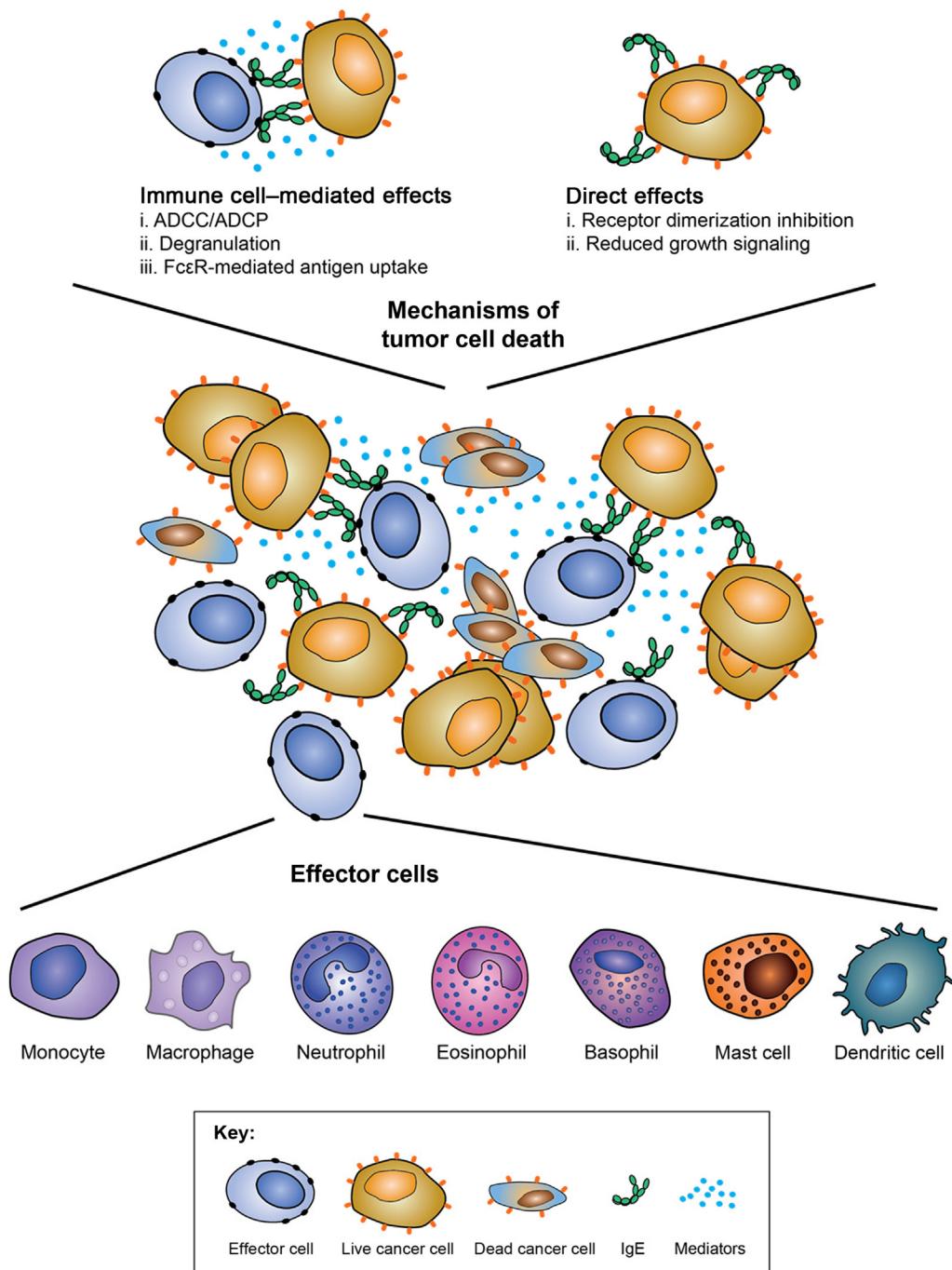


FIGURE 25.4 IgE-mediated functions against tumor cells. Fc-mediated effector functions can be potentiated upon engagement of the Fc receptors on IgE antibodies with effector cells (e.g., monocytes, macrophages, mast cells). Monocytes and macrophages can trigger an antibody-dependent cellular phagocytosis (ADCP) effect. Mast cell degranulation and antibody-dependent cellular cytotoxicity (ADCC) effects can trigger the release of toxic and proinflammatory mediators (e.g., cytokines, chemokines, histamine, proteases), which can contribute to immune cell recruitment and enhanced antitumor effects. IgE antibodies can also engage with antigen-presenting cells (APCs) to enhance antigen uptake and presentation. Direct effects of IgEs include inhibition of receptor-ligand or protein-protein interactions on the surface of cancer cells, leading to reduced downstream signaling and impaired tumor cell growth. Adapted from Sutton, B.J., Davies, A.M., Bax, H.J. and Karagiannis, S.N., 2019. IgE antibodies: from structure to function and clinical translation. *Antibodies* 8 (1); and Jensen-Jarolim, E., Bax, H.J., Bianchini, R., Capron, M., Corrigan, C., Castells, M., et al., 2017. AllergoOncology—the impact of allergy in oncology: EAACI position paper. *Allergy* 72(6): 866–887.

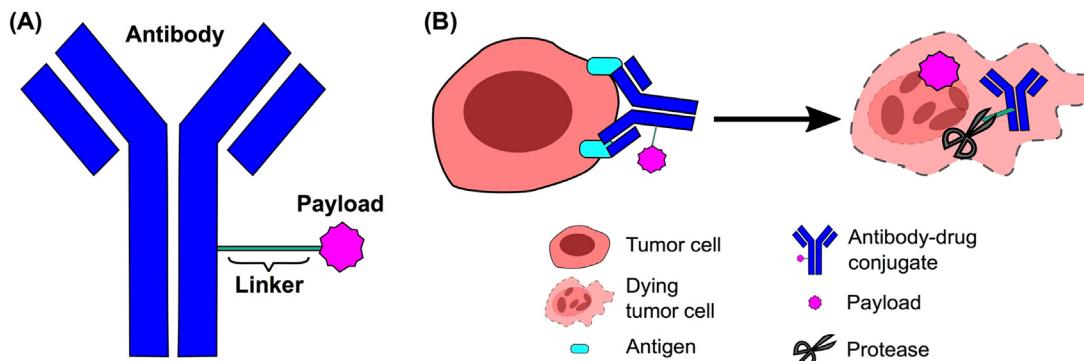


FIGURE 25.5 Antibody-drug conjugates and their mechanism of action. (A) Schematic diagram of an ADC, which consists of a monoclonal antibody recognizing a specific antigen on the surface of tumor cells, joined to a cytotoxic agent (the payload) through a chemical linker. The payload becomes cytotoxic to cells once it is cleaved from the antibody. (B) Schematic diagram of the mode of action of tumor cell killing by ADCs. Target antigen binding of the ADC on the surface of tumor cells triggers internalization of the whole complex. Once inside the cell, proteases cleave the linker, thus releasing the cytotoxic payload and leading to cell death (typically by DNA or tubulin modification or damage). *Modified from Hoffmann, R.M., Coumbe, B.G.T., Josephs, D.H., Mele, S., Ilieva, K.M., Cheung, A., et al., 2018. Antibody structure and engineering considerations for the design and function of Antibody Drug Conjugates (ADCs). Oncoimmunology 7 (3), e1395127.*

are one of the fastest growing classes of oncology therapeutics (Beck et al., 2017). ADCs are designed to combine the selectivity of monoclonal antibodies with the cytotoxic potential of chemotherapeutic agents (Peters and Brown, 2015). They comprise a tumor antigen–specific monoclonal antibody conjugated to a cytotoxic agent via a chemical linker (Fig. 25.5). Thereby, normally intolerable cytotoxic agents can be specifically delivered to cancer cells by taking advantage of the exquisite specificity and high affinity of antibodies for their target antigens, which are often overexpressed on tumor cells.

Mechanism of action of antibody-drug conjugates

The main mechanism of action of ADCs is well described, and involves initial antigen binding of the antibody component of the ADC followed by internalization of the entire complex through receptor-mediated endocytosis (Ritchie et al., 2013) (Fig. 25.5). After internalization, the cytotoxic payload is cleaved from the antibody, typically by proteolytic cleavage of the chemical linker in the lysosome. The payload is then free to kill the tumor cell via its usual mechanisms of action (e.g., typically DNA modification or tubulin inhibition) leading to cell cycle arrest and apoptosis (Kim and Kim, 2015). Almost all payloads in clinical use are small hydrophobic molecules that can cross bio-membranes once cleaved from the ADC (Jain et al., 2015). Therefore ADCs provide an opportunity for a therapy that can directly interfere with critical cellular processes. Although this represents the primary mode of action of ADCs, depending on the antibody isotype, linking chemistry and choice of payload, ADCs may also trigger immune effector functions, or

participate in off-target/on-tumor cell killing (i.e., a “bystander effect”) if the payload is released from the targeted cell or is cleaved from the antibody prematurely in the TME. In this case the cytotoxic payload can then enter tumor cells independently of antigen specificity.

Antibody-drug conjugates in preclinical development and clinical use

There are currently nine FDA- and European Medicines Agency–approved ADCs in clinical use for cancer treatment (Table 25.2). Most approved ADCs feature antibodies of the IgG1 subclass, apart from gemtuzumab ozogamicin, targeting CD33, and inotuzumab ozogamicin, targeting CD22, which are based on IgG4 antibodies. All payloads used for these nine approved products work by interfering with tubulin polymerization or DNA replication. The auristatin-based monomethyl auristatin E (MMAE) payload (e.g., used in brentuximab vedotin targeting CD30, polatuzumab vedotin targeting CD79b, and enfortumab vedotin targeting nectin-4) and the maytansinoid-based DM1 payload (e.g., used in trastuzumab emtansine targeting HER2) both target tubulin (Beck and Reichert, 2014).

The calicheamicins, the payload component of inotuzumab ozogamicin targeting CD33 and gemtuzumab ozogamicin targeting CD22, bind in the minor groove of the DNA and cause strand scission, whereas the DX-8951 and SN38 payloads of trastuzumab deruxtecan and sacituzumab govitecan, respectively, are topoisomerase inhibitors that block the relaxation of DNA strands for replication and transcription, causing single- and double-stranded DNA breaks, ultimately leading to apoptosis (Rowe and Lowenberg, 2013; Kantarjian et al., 2016) (Table 25.2).

TABLE 25.2 Approved ADCs in clinical use.

ADC	Developer	Indication	Target Antigen	Antibody Type/Mutations	Linker	Cytotoxic Payload	Payload Mechanism of Action	Approval Status
Gemtuzumab ozogamicin	Pfizer	Acute myeloid leukemia (AML)	CD33	Humanized IgG4	Acid-labile hydrazine bifunctional linker	Calicheamicin	DNA-cleaving agent	Accelerated approval in 2000 (U.S.), withdrawn in 2010, full approval in 2017
Brentuximab vedotin	Seattle Genetics	Relapsed Hodgkin's lymphoma and systemic anaplastic large cell lymphoma	CD30	Chimeric IgG1 fusion of the variable heavy and light regions of the murine anti-CD30 antibody AC10 with the constant gamma1-heavy and kappa-light regions of human immunoglobulin	Cathepsin cleavable valine-citrulline (approx. DAR = 4)	MMAE	Microtubule inhibitor	Accelerated approval by the FDA in 2011, full approval in 2015
Trastuzumab emtansine	Genentech	HER2 ⁺ breast cancer	HER2	Humanized IgG1 (from mouse)	Noncleavable thioether linker (DAR = 3–4) Hydrazone linker (DAR = 2–3)	DM1	Microtubule inhibitor	Approved by FDA in 2013
Inotuzumab ozogamycin	Pfizer	Acute lymphoblastic leukemia (ALL)	CD22	Humanized IgG4 (kappa)	Hydrazone linker (DAR = 2–3)	Calicheamicin	DNA-cleaving agent	Approved by FDA in 2017
Polatuzumab vedotin	Genentech	Diffuse B cell lymphoma (DLBCL)	CD79b	Humanized IgG1	Cleavable citrulline-valine linker (DAR = 3.5)	MMAE	Microtubule inhibitor	Accelerated approval by FDA in 2019
Enfortumab vedotin	Seattle Genetics	Metastatic urothelial cancer	Nectin-4	Fully human IgG1	Cleavable maleimide linker (DAR = 3.8)	MMAE	Microtubule inhibitor	Accelerated approval by FDA in 2019
Sacituzumab govitecan	Immunomedics (licensed to Seattle Genetics)	Refractory/relapsed triple-negative breast cancer (TNBC)	Trop-2	Humanized IgG1 murine anti-Trop-2 mAb	pH-sensitive cleavable PEG8-triazole PABC-peptide-MC linker (CL2A) (DAR = 7.6)	SN38	Topoisomerase I inhibitor	Accelerated approval by FDA in 2020
Trastuzumab deruxtecan	Daiichi Sankyo	HER2 ⁺ breast cancer	HER2	Humanized IgG1	Cleavable tetrapeptide-based linker (DAR = 7–8)	DX-8951	Topoisomerase I inhibitor	Accelerated approval by the FDA in 2019
Belantamab mafodotin-blmf	GlaxoSmithKline (GSK)	Refractory multiple myeloma (MM)	BCMA (B cell maturation antigen)	Afucosylated, humanized IgG1	Noncleavable, protease-resistant maleimidocaproyl linker (DAR = 4)	MMAF	Microtubule inhibitor	Accelerated approval by FDA in 2020

Source: Information from various sources.

Trastuzumab emtansine (Kadcyla) (containing the maytansinoid payload DM1) was approved in 2013 (Barok et al., 2011); trastuzumab dextruxtecan (Enhertu) and enfortumab vedotin (Padcev) were approved in 2019. These latest approvals provide confidence that ADCs can be designed to treat other solid tumor types (Nagayama et al., 2020).

Treatment of triple-negative breast cancer (TNBC) with ADCs has been recently exemplified with the approval of sacituzumab govitecan. For this ADC the antibody component targets the trophoblastic cell surface antigen 2 (Trop-2), and the SN-38 payload is a topoisomerase I inhibitor that leads to single- and double-strand breaks. Sacituzumab govitecan has an overall response rate in one-third of treated TNBC patients. Another ADC that is in clinical development is ladiratuzumab vedotin, which targets the zinc transporter LIV-1 and utilizes the microtubule-disrupting MMAE payload (McGuinness and Kalinsky, 2020). Similarly, loncastuximab tesirine (Lonca-T), comprising a CD19-targeting antibody and a pyrrolobenzodiazepine (PBD) dimer DNA cross-linking payload (tesirine), is expected to be approved in 2021 for the treatment of relapsed or refractory diffuse large B cell lymphoma (DLBCL).

Overall, despite the failure of many ADCs in the clinic in the early years of the technology, the field of ADCs has grown rapidly since the approval of brentuximab vedotin and trastuzumab emtansine, with approximately 600 ADCs currently in the preclinical phase and over 80 in early- and late-stage clinical trials.

Trastuzumab emtansine

Only about 30% of patients with HER2-positive tumors respond to treatments with trastuzumab, owing to primary and acquired resistance (Narayan et al., 2009). Suggested reasons for trastuzumab resistance include elevated activity of other HER family proteins and crosstalk with other downstream signaling pathways (PI3K/AKT) (Berns et al., 2007; Mohamed et al., 2013). Another proposed mechanism is the accumulation of a truncated form of the HER2 receptor not recognized by trastuzumab that forms dimers with other HER family members and activates downstream signaling pathways (Saez et al., 2006; Scaltriti et al., 2007).

To increase the efficacy of HER2-targeted therapies for breast cancer, the antibody trastuzumab was conjugated to the cytotoxic agent emtansine (DM1). Trastuzumab emtansine (T-DM1) has shown efficacy in patients with HER2-positive breast tumors who do not respond to naked trastuzumab treatment (Verma et al., 2012). The FDA approved T-DM1 in 2013 for the treatment of metastatic HER2-positive breast cancer for patients previously treated with trastuzumab whose disease progressed (Chung et al., 2013). T-DM1 has been shown to

have an overall response rate of 26% with a disease control rate of 78% and a median progression-free survival of 8.4 months (Michel et al., 2015). For patients who do not respond, the clinical, biological, and pharmacological factors that cause poor efficacy are not well understood. It has been shown that the intracellular concentration of the payload emtansine needs to reach a certain threshold in order to cause cancer cell death, and the downregulation of HER2 expression could lead to an insufficient amount of emtansine being delivered to the tumor site.

Even though trastuzumab emtansine treatment has good efficacy, most patients eventually progress (Verma et al., 2012; Hurvitz et al., 2013). It is thought that the development of resistance could be due to numerous factors, including (1) low HER2 expression (Burris et al., 2011; LoRusso et al., 2011), (2) poor internalization of the ADC complexes, (3) defective intracellular and endosomal trafficking of the complex (Barok et al., 2011), (4) defective lysosomal degradation of the conjugate (von Schwarzenberg et al., 2014), (5) masking of the HER2 epitope (e.g., by mucin-4 binding to HER2) (Price-Schiavi et al., 2002; Nagy et al., 2005; Palyi-Krek et al., 2007), (6) a high rate of recycling of the cell surface receptor (Austin et al., 2004), (7) drug efflux pumps (Kovtun et al., 2010), or (8) altered tubulin structures, the main target of the emtansine payload (Kavallaris, 2010).

As one of the first approved ADCs, trastuzumab emtansine is often used as a comparator for other ADCs in preclinical development.

Examples of target antigens for antibody-drug conjugates in preclinical and clinical development

Epidermal growth factor receptor as a potential antigen for antibody-drug conjugate therapy

The EGFR, a transmembrane tyrosine kinase receptor, is known to contribute to cancer cell survival and proliferation. EGFR expression has been associated with 18% of breast tumors, and overexpression has been observed most frequently in TNBC (Rakha et al., 2007) and has been associated with poor clinical outcome (Gazinska et al., 2013) and a worse disease-free survival in patients who received adjuvant chemotherapy. Studies have also suggested that EGFR expression may be associated with resistance to some forms of systemic therapy (Rimawi et al., 2010). In line with these findings, combined inhibition of EGFR and glycolysis has been shown to synergistically suppress the progression of TNBC (Lim et al., 2016), supporting the relevance of EGFR signaling in cancer cell metabolism (Sigismund et al., 2018).

The monoclonal antibody cetuximab (Erbitux), which is specific for EGFR, has been used in the treatment of several forms of metastatic cancer, including colorectal, lung, and

head and neck cancers (Martinelli et al., 2009). However, clinical trials of cetuximab in breast cancer have failed, owing to low response rates. Interestingly, cetuximab has been shown to reduce breast cancer stem cell growth in vitro, perhaps owing to downregulation of EGFR signaling (Tanei et al., 2016). ADCs directed toward EGFR have been developed, such as the EGFR-targeting AVID100, designed to deliver the maytansinoid DM1 to EGFR-overexpressing cancers, including breast cancers. This agent is currently progressing in a phase IIa clinical trial in TNBC patients (NCT03094169). Other EGFR-targeting ADCs have recently been developed for the treatment of pancreatic cancer (Li et al., 2019), esophageal squamous cell carcinoma (Hu et al., 2019), and other solid tumors (He et al., 2019). Other ADCs that use cetuximab as a vehicle have been developed, such as cetuximab-docetaxel (Glatt et al., 2018), which has provided evidence for the suitability of cetuximab as the antibody component for novel EGFR-targeted ADCs.

Folate receptor alpha as a potential antigen for antibody and antibody-drug conjugate therapy

Folate receptor alpha (FR- α) is a member of the folate receptor family and is a single-pass GPI-anchored membrane with high affinity for free folate. It is thought to mediate unidirectional transport of folate into the cell, most likely through endocytosis (Kelemen, 2006). FR- α not only functions as a folate transporter but may also confer signaling and growth advantages on malignant cells, thereby contributing to cancer development in different ways (Vergote et al., 2015). It is normally expressed in epithelial tissues and is usually found in the apical surface (i.e., on the lumen in different organs), so it is not directly available to interact with free circulating folate. Its expression level in normal human tissues is restricted in the apical surfaces of some organs, such as the kidney, lung, and choroid plexus. However, FR- α is overexpressed in different tumor tissues of epithelial origin, such as endometrial, ovarian, lung, and breast carcinomas (Toffoli et al., 1997; Shi et al., 2015; Boogerd et al., 2016). Among the different suggested mechanisms for the function of FR- α in tumor tissues are enhanced cellular proliferation mediated by elevated FR- α uptake or GPI-mediated transduction of proliferation signals. The low or restricted expression of FR- α in normal tissues and overexpression in some solid tumor, render FR- α an attractive target for cancer therapy.

FR- α -targeted agents that are currently in clinical trials include five monoclonal antibodies or vaccines for epithelial ovarian cancer (i.e., farletuzumab, MOv18 IgG1, radiolabeled MOv18 IgG1), TNBC (i.e., FR- α peptide vaccine), non–small lung cancers (i.e., Farletuzumab), and FR- α -positive advanced tumors (i.e., MOv18 IgE). Moreover, the ADC mirvetuximab

soravtansine, developed by ImmunoGen (IMGN853), is an anti-FR- α ADC that has demonstrated notable single-agent activity in patients with FR- α -positive platinum-resistant ovarian cancer. This agent is currently undergoing clinical trials, including a phase III trial (i.e., FORWARD I; NCT02631876) (Ab et al., 2015; Moore et al., 2017a,b).

It has been demonstrated that a proportion of TNBCs can express elevated levels of FR- α , which can be involved in cancer cell signaling pathways and may correlate with a worse clinical outcome (O’Shannessy et al., 2012; Zhang et al., 2014; Cheung et al., 2018). Therefore the clinical and biological significance of FR- α in TNBC and the largely overlapping association of FR- α expression with clinical parameters and outcomes through genomic and immunohistochemical analyses suggest that it could be a promising new therapeutic target candidate.

Choice of antibody

In identifying antibodies for ADC design, key considerations include (1) choosing an appropriate target antigen that is overexpressed by tumor cells and has low or restricted expression in normal tissues and that the antibody recognizes with high specificity and affinity, (2) ensuring that the antigen-antibody interaction results in internalization of the ADC, and (3) selecting an antibody that can still engage immune effector functions such as ADCC and ADCP when converted to an ADC.

The suitability of the target antigen for ADC design plays a vital role in ensuring ADC efficacy. Ideally, the antigen should be homogenously and selectively expressed on the surface of tumor cells with restricted or no expression by normal tissues (Damelin et al., 2015). Moreover, the antigen expression should not be downregulated by endocytosis or by the effects of repeated stimulation during treatment (Diamantis and Banerji, 2016). In exploiting the difference in protein expression between cancer cells and normal cells, off-target toxicity, one of the major side effects of chemotherapy, can in principle be limited through the use of ADC technology. However, many tumors show complex heterogeneity with different cell subpopulations harboring distinct phenotypic diversity (Fisher et al., 2013). These malignancies represent a challenge for ADC development, as subpopulations of non-antigen-expressing cells can exist within a tumor that can survive after the treatment unless there is a significant bystander effect. An alternative strategy to avoid this problem is to target ADCs to antigens that are present within the TME, including those in the neovasculature, the subendothelial extracellular matrix, and the tumor stroma, while utilizing an appropriate linker and payload to ensure a significant bystander effect. Antigens of this type have become potential targets of interest for ADC

development (Mukherjee et al., 2009; Palumbo et al., 2011).

For an ADC to generate a therapeutic effect, antibody recognition of the epitope on the antigen must result in endocytosis (Fig. 25.6). In general, antigens that internalize well and have high expression levels on tumor cells but low expression levels on healthy cells are preferred for an ADC approach, as they minimize potential unwanted on-target/off-tumor toxicity. However, the results of clinical trials indicate that toxicities due to off-tumor/on-target expression are common, and it has proved difficult to predict systemic toxicities based on preclinical studies of target expression in healthy tissue, no matter how limited it appears to be (de Goeij and Lambert, 2016).

ADCs based on just the Fab portion of an antibody, such as diabody-drug conjugates (DDCs) have also produced promising results in vitro. Examples include a CD30-targeted DDC (Kim et al., 2008) and a single-domain ADC targeting prostate cancer cells (Nessler et al., 2020). However, so far, no antibody fragment-drug conjugates have had clinical success.

Importance of the antibody Fc region

The main role of the antibody moiety of an ADC is to deliver a cytotoxic agent selectively to the target cancer cells via its specificity and high affinity for an antigen expressed on the cell surface. Therefore ADC design has focused largely on the Fab portion of the antibody that is responsible for recognizing the target antigen. However, the Fc domains of the antibodies have received relatively little attention (Hoffmann et al., 2018), considering that they have the potential to contribute to the activity of an ADC by activating complement components and immune effector cells (Fig. 25.7).

So far, IgG1 antibodies are the most commonly represented subclass for antibody design and, by extension, for ADC design. This is most likely due to their high abundance in human serum, relatively long half-life (i.e., approximately 2 weeks), ability to fix complement, and

higher affinity for Fc γ Rs expressed on effector cells compared to the IgG2 and IgG4 subclasses. Therefore an ADC may retain effector functions by engaging the immune system as well as having a direct effect on cancer cells by delivering its payload. Even though the IgG3 subclass has superior potential to fix complement and bind activating Fc γ Rs (Bruhns et al., 2009), it is the only subclass that has not yet entered clinical trials, probably owing to the low half-life in serum (approximately 7 days) compared to the other IgG subclasses (typically 14–21 days) and the vulnerability of the longer hinge region to proteolysis (Carter, 2006). Furthermore, aggregate formation during IgG3 production can be a problem and has been linked to immunogenicity (Saito et al., 2019). IgG2 and IgG4 may be used when recruitment of the immune system is not desired. This is because IgG2 and IgG4 antibodies have a low, or sometimes no, capacity to fix complement along with a lower affinity for Fc γ Rs. Interestingly, IgG1 and IgG4 antibodies have similar structural characteristics, with both containing two disulfide bridges in the hinge region, whereas IgG2 antibodies have four (Wiggins et al., 2015). The number of disulfide bridges is important in linking cytotoxic payloads to hinge region thiols after partial antibody reduction, as the number of disulfide bridges correlates both with the amount of payload that can be conjugated per antibody (i.e., the Drug-Antibody Ratio, or DAR) and with antibody stability.

These considerations may be crucial for the design of an ADC that can deliver sufficient cytotoxic payload to trigger cancer cell death without triggering off-target effects in low-target-expressing normal tissues.

Apart from choosing a specific antibody isotype, engagement of an ADC with the immune system can be modulated by engineering their Fc regions. For example, enhancement or reduction of the ability of an IgG1 antibody to trigger ADCC, ADCP, or CDC effects can be achieved by antibody glycoengineering to remove fucose or through the introduction of single, or combinations of, point mutations (Tai et al., 2014). Moreover, the

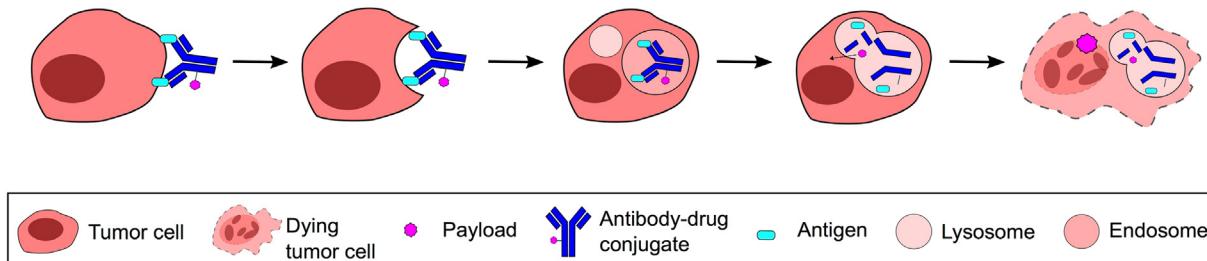


FIGURE 25.6 Antigen binding and internalization of antibody-drug conjugates (ADCs). The Fab region of the antibody is responsible for antigen recognition and binding. Once bound to the antigen on the cell surface, the antigen-ADC complex internalizes, and the ADC then moves to the lysosome, where the payload is released through enzymatic degradation of the chemical linker. The payload is then free to reach its relevant cellular target, leading to cell death. Modified from Hoffmann, R.M., Coumbe, B.G.T., Josephs, D.H., Mele, S., Ilieva, K.M., Cheung, A., et al., 2018. Antibody structure and engineering considerations for the design and function of Antibody Drug Conjugates (ADCs). *Oncimmunology* 7 (3), e1395127.

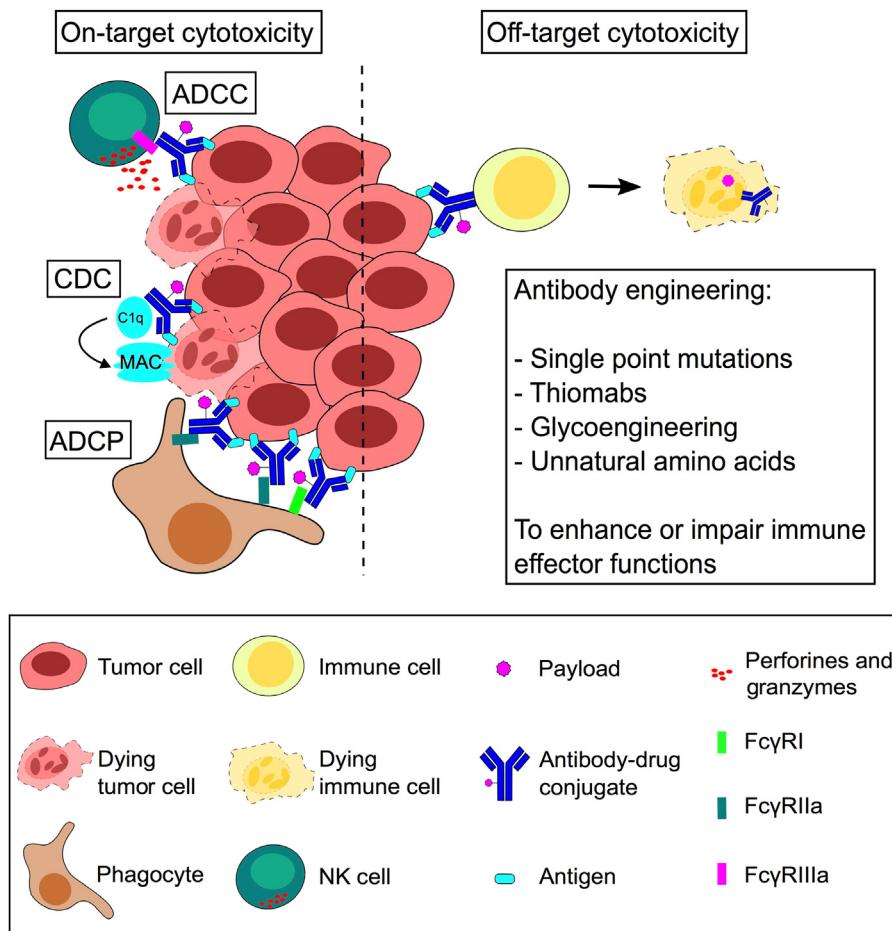


FIGURE 25.7 Impact of the Fc region on ADC development. The Fc region of an antibody can trigger immune effector functions such as the ADCC effect through binding to Fc γ receptors. However, if an ADC is internalized into nonmalignant cells, it can cause off-target toxicity. Antibody engineering can enhance or reduce immune effector functions through, for example, single point mutations, glycoengineering, Thiomab technology or the incorporation of unnatural amino acids. *ADCC*, antibody-dependent cellular cytotoxicity; *ADCP*, antibody-dependent cellular phagocytosis; *CDC*, complement-dependent cytotoxicity; *MAC*, membrane attack complex; *NK*, natural killer. Modified from Hoffmann, R.M., Coumbe, B.G.T., Josephs, D.H., Mele, S., Ilieva, K.M., Cheung, A., et al., 2018. Antibody structure and engineering considerations for the design and function of Antibody Drug Conjugates (ADCs). *Oncioimmunology* 7 (3), e1395127.

introduction of point mutations can provide the benefit of an increased serum half-life. For example, the ADC MEDI-524-YTE was designed to contain three single point mutations that result in a fourfold increase in serum half-life in cynomolgus monkeys compared with the wild type (Saunders, 2019). J6M0-mc-MMAF is another example of an engineered ADC in phase I clinical trials for the treatment of multiple myeloma (Dall'Acqua et al., 2006). It consists of an anti-BCMA (B cell maturation antigen) afucosylated IgG1 antibody that is designed to have enhanced effector functions.

Choice of linker

The linker component of an ADC is designed to connect the cytotoxic payload to the structure of the antibody. One of the key functions of the linker is to maintain the stability of the ADC complex in the blood circulation while allowing toxin release upon ADC internalization by target cells.

The first generations of ADCs relied on linking via an antibody's lysine residues, resulting in heterogeneous

ADCs with poorly defined drug-antibody ratios (DARs) (Damelin et al., 2015) (Fig. 25.8).

A conjugation strategy was subsequently developed that provided a better defined DAR based on the use of maleimide-containing linkers (Schumacher et al., 2014). This technology, known as stochastic conjugation, involves reaction of the electrophilic maleimide moiety of the linker with nucleophilic free thiols on the antibody revealed by partial reduction of the disulfide bridges present in the hinge region of the antibody, thus achieving DAR values of up to 8 for ADCs based on IgG antibodies. Currently, there is a trend toward the development of homogenous ADCs that can be obtained through site-specific linking technologies. These include the integration of additional cysteines or nonnatural amino acids with chemically reactive groups through antibody engineering technologies (Fig. 25.8).

Linker payloads should be conjugated to antibodies at sites where they do not interfere with the Fab or Fc regions, as this may interfere with antigen recognition or immune system engagement, respectively. Also, as most payloads are relatively hydrophobic, they can reduce the

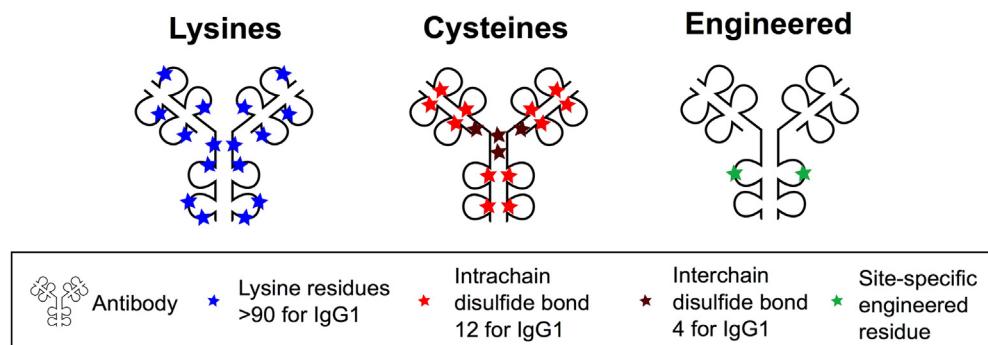


FIGURE 25.8 Different types of antibody-drug conjugate (ADC) linking technologies. Noncleavable linkers rely on the complete degradation of the antibody after internalization of the ADC, whereas most cleavable linkers are cleaved by different mechanisms depending on the linker (i.e., proteases, reduction) and some cleavable linkers do not depend on ADC internalization for payload release and can result in higher off-target cytotoxicity. The hydrophobicity of linkers can play a vital role in the biodistribution of an ADC. Linkers can be attached nonselectively via lysines or the hinge thiols of cysteines, or antibody engineering can be performed for site-specific conjugation.

efficiency of conjugation and cause aggregation of the ADC formed, thus leading to a loss of biological activity. Therefore linker design is focused on decreasing hydrophobicity for manufacturing and stability purposes, but can also help improve pharmacokinetics, solubility and potentially offer a larger therapeutic window (Lyon et al., 2015).

Choice of cytotoxic payload

The basic criteria for selecting the ADC payload are sub-nanomolar to nanomolar cytotoxicity, amenability to conjugation, solubility, and stability (McCombs and Owen, 2015). The toxicity of a payload is a very important factor, as there is often only a limited number of antigens on the surface of tumor cells (approximately 5×10^3 – 10^6 antigens per cell), and the average DAR of most ADCs that reach the clinic is 3.5–4.0. This means that the amount of cytotoxic payload delivered by a typical ADC to a tumor cell may be too low to invoke cell death (Beck et al., 2017). The amenability to conjugation also plays a role in payload selection, as it is crucial that a payload retains its cytotoxic potency if chemical modification is required for attachment to a linker. The payload also needs to have acceptable aqueous solubility (i.e., a manageable hydrophobicity). As many cytotoxic agents are hydrophobic, they tend to induce antibody aggregation, which must be avoided to reduce the chance of immunogenicity and ensure ADC stability. Also, cleavage of the payload from the ADC in the blood must be avoided to prevent off-target cytotoxicity (Fig. 25.9). Therefore use of the lowest-potency payload that can provide a therapeutic effect should be considered in case of premature release. Establishing the most appropriate level of cytotoxicity for the payload is a significant challenge, given that only a small percentage of an

administered ADC will reach the tumor site (Teicher and Chari, 2011).

Payloads used as components in ADCs in clinical trials are based on a limited number of families of cytotoxic agents that target either DNA or tubulin (Pysz et al., 2019). For example, the auristatins, maytansinoids, and tubulysins are antimitotic agents, blocking the polymerization of tubulin. As the microtubules are intimately involved in cell division, these agents are particularly cytotoxic toward highly proliferating cells (Beck et al., 2017). DNA-modifying agents in clinical use as ADC payloads include the duocarmycins (DNA adenine-monoalkylating agents), calicheamicins (DNA-cleaving agents), PBD dimers (DNA cross-linking agents), DNA intercalators (e.g., doxurobicin), and topoisomerase inhibitors (e.g., DX-8951 and SN-38). These agents are not only cytotoxic toward proliferating cells but also capable of killing resting cells. Therefore DNA-interactive agents can provide an advantage over tubulin inhibitors when treating tumors containing dormant cells with stem cell-like properties that may give rise to metastases in the future. However, they are also associated with a higher risk of off-target cytotoxicity against normal cells compared to the tubulin inhibitors. At present, the auristatins represent the largest family of ADC payloads in clinical use, the main examples being MMAE and monomethyl auristatin F (MMAF), which are analogs of the naturally occurring antimitotic agent dolastatin, which was discovered in the sea hare *Dolabella auricularia* but was too toxic for use as a standalone anticancer agent (Senter and Sievers, 2012).

The PBD dimers are a relatively new family of DNA-interactive ADC payloads based on naturally occurring antitumor antibiotics that bind covalently in the minor groove of the DNA (Mantaj et al., 2017). They can form interstrand and intrastrand cross-links and monoalkylated adducts within the DNA helix, which explains their

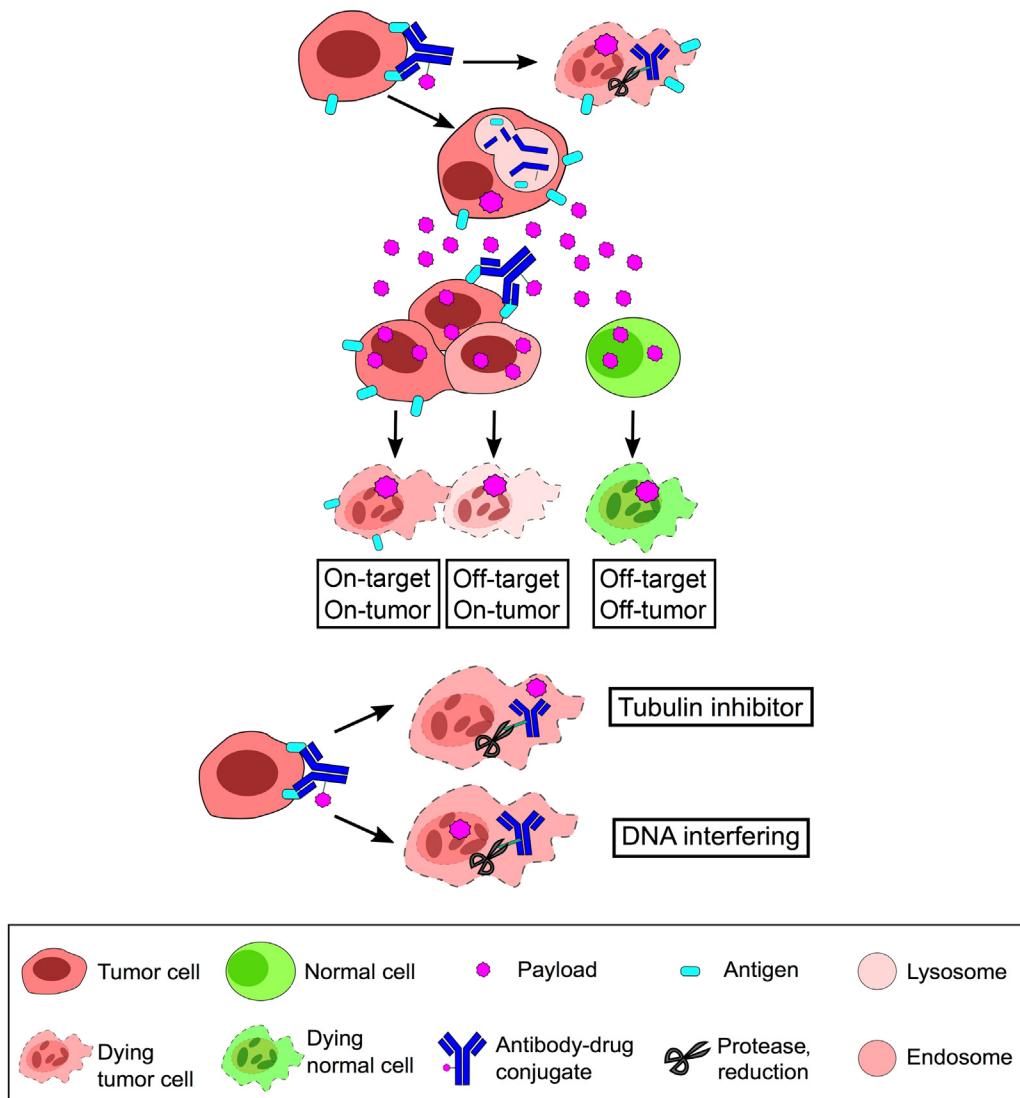


FIGURE 25.9 On- and off-target effects of ADC payloads. The payload is mainly responsible for ADC toxicity. It is usually a small, relatively hydrophobic molecule capable of crossing cell membranes and causing cell death by targeting the tubulin or DNA. Once cleaved from the antibody, payloads can enter other (tumor) cells, resulting in a further anticancer effect (i.e., a bystander effect) as well as off-target cytotoxicity from entering normal cells. Modified from Hoffmann, R.M., Coumbe, B.G.T., Josephs, D.H., Mele, S., Ilieva, K.M., Cheung, A., et al., 2018. Antibody structure and engineering considerations for the design and function of Antibody Drug Conjugates (ADCs). *Oncioimmunology* 7 (3), e1395127.

extreme cytotoxicity. Early ADCs based on PBD dimer payloads were unsuccessful in the clinic because of unacceptable system toxicities. For example, the phase III CASCADE clinical trial of vadastuximab talirine for acute myeloid leukemia (AML) was discontinued owing to safety concerns related to a higher death rate (Winer and Stone, 2019). Furthermore, SGN-CD70A for the treatment of relapsed or refractory non-Hodgkin's lymphomas was associated with thrombocytopenia in patients, and ADCT-401 (MEDI3726) for the treatment of prostate cancer was suspended after a phase I clinical trial. However, later-generation ADCs based on PBD dimers are proving to be more successful. For example,

loncastuximab tesirine (Lonca-T), which is based on a CD19-targeting antibody and a PBD dimer is expected to be approved in 2021 for the treatment of relapsed or refractory DLBCL.

It is worth noting that the PBD dimers have led to the development of two other families of DNA-interactive payloads (Jackson et al., 2018). The indolinobenzodiazepine dimers (IGNs) work by forming DNA monoalkylated adducts rather than cross-links. IMGN632 is a CD123-targeted ADC based on the IGN payload that is currently near the approval stage for the treatment of hematological malignancies blastic plasmacytoid dendritic cell neoplasm, AML, and acute lymphocytic leukemia. The other family

is the pyridinobenzodiazepines (PDDs), which also work by forming monoalkylated adducts in the minor groove of DNA. However, unlike other DNA-interactive payloads, the PDDs are designed to be sequence-selective and to interact at specific locations on the DNA helix, including transcription factor binding sites. This allows PDD-based ADCs to be targeted toward tumors that are known to be dependent on particular transcription factors, thus introducing a second level of overall selectivity and introducing the possibility of patient selection. Also, in preclinical models, PDD-based ADCs have been shown to have a lower degree of systemic toxicity compared to those based on PBD dimer or IGN payloads and so should have a wider therapeutic window in the clinic (Hoffmann et al., 2020).

Challenges in antibody-drug conjugate design, evaluation and translation

ADC design and implementation have presented numerous challenges related to the choice of antigen, conjugation strategy, and payload (Donaghy, 2016). As was described above, many ADCs have reached the clinic but have not progressed to the approval stage, mainly owing to unacceptable systemic toxicities that have not allowed clinically effective doses to be reached (Beck et al., 2017).

The toxicities are mainly attributed to the payloads, although in some cases they are antibody related. Choosing the right target antigen and antibody combination can play an equally important role in ADC development. For example, all four ADCs targeting CD70 have been unsuccessful despite being based on different payload families, including MMAF, DM1, PBD dimers, and duocarmycin. This suggests that some antigens are not suitable as ADC targets.

Conjugation strategies also affect the stability of the ADC, and early release of the payload may occur before the agent reaches the tumor, thus allowing the payload to injure healthy tissue. This has led to a trend toward the design of site-specific ADCs with engineered conjugation sites rather than stochastically conjugated ADCs. For example, although ultimately unsuccessful in the clinic, vadastuximab talirine was the first ADC synthesized with site-specific conjugation through two engineered cysteines, ensuring a uniform DAR of 2.0 (Zhao et al., 2020). Similar site-specific technologies include transglutaminase-mediated conjugation (Strop et al., 2016).

Intrinsic and acquired resistance mechanisms are one of the pitfalls of immunotherapy, and these limitations also apply to ADCs. Mechanisms include resistance to certain payloads; for example, tubulin-binding agents can

alter the levels of proapoptotic proteins. Resistance can also be induced by the increased expression of ATP-binding cassette efflux pumps such as the multidrug resistance protein 1 (MDR1), which can transport the payload out of the cell (Beck et al., 2017). Other resistance mechanisms include downregulation of the target antigen expression and/or antigen-ADC internalization, or enhanced recycling of the ADC complex back to the outside of the cell (Shefet-Carasso and Benhar, 2015).

Therefore it is important to design ADCs carefully, taking each of the three structural components into account. Addressing the biology of the specific tumor type is also important, including an understanding of the antigen density on the cell surface and the sensitivity of the tumor cell type to the chosen payload.

Conclusion

Monoclonal antibodies have contributed significantly to improvements in the therapeutic armamentarium for cancer therapy, based on their high specificity toward a target antigen mediated by the Fab domain and Fc-mediated immune-activating functions. The combined attributes of an antibody, namely, its structural characteristics and isotype, target antigen, epitope specificity, and affinity, together determine the agent's pharmacokinetic and pharmacodynamic characteristics and consequently the functional, efficacy, and toxicity profiles. The functional and toxicity profiles could also be influenced by the anatomical location of the target cells and conditions in the TME. Treatment resistance due to compensatory pathway activation is also known to limit the effectiveness of monoclonal antibody therapies.

Future studies may provide a clearer understanding of the multifaceted mechanistic profile of an antibody. In particular, future studies on the significance of the Fc-mediated effector functions such as ADCC, ADCP, and CDC should prove valuable, as will further studies on how antibody structure and isotype may influence efficacy. Fc-engineered antibodies and those with Fc domains of isotypes different from the conventionally used IgGs may be required for antibodies to function effectively in certain immune-privileged and Th2-biased TMEs. It is noteworthy that checkpoint inhibitor antibodies have shown significant efficacy in some cancer types. Treatment combinations with these antibodies with targeted drugs and chemotherapeutic agents are already benefiting patients with cancers that are challenging to treat, such as TNBC. However, the toxicity that is observed with checkpoint blockade treatments and their combinations will likely continue to present a significant challenge in the clinic.

Similar structure-function considerations will be important for the design of antibodies to serve as vehicles

to specifically deliver toxic cytotoxic payloads to tumors through ADC technologies. Despite some clinical benefits, the toxicities that are observed in the clinic with current generations of ADCs are comparable with those of many standalone chemotherapeutic agents. Reported dose-limiting toxicities are often associated with the mechanism of action of the cytotoxic payload (Coats et al., 2019). Thus many ADCs have been discontinued as a result of a poor therapeutic index. While the promise of a more targeted chemotherapy with low toxicity has yet to reach its full potential with ADCs, gradual improvements in ADC design combined with a wealth of clinical data are helping researchers to create new ADCs with improved therapeutic indices. Through many years of research, it has now been established that the clinical efficacy of ADCs may depend on fine-tuning several parameters, including target antigen selection, antibody structure, epitope specificity, and affinity, alongside cytotoxic payload type and conjugation strategy. These combined properties may be further optimized with the selection of appropriate patient cohorts.

For monoclonal antibodies and ADCs it is likely that identification of biomarkers of both treatment response and toxicity will be important to achieve patient selection for specific treatments and combinations in the future.

Acknowledgments

The research was supported by the National Institute for Health Research (NIHR) Biomedical Research Centre (BRC) based at Guy's and St Thomas' NHS Foundation Trust and King's College London (IS-BRC-1215-20006). The authors acknowledge support by Breast Cancer Now (147; KCL-BCN-Q3); the Cancer Research UK King's Health Partners Centre at King's College London (C604/A25135); The Guy's and St Thomas's Foundation Trust Charity (Melanoma Special Fund, 573); CRUK/NIHR in England/DoH for Scotland, Wales and Northern Ireland Experimental Cancer Medicine Centre (C10355/A15587); Cancer Research UK (C30122/A11527; C30122/A15774). The authors are solely responsible for study design, data collection, analysis, decision to publish, and preparation of the manuscript. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, or the Department of Health.

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Chapter 26

Orphan drugs: why is translation so successful?

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Abstract

Orphan drugs are defined as drugs that are used to treat rare diseases. Rare diseases are defined as those that affect fewer than 1 in 2000 people in Europe or up to 1 in 1000 in other regions. Six to eight percent of the European population, or about one in 17 citizens, suffer from rare diseases. Legislative actions to address this problem, the “Orphan Drug Act” in the United States and the “EU Regulation on Orphan Medicinal Products (Regulation EC 141/2000),” have facilitated the development of orphan drugs by providing both advisory and financial support. At present, there are 2200 drugs designated as orphan drugs that have those benefits assigned to their development, and 160 of them have been authorized for marketing. The numbers indicate that the translational success of about 7% is higher than what we expect in typical drug development scenarios. The most important feature of rare disease is their genetic, mostly monogenic, origin. This guarantees high translational success because the pathways, biomarkers, and clinical trial designs can be streamlined to this feature.

Keywords: Orphan drugs; high translational success; monogenic diseases; clear pathways

Introduction

Orphan drugs are defined as drugs that are used to treat rare diseases. Rare diseases are defined to affect fewer than 1 in 2000 people in Europe and up to 1 in 1000 in other regions. Although patients with such diseases are rare, there are at least 6000 different rare diseases, and together they affect 30 million Europeans (Eurordis, 2020). In other words, although the diseases are rare, patients affected by them are not rare.

These facts have two major implications for drug development:

1. Because most of these rare diseases have distinct pathogeneses, a drug has to be developed for very few

cases of one particular rare disease, and remuneration of costs of development would lead to astronomically high prices that insurers might not pay, and considerable losses to the pharmaceutical company seem to be inevitable.

2. Regular clinical development will fail because companies will not find study cohorts of several hundred or thousand patients for each disease that would be typical study populations in phases II and III of normal drug development.

Furthermore, some drugs aiming at diseases in underdeveloped countries or drugs that may be repurposed to treat rare diseases may be hit by such financial shortcomings.

These obstacles have long kept companies from undertaking major activities in this area, and affected patients, often children, were left without hope of effective treatment. The diagnosis of a rare disease was frequently equivalent to a death sentence or tragic life forecast with the natural course of the disease as the most likely outcome.

The US Food and Drug Administration in 1983 and the European Medicines Agency in 2000 took legislative actions to address this problem: the Orphan Drug Act in the United States and the EU Regulation on Orphan Medicinal Products (Regulation EC no. 141/2000) in the European Union. These laws facilitate the development of orphan drugs by providing both advisory and financial support, for example, the reduction of fees, the counseling on studies, the funding of programs under the auspices of the HORIZON2020 call in the EU or the Orphan Products Grant program in the United States, and the provision of marketing exclusivity for 10 or more years. Fig. 26.1 depicts the numbers of orphan drug designation over the years in relation to such legislative acts.

Such programs have been very successful in turning the scene into a highly proliferative and even economically

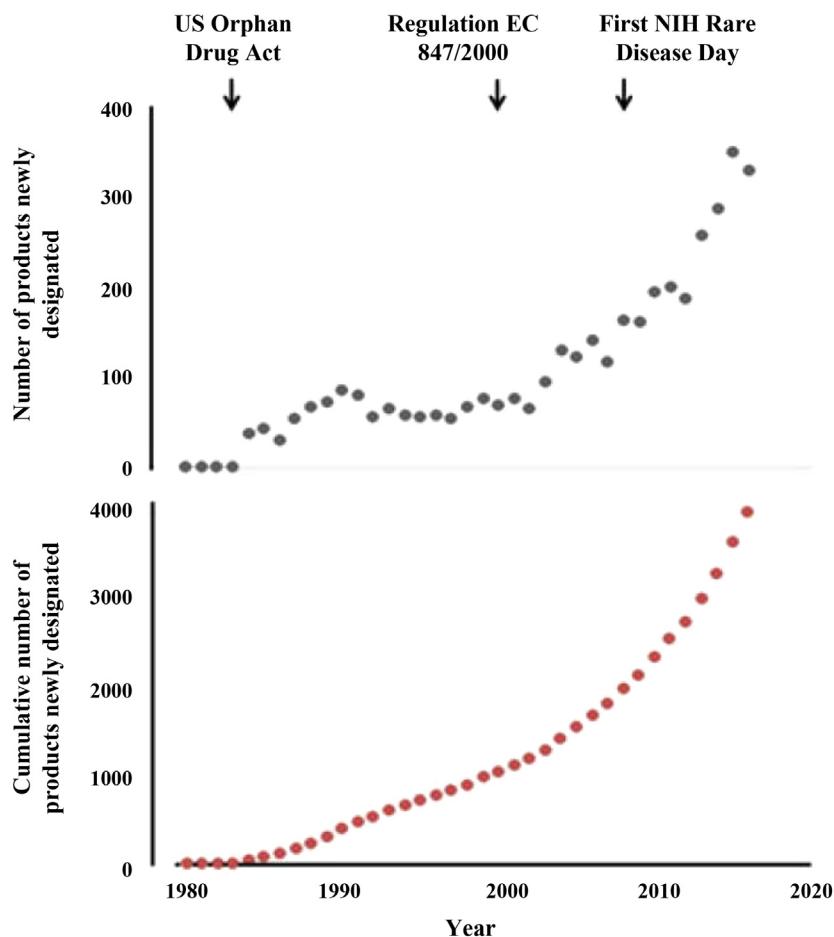


FIGURE 26.1 Numbers of designated orphan drugs in relation to legal and publicity actions in the area. From Cremers, S., Aronson, J.K., 2017. Drugs for rare disorders. *Br. J. Clin. Pharmacol.* 83, 1607–1613, with permission by John Wiley and Sons.

profitable area of drug development. At present, there are 2200 drugs designated as orphan drugs that have the statutory benefits assigned to their development, and 160 of them have been authorized for marketing (EMA: Factsheet, 2020). From 2010 to 2019, 100–200 designations were filed per year, and around 10 market authorizations were issued per year in the same period. This represents 30%–40% of all market authorizations in these years. As can be seen in Fig. 26.2, most designations were in the oncology and musculoskeletal and nervous areas.

Fig. 26.3 illustrates a shift in the distribution of therapeutic areas if marketing authorizations are considered, in that skeleto muscular and nervous applications were less prominent and alimentary tract and metabolism more prominent compared to the designations.

Special characteristics of translational processes for orphan drugs

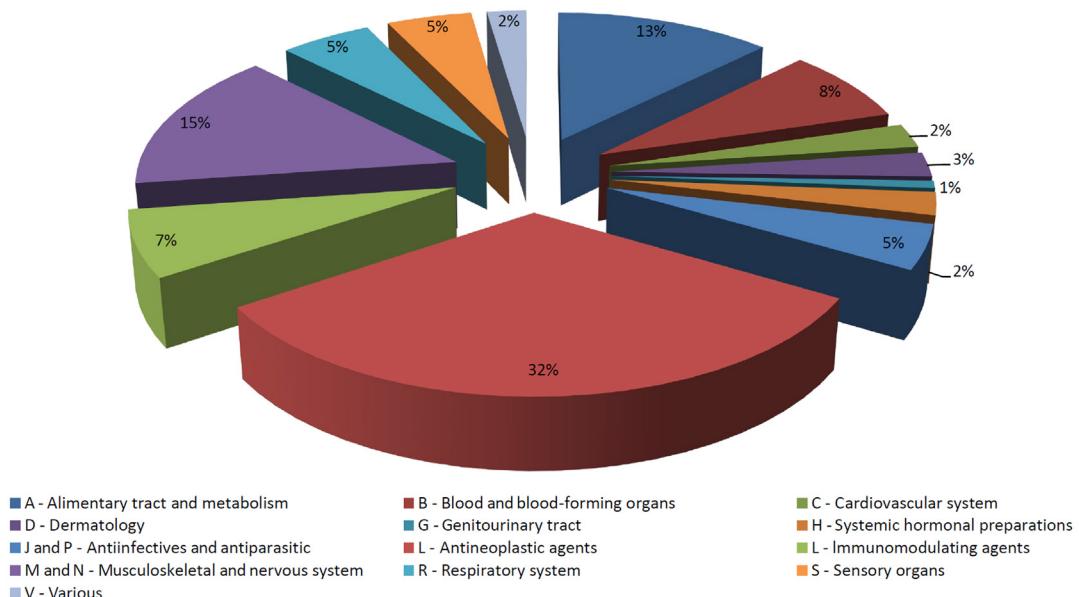
The numbers given in the preceding section indicate a translational success of about 8% (out of 2397

designations, 190 orphan drugs were approved), which is higher than what we expect in typical drug development scenarios. In a 2015 report, only 1.4% of drugs assigned to clinical development made all the way to the market (Waring et al., 2015). Thus it appears that the translational risk is not higher than for drugs treating nonrare diseases.

The most important feature of rare disease is their genetic, mostly monogenic origin. For 80% of rare diseases, genetic abnormalities could be identified as the exclusive pathogenetic origin, 60% being of autosomal-dominant, autosomal-recessive, or X-linked inheritance (Cremers and Aronson, 2017). Obviously, these are inborn errors of genetics, and 75% of affected patients are children (Eurordis, 2020). The reason for the preponderance of genetic diseases in the realm of rare diseases is the fact that such gene mutations are sporadic in many cases or would otherwise be extinct. The large number of rare genetic defects discussed here reflects a selection of those mutations that are not lethal in utero but result in defects early in life after birth. Under natural conditions many of them would not be inheritable by offspring, as

Distribution of opinions on orphan designation by therapeutic area

Period 2000–2020 / Total opinions 2397



Classified as public by the European Medicines Agency

FIGURE 26.2 Distribution of orphan drug designations across therapeutic areas ([EMA. Orphan Medicines Figures 2000–2020, 2020](#)).

the carriers would not reach reproductive age. Thus most of them are sporadic and therefore rare.

The other origins of rare diseases are related to infections, allergies, the environment, or degenerative or proliferative diseases.

As [Table 26.1](#) shows, successful treatments have been developed for diseases that are linked to a single gene defect. In many cases a defective receptor may be blocked, for example, by an antibody, or a missing enzyme or other protein can be substituted for the successful treatment of the monogenic disease.

If we apply the formal assessment of the translatability score (see Chapter 11: Target profiling in terms of translatability and early translation planning) to this typical situation (inherited single gene defect with clinical penetration), we have a large contribution to a high score from the following items from Table 11.2:

- In vitro including animal genetics
- In vivo including animal genetics (e.g., knock-out, overexpression models)
- Human evidence
- Genetics
- Biomarker grading
- Biomarker development

- Concept for proof-of-mechanism, proof-of-principle, and proof-of-concept testing
- Biomarker strategy
- Surrogate/endpoint strategy
- Personalized medicine aspects
- Disease subclassification, responder concentration

These items represent 8 of the 13 items that need to be assessed to create the translatability score. Four of them are related to genetics that are obviously strong predictive parameters in the development of drugs for genetic diseases. Another four are related to biomarkers or surrogate endpoints: If the new drug effectively knocks a gene product in or out as desired (knock-in or knock-out defined by the physiological consequence of gene action), this can be easily measured. Either the gene product itself becomes detectable if it is missing or its action is reduced if an activating mutation needs to be silenced. Such biomarkers may even bridge the gaps between proof of mechanism (PoM: proximal effect of intervention, gene product measurable), proof of principle (PoP: effect of pathophysiological, disease-modifying relevance), and proof of concept (PoC: disease actually improves as the missing gene product has unequivocally been shown to be the only pathophysiological reason, for example). Of

190 Initial orphan marketing authorizations and 34 extension of indication granted to date

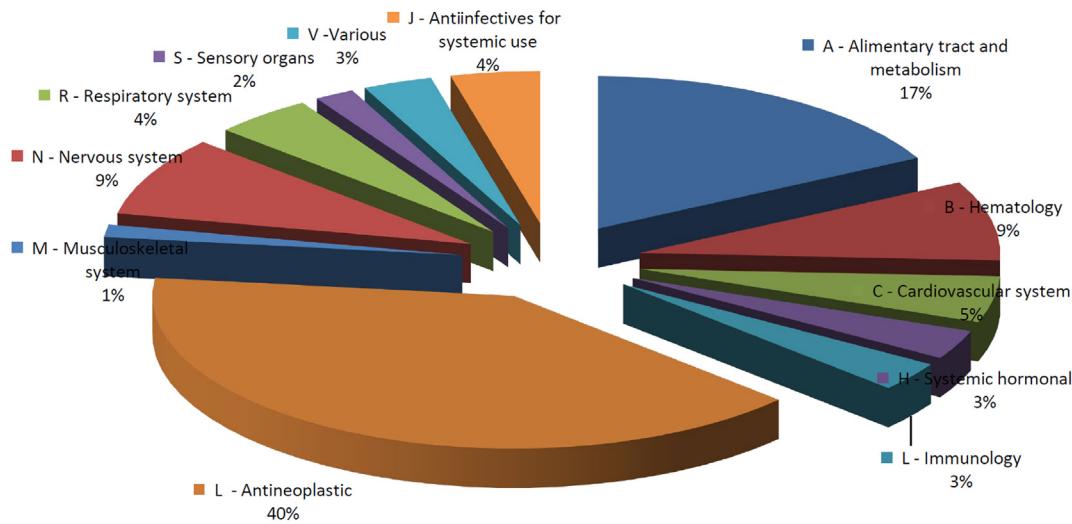


Chart includes 224 products:

117 active initial authorizations; 21 active extensions of indication

23 withdrawals from the register of orphan medicinal products (including 9 extensions of indication)

7 withdrawals from register medicinal products human use/ orphan status expired

1 revoked from register medicinal products human use/ orphan status expired

51 removals of initial MAA from register after expiration of the market exclusivity period

4 removals of extensions of indication

Number of conditions: 133

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FIGURE 26.3 Distribution of orphan drug marketing authorizations across therapeutic areas (EMA. Orphan Medicines Figures 2000–2020, 2020).

TABLE 26.1 Examples of drug treatments for rare diseases.

Drug	Disorder	Gene
Activin A antibody	Fibrodysplasia ossificans progressiva	Activin A receptor type 1 (<i>ACVR1</i>)
Alglucosidase alfa, recombinant human	Pompe's disease	Glucosidase alfa, acid (<i>GAA</i>)
Ivacaftor + iumacaftor	Some forms of cystic fibrosis	Cystic fibrosis transmembrane conductance regulator (<i>CFTR</i>)
Mavoglurant (mGluR5 antagonist)	Fragile X syndrome	Fragile X mental retardation 1 (<i>FMR1</i>)
Nonretinoid retinol binding protein 4 antagonists	Ophthalmic Stargardt's disease	ATP binding cassette subfamily A member 4 (<i>ABCA4</i>)
Quinidine	Therapy-resistant potassium sodium-activated channel subfamily T member (KCNT1)-positive epilepsies	<i>KCNT1</i>

Source: Modified from Cremers, S., Aronson, J.K., 2017. Drugs for rare disorders. Br. J. Clin. Pharmacol. 83, 1607–1613, with permission by John Wiley and Sons.

course, clinical biomarkers on top of PoM markers need to verify the clinical improvement. This is normally trivial, as the rare disease is typically characterized by

clinical or chemical biomarkers that are impressively changed by grave diseases, often leading to death. Thus muscle strength or eye vision as a clinical biomarker for

PoC will normally be readily accessible and very predictive for the course of the disease and the effects of a novel treatment for it.

The genetic background of a disease that has to be established in rare diseases is the ultimate prerequisite for personalization, disease subclassification, and responder concentration. A drug for a rare disease will thus be indicated (in the first place) only for a population of highly concentrated responders and ultimately represents what is typically called personalized medicine. The translatability-scoring instrument will yield high likelihoods of successful translation from early stages of development already.

Even if not formalized by this scoring approach, these features of developing drugs against monogenic diseases will lead to high success rates, as can be seen from **Figs. 26.2 and 26.3**. The increasing numbers of both designated and approved orphan drugs seem to reflect the acceleration of translational success that even appears to overcome the problem of small patient numbers available for clinical trials. The combination of disease severity and the often life-saving efficacy of orphan drugs enables researchers to detect large clinical effects even in cohorts of only five or ten patients.

In recent years, companies are approaching a “niche buster” concept ([Kumar Kakkar and Dahiya, 2014](#)), in contrast to the blockbuster approach of former years. Single orphan drugs are limited to a turnover of 50 million euros per year in Europe or have to go through the normal approval process, as they otherwise lose the orphan status; thus they will not become “busters” by themselves. However, the sum of several products that may be developed in parallel utilizing the same platforms and skills may then become a niche buster in total.

Western societies are willing to pay high prices for orphan drugs, as can be seen from the recent US approval of Zolgensma, the most expensive drug ever (US \$2.1 million), to treat spinal muscular atrophy, though the discussion about such exorbitant prices is still ongoing ([Dyer, 2020](#)).

Orphan drugs now represent an important share of market approvals each year, thousands of previously untreatable patients benefit from the translational success in the field, and supportive measures by governments and marketing authorities have been very helpful in this regard.

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Chapter 27

Translational science biostatistics

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Abstract

Drug development can be described as a series of experiments that span drug discovery, preclinical research, and clinical research. Statistics can be used to describe and explore data and thus generate hypotheses. However, the ultimate goal of many statistical analyses is to support or refute a hypothesis about the effect of a treatment. We first introduce the fundamentals of statistical modeling and some of the most common statistical models, followed by principles of experimental design and introduction of some designs that are particularly relevant to translational medicine. Multiplicity issues, biological modeling, and statistical models are discussed as well.

Keywords: Statistical modeling; biological modeling; experiment design; multiplicity; statistical inference

Statistical problems in translational science

Drug development can be described as a series of experiments that span drug discovery, preclinical research, and clinical research. During these experiments, knowledge about the drug and the disease under study is accumulated and structured. Each experiment has a component of learning and one of confirming, and later experiments are based on what has been inferred from previous ones. More specifically, the decisions as to whether to stop or continue drug development or which experiment to perform next, as well as many details of each individual experiment, are based to a large extent on the information that has been gathered up to that point. However, traditionally, the step from one experiment to the next is done rather informally.

Statistics can be used to describe and explore data and thus generate hypotheses. However, the ultimate goal of many statistical analyses is to support or refute a hypothesis about the effect of a treatment. Traditionally, this is often

done in the context of statistical tests of hypotheses. Within one experiment, statistical tests of hypothesis are set up to control the type 1 error, that is, to keep the probability of rejecting a true null hypothesis below a predefined threshold, as is discussed in more detail in Section “Design and Interpretation of an Experiment.” Statistical tests of hypotheses are widely used in clinical research, as evidenced by the ubiquity of *p*-values in the medical literature, but they are not always optimal for an individual step in a development process. Confirmation of a hypothesis of the efficacy and, as much as possible, of the safety of a potential medicine is essential in phase III of clinical development, in which the public and regulators need to be convinced. In preclinical and early clinical phases, statistical hypothesis testing has a less important role. In these situations, support for the choice between different development strategies is needed, with the option of stopping development.

This point of view can be carried even further if we do not limit ourselves to looking at various development strategies for one compound but ask questions like the following: “Given our current knowledge, which of a number of compounds with similar indications or mechanisms of action should we concentrate on for further development?” A fully decision-theoretic approach would have to specify a cost-benefit relation, uncertainty about potential treatment effects, available resources, prior knowledge about treatment effects, treatment populations, side effects, and the like and then combine these elements into a statistical model. This may be overambitious for an entire drug development program, but elements from this approach can be implemented into parts of the program, as is discussed further later in this chapter.

In summary, we should keep in mind that for preclinical and early clinical development, approaches to statistical data analysis must go beyond classical hypothesis testing. In particular, this includes models that can handle the limited information generated by early clinical phase trials.

Statistical inference and description of relationships are also important in other fields, such as pharmacokinetics and kinetic-dynamic relationships, as well as for dose-response modeling. For biomarkers, inference and confirmation of the relationship with clinical endpoints is important. In all these areas a mathematical model that describes the relationship between the scientific entities is needed. Such models might be mechanistic (e.g., describing the relationship in terms of a biologically motivated set of differential equations) or empirical (e.g., a smoothing spline), but they will almost invariably have a stochastic component. Statistics thus has a role in estimating the parameters of the model, quantifying the uncertainty of this estimation, and selecting the most adequate model. Again, in traditional applications, selection and estimation are performed within one experiment, but statistics can also provide support that spans a series of experiments. Furthermore, in safety assessments we need to demonstrate the absence of an effect of a certain size. The specification of an upper tolerance limit for an effect indicating harm is a paramount prerequisite of any reasonable statistical treatment of drug safety, but in general this specification cannot be obtained by statistical means.

In Section “Statistical Models and Statistical Inference,” we introduce the fundamentals of statistical modeling and some of the most common statistical models. We then turn to the statistical principles of the design of an experiment and introduce some designs that are particularly relevant to translational medicine in Section “Design and Interpretation of an Experiment.” Section “Multiplicity” is devoted to multiplicity, because it is ubiquitous in translational science and because an appropriate understanding of related problems and potential solutions is important. This is followed by Section “Biomarkers,” discussing the concepts of sensitivity and specificity for diagnostic tests. Section “Biological Modeling” essentially concludes the chapter; however, for readers interested in statistical methodology, we have added a short overview of the statistical models used in translational medicine in Section “Statistical Models.” This section uses more technical terminology than the rest of the chapter and contains further reading that requires some knowledge of statistical sciences.

Methods are illustrated with examples from pharmacokinetics and pharmacodynamics. Our goal is to convey the basic ideas and to give references for readers who want or need to understand the details.

Statistical models and statistical inference

The science of statistics is concerned with the analysis of data that are subject to random variation, that is, measurement errors or any other sources of uncertainty that prevent exact reproducibility of an experimental result. An

important basic distinction is made between exploratory statistics, which aims at simply describing the data without any explicit attempt to draw conclusions, and inferential statistics. The distinction can be blurred; for example, linear regression is sometimes viewed as a tool to merely summarize the relationship between two measurements descriptively, but sometimes the objectives may comprise testing for statistical significance of the regression parameters. Means, variances, standard deviations, standard errors, and other measures of the location and shape of the distribution of available data are the mainstay of any statistical analysis. They are reported to summarize and highlight the predominant or most relevant features of an experiment’s outcome. Many graphical tools, such as the boxplot, serve the same purpose (see Hoaglin et al., 1985; Mairdonald and Braun, 2007; Tufte, 2001; Weiss, 2008) and have been implemented in statistical software packages such as the free software R (R Core Team, 2014), (<http://www.r-project.org/>).

Inferential statistical modeling should always start with a clear statement of the scientific questions to be answered. In translational medicine the primary focus of late-stage preclinical and early clinical trials will typically be a decision about continuing a compound into full clinical development (proof of concept, or POC). The decision will rest on the following:

1. The selection of one primary or several coprimary endpoints. These will usually be measurements of treatment efficacy. In translational medicine these will often be biomarkers that are used as surrogates for clinical endpoints, for example, CD4-cell counts, hormone levels, tumor shrinkage, and the like. Safety parameters may also be considered, in particular if they represent “red lights” that would halt any further development of an otherwise efficacious drug.
2. The selection of secondary endpoints. It is usually advisable to restrict the number of primary endpoints to one or a few. Otherwise, multiplicity might pose a challenge (see the following discussion). However, as Senn and Bretz (2007) argue convincingly, concerns over multiplicity should not prevent researchers from acquiring more measurements. All questions of scientific interest that are not covered by the primary analysis may be relegated to the secondary endpoint analyses.

Of course, if the decision is to continue into full development, a number of follow-on questions arise. Although it may not be possible to address all of them in a translational medicine trial, it is important to consider them as early as possible. These may include the following:

1. Selecting a formulation and the type of application (oral, intravenous, subcutaneous, etc.)
2. selecting a dose and a regimen

3. assessing safety and the safety-efficacy tradeoff
4. determining the patients to be treated and subpopulations of special interest.

In preclinical phases the focus may be more on selecting from a number of candidate substances than on a go-or-no-go decision for a single one. Decision making in this setting will similarly be based on one or a few primary endpoints and supportive evidence from secondary endpoints. In toxicology, however, a subset of trials has confirmatory character, because their purpose is to demonstrate to the public that the substance is safe enough to be administered to humans.

Inevitably, these questions require a stochastic model to describe the relationship between a treatment and its effect in the intended patient population. An exhaustive overview of all approaches to the modeling of clinical data is beyond the scope of this text. A model may be extremely simplistic (the percentage of responders to a treatment defined by a threshold on a single lab parameter) or very complicated (a multivariate nonlinear mixed effects model adjusting for several covariates). It may be purely empirical or motivated by biological considerations, or it may be a model that is a hybrid of the two. It may be parametric or nonparametric, longitudinal or cross-sectional, a parallel or a cross-over design, and Bayesian (that is, including prior information) or frequentist (restricted to the data generated in the experiment at hand). However, all such models have two aspects in common:

1. Their stochastic nature. The response to treatment is not deterministic but varies within a range of possibilities (whether this is inherent or just due to the fact that we do not know all influences is a philosophical question that does not concern us here).
2. Their approximate character. A model does not need to be “true”; however, it must be a reasonable approximation of reality. How close to the truth this approximation is depends on the situation at hand and on the primary object of interest. For example, if we are primarily interested in the effect of a treatment and if patients have been randomized to one of two treatment arms, we do not usually try to find many additional sources of variation between patients to put into the model covariates X , say. However, we may consider covariates that are already known to exert an important influence. The objective of including these in the model is to reduce variation in the treatment effect estimate or to explain differences in the treatment effects between individuals, but the interest is not primarily in these covariates themselves.

As a third aspect, one may add that the focus of interest of statistical analyses is usually on a population rather than on the individual, but there can be exceptions.

The generic form of a statistical model will in many cases be

$$y = f(X, \beta, \varepsilon)$$

where y is called the response, X is a set of covariates or factors influencing the response, β is a set of unknown parameters that relate the factors to the response, and ε is an error term inducing variation in the individual responses. The term *response* is a synonym for endpoint and denotes the primary quantity of interest, highlighting its dependence on the factors, in particular the ones that are deliberately modified in the experiment. The factors X might be categorical—and treatment allocation (e.g., new treatment or placebo) will usually be one of them—or continuous, for example, the patient’s age, the baseline (pretreatment) blood pressure, or the dose of an administered drug. Traditionally, the error term was often considered additive, such that the models are $y = f(X, \beta) + \varepsilon$. The classical interpretation of ε is that of a measurement error. Modern developments have generalized this concept to mixed-effects models in which the error structure can be incorporated into the model (e.g., [Diggle et al., 2002](#); [Fitzmaurice et al., 2011](#)). This is of particular interest when repeated measures on a patient are taken. This will be discussed further in Section “Statistical Models.”

The selection of an appropriate model should be made by a team of experts that includes statisticians. However, as is discussed in Section “Biological Modeling,” the decision about the right model is one that a statistician cannot—and hence should not—make alone.

Major research in statistical inference is focused predominantly on estimation of the model parameters β . Just as there are many different models, there is a multitude of estimation methods, and presenting them all is certainly beyond the scope of this text. The most important general principle is probably that of maximum likelihood (ML) ([Cox and Hinkley, 1974](#)). More important than knowing the technical details, however, is having an appreciation of the fundamental challenges of any modeling attempt:

1. The unknown parameters β are estimated from the available data y and X . Hence the estimates are subject to some uncertainty, quantified by standard errors and confidence intervals.
2. A proper statistical analysis will involve some diagnostic checks for goodness of fit, that is, a quantitative assessment of how well the data match the assumed model. The general idea is usually to calculate a measure of deviance between the observed values and those predicted by the model. In addition, the individual contributions to the deviance, which are called residuals, should be checked for poorly predicted outcomes and for nonrandom patterns.

3. A complex model with many parameters may seem to fit the observed data well, but it will often work poorly when applied to predict future responses. This phenomenon is called overfitting. Several techniques are available to assess this. Traditionally, goodness-of-fit criteria that included a penalty for the number of parameters (e.g., Akaike's AIC) have been used. With the advent of powerful computers, more complex models have gained importance. These call for more sophisticated model-checking procedures, such as cross-validation, splits into training and test datasets, and resampling methods. See [Hastie et al. \(2009\)](#) for an overview of model assessment techniques.

Although at several points in the drug development process it is certainly necessary to fit stochastic models to given data, another challenge lies in the acquisition of the data, that is, the design of the experiment. Because the researchers usually have a certain model in mind before the experiment is started, they should set it up in such a way that the model parameters can be estimated as precisely as possible. Hence the stochastic model needs to be considered even before any data are acquired. This entails sample size estimation, collection of covariate information, randomization (simple or stratified), and allocation of total sample size to strata and treatment groups and, possibly, to dose categories and the number of dose levels and their values.

Design and interpretation of an experiment

When planning an experiment, one should have in mind the way it is going to be analyzed. Once the experiment has been performed, it is usually difficult or impossible to make up for deficiencies in design. In addition, any change made to an analysis plan after the data are known undermines the credibility of a confirmatory analysis.

The quality of an experiment can be seen as the degree to which the researchers get correct answers to the questions asked. Lack of quality in this sense can be attributed to two components: systematic error (bias) and random variability. Stated the other way around, the quality of an experiment has two components: reliability measures the degree to which similar results would be obtained if the same experiment was repeated, whereas validity describes the degree to which the experiment actually measures what the researchers want to measure. In designing an experiment, measures should be taken to maximize validity and reliability under the conditions given by ethical and financial constraints as well as by time constraints. Among the most efficient measures for ensuring validity in clinical and preclinical trials are (1) the use of a control group, (2) the use of randomization

for the assignment of subjects to the experimental groups, and (3) the blinding of subjects as well as experimenters. In many cases, further precautions need to be taken to avoid influences from different sources being confounded. As an example, in a crossover design, that is, an experiment in which each subject receives a sequence of experimental treatments (including control) over a number of periods, researchers run the risk of confounding the effect of time (i.e., a period effect) with a treatment effect.

The key measure to ensure reliability in an experiment is standardization. This is a way to reduce variability and thus increase reliability. However, standardization has its limits, and there may be influential factors that cannot be standardized. For example, the effect of a drug may depend on body weight, and performing the experiment with subjects whose body weights are in a narrow range may not be reasonable. Such a factor may then be a candidate for inclusion as a covariate in the statistical model. This augmented model is likely to show reduced variability compared to the model without the factor. The reliability of an experiment can be further increased and the variability of the outcome decreased by increasing the sample size, that is, the number of independent experimental units included. Determining a sufficient sample size is a key step in planning an experiment.

There is an obvious conflict between standardization and the validity of inference. An experiment that is performed under very strict conditions will produce results that can be generalized only to situations with a similarly rigid setting. In a phase III clinical trial, in which we want to obtain results that generalize to the population of all patients with a given disease, every ethically justifiable effort should be made to include a representative sample of this population in the experiment, and restrictions of age, concomitant diseases, and so on should be avoided. However, in early clinical development, such as in a POC study, the subjects who are included in a trial will often serve as models rather than representatives of a larger population. In such cases it often is acceptable to impose restrictions on the populations under investigation in order to increase homogeneity. This, of course, applies a fortiori to preclinical settings.

The most straightforward design of an interventional experiment is the parallel group design. Subjects are randomly assigned to two or more experimental groups. Subjects in all groups are treated in an identical manner, apart from the difference in the intervention to be tested. Because in such a design the only difference between what happens to the subjects in different treatment groups is the intervention under study, there is relatively little room for confounding, and the interpretation of the outcome is usually straightforward. The drawback of a parallel group design is that comparisons between treatments are always comparisons between groups of different subjects. If there is heterogeneity in the subjects under study,

this heterogeneity will affect the reliability of the between-group comparison. One way to reduce this variability is to look at changes from a pretreatment baseline value. As a rule, if, in the population under study, the correlation between baseline values and observations at the endpoint of the study is at least 0.5, looking at the change from baseline will reduce the variability of the measurement, compared to looking at the value at the endpoint only. In some cases and depending on the nature of the measurement, percent change from baseline

$$\frac{x_a - x_b}{x_b} \cdot 100$$

(with x_e being the observation at the endpoint and x_b the one at baseline) or percent of baseline may be an alternative function to look at (see, however, [Vickers, 2001](#)).

In some cases, crossover designs that compare different treatments within one subject can be used. The simplest among them is the two-period crossover design, in which subjects are treated with the two treatments under study in two subsequent periods with a sufficient washout in between. In crossover designs, researchers must take care to distinguish time-related effects from treatment effects. In a two-period crossover design, assigning half of the subjects to the sequence $a-b$ (assuming that a and b are the treatments to be compared) and the other half to $b-a$ allows period effects (e.g., habituation to the experimental situation) to be separated from treatment effects, provided that there is an additive relationship between the two. However, if the effect of the treatment in the first period carries over in any way to the second period, this will introduce a bias into the estimates of both the period and the treatment effect. A carryover effect cannot be identified in a two-period crossover design.

If $k > 2$ treatments are to be compared, the two-period crossover can be extended to a k -period design. In this case, each subject is assigned to one of a number of sequences. In a Latin square, k sequences of k periods are used in such a way that each treatment appears in each period exactly once. This gives the best efficiency for estimating treatment and period effects. It is possible to achieve even more balance: in a Williams design, every treatment will appear in each period the same number of times, and in addition, every treatment will be preceded by every other treatment. For even k , k sequences can achieve this balance; for odd k , $2k$ sequences are needed.

The presence of carryover will always be a problem in a crossover design. If one makes rather unrealistic assumptions regarding the carryover (e.g., an additive effect that affects only the subsequent period), this effect can be estimated in Latin square designs for $k > 2$. However, we follow [Senn \(1993\)](#) and recommend that a crossover design should be only considered if a relevant

carryover can be excluded a priori. Stability over time is another prerequisite for a crossover design. Therefore a crossover trial should rarely be used in progressive diseases or if seasonal effects are to be expected.

Estimation of the dose-response relationship is a common objective in many trials in translational medicine. We will usually use $k + 1$ treatment groups, using k ascending doses and a placebo group. (We use the term *placebo*, although our considerations apply to any form of inactive control. This is to avoid confusion with an active control, which will be dealt with below.) Parallel groups or, if the prerequisites are fulfilled, Latin square crossover designs could be used. A simple way of analyzing such a trial is to compare each dose to the placebo group. If we expect a monotone dose-response relationship, this expectation can be incorporated into the analysis procedure ([Peng et al., 2006](#); [Williams, 1972](#)). In particular, as a general rule, the most efficient design is one with treatment groups of equal size; however, a design with multiple comparisons to the placebo group can be more efficient if the placebo group is larger. This might be considered in particular in trials with healthy volunteers. The methods cited previously assume a monotone dose-response relationship and do not address details of its shape. If we want to estimate the shape of the dose-response relationship and, in addition, if we want to be able to deal with nonmonotone relationships, we can attempt to model the shape and identify the best-fitting model. The MCPmod method ([Bretz et al., 2005](#); [Pinheiro et al., 2014](#)) combines a modeling approach with a many-to-one comparison of dose levels and placebo in a very efficient way.

In pharmaceutical research, some trials include active control groups in addition to a placebo group. There are several reasons why an active control could be used. If the primary purpose is to compare the test treatment to a standard active treatment, researchers must guard against a situation in which the standard treatment does not show an effect for whatever reason. Having a placebo arm in the trial in addition to an active control is the simplest way to achieve this. The placebo arm is even more useful if the researchers are looking for noninferiority rather than superiority of the test treatment in comparison to the active control. Note that in such a case it may be sufficient to assign only a small fraction of the subjects to the placebo group ([Piegot et al., 2003](#)). Even if the goal is a comparison of the test treatment with the placebo, inclusion of an active control may be valuable. In this case the active control will be used to show assay sensitivity. In particular, in early trials exploring a biomarker, an active control can help to distinguish a failed experiment (in which neither the active control nor the test is different from the placebo) from an inactive treatment (in which the active control differs from the placebo but the test treatment does not). The demonstration of assay sensitivity using an active control is

particularly important in trials that are set up to demonstrate safety. As an example, the Thorough QT/QTc Study described in the ICH E14 guidance ([International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, 2005](#)) explicitly recommends the use of an active control for this purpose.

Traditionally, hypothesis testing has played a central role in the inferential analysis of experiments. This paradigm is based on three elements:

1. A statistical model of the experiment. This model describes how the data are generated. It may also describe relationships between measurements or experimental conditions within the experiment. A test statistic that is a function of the data as described in the model is a central part of the model.
2. A null hypothesis H_0 that is related to the test statistic in the model.
3. Data observed.

The test statistic is computed on the basis of the data that are observed, and this value is compared with the predictions of the model. On the basis of this comparison, either we reject the null hypothesis in favor of an alternative or we cannot reject it.

The most common use of hypothesis tests is probably to show the existence of a drug effect. In a placebo-controlled trial, a null hypothesis would state that there is no difference between the test drug and the placebo. To take a more specific example, assume that we are measuring an endpoint Y that we can assume to be normally distributed as $N(\mu_i, \sigma)$ for $i = 0$ (placebo) and $i = 1$ (test) with mean values μ_i and a common standard deviation σ . In this case the null hypothesis would be

$$\mu_1 \leq \mu_0$$

and the alternative hypothesis would be

$$\mu_1 = \mu_0 + \Delta$$

with some relevant difference $\Delta > 0$. However, there are other situations. Let us assume that we are interested in the safety of the drug. In this case the endpoint may be a parameter describing an undesired effect. We essentially exchange the role of our null and alternative hypotheses. Our null hypothesis would be

$$\mu_1 \geq \mu_0 + \delta$$

where $\delta > 0$ is the margin of increase in Y that would be of no concern. Our alternative hypothesis could be

$$\mu_1 = \mu_0$$

if this is what we hope to be able to show. Similar situations can arise in a test for efficacy if we compare the test

drug with an active control and we cannot expect the test to be more efficacious than the control. This is often done in so-called noninferiority trials, in which a new drug might not be more efficacious than some standard treatment but has other advantages, such as increased tolerability, a better safety profile, or easier administration.

Two types of errors might arise in hypothesis testing: the error of rejecting a null hypothesis that in fact is true (type I error) and the error of not rejecting a null hypothesis if in fact an alternative hypothesis is true (type II error). The usual way to proceed is to fix an upper limit α for the probability of a type I error and to derive a threshold for the test statistic that corresponds to this limit. Depending on the choice of an alternative hypothesis, on the variability of the data, and on the sample size, we can then compute the probability of a type II error. In planning an experiment, this relationship can be used to determine the sample size that is needed to make sure that the risk of missing a relevant alternative hypothesis—for example, an effect size of at least Δ —is kept below a predefined threshold β . Whereas the null hypothesis and the alternative hypothesis should be based on scientific—and therefore rarely statistical—arguments and the question to be investigated, the choice of the accepted error probabilities is rather arbitrary. It is very common to set α to 0.025 or 0.05, whereas for β , values as large as 0.1 or even 0.2 are common. Larger values for β have been criticized as unethical ([Halpern et al., 2002](#)). These choices may be reasonable in a setting in which evidence comes from a number of similar trials, and the final conclusion may depend on a formal or informal metaanalysis ([Sutton et al., 2007](#)). In a POC setting, however, in which the outcome of a trial determines the fate of a new drug or therapy, such a choice seems inadequate. A naïve approach could be to look at the expected costs that the decision to stop or continue development entails. On the one hand, there are the costs of the POC trial to be planned, which increase with sample size. On the other hand, there are the costs that are consequences of a type I or type II error, which need to be weighted with the probability for such an error to calculate the expected loss. In a POC setting, the costs of a type I error would essentially be the costs of a phase II program; the costs associated with a type II error are those of a lost opportunity to develop and market an effective drug or those entailed by a switch to a less effective alternative compound in the pipeline of a development portfolio. These costs may be substantially higher than the costs of a phase II program, let alone those of the POC trial itself. If so, it would seem logical to keep α approximately at the range that is currently being used or even to relax the requirements on type I error control but to require a substantially smaller acceptable type II error probability. The increased costs of the POC trial may often be compensated by the reduced risk of missing an opportunity.

This reasoning is very crude. In particular, it considers only one alternative hypothesis, and it does not take into account prior knowledge. Decision theory based on Bayesian statistics offers a much more refined framework. A number of authors have addressed this problem for various settings (Cheng et al., 2003; Halpern et al., 2001; Mayo and Gajewski, 2004; Pezeshk and Gittins, 2002, 2006; Ondra et al., 2016). Yin (2002) specifically addresses the question of sample size calculation for a POC study using a biomarker.

A related concept is that of the expected value of a drug development project (Senn, 1996). This concept has been developed mainly to determine the optimal timing of studies in drug development, but it can also be applied in designing individual studies with respect to their size and error probabilities.

The considerations made here for a clinical POC study apply also to earlier situations if one experiment decides the fate of a drug. We will never know which potent drugs have been lost because of the insufficient power of an early decisive study.

Alternative ways to determine the sample size of a trial include a method that ensures that a confidence interval of at most a certain width is obtained with a given probability. A more interesting concept is that of predictive power. This is a hybrid of frequentist and Bayesian concepts. Essentially, predictive power is the Bayesian probability that a future planned trial will be significant in the frequentist sense. The attractiveness of the concept stems from the fact that it spans more than one trial in a development program (see, e.g., Spiegelhalter et al., 2004).

In recent years a trend toward a more ambitious integration of early- and later-stage clinical development into single clinical trials has emerged. These designs are called platform, basket, or umbrella trials (Woodcock and LaVange, 2017). Meyer et al. (2020) provide an overview of such trials.

Another much more general trend having far wider implications on science in general is marked by the surge of data science, such as machine learning and deep learning using multilayer neural networks. Impressive advances in many fields of science have very recently been made, for example, in image processing. At this time, however, we believe that it is still a bit premature to cover this topic in the specific context of translational medicine. In preclinical and early clinical research and development the amount of available data is usually limited by constraints such as the number of healthy volunteers who are willing to participate in early clinical phase trials. Machine learning approaches usually require enormous amounts of data. These data may in some contexts be generated by computer simulations rather than by non-virtual experiments. However, if assumptions underlying

the simulations are themselves based on slim empirical evidence or if extrapolation of these assumptions from subtly different empirical data is tenuous, highly complex “black-box” algorithms may produce biased results. Their complexity then prevents an inspection of the precise mechanisms by which this bias arises (if it is detected at all). Hence we believe that for the near future, traditional statistical model building will remain an important tool for “small data” and thus will remain important for translational medicine, where “big data” are still hard to obtain. Needless to say, this assessment is preliminary. A comprehensive perspective on the impact of machine learning on the science of statistics is given by Efron and Hastie (2016).

Multiplicity

The issue of multiplicity arises if a single trial is intended to answer more than one research question. For example, if several hypotheses about the effect of a treatment on different biomarkers are investigated simultaneously, then of course the probability of making a wrong decision about at least one of them increases with the number of hypotheses. Likewise, if we look at a number of subgroups within the same experiment, multiplicity comes into play.

As an illustration of the effect of multiplicity, consider independent hypotheses, all investigated with statistical tests at the “local” level $\alpha = 0.05$. If ten out of all investigated hypotheses are true, then the probability of at least one false rejection is

$$1 - (1 - 0.05)^{10} \approx 40\%$$

A closely related problem is that of selection bias. If a trial involves the observation of several biomarkers regarding their response to treatment and if only the results of the ones displaying the strongest reaction are considered, then the treatment effect estimates will overestimate the true treatment effects on these selected biomarkers. This is a particular challenge in gene expression analyses in which thousands of genes are screened for differential expression.

The traditional methods of dealing with this problem have been developed in the context of statistical hypothesis testing. In this context, strong control of the family-wise error rate (FWER) is a frequent requirement: The probability of rejecting *any* true hypothesis is controlled at a fixed level α , usually 5%. Among the many methods of achieving this, probably the best known is the Bonferroni method, which consists of simply doing k tests, each at level α/k . This method is conservative in the sense that it has a tendency to stick with the null hypothesis. Other widespread approaches that are less conservative include those of Scheffé, Tukey, and the Dunnett

many-to-one comparison method (Dunnett, 1955) for normally distributed data. More general approaches to correcting for multiplicity that can be used with almost any statistical test are the closed test principle (Marcus et al., 1976) and the partitioning principle (Finner and Straßburger, 2006; see Hsu, 1996 for an overview).

FWER control is a very strict requirement, especially when one is considering many hypotheses. It places a lot of emphasis on the type I error, invariably at the expense of the type II error, that is, at the expense of the probability of detecting true differences. Hence it is not advisable to attempt strong FWER control with all endpoints of a clinical trial. It is perfectly acceptable to label most of the clinical trial objectives as “exploratory” and analyze the corresponding data without multiplicity adjustment. Findings from such exploratory analyses need to be confirmed in an independent trial, in which they are tested as a primary hypothesis, that is, with control of multiplicity if applicable.

In spite of this, multiplicity adjustment is important if the number of biomarkers is very large. The advances in “omics” technology have multiplied the number of biomarkers assessed in translational medicine. Thus recent years have seen a revival of multiplicity adjustment methods. Advances have been achieved mainly in two directions:

1. Resampling methods make use of modern computer power. To illustrate the general idea, assume that a treatment is to be compared with a placebo and that there are many endpoints (e.g., several biomarkers that all measure treatment effect) per patient. The resampling approach starts by assuming that no one knows to which group (treatment or placebo) the patients belong. It then generates a complete list of all possible, hypothetical allocations of the patients to the two groups and, for each generic allocation, obtains the corresponding value of a predefined test statistic, for example, the minimum of all p -values of the single endpoint comparisons. If the observed value of an endpoint (i.e., the value that is obtained from the real observed patient group allocation) is in the extreme tail of the empirical distribution of this quantity, we have proof of a significant effect of the treatment in this endpoint with strong control of the FWER (Westfall and Young, 1993; Westfall, 2011). If it is not possible to generate a complete list, owing to the computational burden, a random sample can be taken instead. For sparse data, rotation approaches are available (Langsrød, 2005; Läuter et al., 2005). The advantage of these methods is that they automatically adjust for correlations between the endpoints, whereas older methods such as Bonferroni or Simes are based on worst-case scenarios.

2. The concept of false discovery rate (Benjamini and Hochberg, 1995) relaxes the strict FWER requirement. It requires that the probability of falsely rejected hypotheses not exceed a designated percentage of all considered hypotheses, typically 5%. Hence it is now accepted that, for example, among 100 true hypotheses, 5 may be rejected. Most research in gene expression data analysis is focusing on this definition (Efron, 2010; Storey, 2003; Storey and Tibshirani, 2003).

Views on the extent to which formal multiplicity adjustment is necessary in medical research differ in the statistical community (Senn and Bretz, 2007). In the context of translational medicine, in which any findings will have to pass the thorough the test of a phase III development, this is usually an issue only when one is selecting from a large number of biomarkers. In that case, however, some care is required, in particular if the POC is declared on the basis of a selected endpoint.

Biomarkers

The U.S. National Institute of Health Director’s Initiative on Biomarkers and Surrogate Endpoints (Biomarkers Definition Working Group, 2001) defines a biomarker as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention.” As a rule, biomarkers are not clinically relevant by themselves but are used as predictors for clinically relevant outcomes. In translational medicine the ultimate outcome to be predicted is usually the clinical efficacy or safety of a treatment when used in a certain indication. In many cases, only one aspect of efficacy is covered by a biomarker. For example, a necessary condition for a drug to act in the central nervous system is that it crosses the blood-brain barrier. Any unspecific measure of brain function, such as the resting electroencephalogram, can be used to ascertain this, but measuring the action of the drug on its target in the brain may be more valuable. Another use of biomarkers is in diagnostic tests. In this case, biomarker expression levels are often compared to a threshold value to decide on the presence or absence of a disease. In phase III trials the efficacy of a drug is often also measured by a binary outcome, such as remission (the definition of which will, of course, depend on the indication). This setting is somewhat similar to that of a diagnostic test; therefore the concepts introduced in the following have been applied to both situations.

Sensitivity and specificity are to diagnostic tests what type I and type II error are to statistical tests of hypotheses: Sensitivity is the probability of correctly identifying a condition, typically the presence of a disease, by a positive diagnostic test result. Hence it is the equivalent of power = 1 – type II error probability. Specificity is the

probability of correctly identifying the absence of the disease by a negative test and is thus equivalent to $1 - \text{type I error probability}$.

Obviously, sensitivity and specificity depend on the threshold to which the biomarker is compared. For example, if high values of the biomarker indicate the presence of a disease, we can increase the specificity of the test by increasing the threshold. This is done at the cost of a decrease in sensitivity. An extremely useful tool is the receiver operating characteristic (ROC) curve. In this context this is a plot of sensitivity versus 1-specificity. For a perfect diagnostic test, the ROC curve would have an inverted L-shape; a useless diagnostic test (i.e., the equivalent of tossing a coin) would be described by straight line from (0,0) to (1,1).

Of course, diagnostic tests should have both high sensitivity and high specificity, but the right balance between them can be difficult to obtain. For example, consider an extremely rare disease that afflicts only 1 in 10,000 people. Suppose that the sensitivity of the test is 1 and its specificity is 0.999. If 100,000 patients are screened, $10 + 0.001 \times 99,990 \approx 110$ is the expected number of people identified as diseased, but in fact only $10/110 \approx 9\%$ of these do suffer from it. The ratio of true positive results among all results is called *positive predictive value*. Together with the corresponding negative predictive value (true negatives/all negatives), it is an additional measure of the quality of a diagnostic test, supplementing sensitivity and specificity.

If we can increase the specificity to 1 by decreasing the sensitivity to 0.8, we would expect to find 8 diseased people and miss 2 while not having any false positives. Depending on how grave the disease is, this might be the preferable option. (Of course, in this example the right answer is probably not to do mass screening but to restrict testing to high-risk subpopulations.) The example illustrates that if an assay is developed and a threshold is chosen, there is no off-the-shelf solution, such as simply picking the point at which sensitivity + specificity is at a maximum.

Validation of biomarkers can be similarly difficult. Roughly speaking, a biomarker is validated if the accuracy of the prediction of the corresponding clinically relevant outcome derived from it is known and is sufficiently good. If a biomarker is used in a confirmatory context, validation is very important. In such a context we would speak of a surrogate endpoint. Strictly speaking, if a biomarker is validated for the prediction of the efficacy or safety of a therapy, then this validation applies only to the exact conditions that were used when validity was established. These conditions include the patient population, therapy used, and so on. In particular, any new therapeutic approach would require a revalidation of a biomarker. If a new therapeutic approach has an effect on a

biomarker that is similar to that of a standard reference therapy, we thus may not automatically conclude that it will have a comparable effect on the clinical endpoint. To be valid, such a conclusion must be strongly supported by biological and clinical arguments. Blood pressure is one such mechanistically validated biomarker that is generally accepted as a surrogate endpoint. However, the fact that, for new antihypertensive therapies, large morbidity and mortality studies are conducted shows the limitations of even this well-established and well-understood surrogate endpoint. In translational medicine, such an overly strict requirement on validation is not appropriate. If we are looking for efficacy in this setting, it is important to have reasonably sensitive markers so that drugs that have therapeutic potential can be identified. If we are looking for safety, on the other hand, the biomarkers should be reasonably specific. In this way, the most promising drugs can be selected for further development, but obviously, some risk of failure in later phases will still remain. However, if the development of a useful drug is discontinued because of an erroneous biomarker, this is usually an irreversible decision that incurs the cost of a lost opportunity in terms of patient and financial benefit.

Formally, a biomarker that is used to predict a binary outcome can be validated by determining the ROC of the marker. If a biomarker is used to predict another continuous outcome, the simplest measure for validation would be the correlation of the biomarker and the clinical endpoint. There is extensive discussion about more elaborate methods for validating surrogate endpoints, but this is of limited relevance to the field of translational science. For an in-depth discussion, see, for example, [Alonso and Molenberghs \(2007\)](#).

If we have a set of biomarkers with moderate sensitivity and specificity, we may attempt to construct a composite marker with better quality. This approach is promising if the various markers measure different entities so that the correlation between them is at most moderate. For example, several combinations based on logic can be constructed. If we start with two biomarkers, we can derive a test that is positive only if both biomarkers are positive. In this case, specificity will increase over each of the individual markers at the cost of sensitivity. Alternatively, we might look at a test that requires only one of the two markers to be positive. This obviously would increase sensitivity.

We can also look at linear combinations, that is, weighted sums of a set of biomarker values. This leads to linear discriminant analysis or logistic regression. Under the model of multivariate normality for each of the two groups (e.g., normal and diseased) and assuming that the within-group covariance matrix is the same for both groups, the optimal separation of the two groups is accomplished by a linear discriminant function that

maximizes the distance between the groups relative to the within-group variance.

A word of caution is appropriate here: to fit a discriminant function that can be used for future patients, that is, one that is a predictor beyond the sample of data from which it is derived, it is important that the number of observations be much larger than the number of biomarkers considered. If this is not the case, the resulting situation is similar to overfitting (see Section “Biological Modeling”). As a rule of a thumb, some authors recommend that for linear discriminant analysis the number of observations per group should be at least three times the number of biomarkers considered, but recommendations of up to 10 times can also be found (Ferber, 1981; Romeder, 1973).

Biological modeling

As was mentioned previously, stochastic models can be purely empirical or motivated by the subject matter. A purely empirical model is not based on any reasoning about underlying biological mechanisms; it simply tries to emulate a factor-response relationship under some generally mild restrictions on the smoothness of this relationship. (Hastie et al., 2009; Section “Biomarkers”) and give an excellent overview of this topic.

If we have knowledge of the underlying biological mechanisms, such empirical modeling is inefficient. For example, if we know that the concentration of a substance in the blood of a patient, considered as a function of time after intake, follows a one-compartmental model, we should restrict attention to time-response relationships that conform to this model.

Knowledge of such underlying mechanisms is problem specific and ranges from very precise to very vague. Transformation to linearity is a very common example of the latter. In fact, this case is so common that it is usually

not considered a case of biological modeling. However, use of a logarithmic transformation is based on the general observation that growth is often exponential such that a logarithmic transformation renders growth data linear. This observation is then extended to deal with phenomena that reveal a skewed distribution. Many lab parameters, such as creatinine and serum ferritin, are examples of responses that are routinely log-transformed before analysis.

From the statistician’s point of view, a biological phenomenon that can be linearized in this fashion is relatively easy to analyze, since standard linear model theory (ANOVA, ANCOVA, and linear regression) can be applied to the data. Phenomena that are nonlinear in nature and cannot be linearized easily are much more challenging. Rather than attempting an exhaustive overview of the types of nonlinear models used in the various fields, let us consider two illustrative and important examples and discuss some salient features. More complete overviews are given by Davidian (2009), Ratkowsky (1990), and Seber and Wild (1989).

Example 1: pharmacodynamics

Studies have investigated the effect of metformin on the level of hemoglobin A₁C (HbA₁C) in patients suffering from diabetes mellitus. Metformin lowers HbA₁C. In addition, within the range of doses investigated, the lowering effect increases as the dose increases but with a diminishing return. Relatively little is known beyond these rather vague notions. In this situation the so-called E_{\max} model is very popular. It assumes that the response (lowering of HbA₁C) has an expected value given by the Michaelis-Menten equation $E_0 + (E_{\max} \cdot d)/(ED_{50} + d)$, where d denotes dose. The three parameters of the model are E_{\max} , the maximum attainable effect; ED_{50} , the dose at which half of the maximum effect is achieved; and a placebo effect E_0 (this can be set to 0 in many

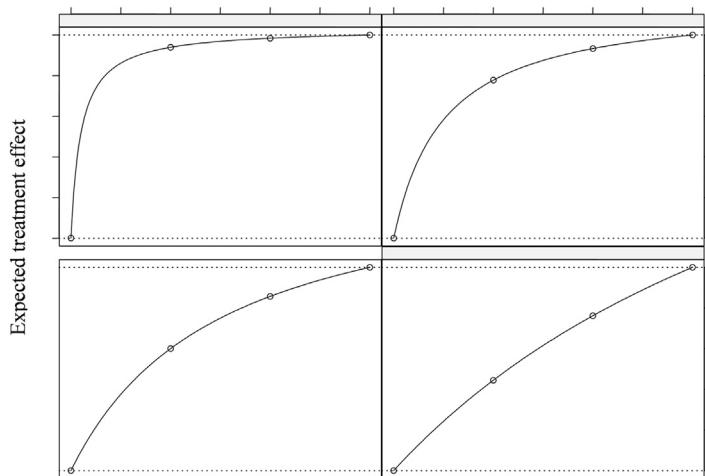


FIGURE 27.1 E_{\max} model shapes for different ED_{50} .

applications). Fig. 27.1 shows the shape of E_{\max} dose-response relationships. The shape is determined primarily by the ED_{50} . The smaller the ED_{50} , the more bent the curve.

The E_{\max} model can be derived from enzyme kinetics following the Michaelis-Menten model. However, the idea behind this is rather heuristic. Such models are sometimes called *semimechanistic*.

Example 2: pharmacokinetics

In pharmacokinetic analyses, more sophisticated approaches are common. This field deals with the modeling of concentration-time profiles. One- and two-compartment models are most frequently used for this purpose (Rowland and Tozer, 1995). The most popular model is the one-compartment model with first-order absorption and elimination:

$$C(t) = d_0 \cdot \frac{f}{V} \frac{k_e k_a}{(k_a - k_e)} (\exp(-k_e t) - \exp(-k_a t)).$$

Here, $C(t)$ denotes the concentration at time t ; k_e and k_a represent the elimination and absorption rate constant, respectively; d_0 represents the initial dose; f represents the fraction of the drug absorbed; and V represents the volume of distribution. This model is derived from the assumption that the rate of absorption (into the compartment) is proportional to the amount available for absorption and that the rate of elimination (from the compartment) is proportional to the concentration. Fig. 27.2 shows a typical concentration-time profile derived by using this model.

Models like these are deterministically based on assumptions about how a biological mechanism will work. In example 1, one of the key assumptions is that the mechanism follows a law of mass action. The assumptions in example 2 include not only the proportionality of rates but also the key assumption of dose proportionality, that is, the assumption

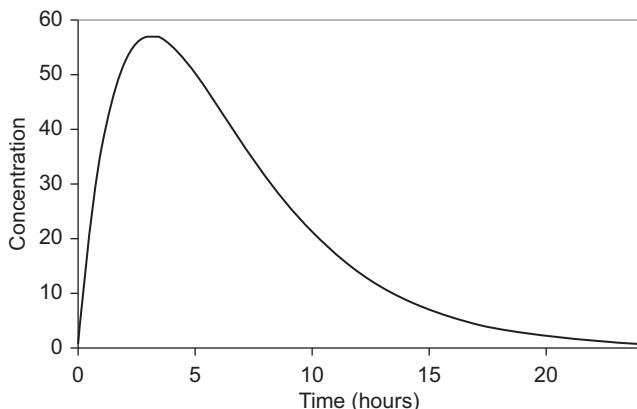


FIGURE 27.2 A one-compartment model.

that doubling the dose, for example, will shift the entire concentration-time curve upward by a factor of 2. In reality these assumptions may or may not be fulfilled. In addition, even if we believe that the models are “true,” the parameters of the models have to be estimated. If we have some concrete data, such as concentration profiles from the plasma samples of a number of test animals, these data will not follow our assumed model perfectly. Hence just as with linear regression, we are forced to find the best fit.

There is much room for sophistication in both the deterministic and stochastic parts of the model. In the deterministic part, we may want to generalize from one-compartment models to multicompartment models (Reddy et al., 2005). In the pharmacodynamic example 1, a Hill parameter can be added if the effect of the dose on the HbA₁C level is below the prediction from the E_{\max} model at low doses.

Regarding the stochastic part, the simplest approach would be to use, for example, the method of least squares to find the E_{\max} curve that shows minimum quadratic deviation from the observed HbA₁C values. This approach requires no specific assumption about the distribution of an error term in the model, but if we assume a normally distributed additive measurement error, then the least-squares solution coincides with the ML estimate of the E_{\max} profile. If patient i receives dose d_i , the model is

$$\text{reduction in HbA}_1\text{C}(i) = E_0 + (E_{\max} \cdot d_i) / (ED_{50} + d_i) + \varepsilon_i$$

with independent, identically normally distributed errors ε_i .

If we desire something closer to reality, we might want to generalize this model. For example, it might be assumed that the errors of repeated measures from the same patient are correlated, or we might regard the model parameters themselves as random variables with an associated random error. The latter assumption leads to so-called (random effects) nonlinear mixed models.

Various software packages are available to fit such models. NONMEM (<https://www.iconplc.com/innovation/nonmem/>) and MONOLIX (<http://lixoft.com/products/monolix/>) allow users to specify a pharmacokinetic model in terms of differential equations. The software solves the differential equations numerically and estimates the parameters by ML (or, to be more precise, by approximations of the ML solutions). In other software packages, such as SAS PROC NLMIXED (<http://support.sas.com/documentation/>) or the R-procedure nlme (<http://cran.r-project.org/web/packages/nlme>), the model must be specified in terms of the model equation. SAS PROC NLMIXED and R nlme do not allow users to specify the model in terms of differential equations, but they use fitting algorithms that are superior to those implemented in NONMEM. Combining nlme and nlmeODE (Tornøe et al., 2004) in R allows the same functionality as NONMEM. However,

readers should bear in mind that software development is in constant flow, especially open source software such as R. Therefore the availability of software implementations and the assessment of their relative strength and weaknesses may change quickly within a few years.

Some further words of caution are in order. As was mentioned previously, there is a tradeoff between the complexity—or rather its absence—of a model and its closeness to the truth. In translational medicine there are usually relatively few observations available to fit a model. With sparse data, however, it is impossible to check the correctness of model assumptions, and problems with overfitting quickly outweigh apparent realism. Ratkowsky (1990) calls this “the trap of overgenerality” and applies the principle of Occam’s razor: “Other things being equal, parsimony is more likely to reflect the truth than its opposite.”

To give a rather simplistic example with observations at just two dose levels, we cannot estimate an E_{\max} model because the number of parameters is larger than the number of dose levels, and the model is overparameterized. Even if we have more dose levels, the data may be such that maximum likelihood estimates do not exist, are not unique, tend to infinity, or are on the boundary of the parameter space, thus invalidating asymptotic properties, which are usually relied on to generate confidence intervals and statistical tests.

If a numerical optimization algorithm fails to converge, the reasons can be manifold. There may be numerical issues with the algorithm itself (e.g., poor start values or inappropriately wide or small steps within the iterations). Often, however, the issue is with the model assumptions (overparameterization or “near” overparameterization). It can even happen that a model describes the data well, but, owing to an unfortunate constellation of observations, it cannot be fit, although there is no

indication that it is “wrong.” Fig. 27.3 gives a simple illustrative example in which the observed response at four dose levels is such that the slope of a logistic (S-shaped) fit through the points becomes infinity and the ED_{50} becomes nonunique, although there is little indication of any lack of fit.

If convergence of the fitting algorithm is successful, overparameterized models might still suffer from a tendency toward instability. Minor changes in the observed data might cause dramatic changes in the model fit with corresponding uncertainty about the conclusions derived from the model. To less-than-careful modelers, this will not be obvious from measures of variation such as standard errors and confidence intervals. These are usually derived under the assumption that the model is correct and are therefore far too narrow in these situations.

In pharmacokinetics the focus of interest sometimes is on a specific parameter rather than on the entire concentration-time profile. The most popular ones are the area under the curve (AUC), the maximum concentration (C_{\max}), the time of maximum concentration (t_{\max}), and the compound’s half-life ($t_{1/2}$). These parameters can be either derived from one of the parametric models described so far (e.g., the one-compartment model) or estimated nonparametrically from observed data. The well-known trapezoidal rule for estimating the AUC is an example of the latter approach. The advantages of such nonparametric methods include less specific model assumptions and hence more robustness. Apart from the limited scope, the disadvantages include less precision if the model is (approximately) correct and some uncertainties regarding the estimated quantities (e.g., is C_{\max} really close to one of the points in time for which we have measurements? Which time points in the tail in Fig. 27.2 should be excluded from calculating the AUC?).

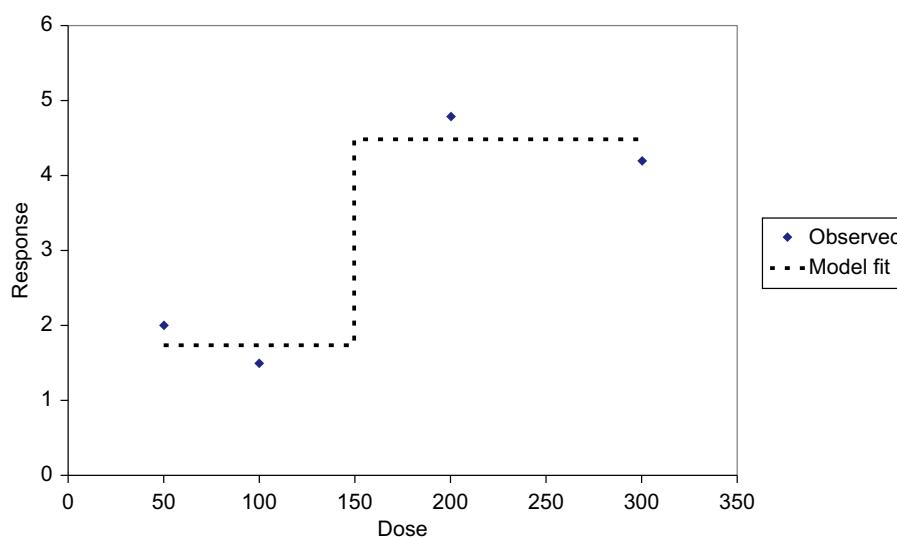


FIGURE 27.3 A logistic model for dose response. The results at the four dose levels are such that the ED_{50} cannot be uniquely estimated, and the slope parameter becomes infinity.

The reasons for the ubiquity of nonparametric estimates are in part historical. Today's computer power enables us to perform numerical optimizations and, if everything else fails, simulations that a generation ago were inconceivable. In past decades a lot of work went into the algorithms to fit more sophisticated parametric models, and many advances were made.

Still, the balance between simplicity and complexity is a delicate one. The availability of powerful computer packages is no safeguard against their misuse, and fitting complex nonlinear models remains an art that requires an indepth knowledge of biology, numerical optimization methods, and mathematical statistics. The combination of these skills in a single individual is rare. In accord with our background in biostatistics, we think that it is appropriate to highlight one specific danger: the illusion that all attention can be focused on development of a "realistic" model, because some black-box optimizer implemented in a software package will churn out the parameter estimates with confidence intervals and significance tests. The reality is not as simple as that. However, a close collaborative effort of teams bringing together experts from all fields can achieve much.

Statistical models

Almost all statistical models used in translational medicine fall into one of the following categories:

1. Models for a continuous and normally distributed response: linear model [regression, analysis of variance (ANOVA), and analysis of covariance (ANCOVA)] (McCulloch et al., 2008): Such models can be written as

$$y = X\beta + \varepsilon, \varepsilon \sim N(0, \Sigma),$$

2. where y is the vector of responses, X is the matrix of covariates, and ε is an error vector that is assumed to be normally distributed with the variance-covariance matrix Σ . The t -test is one of the simplest examples of a test for the significance of a parameter in such a model. The category includes linear mixed effects models (in mixed effects models more than one component of Σ is unknown and must be estimated). The model is often justified asymptotically by the central limit theorem.
3. Models for a response that is continuous but not normally distributed:
 - a. Transformation to normality, such as Box-Cox (see, e.g., Box et al., 2005).
 - b. Nonparametrics or transformation to ranks or rank-based scores (e.g., Hollander et al., 2014).
 - c. Nonlinear modeling (e.g., compartmental models, E_{\max}) (Seber and Wild, 1989).

4. Models for a categorical (often binary) response (Agresti, 2019):
 - a. Generalized linear model (GLM), usually logistic regression, sometimes Poisson regression, or other GLM link functions: cloglog, negative binomial.
 - b. Many tests of contingency tables; for example, the chi-square test, Fisher's exact test, and the Cochran-Mantel-Haenszel test are special cases of parameter tests in these models.

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Chapter 28

Computational biology and model-based approaches in translational medicine

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Abstract

Model building is one of the fundamental knowledge generation concepts in science and medicine. We introduce the concept and its application in Translational Medicine with a series of examples. The Hodgkin & Huxley model for the neural action potential illustrates the power of models in the identification of previously unknown structural properties in biology. Compartmental and Physiology-Based Pharmacokinetics demonstrate how data can be interpreted by mathematical abstraction such that predictions for untested clinical settings are enabled. Based on a computational model of blood coagulation, the mechanistic interaction of different pharmacological principles for anticoagulation is predicted. Quantitative computer simulations are used to inform complex dosing schedules ensuring patient safety when treatment paradigms are switched. Finally, we introduce the framework of model-informed drug development, a novel paradigm in pharmaceutical industry and regulatory practice where direct experimental and clinical evidence is complemented and even substituted by model-based derivations.

Keywords: Model building; simulation; prediction; MIDD; model-informed drug development; PBPK; pharmacokinetics; coagulation

Introduction: Model building in sciences

“... the sciences do not try to explain, they hardly even try to interpret, they mainly make models. By a model is meant a mathematical construct which, with the addition of certain verbal interpretations, describes observed phenomena. The justification of such a mathematical construct is solely and precisely that it is expected to work—that is, correctly to describe phenomena from a reasonably wide area”(von Neumann, 1955).

Simplified, sometimes abstract representations of reality so-called models are among the most important tools in sciences. Model building is one of the fundamental

concepts in the theory of sciences (Gilbert, 1991). While the introductory quote from John von Neumann may appear to be an exaggeration and an oversimplified concept of the sciences, it is difficult to imagine any of today’s disciplines of science without abstraction, that is, the process of formal model building.

Depending on the specific scientific area, models may have very different levels of sophistication and formalization and resulting accessibility. This may be illustrated by the comparison of the so-called calotte model of chemical molecules and the string model in modern particle physics. Both models represent the constituting building blocks of our material world, though at different microscopic scales. The calotte or space-filling model describes molecules as compositions of spherical atoms with radii representing the size of the atoms and distances between spheres representing the typical distances of atoms in a molecule. It is widely used already in secondary schools to introduce the concept of molecules and chemical reactions in a very tangible—literally touchable—way. In string theory, particles are represented as one-dimensional mathematical objects that are known as “strings” and, depending on the specific theory, are embedded in 10, 11, or 26 space-time dimensions, generalizing the three-dimensional space and time we experience in daily life. Although string theory provides a way to analyze, interpret, and predict various phenomena in high-energy physics and cosmology, the required mathematical concepts limit use and understanding to a small group of physicists and mathematicians who have a high degree of specialization and a rare talent for mathematical abstraction.

Irrespective of the level of formalization they require, model building and the resulting models are meant to do the following:

1. support understanding of phenomena in the real world;

2. help to develop precise definitions of real-world entities and processes with minimal ambiguity;
3. characterize entities, processes, and emerging properties of the system of interest;
4. provide quantifications;
5. enable prediction, extrapolation, and forecasting of system properties and behavior, for example, via simulation.

This is also relevant and has found widespread use in life sciences and medicine. Two out of countless examples are the discoveries that were honored by the 1962 and 1963 Nobel Prizes in Physiology and Medicine. In 1962, Francis Crick, James Watson, and Maurice Wilkins received the prize “for their discoveries concerning the molecular structure of nucleic acids and its significance for information transfer in living material” (www.nobelprize.org/prizes/medicine/). Elements of their achievement include the development of the double-helix model of DNA—in a way a very elaborated advancement of the concept underlying the calotte model—and the identification of the hierarchical structure of the genetic coding of information. Just a year later, Alan Hodgkin, Andrew Huxley, and John Eccles were given the prize “for their discoveries concerning the ionic mechanisms involved in excitation and inhibition in the peripheral and central portions of the nerve cell membrane” (www.nobelprize.org/prizes/medicine/). A key element of their discoveries is the so-called Hodgkin–Huxley model for the generation of action potential in nerve cell axons. This model of neural activities provides a differential equation-based representation of the voltage-based gating of ion channels and resulting ion fluxes across cellular membranes. It formally defines the necessary molecular and cellular elements (insulating membrane, concentration gradients, leak channels, gated channels, and ion pumps) for well-defined short electrical excitation, polarization, and repolarization in nerve cells and provides a basis for quantitatively accurate predictions of, for example, pharmacological interventions. The model building performed by Hodgkin and Huxley included postulating three distinct states of the sodium ion channel, a theoretical finding that could not be experimentally confirmed until decades later. The model can also be seen as one of the pioneering works in the field of computational biology, both in the broader sense of providing the concept and mathematical formalization of a model for a complex biological process and in being one of the first works implemented on an (analog) computer to solve the model and study its behavior ([Hodgkin and Huxley, 1952](#)). More than 70 years later, Hodgkin’s and Huxley’s work in the 1940s impresses every computational biologist and provides an illustration of the power of modeling and systems analysis.

In translational medicine, computational modeling has continuously gained dissemination and relevance up to a level at which a comprehensive review would exceed the scope of a book chapter and could easily fill an extensive monograph ([EFPIA MID3 Workgroup et al., 2016](#)). Instead of undertaking a desperate attempt to review the full breadth of computational modeling and model-based approaches in translational medicine, we will first provide a simple but highly relevant exemplary concept of modeling in clinical pharmacology that provides the basis for many translational applications in the clinical development of drug candidates. We will then discuss two specific application areas in translational medicine in more detail before we introduce the framework of model-informed drug development (MIDD) to reflect recent developments regarding the acknowledgment and appreciation of model-based concepts in decision-making processes that immediately affect the well-being of individual patients as well as global patient populations.

Compartmental modeling of pharmacokinetics: A conceptual framework for translational applications in clinical pharmacology

One of the most frequently used modeling frameworks in translational medicine is the compartmental modeling of the pharmacokinetics (PK) of drugs. The aim is to investigate, quantify, and predict the concentration time course of a substance, that is, its PK, after an organism has been exposed to it via the application of an intravenous injection, for example ([Bonate, 2006](#)). The complex interplay of properties of the drug and its formulation in the drug product (solubility, protein binding, molecular weight, polarity, and other physicochemical properties), the organism and physiological processes (anatomy, tissue composition, blood flows, renal excretion, hepatic and extrahepatic metabolism, etc.), and the interaction between drug and organism (e.g., passive glomerular filtration in kidney, oxidative metabolism via cytochrome P450s, binding to proteins) need to be represented in a model such that the resulting PK are adequately captured. Applications of interest, such as dose selection, shall be enabled. The same concepts can also be applied to other xenobiotics, such as environmental chemicals in the context of toxicokinetic modeling aiming at the determination of no-effect exposure levels.

As in many modeling frameworks the compartmental modeling of PK relies on a massive lumping of real-world entities and processes into simplified abstract representations featuring the same phenotypical behavior ([Brochot et al., 2005](#)). For many drugs that are applied intravenously, PK in blood plasma are adequately represented by

a model that describes the body of an animal or human as a single compartment (Fig. 28.1A). The complex organism is described as a container with fluid volume, called a compartment, in which a drug dose is instantaneously distributed, the so called well-stirred hypothesis. The concentration in this theoretical, effective fluid volume represents the concentration of the drug in blood plasma. The effective volume itself depends on both properties of the organism and the drug. The blood volume in mice differs from that in humans. Hydrophilic drugs may distribute only into drug plasma and interstitial fluid, while lipophilic drugs will also permeate across cellular membranes and distribute into larger volumes. It is an abstract volume with no obvious direct link to physiological volumes. For highly lipophilic drugs, eventually binding to proteins at a high degree, the effective volume of the compartment in the model may exceed the factual volume of the body by orders of magnitude, reaching hundreds of liters.

Physiological processes that lead to the degradation and excretion of the drug from the organism are represented by a process called clearance. It is typically assumed to be proportional to the concentration in the compartment, that is, as a first-order process. The proportionality constant for the excretion process is called clearance itself, since it quantifies the rate at which a specific drug can be eliminated by a specific organism. It represents a quantitative property emerging from the interplay of various properties of the drug and the specifics of various physiological processes in the organism.

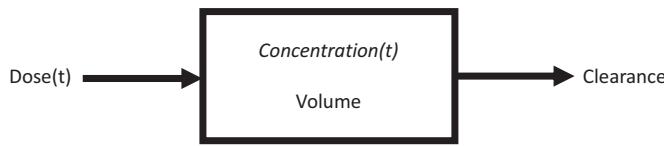
It may be surprising that such an abstract, simplified model can represent PK of real drugs in real organisms. But for many applications in research or clinical practice, measured concentrations of a drug, especially when administered as in intravenous bolus, in blood plasma will

be accurately characterized by the behavior of the compartmental model (Fig. 28.1B). In this regard, compartmental models are fully functional effective representations of PK, suitable and appropriate for many applications. Deviations between a one-compartment model as depicted in Fig. 28.1 and the PK in specific application (e.g., for a lipophilic drug applied as an oral tablet formulation) can be reconciled by introducing further compartments and abstract processes to the model. The modeling principle will stay the same.

Despite the counterintuitive nature of the abstraction underlying the concept of the volume of distribution in a compartmental PK model, it has a high relevance for critical pharmacological questions. Volume of distribution quantifies the dilution effect and thereby the maximum concentrations that can be reached after administration of an arbitrary dose. The volume of distribution also quantifies the level at which drug penetrates the cellular space and tissue as a systemic property. Although the volume of distribution in most cases does not have a simple one-to-one relationship with a physiological volume, it can nevertheless scale with certain body measures, such as weight or height, and thus its change might be predictable between species or for special patient populations, such as children. Along these lines, though abstract and a dramatic simplification compared to the physiological reality, the clearance is of utmost pharmacological relevance. It determines the dose required to reach average concentration levels in blood and the dosing interval suited to stay above minimal trough concentrations at the end of the interval prior to redosing.

Although the graphical and verbal description of the compartmental model in Fig. 28.1 provides all relevant elements of the model which are required to understand the concept and to define its properties, for most

A



B

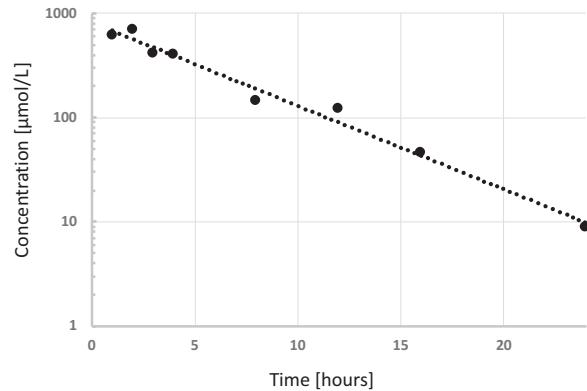


FIGURE 28.1 (A) A one-compartment model representing the pharmacokinetics of a drug after administration to arbitrary organism, such as a pre-clinical animal model or a human patient. The dose is entering a distribution volume (volume) over time at a rate $Dose(t)$, is instantaneously distributed and excreted (clearance). (B) A typical comparison between drug concentrations measured over 24 hours after the intravenous injection of a drug and the concentration time curve resulting from the one-compartment model.

applications another level of formalization is required. The model needs to be formulated as a mathematical equation:

$$\underbrace{\frac{d}{dt} \text{Concentration}(t)}_{\text{concentration change}} = \underbrace{\frac{\text{Dose}(t)}{\text{Volume}}}_{\text{influx by dosing}} - \underbrace{\text{Clearance} * \text{Concentration}(t)}_{\text{outflux by clearance}} \quad (28.1)$$

[Eq. \(28.1\)](#) provides such a mathematical formalization. The differential equation defines the temporal change of concentration at a given point in time (left-hand term) as the consequence of the dosing rate at this point in time (right-hand term: *influx by dosing*) and the instantaneous dilution into the volume of distribution (*Volume*) and the first-order degradation resulting from the clearance process (second right-hand term: *outflux by clearance*).

To use the ordinary differential equation (ODE) model, that is, to solve the mathematical equation and apply it to a specific PK setting, further information is required. [Fig. 28.1B](#) shows typical experimental concentration data from a PK experiment in which a drug was administered as a single intravenous bolus with the dose D to a healthy volunteer at time $t = 0$. Assuming that the concentration was zero prior to the bolus injection, that is, it is the first dose, and that the time required for the bolus is negligible, as we already assumed for the process of distribution, we can solve the ODE and get [Eq. \(28.2\)](#), determining the concentration of the drug at any time $t > 0$:

$$\text{Concentration}(t) = \frac{D}{\text{Volume}} e^{-\text{Clearance} * t} \quad (28.2)$$

[Eq. \(28.2\)](#) is the basis for the curve plotted in [Fig. 28.1B](#). It describes the exponential decline of the drug concentration over time.

Different translational applications of compartmental PK modeling are common practice in drug development. [Eqs. \(28.1\) and \(28.2\)](#) are, for example, the basis for inter-species extrapolation in which PK data from different animal species that are commonly studied in preclinical drug development, such as mouse, rat, dog, minipig, and monkey, are compared and in which prior to first-in-human applications, the PK of the drug candidate in humans is predicted. The bridging between species is achieved by adjusting the volume to the differences in body weight and plasma volume of the different species and by adjusting the clearance to the typical differences in kidney and liver function between the different species. The adjustments are based on so-called allometric models. The models are by nature hierarchical; that is, the solving of a practical scientific challenge (PK in this case) leads to the identification of a new challenge (in this case, the need to develop a model for the differences between species

relevant for PK). On the basis of both theoretical considerations and empirical evidence, the volume of distribution is usually assumed to be proportional to body weight, while the clearance is assumed to be proportional to body weight to the power of three-quarters ([Anderson and Holford, 2009](#)). In concrete applications the assumptions need to be chosen carefully. For instance, in cases of lipophilic drugs that distribute extensively into peripheral tissues, including fat tissue, the volume of distribution is usually assumed to be proportional to body weight. For hydrophilic drugs that predominantly distribute into the aqueous compartments, such as plasma and interstitial organ spaces, the volume of distribution might be better correlated with the lean body mass or height because excess body weight (typically in the form of fat tissue) does not contribute to the distribution volume in such a case.

Physiologically based modeling: prediction of pharmacokinetics in children

In the previous section we introduced the concept of compartmental modeling in PK as a simple example illustrating the types of abstraction and formalization required in model-based, computational approaches. In a general sense, most modeling activities aim at predictions, that is, translational applications. However, compartmental modeling is very often used primarily because of its ability to quantitatively describe experimental data. By contrast, so-called physiologically based pharmacokinetics (PBPK), one of the most sophisticated advancements of compartmental modeling, can be considered the translational model framework in clinical pharmacology. Its applications cover the full spectrum of translational prediction challenges from pharmaceutical research and development into the clinic.

PBPK models support the prediction of pharmacokinetics in humans, in particular when PK in different animal species does not fulfill the assumptions of allometry. PBPK is used to predict the *in vivo* PK performance of new drug formulations based on *in vitro* characterizations such as particle-size distribution measurements or dissolution kinetics assays. In particular in generics development but also during the initial development of drugs when formulation changes need to be introduced, PBPK is used to assess the bioequivalence with previous formulations, such as the marketed innovator drug formulation. Extensions of PBPK can be used to predict the deposition of inhalative medications in different segments of the lung based on laboratory measurements with so-called impactors. *In vitro* metabolism assays, for example, with recombinant CYP P450 enzymes, are the basis for the prediction of so-called drug-drug interactions (DDIs).

DDIs are common, and a drug candidate's PK can be altered as a result of the concomitant use of other drugs (it is then called the victim) and at the same time might also have the potential to modify the PK of other drugs (it is then called the perpetrator). The clinical relevance of DDIs is high, in particular in polypharmacology settings. A victim drug may no longer be efficacious if its concentrations are lowered. Organ rejection as a consequence of the missing effect of immune-suppressive treatments after the uncontrolled use of St. John's wort is a very obviously disastrous example. Increased exposure of a victim can also be as fatal, for example, leading to opportunistic infections in cancer patients experiencing myelosuppression as a consequence of an overdosing of chemotherapy. Despite the critical relevance of DDIs, the predictive performance of PBPK nowadays allows the use of computer simulations instead of clinical studies as an independent information source in the clinical pharmacology section of drug labels.

Another very elaborated application of PBPK is the prediction of PK in so-called special populations. Special populations differ from the general patient population for a drug in specific characteristics, such as age, body mass index, comorbidities, and pathophysiological impairments in particular of the excretion organs kidney and liver. Depending on the properties of the drug, such differences may cause massively different PK, and the evaluation of the impact of age, body weight, gender, ethnicity, diet, and renal and hepatic impairments on PK is a mandatory standard in all drug development programs. Careful design of dedicated clinical pharmacology trials as well as classical outcome trials requires a robust understanding of the potential effects even before first experimental testing in humans. PBPK enables quantitative predictions based on general prior knowledge about anatomy, physiology, and pathophysiology and specific properties of the drug of interest. Physicochemical properties of the drug and PK experimentally observed in clinical phase I settings with young, healthy male adults can be translated into quantitative expectations for the PK in elderly patients, patients with chronic kidney disease or cirrhosis, and many other well-defined special populations. The most prominent application of PBPK-based predictions is pediatric scaling to children, including newborns, premature neonates, and even unborn children exposed to drugs via the placental link with the mother (Wagner et al., 2015; Yoshida et al., 2017; Cole et al., 2020). We will focus on pediatric scaling to illustrate the use of PBPK modeling in a translational context.

Despite the differences in scientific direction, a common denominator of most reviews is the focus on scientific and technological state of the art. Complementary to this rather canonical scope of scientific review articles is

an analysis of factors that either facilitated scientific progress in the field of PBPK modeling and its application to pharmaceutics or played an important role in preventing or delaying progress and application. Such factors themselves may be of diverse nature.

Before we introduce the concept of PBPK modeling in more detail, it is interesting to briefly review the historical development of its application in a pharmaceutics and medicine context. When the concept of physiologically based PK modeling was developed and published by Teorell (1937) in the 1930s, computer systems allowing the numerical integration of larger ODE systems were not available. It consequently took decades before PBPK became more than a theoretical concept and could gain practical relevance. A first series of publications on pharmaceutical applications in the late 1970s coincided with the vanishing of the technological hurdle when microprocessor-based computers became affordable for academic use. Once the technological hurdle was lowered, the scope of these early works was very broad even in comparison to today's PBPK applications. Harrison and Gibaldi (1977a,b) modeled digoxin in rats, dogs, and humans and investigated the effect of renal failure. In 1982, Sugita et al. (1982) published a drug-drug interaction PBPK for tolbutamide-sulfonamide interaction. A peak of complexity was probably reached when Sorensen (1985) published his Ph.D. thesis at MIT in which he described a highly detailed coupled PBPK-pharmacodynamics model of glucose, insulin, and glucagon. Looking back, it is interesting that despite these early proofs of concept, it took more than two decades before PBPK modeling was more widely used in pharmaceutics and the number of publications grew considerably.

Since the concepts, case studies, and affordable computer systems were available, what prevented more widespread use? The answer very likely lies in the fact that PBPK modeling required a very high up-front investment. Models still had to be built and parameterized from scratch, and for scientists with the required technical skills, traditional compartmental modeling approaches offered a far more efficient use of their scarce time. Around 2000 this economic hurdle was surmounted as detailed generic drug-independent models were published (e.g., the CAT model of the gastrointestinal (GI) tract for oral absorption (Yu et al., 1996) and the Poulin–Theil model for partition coefficients (Poulin and Theil, 2000)) and consecutively refined and implemented in the commercial PBPK modeling software tools GastroPlus, SimCYP, and PK-Sim. PK-Sim was later transferred into the noncommercial open science community Open Systems Pharmacology (<https://github.com/Open-Systems-Pharmacology/>). Since then the technical hurdle to PBPK modeling is negligible. Easy-to-use graphical user interfaces allow selection, parameterization,

simulation, and visualization of simulation results literally within minutes.

The commercial nature of the software tools supported relevant efforts in promoting the concept of PBPK modeling, including the training of large groups of academic and industrial pharmacokineticists. These efforts catalyzed the formation of a community and raised the visibility of PBPK modeling. They also contributed to an effective overcoming of cultural hurdles that is still relevant into the late 2000s. A widespread disbelief in predictive model-based approaches in clinical development was proven false by numerous published successful case studies and was finally replaced by a general recognition and acknowledgment even by regulatory bodies (Leong et al., 2012). A striking example of this development is the majority vote of the Advisory Committee for Pharmaceutical Science and Clinical Pharmacology of the U.S. Food and Drug Administration (FDA) in support of the use of PBPK modeling for pediatric

drug development in March 2012, only 7 years after Bjorkman's (2005) first publication on PBPK-based prediction of kinetics in children.

Today, PBPK modeling (and, though still to a lesser extent, the broader concept of systems pharmacology) is an accepted element of the concept of MIDD and model-informed drug discovery and development (MID3) (EFPIA MID3 Workgroup et al., 2016), and both the FDA (<https://www.fda.gov/ucm/groups/fdagov-public/@fdagov-drugs-gen/documents/document/ucm531207.pdf>) and the European Medicines Agency (EMA) (https://www.ema.europa.eu/documents/scientific-guideline/guideline-qualification-reporting-physiologically-based-pharmacokinetic-pbpk-modelling-simulation_en.pdf) have issued dedicated guidelines for the application of PBPK modeling in a regulatory context. We will address the concept of MIDD in the last section of this chapter.

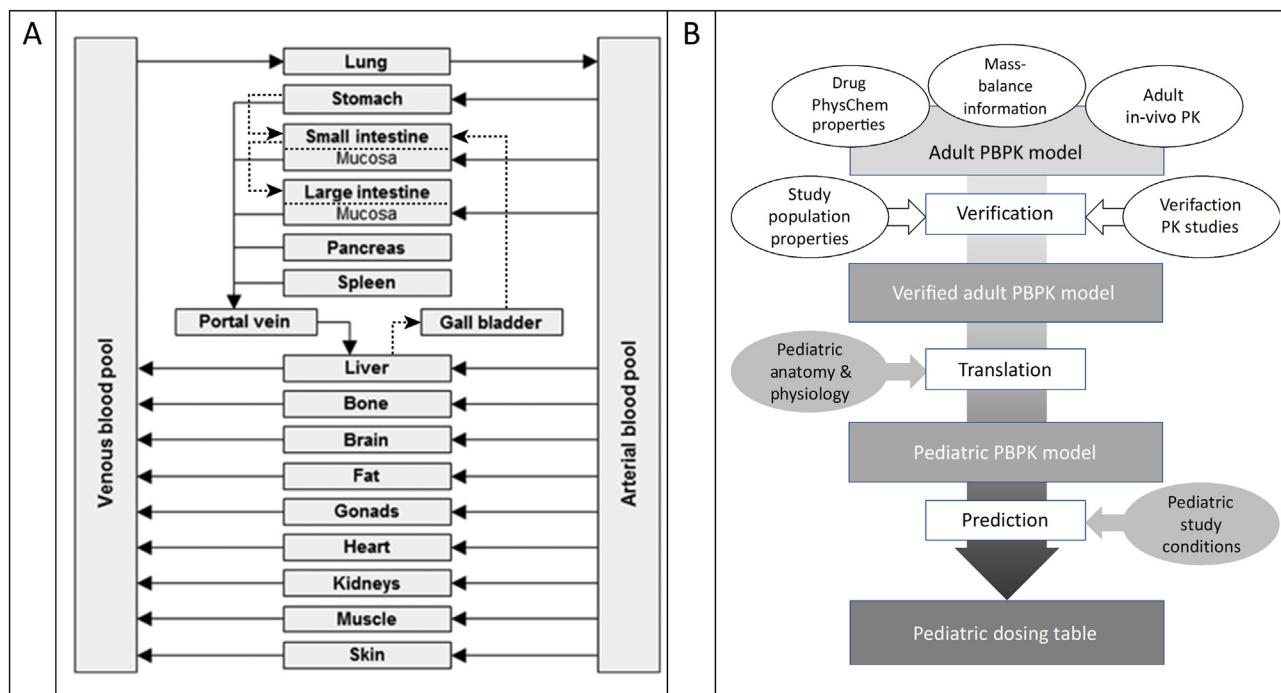


FIGURE 28.2 (A) A sketch of PBPK model. Organs and tissues are represented as boxes. Flows connecting organs with the rest of the body are shown. The venous blood pool, the lung, and the arterial blood pool close the blood circulation at the organism level. Dashed lines indicate the transport of drug doses through the gastrointestinal tract and the excretion of drug and metabolites into the bile in the gall bladder. Not shown is the substructure of the different compartments. Blood consists of plasma and blood cells. Organs are composed of a vascular space with plasma and blood cells, an interstitial space, and a cellular space in typical PBPK models. The volume of the different compartments in the formal model represents the physical volume of the tissues and subcompartments in the real world and is not reflected in the relative size of the boxes in the diagram. (B) A schematic diagram of the workflow for pediatric translation of pharmacokinetics with PBPK models. The translational predictions are based on an adult PBPK model representing available information about adult physiology, a drug, its PK in adults, and the mass-balance information quantifying its metabolism and excretion via different pathways. After verification of the validity of the adult model, information about anatomy and physiology in children of different ages is used to translate known PK in adults into predictions for children of the respective age. Predicted PK can be used to establish pediatric dosing tables such that the expected exposure in future clinical trials with children is matched to the clinical requirements of the patients. A common approach in pediatric development plans is a matching of exposures with those shown to be safe and efficacious in adult patients. *PBPK*, physiology-based pharmacokinetics; *pharmacokinetics*.

Numerous review articles and tutorials on PBPK modeling have been published, the first probably by [Himmelstein and Lutz \(1979\)](#) in the late 1970s, and facilitate further reading.

So what is PBPK modeling about? [Fig. 28.2A](#) shows a schematic diagram of a PBPK model. In a formal sense, PBPK models are nothing but compartmental models of the body representing the PK and all the processes driving it in ODE. The unique feature of PBPK is its explicit representation of anatomical entities and physiological processes. State-of-the-art PBPK models provide one-to-one representations of all relevant organs and tissues characterized by their anatomical and physiological properties such as volume, fraction vascular and cellular space, and tissue composition divided into lipid, proteins, and water. Blood is represented with its cellular mass and the protein and lipid content in blood plasma. Blood flows and lymph flows in organs represent quantitatively measured values for vascular flows rates and lymph flow. Diffusion and convection of drug molecules across the vascular endothelial barrier and cellular membranes are explicitly represented. The passage of a tablet through the GI tract, the disintegration of the tablet, the dissolution of the drug substance, its permeation across the intestinal wall into the portal vein, and its potential presystemic metabolism in enterocytes and transporter protein-mediated reflux into the luminal space are represented with a high degree of detail. Filtration in the glomeruli of the kidney and metabolism of drugs in the hepatocytes by individual liver enzymes allow a very detailed mechanistic description of the process of drug clearance.

Most of the parameters that determine the properties of the different model elements are obtained from information sources independent of PK. Among the typical examples are the 1975 Reference Man, a report of a task group of the International Commission on Radiological Protection, and more recent radiological imaging assessments for GI tract motility, blood flow rates and proteomics measurements of liver and gut wall biopsy material characterizing the enzymatic content in specific tissues. The availability of quantitative information for both typical participants of, for example, a phase I clinical pharmacology study—the proverbial young, healthy male adult—and the members of a special population of interest is the foundation for the predictive power of PBPK modeling.

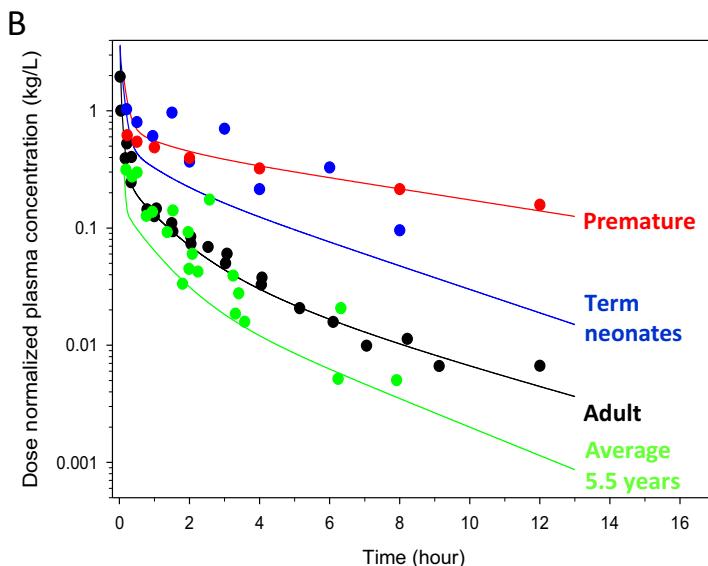
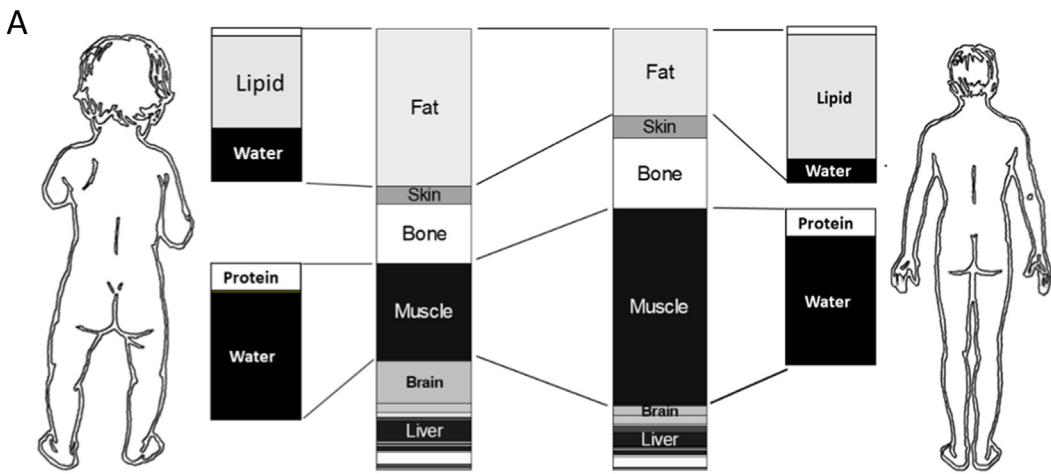
Once an appropriate qualified PBPK model has been established for the PK in the adult population for which experimental PK evidence is available, translation into children is achieved by replacing all anatomical and physiological parameters that differ between adults and children and simulating the model with the modified parameters ([Fig. 28.2B](#)). Whether a model is considered qualified depend on various aspects covered extensively

in both scientific publications (e.g., [EFPIA MID3 Workgroup et al., 2016](#)) and regulatory guidelines (e.g., EMA PBPK guidance). The most important properties in the context of this chapter are as follows:

1. the use of state-of-the-art information for all parameters characterizing the organism;
2. parameter values for drug-related properties of the model in line with in vitro characteristics of the drug substance and its drug formulation;
3. a qualitative and quantitative match between already existing PK data and model simulations.

The investigation of difference between children and adults and its use in pediatric scaling has developed into an independent research field. When growing children are compared with adults, there are many more notable differences than the obvious difference in average body weights. For example, the relative contributions of an organ to the total body weight can be quite different in a child than in an adult, as illustrated in [Fig. 28.3A](#). For example, a 1-year-old child has relatively more fat and less muscle mass than a typical young adult. The composition of the tissue with respect to the water, lipid, and protein content is also different. The fat tissue of a child has a higher water content and lower lipid content than the fat tissue of an adult. Taken together, age-dependent differences in body and tissue composition lead to differences in the (absolute and relative) sizes of aqueous and lipid compartments, which, in turn, can affect the distribution volumes of drugs depending on their lipophilicity in an age-dependent manner. Another important aspect is maturation: organ functions are typically not fully developed at the time of birth but mature over time. The duration until an organ system is fully mature can range from minutes after birth (e.g., changes in blood circulation after ventilation of the lung) to months and even years. From the pharmacokinetic viewpoint, maturation of the major eliminating organs, the kidney and liver, plays a dominant role for understanding the age dependence of clearance processes. The glomerular filtration rate (GFR), for example, has been described by a sigmoid hyperbolic model as function of postmenstrual age, reaching half of the adult reference value at 47.7 weeks and 90% of the adult GFR at 1 year ([Rhodin et al., 2009](#)). Similarly, maturation of different metabolic processes in the liver has been described by several authors in the form of so-called maturation functions, which are typically sigmoidal in shape and describe the relative activity of the respective process in growing children as a function of age or body weight relative to the adult activity ([Edginton et al., 2006; Maharaj and Edginton, 2014](#)).

[Fig. 28.3B](#) shows an example of observed and modeled age-dependent PK of morphine. The modeled



Experimental data

- Baillie et al. *Age and Aging* **18**, 258-262 (1989)
- Skarke et al. *Br. J. Clin. Pharm.* **56**, 228-231 (2003)
- Hain et al. *Br. J. Clin. Pharm.* **48**, 37-42 (1999)
- Mikkelsen et al. *Acta Paediatrics* **83**, 1025-1028 (1994)

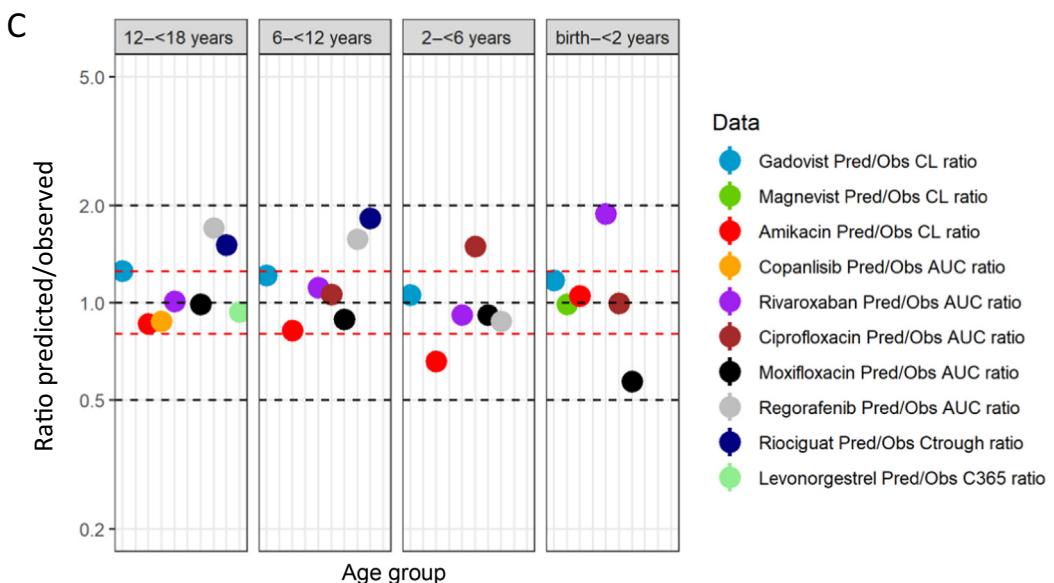


FIGURE 28.3 (A) A schematic diagram illustrating the differences in relative contribution to the total body weight in a 1-year-old child and an 18-year-old young adult. For muscle and fat tissue the different compositions in water, protein, and lipid content are shown as well. (B) Dose-normalized pharmacokinetics of morphine in four patient groups of different ages. Circles show experimental concentrations, while lines show the respective simulations with a PBPK model. For adults the model was adjusted to match the data. For children the predictions are based on adjustment of age-dependent physiological parameter (independent of morphine PK) only. (C) Comparison of derived PK parameters (area under the curve, clearances, Ctrough, C365) for 10 drugs obtained from predictive PBPK modeling and clinical trials. Data are group by age cohorts. *PBPK*, physiology-based pharmacokinetics; *PK*, pharmacokinetics.

curves were obtained as predictions following the workflow in Fig. 28.2B. After adjustment of the adult PK simulations to data, the age-related changes in body composition and known maturation functions of the relevant elimination processes were incorporated into the model, but no other modifications to the adult model were introduced. The model-based predictions therefore represent a knowledge-based translational prediction that reflects all the relevant difference between age groups, including the nonmonotonous nature of the age-dependent PK: Newborns show higher exposure than adults, while children with an average age of 5 years show lower concentrations.

For approximately 15 years, PBPK-based pediatric scaling has been available in pharmaceutical development as a basis for dosing decisions in the pediatric development plan. Fig. 28.3C compares experimentally observed PK parameters for 10 drugs that our company investigated in children over this period. (Note: Today, pediatric development is a legal obligation for pharmaceutical innovator companies in many countries, including the United States and countries of the European Union.) Depending on the drug, clearances (CL), area under the curve (AUC, the integral concentration in blood over a predefined period of time), trough concentrations at the end of the dosing interval (C_{trough}) or concentrations after 365 days of application are compared. Independent of the age group, all model-based predictions deviated from the observed experimental values by less than a factor of 2. A large proportion of the predictions deviated by less than a factor 1.25, which, in light of the typically high variability in PK, illustrates the excellent quantitative performance of the PBPK-based translation.

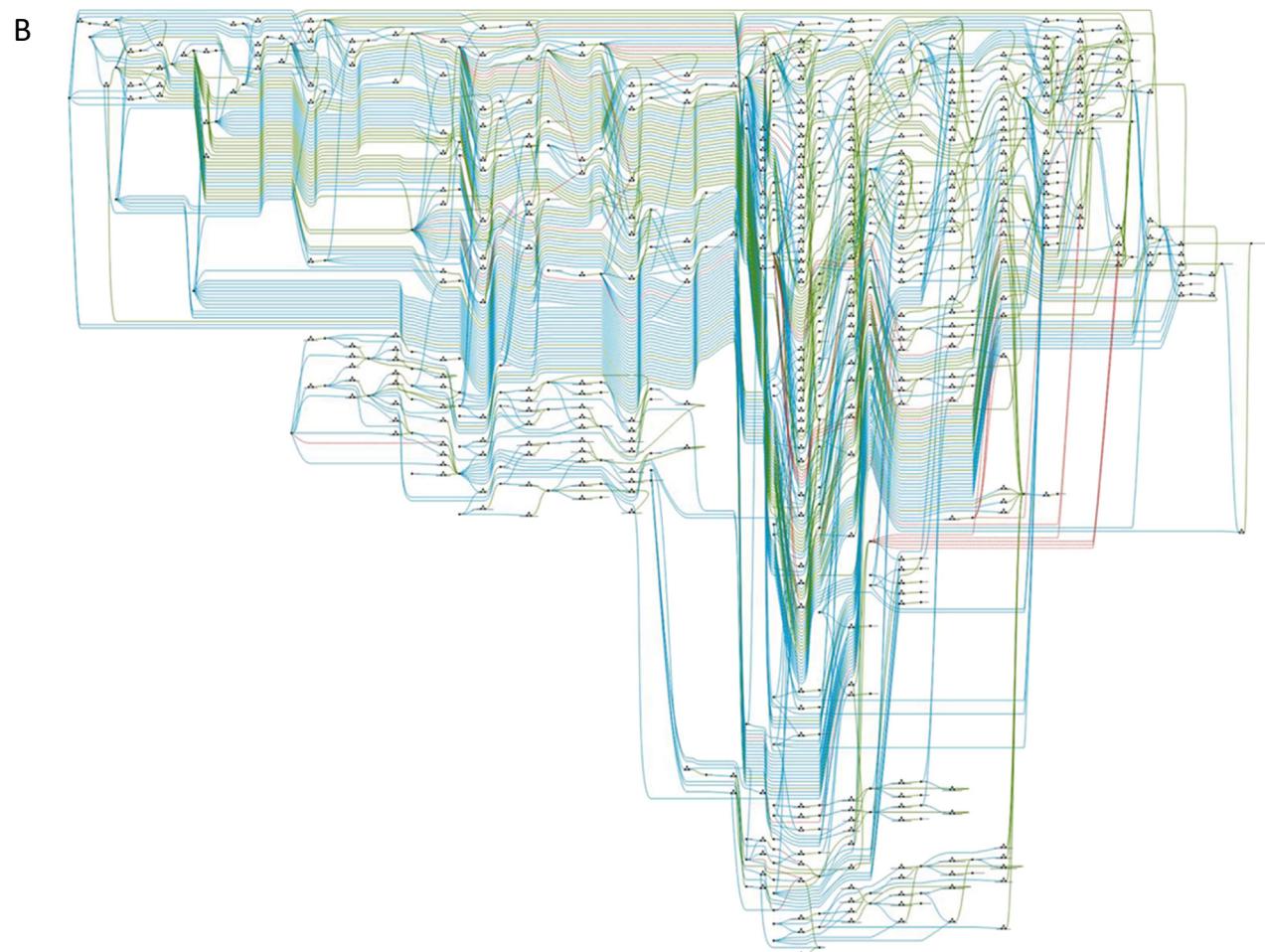
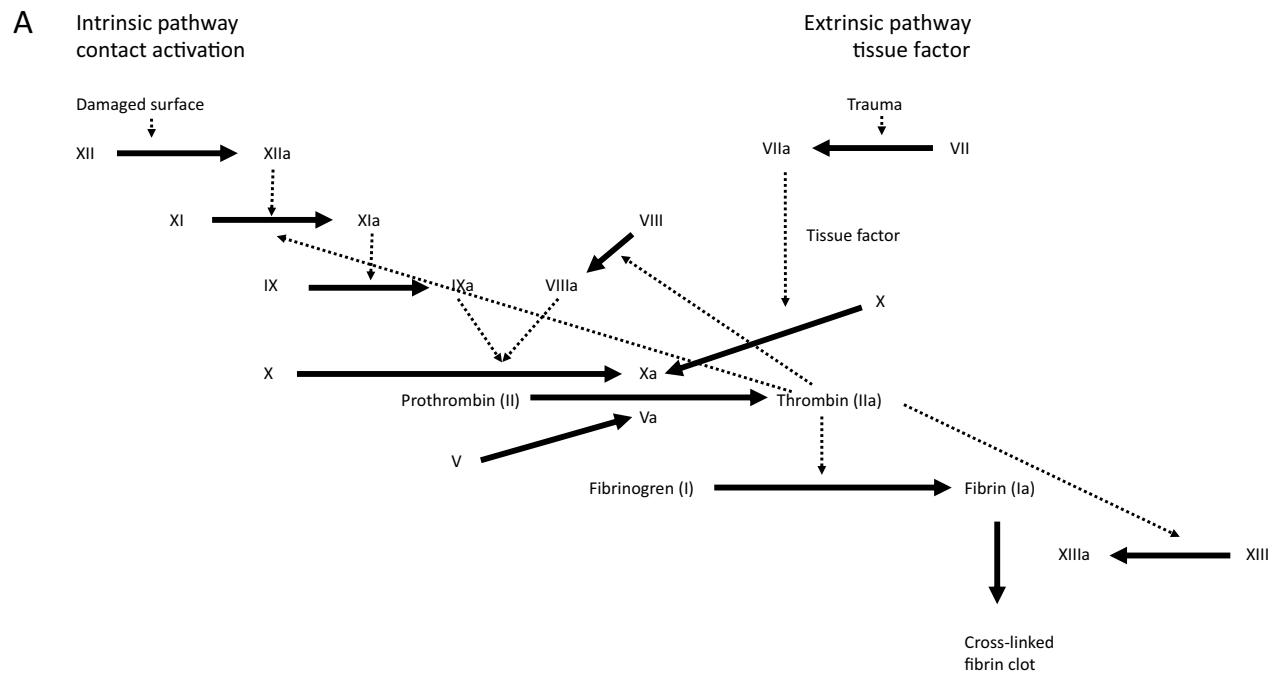
Blood coagulation modeling: Prediction of pharmacodynamic drug interactions

Up to this point, we have focused on different types of PK modeling. It is a well-developed and broadly used framework with proven predictive translational power. In this description of a translational application of computational approaches, we discuss blood coagulation modeling as an example of a computational model representing complex biochemical pathways and their interaction with cellular systems and tissue. Fig. 28.4A shows a schematic diagram of the reaction cascade underlying the clotting process in blood plasma. Triggered by biochemical activations factors such as tissue factors or surface activation, the cascade ultimately transforms fibrinogen into fibrin,

the protein that acts as glue in blood clots. While the coagulation cascade describes the molecular components of the blood coagulation pathway, the physiological clotting process also involves cellular contributions, in particular the activation, transformation, and aggregation of thrombocytes and vascular endothelial cells. Physical processes such as shear stress generated by high flow rates at stenoses and the rupture of vascular endothelial plaques and the resulting release of biochemical factors are also important. However, the model of blood coagulation shown in Fig. 28.4A is nothing but a graphical representation of a computational model. It does not represent these components of coagulation physiology (Hockin et al., 2002; Burghaus et al., 2011). It is another example of the abstraction and simplification that are necessary in model building.

Despite all its obvious differences from PBPK modeling, blood coagulation modeling also belongs to the physiologically based mechanistic modeling approaches. Objects and phenomena to be modeled are represented in a one-to-one fashion wherever possible. Concentrations of zymogens and coagulation factors quantitatively reflect concentrations measured in human blood plasma. The catalytic activity of activated factors reflects the reaction kinetics of factor activation steps measured in *in vitro* assays. Other properties, such as the coenzymatic nature of lipid surfaces of cells *in vivo* or the mimicking effect of pegylated lipids *in vitro*, are parameterized in a so-called effective way. Parameter values are chosen such that the observed quantitative systemic behavior of the model is in line with experimental data obtained in laboratory experiments with blood or plasma samples.

A clinically highly relevant coagulation assay is the so-called prothrombin time (PT) test. It measures the time required to reach a threshold activation level of thrombin (measured with optical or mechanical means) in a blood cell free buffered human plasma after activation with calcium and tissue factor. In anticoagulation therapy with warfarin, a vitamin K antagonist inhibiting the synthesis of coagulation factors II, VII, IX, and X, a calibrated version of the PT test is used for therapeutic drug monitoring. The dependence of the effect of warfarin in both intrinsic factors, such as the genotype of liver enzymes, and extrinsic factors, such as diet, results in high interindividual and intraindividual occasion variability. Since the therapeutic window for warfarin is narrow and underdosing can result in insufficient protection from thrombotic events such as ischemic strokes while overdosing can cause intracranial or GI tract bleeds, patients on warfarin therapy need to be continuously monitored. A PT test is used, and the



(Continued)

prolongation of the PT time relative to a standardized control is reported as the so-called international normalized ratio (INR) value. For stroke prevention the target INR range in patients with atrial fibrillation is 2–3 (Hindricks et al., 2020). In clinical practice, warfarin doses are adjusted by the treating physician when the INR of a patient no longer meets the target corridor for INR.

In clinical drug development of direct inhibitors of coagulation factors (e.g., ximelagatran and dabigatran, which are direct thrombin inhibitors, and apixaban and rivaroxaban, which are direct inhibitors of factor Xa), investigators of these drugs had to switch patients from warfarin treatment to their study drugs when they were enrolled in clinical trials. Two complicating properties of warfarin made this task a clinical challenge with high potential to cause safety issues. Warfarin has a long PK half-life. Relevant drug concentrations circulate even days after treatment discontinuation (Fig. 28.5A). In addition, the recovery time of the coagulation factors inhibited by warfarin have half-life ranging from 1 to 3 days. Even in the absence of warfarin, recovery of fully normal levels of these factors will take up to a week (Fig. 28.5A). Investigators had to decide when to start the treatment with the study drug and at what initial dose to prevent unnecessary periods without anticoagulation, driving up the risk of strokes, and at the same time not introducing an inappropriate bleeding risk. The latter is a potential consequence of the direct inhibition of a still suppressed coagulation cascade with low coagulation factor concentrations.

Blood coagulation modeling can be applied to investigate this involved matter of transient pharmacodynamic drug-drug interaction to derive an appropriate dosing schedule during the time of transition from one pharmacological concept to another. The coagulation model introduced in Fig. 28.4 adequately represents the dependency of the INR and varying coagulation factor levels (Fig. 28.5B).

Adding a direct inhibitor of thrombin such as ximelagatran on top of the residual suppression of the coagulation systems after discontinuation of a warfarin treatment will potentially inhibit coagulation beyond the intended level and lead to exaggerated pharmacology. Fig. 28.5C shows the clotting times resulting from the simulation of the blood coagulation model. A constant ximelagatran concentration leads to massive prolongation of the PT time right after warfarin discontinuation, although its

effect on PT time is almost negligible when warfarin washout has effectively taken place at the end of the simulated interval. A comparison of the predicted synergistic effect with the naïve assumption of an additive effect of the two pharmacological concepts demonstrates the clinical relevance of the interaction (Fig. 28.5C). Based on the assumptions that the effective prolongation of PT times should never exceed values defined by guidelines for warfarin treatment, the computational models can be used to determine target ranges for ximelagatran plasma concentrations as a function of time after discontinuation (Fig. 28.5D). These could then be used consecutively in combination with a PK model of ximelagatran to derive a dosing table for patients who are switching treatment.

The described effects also need to be considered at the end of a clinical study when the reverse switch is required. Burghaus et al. (2014) and Siegmund et al. (2015) investigated both scenarios for warfarin and rivaroxaban, a direct factor Xa inhibitor, in some detail. While our short description focuses on the application in clinical trial settings, the concept can ultimately support a comprehensive drug label when drug candidates receive marketing authorization and become available in clinical practice.

Model-informed drug development and discovery (MID3)

In the preceding sections we introduced the general concept of model building and provided concrete examples for the use of computational modeling in translational applications. Over the past decades the continuous use of computational models in pharmaceutical R&D has led to the publication of numerous applications along the full value chain of research and development into regulatory assessment (Woodcock and Woosley, 2008) and drug life cycle management (Fig. 28.6). In 2016, the MID3 working group of the EFPIA (European Federation of Pharmaceutical Industries and Association, 2016) reviewed approximately 100 primary publications of cases studies addressing very diverse classes of R&D challenges (EFPIA MID3 Workgroup et al., 2016). The rich experiences with model-based approaches are also reflected in a robust understanding of best practices and minimal requirements, ensuring the reliability of model-based translational predictions, the main objective of the MID3 publication. Today, the recognition has reached a

FIGURE 28.4 (A) Schematic representation of a blood coagulation model. The biochemical cascade leading to the activation of coagulation factors and ultimately fibrin is shown as reaction arrows linking zymogens to activated coagulation factors shown with their roman numbers or names. Dotted arrows represent the catalytic activity of enzymes. (B) A screenshot of the graphical representation of the model in MoBi, a computational tool that allows the modeling and simulation of physiologically based pharmacokinetic models and biochemical cascades (<https://github.com/Open-Systems-Pharmacology/>).

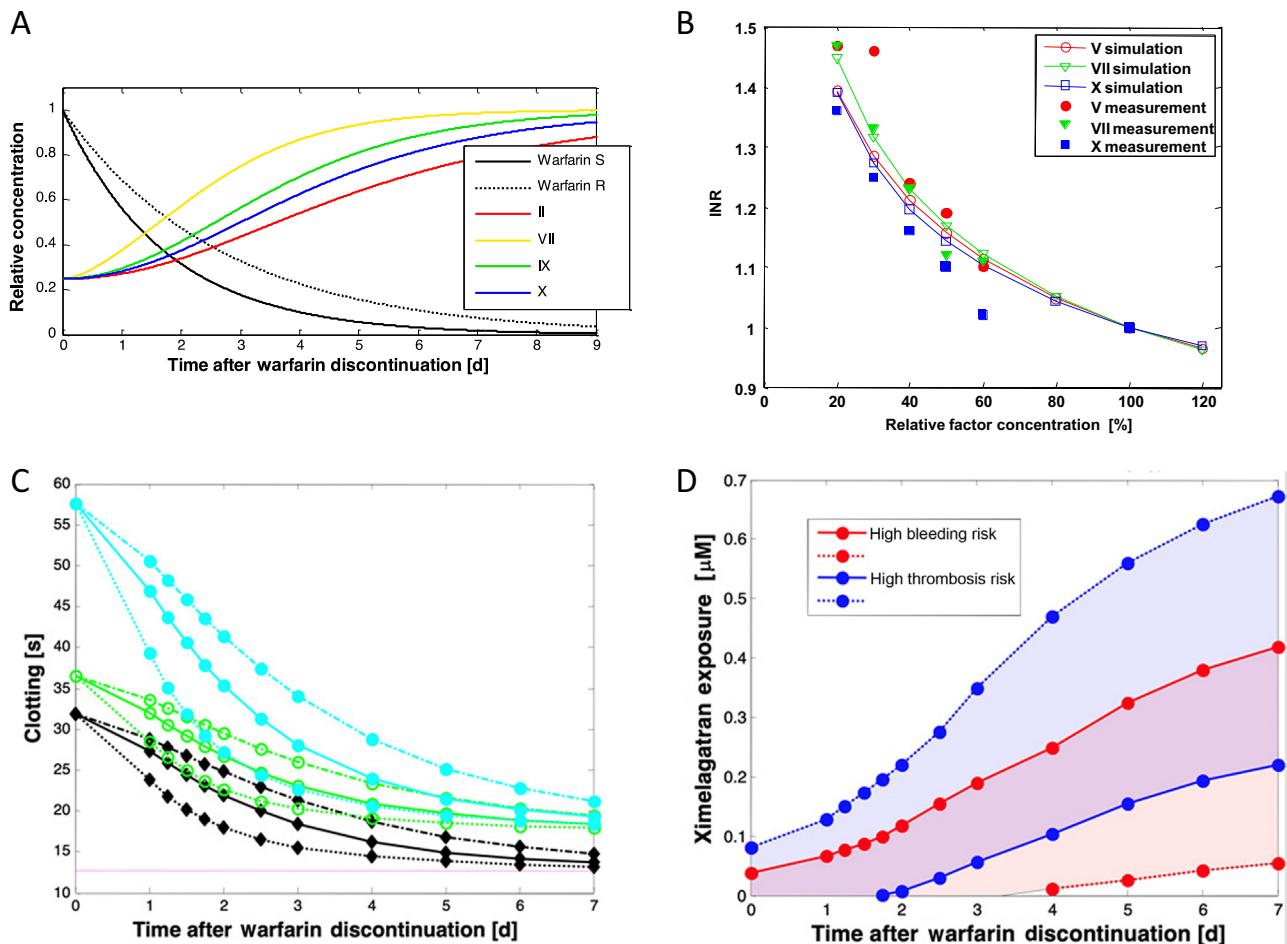


FIGURE 28.5 (A) Pharmacokinetics of the two enantiomers of warfarin after discontinuation of the drug treatment and recovery of the inhibited coagulation factors. The relative decline of the concentrations of S and R warfarin are shown as solid and dashed black lines. Concentrations below 10% of the initial concentrations are only reached after 4–7 days. The recovery of the concentration of coagulation factors II, VII, IX, and X to normal nonsuppressed levels is shown by colored lines. (B) The INR measured with calibrated PT tests is sensitive to coagulation factor levels. Solid symbols show experimental lab data for blood plasma with varying factor V, VII, and X concentrations but otherwise normal composition. Dotted lines and open symbols show results of a simulations with the blood coagulation model under matched conditions. (C) A treatment with the direct thrombin (factor IIa) inhibitor ximelagatran on top of a slowly decaying warfarin effect on PT time after treatment discontinuation starting at an INR = 2.5 (black symbols; solid line, typical decay, dotted line, high clearance scenario, dashed line, low clearance scenario) is simulated with the coagulation model (cyan line and symbols) and compared to the assumption of a strictly additive effect on PT times (green lines and symbol). The ximelagatran concentration is assumed to be constant 0.25 μM . (D) Visualization of target exposures for ximelagatran as a function of time after warfarin discontinuation in patients with starting INR = 2.5. The red area represents ximelagatran concentration range compatible with a low INR range for patients with an assumed high bleeding risk. The blue area represents the corresponding range for patients with an assumed high thrombosis risk. INR, international normalized ratio; PT, prothrombin time.

level at which major regulatory agencies around the globe have issued guidelines for industry regulating the use of models for specific applications, such as DDIs or special population PK, and the general framework of MIDD. The FDA offers a dedicated regulatory track for MIDD applications as part of a pilot program. The International Council for Harmonization has initiated a workstream to develop an MIDD umbrella guideline with the intent to

establish global standards for model-based approaches as an independent source of evidence.

All these developments demonstrate that model building and the use of computational models have become an indispensable concept in the life sciences and also need to be considered in all serious research activities in translational medicine whenever the aim is to provide reliable quantitative characterizations or predictions.

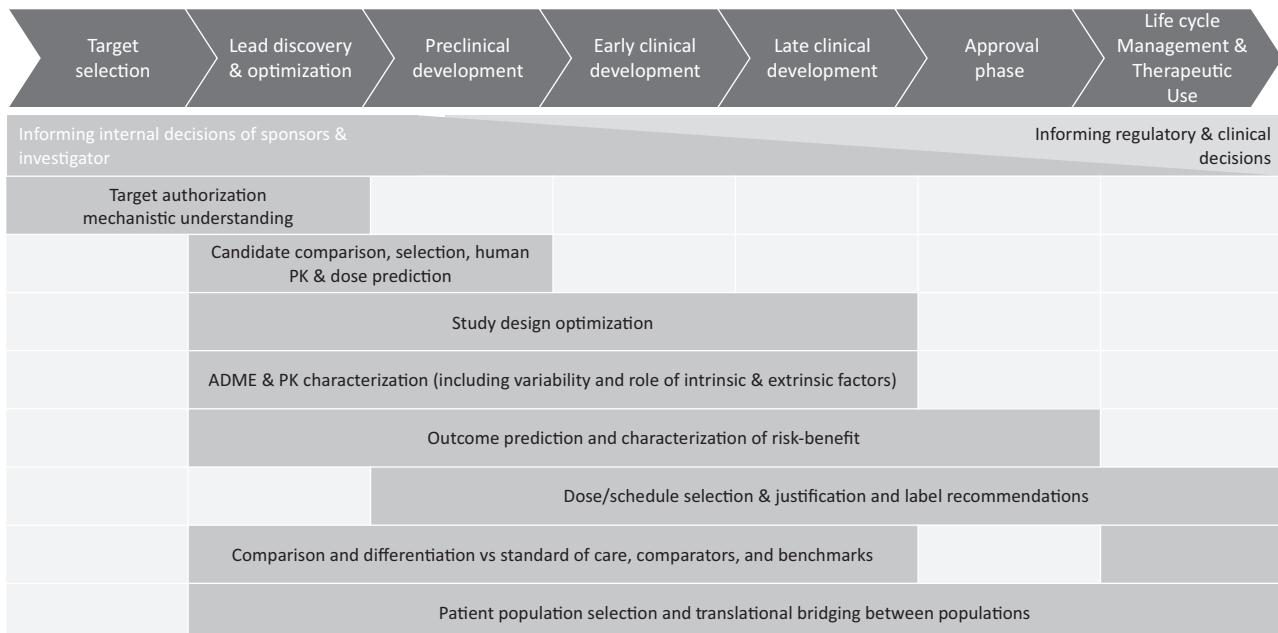


FIGURE 28.6 Typical applications of model-informed drug development and discovery along the value chain of today's pharmaceutical R&D paradigm. Adopted from EFPIA MID3 Workgroup, Marshall, S.F., Burghaus, R., Cosson, V., Cheung, S.Y., Chenel, M. et al., 2016. Good practices in model-informed drug discovery and development: practice, application, and documentation. *CPT Pharmacomet. Syst. Pharmacol.* 5(3), 93–122.

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Chapter 29

Intellectual property and innovation in translational medicine

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Abstract

Translational medicine continuously intends to find faster, less expensive, and more effective ways to translate basic research into clinical applications. Intellectual property can contribute to making this transition. This chapter provides an overview of the way in which intellectual property contributes to the medical translation process, including details about patents both in general and in relation to specific medical tools and products. Since access to information and to research tools as well as collaborations and partnerships are instrumental in this process, this chapter also discusses the importance of combining practices of patenting with principles of open science.

Keywords: Patents; patents in genomic medicine; intellectual property in genomic medicine; innovation in genomic medicine; open science; partnerships in biomedical research; collaboration in biomedical research

Introduction

The large sums of private and public money and time that have been devoted to research and development and the scientific and technological advancements in medicine have not yet translated into many clinical products (Edelman and FitzGerald, 2019; Bornstein and Licinio, 2011; FitzGerald, 2005). Translational medicine intends to find ways to remedy these delays and encourage a faster, less expensive, and more effective translation from basic research done in the labs to clinical applications that benefit patients. Understanding how translational medicine works and finding ways to make it more fluid, responsive, and timely have proved to be of utter importance as the world deals with the COVID-19 pandemic (Edelman and FitzGerald, 2019). Intellectual property, used strategically, can contribute to this process. To provide an overview of the way in which intellectual property can be of assistance in the medical translation process, this chapter will

describe details about patents both in general and in relation to specific medical tools and products. Since access to information and to research tools can be instrumental in translational medicine to further medical innovation, this chapter refers as well to principles of open science that promote such access. Finally, it will also discuss the importance of collaboration and partnerships and of combining practices of patenting with principles of open science to achieve that collaboration and those partnerships.

Context

General description of translational medicine

Translational medicine (TM) is the branch of medical research whose purpose is to facilitate a faster, easier, more fluid, more responsive, more timely, and less expensive transition between preclinical medical research (also known as basic research) and clinical applications that could cure diseases in humans, thus allowing the achievement of the social value of science as the source of useful products that improve people's lives (Edelman and FitzGerald, 2019; Wehling, 2010; Joly et al., 2012). The translation process can be understood as comprising three stages. The first stage translates from basic science (laboratory, *in vitro* and *in animals*) to potential clinical applications. The second stage involves the translation of the proposed human applications into proven clinical applications. In this stage, drug development and safety and efficacy evaluations (e.g., clinical trials) take place. The third stage translates the proven clinical applications into the implementation and adoption of clinical practices (Drolet and Lorenzi, 2011). The list of issues that need to be addressed in the execution of TM includes scientific research (e.g., biological, genetic, medical, biomedical, etc.), funding and business decisions, regulatory systems (including clinical trials, among other issues), legal and ethical frameworks, communication and networking among the

different parties, product development, and intellectual property (Albani and Prekken, 2009; Prestwich et al., 2012). However, TM should not just focus on enhancing the interconnectedness between academia and the industry to enable and improve the development of drugs, devices, and diagnostics; it should also extend to training the next generation of translational scientists, maintaining an adequate level of investment throughout the translation pipeline, and facilitating access to the drugs, devices, and diagnostics that result from translation medicine (Edelman and FitzGerald, 2019).

One of the most common barriers to the translation of biomedical research is that many researchers, particularly those in academia, tend to focus on areas or projects in which they are interested based on their scientific value (*l'art pour l'art*) and on writing papers with a strong scientific impact. This research activity does not necessarily result in clinical applications (Wehling, 2010). TM could influence the focus of some researchers by suggesting areas or projects with higher probabilities of translation into clinical applications or with higher social value. Another problem is the high costs associated with the development of innovative drugs, diagnostic tests, and medical devices. On the one hand, academic research is expensive and usually publicly funded. The high costs associated with academic research are likely to make exclusive public funding unsustainable. On the other hand, among the sources of the highest costs taken on by the industry are typically the clinical trials that must be undertaken to determine the efficacy and safety of a particular product on human beings. TM can help to broaden the sources of funding by facilitating early collaboration and partnerships between academia and industry, making the process less burdensome for the parties involved. Another difficulty is the lack of awareness of a significant confusion about the benefits and limitations of the different options of protection, commercialization, and collaboration that are available at the various stages of the research and development (R&D) process. Hence whereas some researchers and companies resort to secrecy, others focus on pursuing excessive proprietary rights, thus limiting the possibility for early and fruitful collaborations. TM can help to raise the awareness of researchers and companies about these options and train them on the potential routes and the opportunities of using those alternatives (Granados Moreno et al., 2018; Spithoven et al., 2010; Tuomi, 2016; Lampert et al., 2017; Ali-Kahn et al., 2017, 2018).

The costs associated with R&D and market approval are usually transferred to the patient. In the United States, for example, in 2017 the retail prescription drug bill was \$333 billion, and government spending on pharmaceutical products was expected to rise by an average of 6.3% a year from 2017 to 2026 (Edelman and FitzGerald, 2019). Alternative and

mixed models of protection, collaboration, and commercialization that are more efficient can be used by actors in TM to reduce the costs of R&D and market approval, consequently lowering prices for the patients (Granados Moreno and Joly, 2020; Granados Moreno et al., 2018).

Currently, the COVID-19 pandemic has made it clear that governments, academia, and industry need to engage in a sustained effort to have a faster, more responsive, more timely, and more cost-controlled translational pipeline, not only to treat localized or identified diseases, but also to avert future pandemics. It has also highlighted the importance of providing affordable, widespread, and global administration of vaccines and therapies when they are discovered (Zerhouni et al., 2020).

Strategies and practices of intellectual property (as part of a broader scheme of commercialization)¹ and principles of open science are tools of TM that can facilitate collaborations and partnerships among research groups and thereby contribute to the achievement of the tasks and objectives of TM (Granados Moreno and Joly, 2020; Granados Moreno et al., 2018; Gold, 2016). In addition to the obvious scientific and technological background required, execution of such a combination involves knowledge and concepts from health policy, health economics, law, and social marketing communication (Glasgow, 2009). Researchers, government, the biotechnology and pharmaceutical industries, physician scientists, regulators, policymakers, investors, business developers, nongovernmental organizations, and funding agencies are some of the parties that would benefit from these practices and strategies (Translational Medicine, n.d.; Bubela et al., 2012; Wehling, 2010; Gold, 2016).

Intellectual property and translational medicine

One of the most successful ways to translate basic research into clinical applications is through collaboration and partnerships from the early stages of basic research (bench) to the stages of production of clinical applications (bedside). Early partnerships of biotechnology and pharmaceutical industries with universities and public research centers could enable all the parties to jointly make decisions on which research projects to develop and the steps that should be taken, despite their well-known differences in incentives and viewpoints concerning innovation. This could lessen the problem of having many academics focusing on research that does not necessarily translate into clinical applications (Bornstein and Licinio, 2011; Gold, 2016; Edelman and FitzGerald, 2019; Granados Moreno and Joly, 2020). Early collaborations between basic and clinical researchers that enable them to share knowledge and findings obtained from the in vitro or animal and human stages of the research may permit researchers to implement

1. Commercialization is defined as “the process of extracting economic value from new products, processes and knowledge through the use of intellectual property rights, licensing agreements, and the creation of spin-off companies” (Joly et al., 2010).

shorter, less expensive, and more successful clinical trials ([Shaw, 2017; Gold, 2016; Wehling, 2006](#)). Collaborations and partnerships could also enable the sharing of costs and risks associated with the R&D process between basic researchers (e.g., universities) and clinical researchers (e.g., biotechnology and pharmaceutical companies), which would eventually make it less burdensome for both. Partnerships and commercialization strategies can encourage continued public funding and attract other sources of funding, including private ones ([Granados Moreno and Joly, 2020](#)).

Forms of intellectual property (e.g., patents, licenses, patent pools²) developed to protect the findings and resulting inventions to facilitate collaborations and partnerships ([Joly et al., 2012; Gold, 2008; Caulfield et al., 2012; Granados Moreno and Joly, 2020; Gold, 2016](#)). Intellectual property rights also help to assign market value to different products resulting from research. They do so by conveniently packaging information, knowledge, and products in easily tradable units, such as patents or copyrights ([Hope, 2008; Granados Moreno and Joly, 2020](#)). Market value can enable market expectations regarding the outcomes of research, thereby incentivizing actors to create and join collaborations and partnerships. The market value that is assigned to the various products also facilitates negotiations during the creation and development of collaborations and partnerships by determining the value of the collaborators' and partners' contributions³ ([Bornstein and Licinio, 2011; FitzGerald, 2005; Granados Moreno and Joly, 2020](#)).

Basic functioning of patents⁴

Patents, along with trade secrets and licensing agreements, are the dominant forms of intellectual property and the most commonly used tools associated with biotechnology and pharmaceutical research, development, and commercialization ([Zerhouni et al., 2020; Bubela et al., 2012; Joly et al., 2012](#)). Patents grant their holders a 20-year⁵ set of exclusive rights to make, use, sell, and import the invention they protect. Inventions⁶ can be patented provided that they are new, result from an inventive activity, are useful (or have

a utility or industrial application in some jurisdictions), and fall within what constitutes patentable subject matter ([Mexican Industrial Property Law, 2020; U.S. Patent Act USC Title 35, 1952; European Patent Convention, 2016; Canadian Patent Act RSC 1985c. P-4, 1985; Canadian Patent Office, 2019; Vaver, 2011](#)).

An invention is considered new if it has not been described, disclosed, or predicted in the national or international prior art by the day on which the patent application is filed or within the 12 months prior to that filing date. Prior art in medical research includes written documents (e.g., scientific or technical articles, books, newspaper or magazine commentaries, teaching materials), patent applications, oral presentations, conferences, and Internet posts available to the public ([Mexican Industrial Property Law, 2020; Canadian Patent Office, 2019; European Patent Office, 2019; U.S. Patents and Trademarks Office, 2014](#)). To maintain the invention's novelty, it is recommended that the researcher make information describing the invention available to the public only when the patent application has been filed or within the 12 months prior to such a filing. It is also recommended that the researcher use confidentiality agreements to protect the novelty of the invention by contractually preventing those individuals with access to the information related to the invention from disclosing it.

An invention results from an inventive activity⁷ (or it is nonobvious⁸) when the inventor employed some creativity or ingenuity in its conception and production. It also requires that an unimaginative person skilled in the art does not see the invention as the next obvious thing to happen in the technical or scientific field at the date on which the application is filed. In this context a person skilled in the art would be a fictional researcher, physician, or clinician with ordinary common scientific expertise and access to publicly available information associated with the invention. As in the case of novelty, this requirement is evaluated at the date on which the patent application is filed ([Canadian Patent Office, 2019; European Patent Office, 2019](#)).

2. A patent pool is a set of agreements between two or more patent holders to license one or more of their patented inventions to one another or to third parties ([Andrews, 2002; Zimmeren et al., 2011](#)).

3. The drawbacks of certain practices of intellectual property are introduced at the end of the following section.

4. The overview herein described covers the way in which patents are regulated in the United States, Canada, Mexico, and Europe. It makes references to the [Canadian Patent Act RSC 1985 c. P-4, 1985](#), the [European Patent Convention \(2016\)](#), the [Mexican Industrial Property Law \(2020\)](#), the [U.S. Patent Act USC Title 35, 1952](#), the [Canadian Manual of Patent Office Practice \(Canadian Patent Office, 2019\)](#), the [European Directive 98/44/EC on biotechnological inventions \(European Parliament, 1998\)](#), and the [European Patent Office Guidelines \(European Patent Office, 2019\)](#).

5. This 20-year period starts on the date on which the patent application is filed ([U.S. Patent Act USC Title 35, 1952; Mexican Industrial Property Law, 2020; Canadian Patent Act RSC 1985 c. P-4, 1985; European Patent Convention, 2016](#)).

6. "Invention" is defined as any human creation (e.g., art, process, machine, manufacture, composition of matter) that transforms matter or energy that already exists in nature for the benefit of men so they can satisfy their needs ([Mexican Industrial Property Law, 2020; U.S. Patent Act USC Title 35, 1952; Canadian Patent Act RSC 1985 c. P-4, 1985; Canadian Patent Office, 2019](#)).

7. ([European Patent Convention, 2016; Mexican Industrial Property Law, 2020](#)).

8. ([U.S. Patent Act USC Title 35, 1952; Canadian Patent Act RSC 1985 c. P-4, 1985; Canadian Patent Office, 2019](#)).

An invention is considered useful⁹ (or susceptible to having an industrial application¹⁰) when it has a practical utility or it can be produced or used for the purposes described in the patent application. The utility, usefulness, or industrial application either is demonstrated or can be soundly predicted at the date on which the patent application is filed (Canadian Patent Office, 2019). For instance, a drug is patentable as a product and its active ingredient as a composition of matter provided that they treat or are predicted to be able to treat a specific disease in a new and nonobvious way. This association with a specific disease or disorder constitutes the utility or industrial application.

There are four categories of innovations relevant to medical innovation that are commonly considered outside the patentable subject matter and are therefore excluded from patent protection in most jurisdictions (Canadian Patent Office, 2019; European Patent Office, 2019; Mexican Industrial Property Law, 2020; World Trade Organization, 1994; Vaver, 2011). The first category of innovations excluded from patentability includes essentially biological processes for the production, reproduction, and propagation of plants and animals.¹¹

Higher life forms are the second category. However, this exception does not exist in all jurisdictions. Whereas Mexico and Canada consider higher (multicellular) forms live organisms, such as the oncomouse,¹² unpatentable (Harvard College v. Canada Commissioner of Patents, 2002; Mexican Industrial Property Law, 2020), in the United States, nonnaturally occurring, nonhuman multicellular living organisms, including animals such as the oncomouse, are patentable (Diamond v. Chakrabarty, 447 U.S. 303, 1980).

The third category of innovations excluded from patentability includes biological and genetic material as found in nature and discoveries that merely reveal something that exists in nature, regardless of whether they are known. This includes the human body and its parts in any of its different states of development. The exclusion from

patentability in these two categories (higher life forms and biological and genetic material) is explained by the lack of human inventiveness or ingenuity in materials and discoveries that already exist in nature. As we will discuss later in the chapter, this category of innovations is relevant in TM because the main issue debated in cases involving gene patents has been whether isolated gene sequences fall within this exception.

The last category of exceptions includes surgical and therapeutic methods¹³ used in human beings or animals to cure, prevent, or ameliorate an ailment or to treat an abnormality or deformity. The reason behind this exception is to prevent having patent rights interfering with saving a human or animal life. Methods involving the excision of tissue, organ, or tumor samples from the body are considered forms of surgery and therefore unpatentable (Canadian Patent Office, 2019). However, this exception does not extend to drugs, products, or instruments used for treating human or animal illnesses or to purely cosmetic treatments, all of which are patentable (European Patent Office, 2019; Guerrini et al., 2016).

Patents are applied for on a country-by-country basis, and they have only national coverage of protection. In each country the application has to comply with national legal and administrative requirements. This is true even in the specific case of a European patent.¹⁴ Even though there are no patents with international coverage, a PCT application¹⁵ filed at the moment of filing the first patent application allows all the patent applications filed in different countries to have the same filing (priority) date (Vaver, 2011; Patent Cooperation Treaty, 2001).

There are four types of patents associated with medical research: patents on products (e.g., drugs, diagnostic tests, medical devices, vaccines, 3D-bioprinters), patents on processes or methods (e.g., methods that test genetic alterations or abnormalities, methods of extraction, methods to manipulate or edit genes), patents on compositions

9. (Canadian Patent Act RSC 1985 c. P-4, 1985; Canadian Patent Office, 2019; U.S. Patent Act USC Title 35, 1952).

10. (Mexican Industrial Property Law, 2020; European Patent Convention, 2016).

11. Plants breeders' rights (another form of intellectual property) usually protect this type of processes. This exclusion prevents multiple intellectual property rights on the same advance or invention.

12. The oncomouse was a genetically modified mouse created at Harvard University to carry an activated gene that significantly increases the mouse's susceptibility to cancer used in cancer research.

13. Some jurisdictions, such as Europe and Mexico, also exclude diagnostic methods practiced on the human body from patentability. However, this exception does not extend to diagnostic tests performed on body tissues or fluids after they have been removed from the human body, provided that those tissues or fluids are not returned to the human body (European Patent Office, 2019; European Patent Convention, 2016; Mexican Industrial Property Law, 2020) The United States, by contrast, allows all medical and surgical methods (U.S. Patent Act USC Title 35, 1952; U.S. Patents and Trademarks Office, 2014; Mayfield, 2016a,b).

14. A European patent is filed before the European Patent Office (EPO) in accordance with the European Patent Convention. A European patent does not grant international protection. For a European patent to have effect and be enforceable in any European country, it is required that a translation of the European patent granted be filed, evaluated, and granted in accordance with national law by each of the European countries where patent protection is sought.

15. A PCT patent application is a patent application filed in accordance with the Patent Cooperation Treaty. For more information, see PCT FAQ at World Intellectual Property Organization online: <http://www.wipo.int/pct/en/faqs/faqs.html>.

of matter (e.g., inventions that combine different types of genetic material, inventions concerning genes and the role they play in the development of diseases or in regulating bodily functions, bioink), and patents on improvements (Mayfield, 2016a,b; Cook-Deegan, 2008; Yoo, 2015; Li, 2014; Vaver, 2011; Resnik, 2004).

A patent holder can be a person, a group of people, or a company, organization, or institution. The patent holder has the right to exclusively make, use, sell, and/or import the patented invention. Patent rights may be assigned¹⁶ or licensed¹⁷ to third parties, thus allowing patent holders to profit from and commercially exploit their patented inventions.

Users (e.g., researchers) are allowed to use patented inventions without needing to obtain the patent holder's authorization (known as users' rights) in the following cases. Researchers can use a patented invention for experiments and research; however, the concept of "research" in this context is vague, and the extent of use permitted varies from jurisdiction to jurisdiction.¹⁸ Generic manufacturers can use the patented invention to apply for a generic drug's regulatory approval to enter the market and to submit supporting samples before the patent of the original drug expires.¹⁹ Developed countries are allowed to export and less developed countries may obtain a compulsory license to import patented drugs.²⁰ The purpose of users' rights in

general is to balance the interests of both parties (patent holders and users), limit the extent of control granted to the patent holders, and ensure the promotion of innovation. Additionally, in the area of medicine, users' rights also intend to promote access to healthcare. In addition to the above-mentioned users' rights, the patent system provides two cases in which a compulsory license might be granted: when the government needs to address a national emergency or extreme urgency (e.g., a public health crisis) and when the patent holder is considered to be abusing his or her patent.²¹

The patent application comprises a description (also known as disclosure) of the invention,²² drawings, graphics, or blueprints that complement the description; a summary or abstract of the description; and one or more claims that define and delimit the scope and extent of the protection requested. In patent applications in which gene sequences are involved, it is necessary to include a list of the relevant nucleotide or amino acid sequences. If the invention refers to or includes biological material that cannot be described, some patent offices require a deposit of such biological material with the International Depository Authority (Canadian Patent Office, 2019; European Patent Office, 2019; Mexican Industrial Property Law, 2020; Regulations for the Implementation of the European Patent Convention, 2015).

16. Assignments transfer ownership on the patented invention (and all the rights granted by the patent) to a third party. They may be partial or total and must be in writing.

17. Licenses grant a third-party consent or permission to use, make, sell, or import the patented invention. They may be sole, exclusive, or nonexclusive. Exclusive licenses need to be in writing, whereas others may be oral. Licenses may be gratuitous or for a royalty.

18. The reason behind this right is to allow and encourage the use of the information and knowledge disclosed in the patent application to promote further innovation. This exception is valid provided that it does not conflict with the patent holder's right to commercialize his or her invention. The uncertainty on the definition of "research" for purposes of this exemption is reflected in the different approaches taken in different jurisdictions. In Canada, fair research, development, and experimentation using a patented invention is allowed without the need for the patent holder's authorization until the moment at which the product is ready to be shown to a prospective customer or the product is approved to be commercialized (which is when commercialization is considered to begin). In the United States, by contrast, university research does not fit in this category of allowed use because faculty members' research is considered to be furthering the university's business (Vaver, 2011; Resnik, 2004; *Madey v. Duke University*, 2002; Organisation for Economic Co-operation and Development, 2004).

19. With this users' right, the generic manufacturer can start marketing the generic version of the patented drug right after the patent expires. The purpose of this users' right or exception is to avoid extending the monopoly that a patent grants beyond the stated term of 20 years [Drug Price Competition and Patent Term Restoration Act (Hatch-Waxman Act), 1984], [*Roche Products, Inc. v. Bolar Pharmaceutical Co.*, 733F.2d 858 (Fed. Cir. 1984)], (EC Directive 2004/28/EC) (EC Directive 2004/27/EC).

20. The purpose of this right is to address problems in accessing healthcare in least developed countries (Vaver, 2011; Doha Declaration on the TRIPS Agreement, 2001; Agreement on Trade Related Aspects of Intellectual Property Rights, 1995).

21. Patent holders are considered to be abusing their patent when they fail to meet the local demand for a patented product, when they unreasonably refuse to grant a license, or when they impose abusive terms in a license. In these cases the government could grant a nonexclusive compulsory license to use the patented invention without the patent holder's authorization for the scope and duration of the purpose for which it was authorized (e.g., national health emergency) and give the patent holder a reasonable fee (Vaver, 2011; Andrews, 2002; Agreement on Trade Related Aspects of Intellectual Property Rights, 1995).

22. The description gives details about the invention and how to put it to use. It includes a recount of the components of the invention, its use, and the best-known method to reproduce it. All the information included in the description should be clear and thorough so as to allow a person skilled in the art to fully and unambiguously understand the invention, know its utility, and be able to reproduce it if necessary or desired. To comply with this part of the description, it is recommended that the researcher keep a journal of the invention, documenting every step followed throughout the whole process that led to the final outcome of the invention and the reasons for asserting the alleged industrial application. References to the current state of the art (e.g., articles, books, commentaries, patents) associated with the invention help to show that the invention is in fact new and nonobvious.

Importance of the patent system

The importance of the patent system lies in the innovation that it is expected to promote. First, the patent system is said to promote innovation by giving researchers and investors economic incentives to engage in innovative research projects. For investors the economic incentive is having the exclusive right to make, use, sell, or import the patented invention or to authorize others to do so in exchange for a royalty, which allows the investors to recoup their investments. For researchers the incentive is having the exclusive right to make, use, sell, or import the patented invention and thereby be economically rewarded for their work (Gold et al., 2019; Zerhouni et al., 2020; Granados Moreno et al., 2018; Granados Moreno and Joly, 2020; Mayfield, 2016a,b; Sumikura, 2009; Castle, 2009; Vaver, 2011; Jensen and Murray, 2005). Second, the patent system is said to promote innovation by creating a state of the art or knowledge commons from which to build up innovation. It does so by requiring the patent holder, as part of the patent application, to disclose the description, details, components, uses, and functionality of the invention to the extent that it allows any person skilled in the art to reproduce the invention (Granados Moreno and Joly, 2020; Granados Moreno et al., 2018; Andrews, 2002; Jensen and Murray, 2005). Third, the patent system is said to promote innovation by facilitating information markets. Commodities such as the information and knowledge related to inventions can be integrated into patents (i.e., as exclusive economic rights with a recognized value assigned). This acquired form of packaging makes it easier for such commodities to be exchanged and to circulate in markets (Hope, 2008; Granados Moreno and Joly, 2020; Granados Moreno et al., 2018).

Despite the benefits that the patent system may bring, there are concerns about the negative impact that patents may have on research and development and, particularly with respect to medicine, on access to health care²³ (Gold et al., 2019; Granados Moreno et al., 2018; Heller and Eisenberg, 1998; Gold, 2008; Hope, 2008; Canadian HIV-AIDS Legal Network et al., 2010; Stiglitz, 2006). These concerns have contributed to a renewed interest in open science approaches (Gold et al., 2019; Granados Moreno et al., 2018; Gold, 2016).

Open science

Definition and principles

Open science is a movement characterized by principles of open data sharing, cumulative research, cooperation, and fast dissemination of knowledge in order to foster scientific process, maximize the impact of research, and meet humanitarian goals (Gold, 2016; Hope, 2008; Granados Moreno et al., 2018; Rouleau, 2017; Owens, 2016; Caulfield et al., 2012). Many of these principles have been integrated into a number of international policies and guidelines, such as the Organisation for Economic Cooperation and Development (OECD) Principles and Guidelines for Access to Research Data from Public Funding, Organisation of the Human Genome (HUGO) Statement on Human Genomic Databases, Genome Canada, and the U.S. National Institutes of Health Final Statement on Sharing Research Data, among others.²⁴

The concept of open science originated in the late 16th and early 17th centuries to make scientific research, data, and dissemination accessible to all, departing from the regime of secrecy of “nature’s secrets,” whose norm was to withhold knowledge from the vulgar multitude (David, 2004). According to the open science movement, seeking truth is a communal activity: “The only way in which a human being can make some approach to knowing the whole of a subject is by hearing what can be said about it by persons of every variety of opinion, and study all modes in which it can be looked at by every character of mind” (Mill, 1879). The principles that characterize open science subscribe to Merton’s norms of science (communalism,²⁵ universalism,²⁶ disinterestedness,²⁷ originality,²⁸ and skepticism²⁹), which represent ideal institutional values to which science should attend (Merton, 1973; Hope, 2008).

The model of open science aims to uphold the aforementioned principles by circumventing the potential structural barriers and restrictions that intellectual property, particularly patents, can impose. One way in which the model of open science has done this is by eschewing patent protection (Granados Moreno et al., 2018; Gold, 2016). The model also adopts a policy of open data access (Shaw, 2017).

Benefits of open science

Some of the arguments in favor of open science mention that sharing and making data and knowledge openly available lead to better data quality, the formation of good

23. Some of these concerns are described more thoroughly in the following sections.

24. For more examples, see Caulfield et al. (2012).

25. Communalism emphasizes the importance of commonly owned knowledge.

26. Universalism encourages keeping information open to all persons, regardless of their personal characteristics (e.g., race, class, gender, religion, nationality, profession.).

27. Disinterestedness refers to scientists’ selfless approach to advance science and research.

28. Initially not included, originality alludes to the novelty of the contribution.

29. Skepticism encourages subjecting all knowledge to rigorous and structured trials of scrutiny, replication, and verification.

practice, and a sharing culture (Hope, 2008). For instance, a large network of researchers from a variety of backgrounds focusing on a specific topic from different angles and through different lenses is more likely to get more feedback, reach new findings, identify more mistakes, and discover more or better ways to reach the same results than a small homogenous group of people. Open science is also helpful in avoiding unnecessary duplication of research and inefficient allocation of resources (Gold, 2016; Hey and Kesselheim, 2016; Rouleau, 2017). Openly sharing research tools and results creates an information commons, which everyone can use and build on. Fast dissemination allows this commons to be created, expanded, and updated in short periods of time. Since researchers can access what others are doing and reuse what others have discovered, they can start where others left off and devote their resources to further advancement in innovation (Granados Moreno and Joly, 2020; Caulfield et al., 2012). Transaction costs can also be reduced with an open science model, as having less restrictive access decreases costs, time, and other resources usually associated with contract negotiations, court challenges, and intellectual property management (Gold, 2016). Furthermore, the more that information, platforms, and data are used, openly shared, and collaboratively exchanged, the more open, creative, and useful said information, platforms, and data will become (Giustini, 2006). These characteristics of open science can be helpful in solving or at least reducing some of the problems of lengthy, complex, and uncertain translational processes. For instance, considering that “omics” data are large, constantly evolving, and still not fully understood, open science provides researchers with the opportunity to have less restrictive access to genetic data, products, and tools, which enables them to work more collaboratively and form partnerships (Hey and Kesselheim, 2016; Granados Moreno et al., 2018; Gold, 2016).

However, there are some concerns associated with the open science model. The first concern that emerges in implementing an open science model is that third parties could appropriate (e.g., via patents) and enclose the information, works, and inventions that were originally released under the open science model. The second most common concern is that researchers, inventors, and

investors will not be able to obtain patents and therefore to commercially exploit their invention and recoup their investment, which could ultimately affect the sustainability and growth of the innovation field. There are also concerns about the way in which the model can interact with other parties using patents to commercialize their inventions and information associated with them (Granados Moreno and Joly, 2020).

Renewed interest in open science

With the emphasis given to the intellectual property system as the main source of incentives for researchers to engage in research projects and for investors to devote resources to those projects, the open science model and its principles have been sidelined, or at least restricted. For instance, certain practices with patents are deemed contrary to principles of scientific production and diffusion for the following reasons. Given that patents assign individual exclusive property rights to use and control the patented invention (and the associated scientific and technological knowledge), patent holders have the possibility of preventing others from using the invention and the knowledge associated to it. This goes against the principle that science should be shared, cumulative, and cooperative. Moreover, researchers may delay publications related to the invention until the patent application has been filed, thus withholding important information about the invention. This action is contrary to the principle that scientific products should be rapidly shared (Hope, 2008; Bagley and Tvarnøe, 2014; Jaffe and Lerner, 2007; Rai et al., 2008).

The open science model regained popularity and its support became stronger with the occurrence of two events. The first was the development of technological tools (advanced computers, internet, storage tools, etc.) that allow faster and easier ways to share and disseminate knowledge, findings, and data (Rentier, 2014). The second was the rise of the open source movement in the late 20th century.³⁰ The open source movement highlighted the potential negative effects that the proliferation of intellectual property rights could bring to innovation³¹ and therefore questioned the need for and convenience of excessive individual intellectual property rights (Hope, 2008). Particularly in the field of medical research, important world health issues, such as the HIV-

30. Open source is a production and development model based on open, free, and universal access. It stemmed from the free software movement at the end of the 20th century. While it originally focused on the software industry, exalting the importance of maintaining the source code of a software program free (as in freedom) for everyone to use, develop, and build on, it has also been proposed in other areas such as health and agricultural biotechnology. The model is implemented by asserting intellectual property rights over the creative work and granting an open license that authorizes the nonexclusive use of the copyrighted (and sometimes patented) creative work, either to review the information associated with the work (e.g., source code), to use the work or invention, to modify it and adapt it to one's needs, or to build on it and create derivative works. As part of this open source license, the work that results from using the copyright or /patented work (derivative work or improvement) needs to be licensed under these same terms of openness and nonexclusivity. The benefits suggested by this model include large and assorted collaboration, decentralized open production, low engagement costs, and peer review and feedback loops that will potentially lead to high-quality outcomes (Zittrain, 2004; Hope, 2008).

31. This issue is developed more thoroughly later in the chapter.

AIDS crisis in South Africa,³² have also influenced the return to principles of open science and a different approach to intellectual property. As of today, several projects and institutions have adopted open science models, including the Structural Genomics Consortium, Sage Bionetworks, and the Canadian Open Neuroscience Platform (Granados Moreno et al., 2018; Gold, 2016; Granados Moreno and Joly, 2020; Rouleau, 2017; Morgan-Jones et al., 2014). The foregoing conditions have favored the adoption and implementation of different models of collaboration and partnership as new ways to combine the principles of open science and forms of intellectual property with the purpose of achieving greater levels of innovation and better access to new products and services (Gold, 2016, 2008).

Open innovation

Definition and objectives

Open innovation is a model of collaboration that recognizes that innovation is both more efficient and effective with the participation and collaboration of different parties. The term *open innovation* is based on the assumption that an efficient R&D process incorporates parties, ideas, resources, and internal as well as external strategies to the company. The purpose of the suggested collaboration is not only to accelerate the R&D process, but also to expand the markets and to maximize revenues. The commercial aspect of these two last purposes constitutes the main difference with open science, as the latter usually focuses only on the advancement of science and the R&D process. It is also why this model more often suits the expectations of the private sector and therefore is easier to adopt. To achieve its purposes, the model proposes the use and trade of intellectual property rights (i.e., copyrights, patents, and trademarks) to establish collaborations and partnerships throughout the different stages of the R&D process.

Benefits of open innovation

Through the collaborations and partnerships that the open innovation model enables, the cooperating parties are able to share ideas, information, research tools, and strategies, which is likely to allow for a more cumulative and collective perspective of the R&D process. This could result in a more efficient and faster R&D process, as duplication of efforts and work could be avoided and different stages of the R&D could be worked on simultaneously. The openness to external collaboration contributes as well to more diverse

collaborators, which can enrich and speed up the innovation process. Furthermore, the use and trade of intellectual property rights capitalize the R&D process through the royalties generated by the use of the protected inventions. Nevertheless, the open innovation model has some important drawbacks. The first drawback is the complexity in determining the ownership of all the parts that make up the invention when it is created in collaboration with multiple parties. Likewise, practices of licensing among numerous parties can become complicated and confusing, as the many terms and conditions may be difficult to fully understand and follow. A third limitation is the risk of disclosing essential and commercially valuable information while sharing information as part of the collaboration (Granados Moreno and Joly, 2020; Hagedoorn and Zobel, 2015; Chesbrough et al., 2006; Joly, 2019). One example of the open innovation model in medical research is the collaboration between AstraZeneca and MRC Laboratory of Molecular Biology that was created to understand the mechanisms of human diseases and to develop new therapies for Alzheimer's disease, cancer, and rare diseases. The European Lead Factory also adopted an open innovation model to provide access to its 500,000 novel compounds for its pan-European drug discovery program (Astra-Zeneca Global, 2012; Bubela et al., 2017).

Public-private partnership models

Definition and objectives

A public-private partnership (PPP) is a cooperative arrangement between the public and the private sectors³³ in which both sectors work together, sharing their resources (e.g., economic funds, knowledge, data, sample, clinical expertise, patient base, infrastructure), risks, and responsibilities in order to achieve a common or compatible objective. This model recognizes that the innovation process requires both the participation of and contributions from members of the public and the private sectors (Granados Moreno and Joly, 2020; Kwak et al., 2009). PPPs can be national and international. PPP models emerge from the idea that neither the public sector nor the private sector is able, by itself, to successfully and efficiently see the innovation process from bench to bedside and sustain it in the long term. The R&D process developed solely in the public might be too slow and ineffective (Kwak et al., 2009). The process maintained only in the private sector might be too burdensome for the parties involved and bring about unequal distribution of the final

32. In 1998, pharmaceutical companies that held patents on antiretroviral drugs used to treat HIV-AIDS sued the government of South Africa for importing and manufacturing their patented drugs without their permission in order to fight the AIDS epidemic (Gold, 2008).

33. In the TM context the public sector comprises publicly funded or not-for-profit organizations, research centers, and universities, and it usually focuses on basic research. The private sector refers to for-profit organizations, such as biotechnology and pharmaceutical companies, and it usually focuses on clinical research (Reich, 2000).

products and services. Hence PPPs aim to facilitate the translation of basic research into clinical products, including new treatments, drugs, and vaccines, by engaging in the different stages of the innovation process conjointly to facilitate the transition between those stages and make them more efficient (Granados Moreno and Joly, 2020; Mittra, 2013; Reich, 2000). PPPs also intend to fulfill a social and moral obligation to improve the health of people in developing countries. PPPs differ from open innovation in that in a PPP the parties involved act as partners when they share resources, risks, and responsibilities, which is not the case in an open innovation model (Reich, 2000; Bubela et al., 2012; Granados Moreno et al., 2017).

Benefits of public-private partnership models

The main benefit of a PPP is that the public and private sectors share resources (e.g., economic funds, knowledge, data and samples, clinical expertise, patient base, infrastructure), risks, responsibilities, and rewards as partners. This creates a more reliable and stable collaboration among the parties involved. PPPs also create greater networks of experts with different insights. Not only can a PPP alleviate the burdens inherent to the innovation process and make the process more efficient, less costly, and faster, but more important, it can facilitate and make the R&D process more fluid and holistic. Finally, sharing resources such as knowledge, clinical expertise, data, samples, and patient base expands the partners' training (Granados Moreno and Joly, 2020; Kwak et al., 2009; Mittra, 2013). The main problems encountered by PPPs are the inherent complexities of basic and clinical science, the high and sometimes unrealistic expectations, and the different objectives and interests originating in both the public and private sectors. Moreover, since PPPs can adopt any model, including open science or traditional proprietary commercialization, they can still have the challenges associated with those models (Granados Moreno and Joly, 2020; Mittra, 2013).

In the area of medical research and health, PPPs have proved to be more efficient and faster and capable of achieving better outcomes than the industry alone,

improving access to healthcare services as well as better spurring R&D in the field (Granados Moreno et al., 2017; Kwak et al., 2009). Examples of successful PPPs include MSSNG (which focuses on autism), ICGC and the Cancer Genome Atlas (which focus on cancer), 100,000 GP (which focuses on rare disorders, infectious diseases, and common cancers), and SGC [which focuses on three-dimensional (3D) structures of proteins and chemical tools] (Granados Moreno and Joly, 2020; Granados Moreno et al., 2017; Bubela et al., 2012; Poupon et al., 2020).

Trends in translational intellectual property

Patents are the main form of intellectual property used in TM to protect inventions and commercialize products. Patents can be obtained on research tools, on new and repositioned drugs, on genetic tests, on risk prediction models, on personalized therapies, and on medical devices and other instruments such as 3D bioprinters and some of their associated outputs.

Patents and research tools

Genes (gene sequences), cDNA,³⁴ and biomarkers³⁵ (genes, proteins, metabolites, enzymes, etc.), as research tools, can be the basis for the development of genetic tests, new and repositioned drugs, gene therapy, gene editing, and personalized medicine³⁶ when they are found to be linked to a disease or disorder (Sumikura, 2009; Chandrasekharan and Cook-Deegan, 2009). The role that these research tools play in the development of clinical products has lured investors (private and some public) and some researchers to pursue patents on research tools, not only to commercialize the inventions associated with them, but also to maintain a competitive position in the field by excluding their competitors from using those research tools (Kesselheim and Shiu, 2014).

For purposes of this patent analysis we will differentiate gene sequences and biomarkers from cDNA. Whereas

34. Complementary DNA (cDNA) is DNA that complements or matches mRNA. cDNA is synthesized by copying an mRNA chain using the enzyme reverse transcriptase. As mRNA is formed after all introns have been removed, cDNA contains only the exons (portion of a gene that codes for amino acids) of DNA, omitting the introns. Scientists use cDNA to express a particular protein in a cell that does not normally express such a protein (Bradley et al., 2006; Association for Molecular Pathology v. Myriad Genetics, Inc., 2013; HumanGenes.org, 2014).

35. A biomarker (or biological marker) is defined as a "characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention." There are different types of biomarkers. A prognostic biomarker provides information on the likely course of a disease in untreated individuals. A predictive biomarker advises on the subpopulations of patients who are most likely to respond to a given therapy. A diagnostic biomarker indicates the existence of a disease. A drug-related biomarker helps to determine whether a drug will likely be effective in a specific patient or how the patient's body will likely process the drug. Examples of biomarkers include genes, proteins, metabolites, and enzymes (Strimbu and Tavel, 2010; Wehling, 2010; Wehling, 2008; Fitzgerald, 2005; Kesselheim and Shiu, 2014).

36. Personalized medicine is a practice of medicine that takes into account the individual's genetic profile to influence medical decisions concerning the prevention, diagnosis, and treatment of a disease ("Personalized Medicine" National Human Genome Research Institute).

gene sequences and biomarkers are found in the human body and subsequently isolated from it, cDNA is created in a laboratory. The compositions of gene sequences and biomarkers also differ from those of cDNA. The differences among these research tools affect the way in which they meet the patent requirements and therefore their patentability (Canadian Patent Office, 2019; European Patent Office, 2019; Mexican Industrial Property Law, 2020; Association for Molecular Pathology v. Myriad Genetics, Inc., 2013).

Gene sequences and biomarkers found in the human body are not patentable. Merely identifying, locating, and correlating gene sequences and biomarkers to a disease, disorder, or reaction is considered a discovery. The reason for this assertion is that, regardless of the complexity and hard work involved in identifying, locating, and finding that correlation, those identified, located, and correlated genes and biomarkers have not been transformed into anything new; they are exactly the same as found in nature and therefore are excluded from patentability (Canadian Patent Office, 2019; European Patent Office, 2019; Mexican Industrial Property Law, 2020; Association for Molecular Pathology v. Myriad Genetics, Inc., 2013; Servick, 2019; Simoncelli and Park, 2015).

The patentability of isolated gene sequences is a more complex issue. Provided that the patentability requirements are met (novelty, nonobviousness, and industrial application), a gene isolated from the human body has been commonly considered patentable for the following two reasons. First, an isolated gene's chemical qualities, characteristics, and structure are different from those of a gene in the human body because the process of isolation breaks the natural bonds of that segment from the rest of the genome. This difference was deemed by the courts and patent manuals and regulations of most jurisdictions sufficient to make isolated genes different from how they are found in nature and therefore patentable, provided that they met the other requirements (e.g., new, nonobvious, and utility or industrial application). Second, this "new" isolated gene was "created" by a human-invented process of isolation. The process of isolating a gene was considered to require human ingenuity; therefore the resulting gene was also considered to be a product of human ingenuity.

According to the European patent regulation,³⁷ unlike discoveries of natural substances such as the sequence or partial sequence of a gene, biological material that has been isolated from its natural environment is patentable. Canada and Mexico have a similar approach. In these jurisdictions, isolated gene sequences (considered biological material) are patentable as compositions of matter as long

as they meet the patentability requirements: novelty, nonobviousness, and industrial application (Bradley et al., 2006; European Patent Office, 2019; Canadian Patent Act RSC 1985c. P-4, 1985; Mexican Industrial Property Law, 2020; European Parliament, 1998).

In the United States, by contrast, the Supreme Court stated in 2013 that isolated gene sequences should be considered discoveries and therefore are not patentable. The U.S. Supreme Court justified its decision by explaining that the isolated genes maintain the same molecules as the genes found in the human body and that the differences found at the ends of the segments of the isolated genes are merely ancillary to the isolation (Association for Molecular Pathology v. Myriad Genetics, Inc., 2013; Shanahan, 2014).

Contrastingly, cDNA is held to be patentable because it is synthetic DNA created in the laboratory with human ingenuity and is considered different from the DNA existing in nature. While the natural human DNA has exons and introns, the cDNA includes only the exons and excludes the introns (Association for Molecular Pathology v. Myriad Genetics, Inc., 2013; European Patent Office, 2019).

Isolated gene sequences and biomarkers (in some jurisdictions) as well as cDNA can be patented as part of the drugs, therapies, and genetic tests in which they are used. This association is what provides the element of utility, usefulness, or industrial application. They can be patented as an essential part in a process or as compositions of matter with the purpose of being used in a specific genetic test, drug, or therapy and be mentioned as such in the claims that make up the drug, therapy, or genetic test patent. The description or inclusion of gene sequences, biomarkers, or cDNA in these terms could give the patent holder exclusive rights not only over the invention (e.g., the process followed in determining the presence of an abnormal or mutated gene), but also over the research activities associated with the isolated gene sequence, biomarker, and/or cDNA (e.g., any research activities associated with an already identified disease and with the discovery of any other disease that could be potentially linked with that gene sequence and/or cDNA) (Andrews, 2002; European Patent Office, 2019; Offit et al., 2013; Simoncelli and Park, 2015; Ali-Khan and Gold, 2017).

Patents on genetic tests and personalized therapies

Genetic tests

Genetic tests allow physicians and clinicians to detect in patients the existence or the likelihood of developing a

37. For the purposes of this chapter the term *European patent regulation* comprises the European Patent Convention, the EPO Guidelines for examination in the European Patent Office, and the Directive 98/44/EC of the European Parliament and of the Council of 6 July 1998 on the legal protection of biotechnological inventions.

chromosome or gene abnormality or mutation and therefore of developing one or more genetic diseases or disorders. Genetic tests can also help to detect the existence, effect, and function of biomarkers responsible for the processing of drugs or treatments, thereby contributing to the development of personalized medicine. Four steps are required to perform a diagnosis test. The first step is to identify and locate the specific chromosomes, genes, or biomarkers associated with the disease, disorder, or reaction. Then the standard sequences or state of those genes or biomarkers to be used as a reference have to be determined. The third step consists of examining whether the individual has those genes, mutations, or biomarkers. The last step analyzes the likelihood of the individual developing the associated genetic disease, disorder, or reaction (Sumikura, 2009; Andrews, 2002; Bradley et al., 2006).

Genetic tests can be patented as processes or as products. A genetic test can be patented as a process if it describes a new and nonobvious method for detecting a genetic abnormality or mutation in association with a genetic disease or disorder or as a process to detect a biomarker to determine the reaction to a drug or treatment (Canadian Patent Office, 2019). For the method to be patentable, it cannot consist merely of observing, comparing, and stating laws of nature; it needs to go beyond simply stating correlations between genetic variants and clinical phenotypes and beyond comparing an individual's DNA sequence with a reference sequence. The way in which the steps of the genetic test are executed, the instruments used, and the way in which the results and correlations are interpreted and disclosed need to be new, nonobvious, and specific to a utility or industrial application for the test to be patentable (Klein, 2007; Mayo Collaborative Services v. Prometheus Laboratories, Inc., 2012; Association for Molecular Pathology v. U.S. Patent and Trademark Office and Myriad Genetics Inc., 2012; Bakshi, 2014). Some jurisdictions, such as Europe and Mexico, exclude diagnostic methods practiced on the human body from patentability. This exclusion does not apply to methods that require that the diagnosis be performed outside of the body; therefore gene tests performed in vitro, for instance, are considered patentable (Mexican Industrial Property Law, 2020; European Patent Office, 2019). A genetic test can be patentable as a product when it is embodied as a kit. Instruments or apparatuses or compositions used in these tests are also patentable as products (Canadian Patent Office, 2019; European Patent Office, n.d.; European Patent Office, 2019).

Personalized therapies

Personalized or precision medicine is defined as a medical approach wherein clinicians make diagnostic or therapeutic decisions specific to a particular individual's unique

biological, environmental, genetic, and lifestyle profiles by predicting whether a newly prescribed drug will likely be effective and/or safe in the treatment of a disease in that patient (National Institutes of Health, 2018; Granados Moreno et al., 2018). Personalized therapies will often combine three main elements. The first consists of using a genetic test to detect whether the patient has the specific genes responsible for the processes of production of the biomarkers (e.g., enzymes) that regulate the analysis and processing of drugs (e.g., chemotherapy, heart or antidepressant medications). The second is predicting the likely effect of a drug, dosage, therapy, or treatment, taking into account and interpreting the results obtained from the test. The last element consists of making a decision to prescribe a specific drug, dosage, or treatment on the basis of the prediction made with the genetic test. Since any change or abnormality in any of these genes may impact the effect that a drug may have on the patient, screening for these genes and knowing the potential effects in that particular individual can help clinicians to better prescribe a treatment (Kesselheim and Shiu, 2014). Precision medicine provides the opportunity to improve disease prevention, optimize healthcare services, and reduce associated expenditures. The potential success of this approach, while modest, is increased with the scientific advancement of genetic testing and the greater access to omics data (Alyass et al., 2015; Granados Moreno et al., 2018).

The human ingenuity of the invention that makes it patentable is found in the combination of the three elements. Therefore in this case the invention related to personalized therapies is patentable if, in addition to being new, nonobvious, and having a specific utility or industrial application, it detects a specific gene or biomarker, determines the likelihood of developing a particular reaction, and, on this basis, also suggests the proper treatment considering the specific genetic conditions of the patient (Kesselheim and Shiu, 2014; Mayo Collaborative Services v. Prometheus Laboratories, Inc., 2012). The patent would grant protection over the process that integrates the genetic screening and the specific administration of a treatment. A kit that allows the aforementioned analysis to be performed is often also manufactured and patented as a product.

Patents on risk prediction models

Risk prediction models help to determine an individual's risk of developing an adverse outcome (e.g., cardiovascular disease, cancer) on the basis of statistical analyses and the existence of certain characteristics or predictors. Biomarkers, along with family history, age, sex, reproductive factors, hormones, and lifestyle, are among the predictors used for risk prediction models. Risk prediction models will help to determine the need for preventive strategy (e.g., changes in the individual's lifestyle) or

even treatment. These models can also monitor the success of a specific treatment in subjects with different predictors (Ahmed et al., 2014; Lee et al., 2014, 2019).

The ingenuity in risk prediction models lies in the multielement statistical analysis to determine the likelihood of developing an adverse outcome or the success in a specific treatment, which goes beyond merely stating the presence of selected biomarkers or any laws of nature. The patent that is obtained in this case is on the process for predicting the likelihood of the outcome. In addition to the method, the invention often comprises a kit for which a product patent can be obtained (Ahmed et al., 2014).

Risk prediction models may also be protected by copyright. In this case the computer program that is created and used for the analysis of predictors to anticipate a condition or response is protected as a literary work by copyright. The protection granted by copyright covers the source code (i.e., selection and arrangement of instructions and algorithms used) as well as the program's screen display. The program's concepts and ideas are not protected by copyright. The term of protection extends for the life of the author plus 50 years³⁸ after the author's death. Since registration is not required, protection starts automatically once the work has been generated. The copyright owner has the exclusive right to distribute, reproduce, rent, modify, and license (i.e., authorize others to exercise these rights) the program (Canadian Copyright Act RSC 1985c.C-42, 1985; Mexican Copyright Law, 1996; U.S. Copyright Law USC Title 17, 1947).

Patents on new and repositioned drugs

Repositioned drugs are known drugs and compounds (patented or not) paired with new uses, diseases, or conditions. They can also consist of a different formulation or combination of compounds. These new uses or formulations are the result of systematically screening compound libraries and drug targets with the use of advanced principles and methods of chemical genomics, computational biology, systems medicine, and text mining. In other words, drug repurposing provides a new scientific and clinical insight into existing drugs and compounds. This new insight is beneficial not only in terms of the repurposed drug targeting untreated diseases or conditions, but also in terms of the pharmacological and developmental information that offers. The advantage of drug repositioning over regular

drug development is that in the former, the drug has already passed a number of tests for safety and efficacy. This can reduce the risks of failure, decrease the costs and process of R&D, and make the safety approval shorter. A repurposing approach can lead to a number of new or unforeseen biotechnology products that include not only drugs, but also vaccines (Chong and Sullivan, 2007; Mucke, 2017). The COVID-19 pandemic has brought about a renewed interest in repositioned drugs. Studies have suggested that remdesivir, a drug that was originally developed to treat Ebola, could be helpful fighting coronaviruses such as MERS and COVID-19 (Bonadio and Baldini, 2020). Repositioned drugs can be patented as products. Patents in these cases cover the new use combined with a frequently new combination, which may involve a new form of administration (Mucke, 2017).

Drugs (new and repositioned) can be patented as products, and their active ingredients can be patented as compositions of matter. The drug and the active ingredient have to comply with the patent requirements: new, nonobvious, and have a utility or industrial application.³⁹

For repositioned drugs to be patented, the new use, disease or condition (industrial application or utility) and related combination, besides being different from the one associated with the known drug or compound, has to be new and nonobvious. This means that the new use, disease, or condition cannot have been described in the state of the art or cannot have been obvious to a person skilled in the art. Furthermore, given that the repositioned drug or compound is related to or is derived from a potentially already patented drug or compound, in order for the repositioned drug to be patentable, it is necessary to verify that it does not infringe on any third party's patents. Therefore it is required to determine whether there are patents over any of the parts or components of the repositioned drug or compound held by any third party. If there are patents, it is necessary to obtain a license to make, use, and/or sell that known drug or compound from its patent holder. These actions are required not only to obtain a patent, but also to commercialize the repositioned drug or compound (Ashburn Ted and Thor, 2004; European Patent Office, 2019).

3D bioprinters

Three-dimensional (3D) printing or additive manufacturing is a computer-aided process that consists of building 3D solid structures and components by printing them

38. This term of protection varies between 50 (Canada), 70 (United States and Europe), and 100 (Mexico) years after the author's death, depending on the specific jurisdiction (Mexican Copyright Law, 2014; U.S. Copyright Law, 2011; Canadian Copyright Act, 2012; European Directive 2009/24/EC, 2009; European Patent Convention, 2016).

39. In the United States the patents granted to new drugs are independent from the exclusive marketing rights granted by the U.S. Food and Drug Administration after the drug has been approved (U.S. FDA/CDER SBIA, 2015. Patents and Exclusivity).

layer-by-layer. The layers follow the patterns determined by computer-based images/models. This process uses inorganic materials. 3D bioprinting uses 3D printing technology for the manufacture of complex tissue structures or components. These structures or components are created by applying biological (organic) material on a pre-shaped supporting scaffold based on a computer-scanned image or design. The scaffold then dissolves, and the biological material (living cells) is treated to enable its “natural” development and physiological organization. The main applications of 3D bioprinting are in reconstructive surgery and regenerative medicine. Current 3D bioprinting technology makes it possible to create 3D objects that are multimaterial and multicolored, including human-like tissues, such as skin, bone, cartilage, bladders, and heart valves and even potentially organs.

The clinical application of the 3D bioprinting technology lies in the 3D printed objects that it helps to create. As the state-of-the-art of 3D printing technology currently stands, 3D printed objects can have a number of clinical applications. For example, 3D printed models of organs, tissues constructs, and body structures and apparatuses can be used for surgical planning, as they can help surgeons and medical staff to visualize, define, and plan the essential surgical steps and needs prior to the actual surgical intervention in a very precise way. They can help visualize the vascular anatomy; determine stent position, size, and length; and, in cases of congenital heart diseases, provide dimensions and distances of structures such as valves to better plan, predefine, and rehearse the necessary steps in realistic textured environments prior to the surgery (Vijavavenkataraman et al., 2017). More graphic and accurate training of surgeons, medical staff, and medical students is also possible with the use of 3D printed models. For example, tracheobronchial tree models for neonates’ or infants’ lungs can be 3D printed for more accurate training on children’s lungs in contrast to adult-size lungs (Varkey and Atala, 2015; Vijavavenkataraman et al., 2017). Prostheses⁴⁰ can also be 3D printed, and 3D printed prostheses are more customized than regular ones, as the former can adapt to the patient’s specific anatomy and needs (e.g., dimensions, weight, and coloring). This is even more evident in hand, face (e.g., jawbones), and eye prostheses. Moreover, in the case of pediatrics, 3D printed prostheses can be less expensive in the long term when children outgrow them, given that these prostheses can be easily replaceable at lower costs than regular prostheses. For instance, the cost for eye prostheses is reduced from approximately \$5200 CDN to \$200 CDN (Vijavavenkataraman et al., 2017; Vaver, 2011). 3D printing can also be helpful in

designing drugs that can be customized in terms of size, dose, appearance (design, color, and shape), and delivery rate of the pill. This is particularly useful in drugs for children. Additionally, drugs can be personalized to the patient’s mass and metabolism, or they can be printed to comprise more than one medication in one pill (Vijavavenkataraman et al., 2017).

There are special requirements and technical challenges in 3D bioprinting that do not apply to regular 3D printing. The materials that will constitute the “ink” to be used in the printing process have to be soft and allow cell viability and spatial accuracy. Hydrogel or any other biocompatible scaffold materials are used as the base or environment to which cells will be added to constitute the “ink.” The hydrogel must be able to provide structural support and adequate environment. A specific type and amount of living cells are then added to the hydrogel. Most common materials for this process are synthetic or natural polymers. The cells can be acquired from the same or a different individual. Ongoing R&D in stem cells suggest that induced pluripotent, embryonic, and multipotent stem cells can be used in 3D bioprinting to increase immunocompatibility. However, finding or creating the most appropriate and effective “ink” requires further research (Vijavavenkataraman et al., 2017; Varkey and Atala, 2015; Vermeulen et al., 2017; Ortega and Granados Arriola, 2015; Yoo, 2015).

The 3D bioprinting technology (cellular printed prostheses) has the potential of enabling 3D printed prostheses to be integrated in the brain communication system and exhibit more biomimicry⁴¹ with tissue and organ functionalities. Similarly, skin tissues can be printed to replace skin in burned victims (Varkey and Atala, 2015; Vermeulen et al., 2017; Vijavavenkataraman et al., 2017). Bioprinted tissue constructs can act as testing beds for cosmetic products. They can also be used to detect drug reactions or side effects in specific tissues (e.g., liver tissue). This could facilitate drug discovery and help to determine the toxicity and efficacy of those drugs both in a whole population and in individuals. Hence this new testing could promote the development of personalized medicine, as the reaction that an individual may have to a drug or to a dosage may be determined by testing that drug on that individual’s tissue. This would then reduce the possibility of that individual directly suffering adverse reactions to that drug and increase the possibility of determining the correct dosage for the individual. It could also accelerate and lower the costs of drug delivery, as the costs and timelines of clinical trials may be reduced. Bioprinted tissue constructs could also benefit animals by eliminating the need for animal testing (Yoo, 2015; Varkey

40. A prosthesis is a device that is designed to replace a missing body part or to enhance the functionality of a damaged body part (Vijavavenkataraman et al., 2017).

41. Biomimicry is defined as “the imitation of natural biological designs and processes in engineering or invention” (Merriam-Webster, online ed., n.d.).

and Atala, 2015; Vermeulen et al., 2017). Another potential and highly expected application of 3D bioprinting is the creation of organs. Although donated organs are a viable and accepted way to treat organ failure, the number of donated organs does not meet the number of organs that are needed. As of August 2017, 116,000 Americans were still on waiting lists to receive an organ, and 20 people die each day waiting for one (Organ Donation Statistics: Why be an Organ Donor?, 2017). Tissue engineering⁴² has become a feasible method to replace damaged tissues and restore compromised organs. Examples of engineered tissues include skin, cartilage, urinary bladder, trachea, and vagina (Varkey and Atala, 2015; Vermeulen et al., 2017; Ortega and Granados Arriola, 2015; Li, 2014).

The methods (and all of its steps) of the bioprinting process; the methods to build the different layers into one solid object; the formulation of hydrogel precursor materials; the methods to derive or synthesize those materials; the methods to derive cells or prepare cells for printing; the maturation process; the methods to enable an organ to grow after being printed by different and innovative printing techniques; the methods to improve speed or accuracy of printing; the methods and materials to better ensure viability of cells; the methods to achieve vascularity, innervation, and biological functions of tissues and organs; the software/interfaces for 3D bioprinting (in some jurisdictions such as the United States); and the hardware itself (the 3D bioprinter and any of its parts or components) are and can be protected by patents, provided that these methods and products are not deemed to fall within the medical treatment exception⁴³ and that they fulfill the new, nonobvious, and utility requirements. Bioprinting materials and the bioink are also patentable. It has also been suggested that bioprinted tissues and organs may be patentable, provided that they are not considered products of nature and have been artificially created, similar to cDNA. This last possibility, however, would still be subject to the interpretation of the patent agent. This uncertainty stems not only from the nature of the invention (i.e., whether these tissues and organs are products of nature or artificially created), but also from the possibility that the invention or research would involve the manipulation or destruction of human embryos (i.e., higher life forms or the human body and its components), depending on the type of cells being used (Li, 2014; Yoo, 2015; Vermeulen et al., 2017; Varkey and Atala, 2015; Mexican Industrial Property Law, 2020; Canadian Patent Office, 2019).

Secrecy

Finally, alternatively, and sometimes even in addition to patenting, academic researchers and the industry have used trade secrets and confidentiality agreements (also known as nondisclosure agreements) to protect certain information (e.g., formulas, designs, patterns), know-how (e.g., processes, practices), instruments, compilation of information, and data associated with collected samples. This information has to comply with three requirements in order to be considered protectable by trade secrets: It is generally unknown to the public, it confers economic benefit, and its owner must make reasonable efforts to maintain its secrecy (Mexican Industrial Property Law, 2020; Civil Code of Quebec CCQ-1991, 1991; Uniform Trade Secrets Act, 1979; Directive EU 2016/943, 2016; Trade Secrets Act, 1989).

Secrecy is particularly useful not only as a means of protection per se, but also as a preliminary phase of patents. As was mentioned earlier in the chapter, two of the requirements for an invention to be patentable are that said invention is (1) new and (2) nonobvious at the moment the patent is filed (or within the 12 months previous to that filing date). While an invention is being developed, a lot of information is created, and a lot can be disseminated through publications, collaborations, academic presentations, and more. This dissemination could destroy the invention's novelty. Confidentiality agreements can maintain the invention's novelty by requiring that the parties involved in the development process enter into a contractual obligation to not disclose any of the information related to the invention until the patent has been filed (Andrews, 2002; Caulfield, 2010; Joly et al., 2012; Levine and Sichelman, 2018).

Secrecy is an attractive option and serves to protect information and inventions in medical research. Its requirements are simple, the time of its protection is unlimited, it can cover anything that has not been made available to the public (as opposed to only patentable subject matter), and its associated costs are less than those relative to patents (e.g., there are no registration fees). This can be particularly useful for startups. Moreover, secrecy can be used not only as a substitute for patents, but also as complementary, providing an additional layer of protection to patented inventions (Levine and Sichelman, 2018). The advantages and disadvantages of secrecy are discussed further in the following section.

42. Tissue engineering consists of taking a small amount of tissue via biopsy from the patient, isolating the cells that compose that tissue, expanding the isolated cells, and transferring them to a natural or synthetic scaffold, where they reproduce, resulting in a tissue construct that matures in a bioreactor and then is used in transplantation Varkey and Atala, 2015).

43. As was mentioned earlier in the chapter, surgical and therapeutic methods are excluded from patentability. In this case, if the technique were intended to print directly onto or into the body, it would be excluded from patentability. It is also worth mentioning that in jurisdictions such as Mexico or the European Union, 3D bioprinting technology will have to pass the morality (ordre public) test in order to be patentable.

Discussion

A perspective on the future of genetic patents

The most controversial discussions surrounding intellectual property and medical innovations in recent years have been those involving gene patents. The controversy continues, even years after the Myriad case ([Ali-Khan and Gold, 2017](#); [Guerrini et al., 2016](#); [Servick, 2019](#)). There have been three major debates in association with the patenting of gene sequences that have a direct bearing on TM and intellectual property.⁴⁴ The first refers to the patentability of gene sequences and whether they constitute mere discoveries or involve human ingenuity. The second focuses on the impact of gene patents on the promotion of innovation. The third revolves around access to the clinical products associated with those patents and therefore healthcare services in general.

Patentability of gene sequences

The debate around the patentability of gene sequences centers on whether they are patentable subject matter. As was mentioned earlier in the chapter, patent systems in most jurisdictions exclude genetic material as found in nature and discoveries that merely reveal something that exists in nature because they lack human ingenuity. Gene sequences found in the human body fall within this unpatentable category; they are genetic material as found in nature. cDNA, by contrast, is artificially created in laboratories, and it has structural and molecular differences from the DNA in the human body, which make it patentable. However, whether isolated gene sequences are excluded from patentability has changed over time and varies from one jurisdiction to another.

Before the Myriad case ([Association for Molecular Pathology v. Myriad Genetics, Inc., 2013](#)),⁴⁵ in the United States an isolated gene sequence could be

patented because isolating a gene sequence or DNA segment from the human body was enough to allege that it was different from how it exists in nature.⁴⁶ The reasoning behind this criterion was that such a gene sequence or DNA segment existed in nature only inside the human body. When it was isolated and removed from the human body, it was stripped of the conditions that surrounded it, changing some of its chemical qualities, characteristics, and structure. These new conditions were created not by nature itself, but by techniques created by human ingenuity.

However, the patentability of isolated gene sequences changed with the Myriad case. In June 2013 the U.S. Supreme Court refuted the isolation criteria asserting that isolated DNA sequences are not patentable because they are the same as the ones naturally found in the human body and the differences between them are just ancillary to the isolation.

The impact of this decision has varied. In the United States the decision laid some groundwork for patentable subject matter for future genetic inventions. Given that the U.S. Supreme Court ruled Myriad's isolated gene sequences of BRCA1 and BRCA2 unpatentable and therefore invalid, subsequent patent applications on isolated gene sequences filed in the United States have been deemed equally unpatentable. With respect to already granted U.S. gene patents, notwithstanding that this decision does not automatically invalidate them, as it dealt only with and ruled on the facts and matters of the Myriad case, it did set a precedent concerning how isolated gene sequences shall be interpreted in terms of patentability for purposes of the patent system in the United States if holders of these patents tried to enforce them. Thus this decision made other existing U.S. patents on isolated gene sequences practically unenforceable.

Outside the United States, however, except for Australia ([D'Arcy v. Myriad Genetics Inc., 2015](#); [Dayton,](#)

44. There another debate regarding gene patents. This debate focuses on the morality of conferring property rights on genes, as they are considered life. This debate will not be discussed here because, although important, it does not have direct relevance to TM or intellectual property.

45. *Association for Molecular Pathology v. Myriad Genetics, Inc.*, 569 U.S. 12–398 (2013). As a mere reference, this case involved the U.S. patents 5,747,282, 5,693,473, and 5,837,492 granted to Myriad for the BRCA1 and the BRCA2 genes, whose mutations are associated with an increase risk (50%–80% risk for breast cancer and 20%–50% risk for ovarian cancer in contrast to the 12%–13% average risk) of developing breast and ovarian cancer. Myriad discovered the exact location of the BRCA1 and BRCA2 in chromosomes 17 and 13. With this information it determined the “normal” nucleotide sequence of the BRCA1 and BRCA2 genes and the specific mutations associated with breast and ovarian cancer. The Myriad patents’ claims asserted ownership on isolated DNA sequences of BRCA1 and BRCA2 genes, a list of 15 “normal” nucleotides contained therein, and the specific mutations in those genes associated with breast and ovarian cancer. They also asserted ownership on cDNA nucleotide sequences (synthetic DNA) that code for the BRCA amino acids. These patents would give Myriad the exclusive right to isolate a person’s BRCA1 and BRCA2 genes or any strand that contains the 15 nucleotides listed or of any other within those genes and to synthetically create BRCA cDNA ([Association for Molecular Pathology v. Myriad Genetics, Inc., 2013](#)).

46. Biotechnological inventions such as inventions involving human genes or DNA sequences are patentable if the biological material (human genes or DNA sequences) is isolated from its natural environment or produced by means of a technical process even if it previously occurred in nature ([European Patent Office, 2019](#)).

2015; Nicol et al., 2019), the U.S. Supreme Court decision in the Myriad case does not seem to have influenced other court rulings.⁴⁷ The European patent regulation still states that biological material that has been isolated from its natural environment is patentable.⁴⁸ Canada⁴⁹ and Mexico follow a similar policy (European Patent Office, 2019; Mexican Industrial Property Law, 2020; Canadian Patent Office, 2019; European Parliament, 1998). These jurisdictions still have gene patents validly granted over isolated gene sequences, and they seem to uphold that isolated gene sequences are patentable as long as they have a utility or industrial application (e.g., diagnostic tests, drugs and personalized medicine, and gene therapies), they are new and have certain level of human ingenuity, and they are nonobvious or involve an inventive step⁵⁰ (Canadian Patent Office, 2019; Mexican Industrial Property Law, 2020).

As of this writing, the number of gene-related patents continues to rise and remains relevant, particularly because of the extent to which these patents affect the development of gene-based technologies and access to healthcare products and services. Furthermore, the genetic tests that are being developed today involve advanced types of sequencing, tens and even hundreds of loci, the simultaneous assessment of several genes, and details of medical histories. The resulting increased complexity of these tests then increases the chances that researchers and companies will have a greater interest and/or pressure to obtain patent protection (Ali-Khan and Gold, 2017; Nicol et al., 2019). In 2019 the American Civil Liberties Union released a letter to the U.S. Congress to voice its opposition to a congressional proposal that, if passed, could overturn parts of the U.S. Supreme Court decision in the Myriad case banning human gene patenting and could ease other restrictions on patenting software and biomedical inventions (Servick, 2019). The debate concerning gene patents therefore remains open, controversial, and relevant.

Patenting of gene sequences and promotion of innovation

The purpose of the patent system has been stated as the promotion of innovation for the benefit of society. However, some literature and even some cases have raised a concern that the current proliferation of patents could in fact do the opposite of what patents are supposed to do and instead could hamper innovation (Granados Moreno and Joly, 2020; Simoncelli and Park, 2015; Ali-Khan and Gold, 2017; Mayfield, 2016a,b).

The concern known as the tragedy of the anticommons⁵¹ is that excessive patent rights in genetic research, particularly in gene sequences, will lead to underuse and/or wasteful use of resources, thus hindering innovation. Excessive patent rights create what is called a patent thicket.⁵² Areas that are burdened with patent thickets require researchers and innovators to engage in arduous negotiations and bear high transaction costs to clear all the intellectual property rights. First, the researchers have to understand the patent landscape around the research field. For instance, in a research field (e.g., medical genetics) in which there are multiple patents associated with research resources and tools (e.g., gene sequences), identifying which patents are relevant to the development of a particular product or technology, what is the scope of ownership of those patents, and who owns them are matters that can be highly technical, ambiguous, time consuming, and costly (Hope, 2008; Heller and Eisenberg, 1998; Chandrasekharan and Cook-Deegan, 2009). Then the researchers have to negotiate contracts and licenses with all the patent holders identified and pay the corresponding licensing fees in order to use the patented research tools and resources relevant in a given R&D process (Gold, 2016; Rouleau, 2017; Granados Moreno et al., 2018; Hey and Kesselheim, 2016; Makker, 2011). Researchers and investors in fields with such multilayered patent thickets may be dissuaded from

47. Australia's High Court ruled in October 2015 that the key part of a gene is the information in it and that that information is not human-made and therefore not patentable. Moreover, the Court noted that gene patents could have a chilling effect on health care and research and that this is a factor that should be considered in dealing with a new extension to the patentable subject matter. With respect to isolated DNA and cDNA, the High Court ruled neither of them are patentable, as both lack novelty when all they do is replicate the genetic information of a naturally occurring organism (*D'Arcy v. Myriad Genetics Inc.*, 2015; Nicol et al., 2019).

48. In Europe a Germany patent court in 2016 even upheld the patents on isolated genes in the case Receptor Tyrosine Kinase, X ZR 141/13 (Nicol et al., 2019).

49. In Canada the Children's Hospital of Eastern Ontario started a legal action to invalidate gene patents associated with long QT syndrome owned by the University of Utah in 2014. However, this case was never decided in court, as the parties reached an agreement by which a license was granted to allow any public health provider to provide a genetic test related to the patented genes without the need of any payment (Nicol et al., 2019).

50. The European and Mexican patent regulations provide an additional requirement for an invention to be patentable: For an invention to be patentable, it cannot be against public order. This makes, for instance, inventions for human cloning or that uses human embryos unpatentable (European Patent Office, 2019; Mexican Industrial Property Law, 2020).

51. Anticommons can be understood as the underuse of a resource because said resource has multiple owners, each of which has a right to exclude others from using it (Heller and Eisenberg, 1998).

52. A patent thicket is defined as "a dense web of overlapping intellectual property rights [in this case, patents] that must be hacked through in order to actually commercialize new technology" (Shapiro, 2001).

continuing their original projects and opt to shift their focus to a less patent-populated topic in order to avoid these hassles (Contreras, 2018; Shapiro, 2001; Granados Moreno and Joly, 2020). Even if researchers and investors are not diverted from the overly patented fields, the resources that must be allocated to obtaining the necessary licenses could have been used for the research activities instead. This reduction of researchers and resources would delay further development or improvement of current innovation. These concerns are particularly strong in the biomedical community, given the cumulative nature of biomedical research and the importance that gene sequences play in the field (Hope, 2008; Contreras, 2018).

However, regardless of the extensive theoretical and academic debate about whether the patent system truly promotes or hinders innovation, the empirical evidence that is available is ambiguous. Empirical evidence supporting the idea that patents have actually promoted innovation in the biotechnology industry or that patents have harmed innovation is inconclusive (Attaran, 2004; Contreras, 2018). Intellectual property rights seem to have both incentive and inhibitory effects that depend of the economic and political contexts, the industry, and the stage of development (Gold et al., 2019).

Notwithstanding this debate, it has been observed that the tragedy of the anticommons can be avoided if transactions costs are kept low by, for instance, developing infrastructures or frameworks in which intellectual property owners grant each other licenses, such as patent pools or intellectual property clearinghouses⁵³ (Hope, 2008; Merges, 2001; Caulfield, 2009; Contreras, 2018). For instance, in other areas of biomedicine, members of the scientific community have created partnerships and intellectual property pools to generate drugs, diagnostics, and vaccines in various stages of development during the COVID-19 pandemic (Courage, 2020; Zerhouni et al., 2020; OpenCovidPledge, 2020; Thomas, 2020; WHO, 2020a,b).

Patenting of gene sequences and access to healthcare

Another common concern regarding the patenting of human genes refers to a hindering effect on access to health care, an effect that can promote health inequality. As was mentioned earlier in the chapter, genetic testing provides essential information to patients. It can inform a patient about his or her risk of developing a hereditary disease or condition, and it can help to determine appropriate preventive care or treatment options.

Because developing these diseases or conditions can have a great impact on the patient's quality and extent of life, having timely and reliable access to these diagnostic tools and treatment will become increasingly important.

The patent system enables patent holders to set the prices of the diagnostic tools and therapies that use patented genes. If these prices are set high enough, they can make diagnostic tools and therapies inaccessible for the public or difficult to introduce and renew in the public healthcare system. The patent system also allows patent holders to set the conditions under which third parties can use, manufacture, or sell their patented genes or gene-associated inventions. Patent holders can either simply prevent others from providing genetic testing or impose burdensome conditions on those who want to provide those services. This allows patent holders to control, to a certain extent, who can manufacture, sell, or import the testing, which ultimately influences the supply of the actual test and the entry of new competitors. Controlling the supply of the actual tests and the entry of competitors can lead not only to a shortage of testing, but also to a greater control over the price (Granados Moreno, 2019; Gold, 2010; Clague, 2006; Simoncelli and Park, 2015; Ali-Khan and Gold, 2017). Examples of these effects include the case of Myriad and its patents on the BRCA 1 and BRCA2 genes (Gold and Carbone, 2010) and the case of University of Utah's patent on the SCN5A and HERG genes related to long QT syndrome (Ali-Khan and Gold, 2017). Thus limiting access to timely genetic testing to those who can afford them in fact limits access to a good quality of life to those with the resources to afford genetic testing.

Finally, it is worth noticing that in the previous edition of this chapter we stated that the debate on access to health care was not relevant to TM and intellectual property. However, as COVID-19 has quickly killed millions of people around the world and greatly affected the livelihood of many millions more, it became obvious that correlation between TM and intellectual property should focus not only on the innovation process, but also on ensuring affordable and widespread access to medical products (Zerhouni et al., 2020).

Trade secrecy as an option alternative to patents

Trade secrecy has been used as an option to protect an invention when patents do not protect it, when there is an improvement or alteration of the invention that is not

53. A clearinghouse, in the context of the patent system, is a neutral platform that brings together and matches patent holders and licensees by facilitating license agreements between them. The benefits of a patent clearinghouse for patent holders and licensees include low negotiation costs, as standard licenses are frequently used, maximized dissemination of the patent holders' inventions, and low search costs for licensees. A clearinghouse can also help to monitor and enforce the invention's terms of use, to collect and distribute royalties, and to mediate and arbitrate in the case of disputes (Zimmeren et al., 2011).

covered by the existing patent, or when there are limitations to what can be patented (Contreras, 2018). A trade secret is defined as information (e.g., formulas, know-how, processes, information, patterns, designs, instruments, practices, or compilation of information) with economic value that its owner keeps confidential to maintain a competitive advantage. The owner of the trade secret needs to take all the necessary steps to keep the information confidential in order for it to be considered a trade secret. The main instrument used for these purposes is a confidentiality agreement (or nondisclosure agreement). A confidentiality agreement can include a thorough description of the information that shall be considered secret and the penalties provided in the case of breach of confidentiality. In general, violation of trade secrets is punished by a fine, imprisonment, or both (Mexican Industrial Property Law, 2020).

The main advantage of using trade secrets as an option to protect an invention and/or its improvements is the low cost involved in acquiring protection. Since there are no registration fees, the only cost is that of taking the necessary measures to keep the information and the invention confidential, which is very low, particularly in comparison to the cost required to obtain a patent. Another advantage of trade secrets is that they allow their owner to protect information that would not otherwise be protected by patents. Moreover, the protection granted by trade secrets does not have a time limitation, as patents have (Contreras, 2018).

There are nonetheless some significant disadvantages associated with using trade secrets to protect inventions. The first disadvantage of trade secret protection is that, regardless of the measures that the owner of the trade secret may take to protect the trade secret, the information that the owner is trying to protect may still be discovered by independent research or reverse engineering. This leads to the second disadvantage: that the protection is usually short-lived and difficult to preserve (Resnik, 2004). A third disadvantage is the negative impact that the secrecy may have on innovation. Disclosure and sharing of knowledge have social value per se. They are the basis and source of innovation. Without disclosure of knowledge there is no basis to build on and no rich source of inspiration. Particularly in the area of molecular diagnostic testing, which relies on interdependent observation, if laboratories do not disclose patient genetic variant data, researchers will not be able to combine and cross-analyze those data and the results that are obtained. This fragmentation of data and information could result in less accurate diagnostics and fewer therapeutics (Williams, 2013; Contreras, 2018). Furthermore, keeping the state-of-the-

art of research secret and undisclosed may lead to duplication of research and a waste of economic resources, time, and effort.

Toward balanced innovation environment

Both commercialization and open science can play a key role in TM. On one hand, commercialization is a very useful, and in some cases even essential, way to fund basic research and to translate it into clinical applications. Patents are the most commonly used strategy to achieve commercialization in the biotechnology and pharmaceutical industries. The trust given to the role that patents play in the innovation in general and in TM in particular strengthens the importance of traditional commercialization (Mayfield, 2016a,b; Gold et al., 2019; Kaye et al., 2007; Castle, 2009). On the other hand, principles of open science are the basis for further medical innovation, given the cumulative nature of science, and can therefore make the translational process faster, more efficient, and less costly (Granados Moreno and Joly, 2020; Poupon et al., 2020; Gold, 2016; Granados Moreno et al., 2018; Rouleau, 2017; Low et al., 2016; Morgan-Jones et al., 2014; Caulfield et al., 2012). Likewise, open data sharing, fast dissemination of information, common ownership of knowledge, and cumulative research building are already integrated not only in day-to-day scientific practices, but also in national and international policies and guidelines.

However, strong and exclusive emphasis on commercialization has been shown to lead to data withholding, delayed publication, and, in some cases, impediments to collaboration (Granados Moreno and Joly, 2020; Simoncelli and Park, 2015; Joly et al., 2012; Heller and Eisenberg, 1998; Shapiro, 2001; Regenberg and Mathews, 2011; Caulfield, 2010; Jensen and Murray, 2005). Similarly, exclusive practices of open science can lead to problems with incentives and sustainability, particularly considering the substantial investment required for lab equipment and clinical trials as well the long-lasting processes (Morgan-Jones et al., 2014; Granados Moreno et al., 2018). Consequently, it seems advisable to design infrastructures that balance the use of both strategies.

As was mentioned earlier in the chapter, the innovation process comprises several stages. Even though all stages are interconnected, each one focuses on different aspects of the process and therefore has different needs to achieve the goals set. A framework that balances commercialization and access to information and to research tools could involve the combination of patenting practices with tools for collaboration such as the creation of patent pools, cross-licensing,⁵⁴ and patent clearinghouses at different

54. Cross-licensing is a form of bilateral license in which two patent holders grant each other a license for the use and exploitation of their respective patented inventions (Zimmeren et al., 2011).

stages of the innovation process (Granados Moreno and Joly, 2020; Low et al., 2016; Morgan-Jones et al., 2014; Bubela et al., 2017). This combination could help to ease negotiations, lessen the problems that patent thickets may cause to research, and keep transaction costs low in the early stages of the innovation process (e.g., the precompetitive stage) while still promoting commercialization in the late stages (Bubela et al., 2017; Morgan-Jones et al., 2014; Andrews, 2002; Gold, 2008; Sumikura, 2009). This balance could also incorporate principles and practices of open science (e.g., open sharing and rapid dissemination) that would favor knowledge exchange, knowledge translation, partnerships, and collaboration⁵⁵ (Joly et al., 2012).

In addition to structuring a framework that balances commercialization and open science, TM strategies should include properly trained individuals (experts), throughout the whole process from bench to bedside. For instance, there can be significant uncertainty and confusion about the meaning, benefits, costs, and effectiveness of each model. This uncertainty can deter parties from adopting the generally more balanced framework we suggest. Having experts who are knowledgeable about both models, the best way and time to implement them and interconnect them, and the governance of the framework could increase the prospect of success (Granados Moreno et al., 2018; Guerrini et al., 2016). These experts should be knowledgeable not only in terms of health and safety regulation, government-related administrative processes, science, and patents (Mankoff et al., 2004), but also in terms of how best to implement the balancing framework suggested above. Particularly regarding patents, the translation process would benefit from having, as in-house experts, lawyers, and patent agents with training in medical science (in the particular research field at hand) and medical scientists with training in intellectual property. Their knowledge and contribution could help to create a more effective patent landscape that would inform researchers and those in charge of commercializing the invention about existing patents and advise on filing for new ones (Guerrini et al., 2016). Likewise, these in-house experts need to be knowledgeable about the principles, models, frameworks, and strategies of open science and open innovation. This expertise would allow them to design and implement frameworks that combine obtaining patents and protecting the economic aspects of the inventions with enabling fast and open access to information and research tools and promoting collaborations and partnerships, thus attaining the desired balance. For instance, these experts could inform researchers in the preclinical and clinical stages about the existing patents that would

need to be negotiated and cleared in order to avoid future litigation and other type of liability (Guerrini et al., 2016). They could also advise the researchers on which patents it could be advantageous to obtain and help to prepare the applications by collecting information throughout the R&D process and identifying the relevant state-of-the-art. These experts could also advise on the creation of patent pools, cross-licensing, patent clearinghouses, and partnerships. Policies of rapid sharing that simultaneously promote access to information, maintain medical inventions protected, and encourage building collaborations could also be developed. Finally, their expertise could be helpful in patent infringement proceedings in which researchers may be involved at any stage of the translational process (Kaye et al., 2007).

Conclusion

TM, commercialization (patents), and open science (access to information and research tools) aim to promote and enable the advancement of medical science and innovation. TM advances medical science by facilitating the translation of basic research into clinical applications that would ultimately benefit patients. This translation requires rigorous scientific research, sufficient funding, timely and appropriate business decisions, compliance with regulatory systems and legal and ethical frameworks, extensive communication and networking among the different parties, and product development. As we mentioned at the beginning of this chapter, the COVID-19 pandemic emphasized the importance of having a TM process that meets these requirements and that is therefore able not only to respond to medical needs in regular times, but also to more efficiently address pandemics (Zerhouni et al., 2020).

Patents, being the most common form of intellectual property used as strategy of commercialization in the field, advance science in three ways. First, patents may entice researchers and investors into engaging in medical research by providing economic rights that allow them to recoup their investment and to profit from the medical invention. Second, patents, used as commodities, help to assign a market value to the medical inventions they protect, therefore facilitating their trade and commercialization in the information market. Third, patents favor the creation of a state-of-the-art or creative commons by requiring a thorough description of the invention that enables any person skilled in the art to reproduce it and by releasing all that information into the public domain after the 20-year period of protection (Mayfield, 2016a,b).

55. In some places, such as Quebec, these strategies, combined with people exchange, entrepreneurship, and commercialization, are encompassed in the term *valorization*. This term is considered broader than the term *commercialization* because it is not motivated primarily by profit, as the latter is (Joly et al., 2012).

Principles of open science (e.g., open sharing of data, fast dissemination of knowledge, and the importance of commonly owned knowledge to build cumulative research) promote innovation by facilitating access to information and research tools (Morgan-Jones et al., 2014; Rouleau, 2017; Gold, 2016). However, strong and exclusive emphasis on each one of them has its drawbacks.

Developing and implementing a TM framework that combines practices of commercialization, such as patents, with principles of open science can maximize the benefits and mitigate the unintended consequences of intellectual property strategies and of open science principles. For instance, the COVID-19 pandemic has mobilized the scientific community to build partnerships, work collaboratively, and more broadly and openly share data to generate drugs, diagnostics, and vaccines in various stages of development (Courage, 2020; Zerhouni et al., 2020; OpenCovidPledge, 2020; Thomas, 2020; WHO, 2020a,b). The framework that we propose should be designed and executed in ways that encourage and favor collaboration and partnership between preclinical (academics) and clinical (industry) R&D, thereby reaping the optimal social and economic value of current medical research (Morgan-Jones et al., 2014; Bubela et al., 2017; Caulfield et al., 2012). Some of the strategies that the proposed framework could include in order to achieve collaborations and partnerships are, on the one hand, cross-licenses, patent pools, and clearinghouses, and on the other hand, policies of fast dissemination and open sharing among the parties to such partnerships. The proposed framework would also benefit from training and employment of in-house experts that implement the framework and integrate practices and principles of open science and of patents in the relevant medical research area. Implementing this framework throughout the R&D process, beginning at its early stages, could favor a more efficient, fair, and productive collaboration among the different parties and actors involved in the R&D process (Williams, 2013).

Acknowledgments

The authors would like to acknowledge the editorial assistance of Katie Saulnier and Sina Faraji from the Centre of Genomics and Policy, McGill University.

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Chapter 30

Translational research in the fastest-growing population: older adults

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Abstract

Aging is *the* major risk factor for morbidity and mortality due to nearly all adult diseases, including diabetes, cardiovascular disease, dementia, and cancer. Strategies for understanding and addressing age-related chronic diseases and geriatric syndromes require a root cause approach: to look beyond the individual diseases to seek understanding of aging itself as the shared driver of multiple conditions. This approach is likely to influence not only lifespan, but also healthspan, that is, the number of years one spends in good health, free from disease or disability. This goal is the foundation of an emerging subdiscipline, geroscience, which posits that common biological mechanisms of aging drive susceptibility of aged individuals to multimorbidity and functional decline. Further, geroscience postulates that some diseases (e.g., HIV infection, a prolonged ICU stay) and/or treatment of those diseases (e.g., cancer chemotherapy) may accelerate the underlying mechanisms of aging, leading to further functional decline. The promise of the geroscience approach is supported by specific examples of translational research models. In addition, an overview of promising geroscience interventions and considerations for trials to test specific approaches are discussed. Animal models and human cohorts are useful in translational research and interventions testing, but important risks and limitations exist.

Keywords: Aging; older adults; geroscience; senolytics; healthspan

Introduction

Why study aging?

Aging is not a disease state, so why devote a chapter to aging in a textbook on translational science in medicine? There are a number of convincing arguments for doing so, but perhaps most important is that aging research has the potential to dwarf the return on investment for research focused on any single disease. Age is *the*

strongest risk factor for multiple illnesses across many organ systems; addressing aging itself has a greater potential benefit for extending the human lifespan than curing any single disease (Miller, 2002). The average lifespan of a 50-year-old human in the developed world is about 31 additional years (i.e., a median life expectancy of about 81 years). It has been estimated that curing cancer would add only 4–5 years of life; a similar number of years would be gained from eliminating all heart disease. However, if one could retard aging to a degree first identified in the 1930s through caloric restriction studies in animals and, in so doing, mitigate the impact of age on multiple diseases, the median life expectancy for those at age 50 would be expected to increase by 29 years, from 81 to 110. That is nearly double the impact of curing cancer, heart disease, stroke, and diabetes combined (Miller, 2002). Thus investing in aging research is likely to at least rival, if not exceed, the impact of research aimed at cure or prevention of any specific disease.

A second reason to study aging is that older adult populations worldwide are growing rapidly. Improved sanitation, reduced maternal and traumatic deaths, and advances in treating and preventing infections have increased life expectancy to 70–80 years even in “developing” countries such as China, Brazil and Colombia (Organisation for Economic Co-operation and Development, 2020). In industrialized nations, modern medical care has further increased the lifespan; as a result there are rapidly increasing populations of older adults in the United States, for instance. The most recent U.S. Census showed that the 65-and-older population had grown by over one-third since 2010; no other age group saw such a fast increase (US Census, 2021). Currently, more than one in every seven people, or 15.6% of the U.S. population, is over the age of 65 years (Administration on Aging, 2018). This segment of the population accounts for the majority of healthcare expenditures in the United

States and other developed countries. The average yearly expenditure for health care of individuals age 65 years and over increased by 43% from 2007 to 2018 to almost \$10,000, and more than double that figure for those age 85 years and over ([US Census, 2021; Administration on Aging, 2018](#)).

Lifespan versus healthspan

Despite marked increases in median lifespan in the last two centuries, the maximum human lifespan has remained essentially constant at about 110 years. Further extending lifespan beyond that level is a problem of enormous scientific *and* ethical consideration. Further, with improved survival of acute illnesses that previously resulted in early to middle-aged adult deaths, there has been a concomitant increase in those living—and aging—with accumulating chronic conditions ([Centers for Disease Control and Prevention, 2021](#)). This realization has led to the concept of “healthspan” [reviewed in [Kirkland \(2013\)](#)], that is, the period of time one spends in healthy, active life before the occurrence of functional limitation and dependence. Extending healthspan rather than lifespan is a major goal of aging research. Focusing on healthspan may also have a greater impact on healthcare costs than disease-focused or lifespan extension will. Surviving one illness essentially means that we live to experience another and another and another, increasing the lifetime cost of health care for an individual. However, improving healthspan has the potential for considerable cost savings resulting from pushing severe, debilitating illnesses to the very end of life, reducing the time during which a person requires high-cost, labor-intensive care and support.

Translational aging research

Using a geroscience approach

Changing demographics portend unprecedented levels of chronic diseases and healthcare burden. This challenge motivates efforts to extend human *healthspan* informed by the so-called geroscience hypothesis. It posits that common biological mechanisms of aging play important roles in the susceptibility of aged individuals to multiple chronic diseases. Further, certain severe illnesses or exposures can result in the accumulation of biochemical and cellular features of aging that may reciprocally accelerate underlying mechanisms of aging ([Kohanski et al., 2016](#)). Such individuals may be at risk for prematurely developing an aged phenotype. Geroscience bridges the interests of biologists focused on understanding basic mechanisms that drive aging and geriatricians attempting to improve the health and quality of life of older patients. The promise of geroscience is that the incidence of aging-related

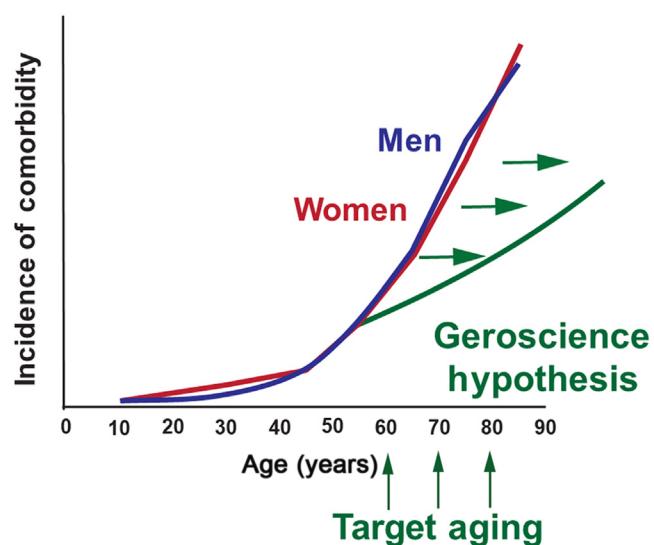


FIGURE 30.1 The geroscience hypothesis. Targeting biological aging processes could delay the incidence of multiple age-related diseases. Adapted from Justice, J.N., Niedernhofer, L., Robbins, P.D., Aroda, V.R., Espeland, M.A., Kritchevsky, S.B. et al., 2018b. Development of clinical trials to extend healthy lifespan. *Cardiovasc. Endocrinol. Metab.* 7(4), 80–83 with kind permission by Wolters Kluwer Health, Inc.

chronic disease can be prevented, delayed, or attenuated by therapeutically targeting fundamental biology shared by the aging process and common chronic diseases (Fig. 30.1) ([Burch et al., 2014; Kennedy et al., 2014; Sierra, 2016; Sierra and Kohanski, 2017](#)).

A translational approach is required to facilitate development of therapeutics targeting aging biology or based on geroscience principles. The term *translational research* has different connotations for different investigators. Some scientists hold the traditional perspective of a unidirectional process in which basic science laboratory discoveries are extended to humans, often culminating in a clinical trial. In contemporary views, however, this research continuum is intended to be dynamic and bidirectional, with insights gained at the translational level informing refined selection of preclinical models and new discovery at the bench ([Seals, 2013; Seals and Melov, 2014](#)).

Extending this concept to establishing the therapeutic potential of targeting aging biological processes, research activities include investigations from molecular signals and *in vitro* studies of senescence to evaluating interventions in model organisms to clinical trials in patient populations that could eventually influence clinical practice guidelines and community-level health ([Seals, 2013](#)). These investigations are intended to spur discovery at the bench, refine interventions, improve animal models, identify biomarkers, and develop methodologies to measure biological aging versus chronological aging in a clinical setting.

The potential of gerotherapeutics to improve health and function (physical and mental) in older adults has resulted in rapidly growing academic translational research programs as well as interest from industry in aging as a therapeutic target.

Biology of aging: hallmarks, pillars, and health

Geroscientists have framed a set of tightly interrelated cellular biological processes that are manifested with aging and could be potential targets to extend the health-span. These processes are collectively known as hallmarks or pillars of aging (Lopez-Otin et al., 2013; Kennedy et al., 2014). Candidate hallmarks or pillars meet the following criteria: (1) They are manifested during normal chronological aging; (2) their experimental aggravation accelerates phenotypes of aging (functional decline, frailty, disease, death); and (3) their experimental amelioration retards the normal aging process and hence increase the healthy lifespan. Using this framework akin to Koch's postulates for assigning the "cause" of disease to an infectious agent, a current list of such biological processes include the following:

- Genomic instability and buildup of DNA damage
- Epigenetic alterations, or changes in how genes are turned on or expressed
- Proteostasis and the accumulation of damaged and misfolded proteins
- Changes in pathways that regulate growth, metabolism, and nutrient sensing
- Loss of adult stem cells and ability of cells or tissues to regenerate
- Mitochondria with reduced energy production and greater damaging, oxidative byproducts
- Increased maladaptive inflammation and disrupted cell-to-cell communication
- Accumulation of senescent cells that are damaged and highly proinflammatory

These mechanisms of aging can be conceptualized in broad themes.

Damage accumulation over time

This can result from misfolded proteins, oxidative membrane damage, or accumulation of genetic damage resulting in genomic instability, epigenomic alterations, somatic mutations, altered gene expression, or mutation and/or deletions in mitochondrial DNA.

Failure of endogenous repair mechanisms

The age-related loss of a cell's protein quality control systems lose activity, that is, the autophagy-lysosomal system and the ubiquitin-proteasome system have reduced

ability to repair the damage that occurs over time or with exposure to cellular stressors. This can lead to impaired protein homeostasis, greater accumulation of misfolded proteins, diminished efficacy of the mitochondrial respiratory chain, and increased electron leakage, resulting greater oxidative stress.

Higher-order consequences of damage

Damage processes can result in cellular dysfunction, altered intracellular signaling pathways, or even "bystander" cell damage. For example, mitochondrial dysfunction, loss of stem cells, accumulation of DNA-damaged senescent cells, diminished nutrient sensing, and dysregulated intercellular communication can lead to persistent, local, or low-level systemic inflammation.

From biological mechanisms to clinical aging phenotypes

Conceptualizing the biological hallmarks or pillars of aging under these broad themes makes clear three ideas: (1) The molecular pathways and biological mechanisms that underlie aging are not independent but rather are highly interconnected and not easily disentangled. (2) Dysregulation in molecular and biochemical processes of aging are integrative and have unique consequences across levels of increasing complexity from the molecular and cellular to the system and organismal levels. (3) How these aging processes manifest across levels of biological organization is complex with new properties emerging at higher levels of organization. Thus clinical aging phenotypes are emergent properties, with a spectrum of highly interdependent changes in the biological aging processes that manifest as organ-level dysfunction, disease, and geriatric syndromes.

An example is cellular senescence. Cells regularly experience potentially oncogenic insults, oxidative damage, and other stressors that can lead to DNA damage. This damage can lead to genomic instability and somatic mutation, or it can be recognized by a damage sensor, which initiates a reversible cell cycle arrest to attempt repair. If the repair mechanisms of the cell are themselves damaged or cannot be recovered, the cell may permanently exit the cell cycle, so the damaged DNA is not replicated; this is a strategy to avoid carcinogenesis. This permanent cell cycle arrest is called cellular senescence. For a senescent cell to maintain cell cycle arrest, certain cell cycle genes must be activated. In some cell types and depending on the source of initiating damage, a number of factors can be secreted, including proinflammatory cytokines, chemokines, profibrotic factors, and signaling molecules. These factors are collectively referred to as the senescence-associated secretory phenotype (SASP). The

cytokines may promote recruitment and activation of immune cells to the local environment, but senescent cells are resistant to cell death, and the immune cells are unable to be effectively cleared, leading to accumulation of cells and a chronically prosenescence and proinflammatory environment. This can lead to chronic tissue inflammation, organ-level dysfunction, and even, paradoxically, carcinogenesis and tumor growth (Coppe et al., 2010; Tchkonia et al., 2013; Ovadya and Krizhanovsky, 2014). In a landmark study by Baker et al. (2011), elimination of senescent cells in a progeroid mouse by selectively deleting senescent cells (targeted by their expression of p16) resulted in marked mitigation of the accelerated aging phenotype. The effect was presumably achieved through elimination of the senescent cells *and* improved function of surrounding cells that had previously been impaired by cytokines produced by cells that had “aged” into the SASP (Pepper, 2011).

Evidence suggests that even relatively few senescent cells can exert detrimental effects *in vivo* (Xu et al., 2017, 2018). A striking example is a model of senescent cell transplantation. Xu et al. harvested adipose progenitors or cells from ear cartilage from luciferase-expressing mice, then induced senescence by ionizing radiation. The senescent cells were transplanted into wild-type mice; 40 days posttransplant these cells were highly metabolically active, secreted potent secretory factors, and were positive for several biomarkers of cellular senescence. Importantly, small number of cells were transplanted such that only 1 in 7000–15,000 cells (or 0.01%–0.03%) in the whole body, or 1 cell in 350 cells (0.28%) locally at the site of transplantation was sufficient to elicit a host of common age-related phenotypes, including decreased grip strength, walking speed, and hanging endurance (Xu et al., 2018). In 17-month-old mice transplanted with senescent cells, there was a 5.2-fold greater risk of death compared to mice injected with nonsenescent cells (Xu et al., 2018).

Animal models in translational aging research

Animal models play a critical role in translational research, and there are important considerations when such models are applied to aging and diseases of older adult humans. The most commonly used animal model is the mouse model. There are distinct reasons to use—and not use—mice for translational research for investigating human aging. To illustrate the relative pros and cons, we will use examples of immune senescence, that is, the gradual waning of immunity with advancing age. Maue and colleagues reviewed the characteristics of human T cell aging that are well represented by mouse models, demonstrating relative parallels of age-related changes in mice with those in humans (Maue et al., 2009). This

general conservation of immune senescence across mice and humans makes the mouse model an excellent resource for experiments that are achievable only in mice.

However, rodent models frequently do not translate to human aging (Akbar et al., 2000; Fauze et al., 2008), including failure in humans of vaccine approaches that were validated in rodents (MacGregor et al., 1998). Extrapolation of data between species must therefore be made with caution. In a review titled “Of Mice and Not Men: Differences Between Mouse and Human Immunology,” Mestas and Hughes detailed the differences between these species (Mestas and Hughes, 2004). Major differences include expression level, diversity and response of pattern recognition receptors [e.g., toll-like receptors, further explored by Copeland et al. (2005), Barreiro et al. (2009), and Munford (2010)], distinct differentiation signals for T cell subtypes, and a large number of variances in cell surface marker and/or receptor ligand specificity that dictate migration and function of immune cells. Further, humans live much longer than mice, and the memory immune cells of humans must be maintained for a substantially greater time. Persistence of human memory T cell pools is likely subjected to additional constraints that may not apply to murine experimental systems (Akbar et al., 2000). Another key difference between mice and humans is their prior exposure history. All humans age in the presence of persistent viral infections, but the combination of viruses present is as unique as the number of people on earth (Virgin et al., 2009). Mice are raised in specific pathogen-free conditions to ensure that viruses such as murine hepatitis virus do not confound the experimental design. Variable exposure and immune activation to persistent viral infections carried by laboratory mice are rarely if ever included in reports of animal models of immune senescence, and it is commonly assumed, almost certainly falsely, that viral exposure and immune response to chronic virus infection in older mice is similar to that of young adult mice. In such experiments, changes attributed to age may reflect the impact of chronic viral infection as the true underlying cause.

To address some of these issues, researchers have transplanted human bone marrow into immunocompromised mice to reconstitute human immune cells or to accept fully differentiated human peripheral blood cells. These “humanized” mouse models have promise for immunology studies [reviewed in Shultz et al. (2007)], and some age-related illnesses [e.g., Alzheimer’s disease (Bruce-Keller et al., 2011)] have been substantially investigated by using humanized mouse models. A note of caution is in order, however: While intrinsic immune cell aging may be relatively well represented by humanized mice, extrinsic forces (human physiology of the liver and kidneys, species variation in response to stressors, diet,

etc.) are not represented in this model. The importance of this was shown in a study by Warren and colleagues using serum or mononuclear cells from different species (Warren et al., 2010; Bruce-Keller et al., 2011). In that study, inflammation in response to lipopolysaccharide was better predicted by the species from which serum was derived than in the species of origin for the cell, suggesting that there are circulating factors that may influence responses more than intrinsic cell properties.

The mouse is the most common model used for the pre-clinical evaluation of geroscience-inspired therapies. The NIA-funded Interventions Testing Program (ITP), funded by the National Institute on Aging (NIA) tests drugs and other interventions to determine whether they prevent disease and extend the lifespan in genetically heterogeneous (outbred) mice (NIA Interventions Testing Program, 2021; Miller et al., 2007; Nadon et al., 2017). The ITP is conducted at three centers to control for laboratory-specific environmental differences. It has shown that of more than 30 compounds with the potential to target biology of aging, seven extend lifespan significantly: nordihydroguaiaretic acid, aspirin, acarbose, protandim, rapamycin, 17- α -estradiol, and glycine. Most compelling are the studies of rapamycin [an mechanistic target of rapamycin (mTOR) inhibitor]. These show that its administration increases the lifespan and healthspan in a dose-dependent manner. Acarbose, nordihydroguaiaretic acid, 17- α -estradiol, aspirin, and protandim are mainly in effective males (Strong et al., 2008; Harrison et al., 2014). Rapamycin significantly increases lifespan in both sexes but may have a larger effect in females, and its effect appears to be enhanced by coadministration of metformin (Miller et al., 2011, 2014; Strong et al., 2016). Lifetime administration is not necessary for significant effects on longevity. For example, glycine started at 9 months increases lifespan in both male and female mice, and rapamycin started at both 12 and 22 months had significant effects (Harrison et al., 2009; Miller et al., 2019). Surprisingly, only 3 months of rapamycin treatment was sufficient to increase life expectancy by up to 60% and improve measures of the healthspan in middle-aged mice. Rapamycin works through the mTOR, and subsequent investigation in small human trials of short duration (6 weeks) of more potent mTOR inhibitors demonstrated increased influenza vaccine responses and even reductions in self-reported infections (Mannick et al., 2014, 2018)—in essence, partially reversing clinically relevant immune senescence.

Human approaches to translational aging research

Observational studies are frequently used to suggest pathways that might be important to the aging process as a

method to develop intervention targets. The most rudimentary approach is to compare young and old individuals. This approach can be unreliable and nonspecific. It is often difficult to ensure the representativeness of the two groups, so results can be difficult to replicate. It is also difficult to separate age-related changes from the effects of common comorbidities and disease conditions common among older adults. In an attempt to remedy this problem, some investigators have identified samples of older individuals with no identifiable clinical conditions. This is difficult to do, primarily because many older people without a clinical diagnosis may have substantial subclinical pathology. For example, about one-third of older individuals have clinically manifest cardiovascular disease, and another one-third have substantial subclinical disease, as identified by using extensive noninvasive imaging. Thus the definition of “disease free” will vary depending on the intensity of disease detection.

A variety of “omics” approaches are increasingly being employed to identify both intervention targets and potential biomarker readouts for use as clinical trial outcomes. Several groups have compared old and young using aptamer-based assays (Menni et al., 2015; Sathanay et al., 2020; Tanaka et al., 2020). While there are many differences in the proteins that are detected, a few (e.g., pleiotrophin, chordin-like protein 1) have been consistently identified. One group has examined the pattern of gene expressions in monocytes collected in the Multi-Ethnic Study on Atherosclerosis (MESA). The gene expression networks most associated with aging were related to mitochondrial ribosomal synthesis, oxidative phosphorylation, and autophagy (Reynolds et al., 2015). Such comparisons cannot distinguish between the consequences of accumulated age-related damage and the pace of the ongoing aging processes. Ideally, a biomarker would reflect the rate of aging at the time of measurement. If a person ages 15% faster than average, a 15% higher biomarker level might not be recognized in such old-versus-young comparisons.

Interventions inspired by young-old comparisons have frequently failed. Older adults have lower levels of insulin-like growth factor-1 (IGF-1), growth hormone, and DHEA-S, but repletion experiments have generally shown no functional benefits and some unexpected harms.

Long-lived individuals and populations have been studied in the attempt to identify physiologies and pathways associated with longevity. Such populations can provide clues to habits that may enhance longevity, but they cannot be used to test mechanistic hypotheses. Many research groups have studied centenarians to try to identify the basis for their extreme longevity. This approach has met with limited success, and no clear risk factor profile has emerged. However, it is clear that longevity runs in families, so many groups are employing genomic

approaches to identify molecular pathways important to human longevity. Many candidate loci have been identified within given populations, but few have been consistently replicated. Current best evidence implicates genetic variability at the *APOE* and *FOXO3* loci (Broer et al., 2015). The *APOE4* genotype is strongly associated with Alzheimer's disease, and the *APOE2* allele may exert a protective effect. *FOXO3* is involved in IGF-1/insulin signaling. It has a homolog in the *Caenorhabditis elegans* model *Daf-16* gene, where its expression is important to lifespan regulation. The issues and complexity in conducting and interpreting genetic studies of human aging and longevity are well summarized elsewhere (Brooks-Wilson, 2013).

Longitudinal cohort studies, such as the Health Aging and Body Composition Study and the Cardiovascular Health Study (CHS), have proven to be productive for addressing new hypotheses regarding the aging process, healthspan, and lifespan. These studies enumerate and carefully phenotype large samples of older individuals, who are then followed and reexamined at intervals over extended periods of time. The studies also typically have biospecimen repositories to allow the efficient examination of new hypotheses as well as identification of individuals of similar ages and overall health statuses to test factors predicting the age-related outcomes. The repeated measures approach allows investigators to parse out physiological and molecular differences that are associated with longer life from those that may change in the anticipation of death. One can look at early deaths (within the first few years) separately or look at risk factor trajectories until the time of the event. Such an analysis suggests that many of the cognitive changes underlying Alzheimer's disease begin to manifest themselves 8–10 years before a diagnosis is made.

Testing treatments to extend healthspan and lifespan

The goal of geroscience is to increase the human healthspan. A common framework for approaching this goal is presented in Fig. 30.1. Many physiological parameters decline with age, including skeletal muscle mass, bone mineral density, maximum heart rate, forced expiratory volume in 1 second, and glomerular filtration rate. These declines can be exacerbated by disease pathologies. These changes contribute to declines in functional performance, which are reflected in things such as usual walking speed, peak exercise capacity, psychomotor speed, and working memory. When severe enough, these declines lead to disability and death.

The framework suggests two primary strategies for extending healthspan: building functional reserve and

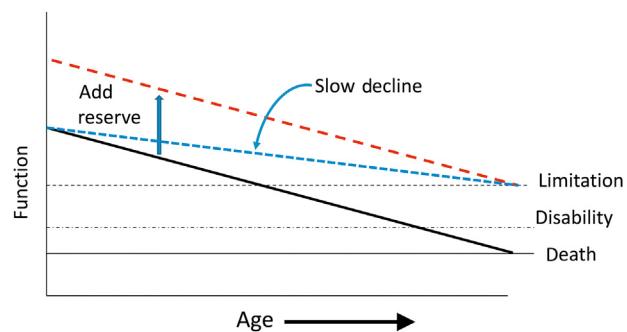


FIGURE 30.2 A model for how age-related physiological changes can affect the healthspan and lifespan. Alternative strategies to delay the onset of disability and death include adding physiological reserve or slowing physiological decline.

slowing rates of decline (Fig. 30.2). Reserve reflects “spare” physiological capacity. One can bring reserve to old age, or one can build reserve even in old age. For example, muscle weakness is a major risk factor for physical disability. On average, strength declines about 1% each year from about age 40 and accelerates after about age 70. A 50-year follow-up of 2775 British infants born in 1946 showed that participants who were larger babies had stronger grips as middle-aged adults (Kuh et al., 2002). Data from a cohort of Hawaiian men of Japanese descent showed that grip strength at age 50 was a strong risk factor for disability 25 years later (Iwaoka and Tannebaum, 1976). Thus it can be inferred that larger infants bring more muscular reserve into old age. On the other end of the age spectrum, Fatarone et al. (1990) showed that in nonagenarians a weight-training regimen increased their strength by an average of 174% and improved their gait speed by 48%, indicating that reserve can be added even at very old age.

Studies of risk factors for the rate of decline of physiological systems and functions require multiple measurements often over an extended period of time. The best established ongoing study of the aging process is the Baltimore Longitudinal Study on Aging (BLSA), which has followed some participants for more than 40 years (BLSA Baltimore Longitudinal Study of Aging, 2021; Normal Human Aging: The Baltimore Longitudinal Study of Aging, 1985). The BLSA shows that while on average many physiological systems decline with age, there is substantial person-to-person variability in how these systems change through time with some persons declining very quickly and others maintaining a level of physiological function with little evident decline over decades. Strength training and high-intensity aerobic exercise are associated with increased strength and aerobic reserve, but they do not seem to slow the rate of decline of these capacities. Tobacco use accelerates decline in lung function. Poor diet is associated with faster declines in

cognitive function. Declines in physiological function can be accelerated by disease events and associated periods of physical inactivity/bed rest. HIV/AIDS and/or its treatment accelerate declines in physiological function; a myocardial infarction can permanently impair the heart's ability to deliver blood. Interestingly, rates of change do not appear to be strongly correlated across physiological systems. The CHS has looked at longitudinal changes in several physiological measures representing different organ systems, and the correlation in these changes over time is low (Newman et al., 2016).

Clinical trials in geroscience

The ITP approach provides the preclinical foundation for testing such interventions in humans (NIA Interventions Testing Program, 2021; Miller et al., 2007; Nadon et al., 2008). ITP paradigm would be very difficult to translate directly into humans. The typical design involves both male and female mice with treatment initiation between 4 and 10 months of age. Mice are followed until 90% have died. This is roughly equivalent to starting these treatments in humans at between 16 and 40 years of age and following them until age 90. It would be logistically and financially infeasible to conduct a 40- to 60-year experiment in a sufficient number of people to evaluate an intervention's effect on life expectancy. There are also important ethical considerations. Participants would be asked to take a test compound for the next 40–60 years. The intervention would not treat any particular complaint or disease, so the immediate benefits to those receiving the test intervention would be expected to be small. The study would be ethical only if the long-term safety of the intervention was virtually certain, because the risk of potential harm would outweigh uncertain long-term benefits. Nevertheless, geroscience-inspired clinical trials are underway with outcomes ranging from frailty and Alzheimer's disease to multimorbidity and immune function (Table 30.1). Most of these trials are relatively short-term and focus on aging diseases or syndromes or biomarkers of aging.

Outcomes for clinical trials testing geroscience-inspired interventions

There is no single accepted endpoint for clinical trials testing geroscience-inspired interventions. Different teams have employed or proposed a variety of healthspan measures. The ASPREE study's primary endpoint was disability-free survival (time until death or incident dementia or persistent physical disability) (McNeil et al., 2018). The Targeting Aging with Metformin (TAME) Trial design team proposed a composite endpoint focused of slowing multimorbidity (death, incidence of mild

cognitive impairment or dementia, myocardial infarction, stroke, cancers, or decompensated heart failure) (Justice et al., 2018b; Espeland et al., 2020). A frailty deficit index has also been proposed and has been evaluated in post hoc analyses of the Look Ahead Trial (Simpson et al., 2020). A deficit index is the proportion of health deficits accumulated by a person out of a long list of potential deficits. Originally proposed by Rockwood and Mitnitski (2007), it has been found to be a powerful predictor of mortality. The Look Ahead Trial randomized more than 5000 individuals with type 2 diabetes to receive either health education or an intensive lifestyle intervention focused on weight loss and increasing exercise. The intervention was highly effective in reducing the average deficit proportion in the intervention group (Simpson et al., 2020).

It is impractical to expect every intervention to be evaluated by establishing its impact on the healthspan. Therefore a great deal of effort has gone into developing and validating biomarkers that might change over a much shorter period of time that would serve to identify promising intervention candidates (Fig. 30.3). Biomarkers have a strong statistical link to the desired outcome; although not necessarily in the causal pathway, they move in a direction after interventions that predicts clinical outcomes. For example, high serum LDL cholesterol is a biomarker for atherosclerotic heart disease. Its elevation is related to higher coronary heart disease risk, and interventions that lower LDL cholesterol also lower the risk of heart disease. The link is so well established that new drugs can be approved on the basis of their cholesterol-lowering properties rather than having to prove a reduction in heart disease risk. The strength of LDL cholesterol as a biomarker for heart disease is based on its causal role in the promotion of atherosclerosis. The search for biomarkers of longevity is complicated by the fact that a single pathway or mechanism that leads to long life has not been identified. Nevertheless, it is possible to propose criteria for biomarkers such that if they were positively affected by a new treatment, one would expect life extension with some confidence (Box 30.1).

These biomarker criteria were put to the test in a use case to develop the aforementioned TAME trial (Justice et al., 2018a). TAME was conceived as a clinical trial to determine whether metformin exerts effects on multiple aging pathways versus a specific disease or mechanistic pathway. Therefore evaluating single disease specific biomarkers may not be sufficient. A multivariable biomarker panel that cuts across cellular pathways, organ systems, and disease states is necessary (Justice et al., 2018b). The components of this biomarker panel were selected on the basis of current best-available evidence filtered through the biomarker criteria (Box 30.1). Of the 258 candidate

TABLE 30.1 Interventions into biological aging processes are being advanced to clinical trials targeting a range of age-related diseases and geriatric syndromes.

Intervention	Mechanism or target	Population	Duration	Outcome	Trial phase and identifier
<i>Nutrient-sensing pathways</i>					
Acarbose	<ul style="list-style-type: none"> ● Inflammatory markers ● Glucose metabolism 	Men with Impaired fasting glucose > 60 years N = 34	10 weeks	<ul style="list-style-type: none"> ● Tissue gene expression 	Phase II; NCT02953093 Study of Acarbose in Longevity (SAIL)
Acarbose	<ul style="list-style-type: none"> ● Alter microbiome ● Glucose metabolism 	Nondiabetic 75–95 years N = 8	12 weeks	<ul style="list-style-type: none"> ● Change in microbiome 	Phase II; NCT02865499 Acarbose antiaging effects in geriatric subjects
Calorie restriction	<ul style="list-style-type: none"> ● Nutrient sensing ● Inflammation/immune ● Oxidative stress 	Nonobese 21–50 years N = 238	24 months	<ul style="list-style-type: none"> ● Body temperature ● Resting metabolic rate 	Phase II; NCT00427193 Comprehensive assessment of long-term effects of reducing intake of energy (CALERIE)
Calorie restriction	<ul style="list-style-type: none"> ● Nutrient-sensing pathways ● Cellular stress resistance 	Acute kidney injury after cardiac surgery > 18 years N = 81	7 days preoperative	<ul style="list-style-type: none"> ● Serum creatinine 24 hours after induction of ischemia 	Phase II; NCT01534364 Effect of a preoperative calorie restriction on renal function after cardiac surgery (CR_KCH)
Metformin	<ul style="list-style-type: none"> ● AMPK activation ● Mitochondria and energetics ● Insulin/IGF-1 signaling 	Nondiabetic with slow gait 65–70 years N = 3000	48 months	<ul style="list-style-type: none"> ● Composite of incident disease (cardiovascular disease, cognitive impairment, cancer) or death 	Phase III; (proposed) Targeting Aging with Metformin (TAME)
Metformin	<ul style="list-style-type: none"> ● Activate AMPK ● Mitochondria and energetics ● Insulin/IGF-1 signaling 	Impaired fasting glucose 65–90 years N = 120	24 months	<ul style="list-style-type: none"> ● Frailty phenotype 	Phase II; NCT02570672 Metformin for preventing frailty in high-risk older adults
Metformin	<ul style="list-style-type: none"> ● Glucose metabolism ● Insulin/IGF-1 signaling ● Modulate immune system 	Nondiabetic with chronic obstructive pulmonary disease 40–75 years N = 30	12 weeks	<ul style="list-style-type: none"> ● Sputum glucose concentration 	Phase I; NCT03651895 Metformin to reduce airway glucose in COPD patients
Metformin	<ul style="list-style-type: none"> ● Glucose metabolism ● Insulin/IGF-1 signaling ● Modulate immune system 	Nondiabetic > 65 years N = 26	20 weeks	<ul style="list-style-type: none"> ● Immune responses to flu vaccine 	Phase II; NCT03713801 Vaccination efficacy with metformin in older adults: a pilot study
Metformin	<ul style="list-style-type: none"> ● Glucose metabolism ● Modulate immune system 	Nondiabetic 63–89 years N = 50	12 weeks	<ul style="list-style-type: none"> ● Antibody responses 	Phase II; NCT03713801 Impact of metformin on immunity

(Continued)

TABLE 30.1 (Continued)

Intervention	Mechanism or target	Population	Duration	Outcome	Trial phase and identifier
Metformin	<ul style="list-style-type: none"> • AMPK activation • Insulin/IGF-1 signaling • Modulate immune system 	Nondiabetic, amnestic, mild cognitive impairment 55–90 years <i>N</i> = 370	24 months	<ul style="list-style-type: none"> • Cognitive function 	Phase II; NCT04098666 Metformin in Alzheimer's dementia prevention
Nicotinamide riboside (NR)	<ul style="list-style-type: none"> • NAD + precursor • Energetics 	<ul style="list-style-type: none"> • 50–79 years • Hypertension, aging • <i>N</i> = 118 	3 months	<ul style="list-style-type: none"> • Systolic blood pressure 	Phase II; NCT03821623 NR for treating elevated systolic blood pressure and arterial stiffness
Nicotinamide mononucleotide	<ul style="list-style-type: none"> • NAD + precursor • Cardiovascular and metabolic functions 	Women with impaired fasting glucose 55–70 years <i>N</i> = 25	8 weeks	<ul style="list-style-type: none"> • Muscle insulin sensitivity 	Phase N/A; NCT03151239 Effect of "nicotinamide mononucleotide" on cardiometabolic function (NMN)
Resveratrol	<ul style="list-style-type: none"> • Nutrient sensing pathways • Activate sirtuins • Lower neuroinflammation 	Alzheimer's disease >50 years <i>N</i> = 120	12 months	<ul style="list-style-type: none"> • Adverse events • Volumetric MRI 	Phase II; NCT01504854 Resveratrol for Alzheimer's disease
Rapamycin analog (Everolimus)	<ul style="list-style-type: none"> • Inhibit mTOR • Modulate immune system 	>65 years <i>N</i> = 240	4 weeks post flu vaccine	<ul style="list-style-type: none"> • Immune response to vaccination 	Phase II; ACTRN12613001351707 Effects of low dose everolimus and/or BEZ235 on vaccine response in elderly
RTB101 (dactolisib)	<ul style="list-style-type: none"> • Inhibit mTOR • Modulate immune system 	>65 years <i>N</i> = 1024	16 weeks	<ul style="list-style-type: none"> • Respiratory tract infections 	Phase III; NCT04668352 Effect of RTB101 on illness associated with respiratory tract infections in the elderly

Cellular senescence and senescence-associated secretory phenotype

Dasatinib + quercetin	<ul style="list-style-type: none"> • Lower inflammation and profibrotic factors 	Idiopathic pulmonary fibrosis ≥ 50 years <i>N</i> = 26	3 weeks	<ul style="list-style-type: none"> • Feasibility • Biomarkers 	Phase I/II; NCT02874989 IPF Study
Dasatinib + Quercetin	<ul style="list-style-type: none"> • Clear senescent cells • Lower inflammation 	Hematopoietic stem cell transplant survivors ≥ 18 years <i>N</i> = 10	3 days	<ul style="list-style-type: none"> • Biomarkers of cell senescence and inflammation • Frailty phenotype 	Phase I/II; NCT02652052 Hematopoietic Stem Cell Transplant Survivors Study
Dasatinib + Quercetin	<ul style="list-style-type: none"> • Clear senescent cells • Lower inflammation 	Chronic kidney disease and diabetes 40–80 years <i>N</i> = 16	3 days	<ul style="list-style-type: none"> • Biomarkers of cell senescence and inflammation • Frailty phenotype 	Phase II; NCT02848131 Senescence in chronic kidney disease

(Continued)

TABLE 30.1 (Continued)

Intervention	Mechanism or target	Population	Duration	Outcome	Trial phase and identifier
Dasatinib + Quercetin	<ul style="list-style-type: none"> Clear senescent cells Lower inflammation 	Alzheimer's disease ≥ 65 years N = 48	12 weeks	<ul style="list-style-type: none"> Adverse events Biomarkers of cell senescence and inflammation 	Phase I/II; NCT04063124 Senolytic Therapy to Modulate the Progression of Alzheimer's Disease Study (SToMP-AD)
Fisetin	<ul style="list-style-type: none"> Clear senescent cells Lower inflammation 	Diabetic kidney disease 40–80 years N = 30	2 days	<ul style="list-style-type: none"> Biomarkers of cell senescence and inflammation 	Phase I/II; NCT03325322 Inflammation and stem cells in diabetic and chronic kidney disease
Fisetin	<ul style="list-style-type: none"> Clear senescent cells Lower inflammation 	Women with frailty ≥ 70 years N = 40	2 months	<ul style="list-style-type: none"> 6-minute walk distance Gait speed 	Phase II; NCT03675724 Alleviation by fisetin of frailty, inflammation, and related measures (AFFIRM-LITE)
UBX0101 (injection)	<ul style="list-style-type: none"> Clear senescent cells Lower inflammation 	Knee osteoarthritis 40–85 years N = 78	12 weeks	<ul style="list-style-type: none"> Safety Reported pain Biomarkers 	Phase I/II; NCT03513016 A safety and tolerability study of UBX0101
Unity Bio	<ul style="list-style-type: none"> Clear senescent cells Lower inflammation 	Knee osteoarthritis N = 161	1 year	<ul style="list-style-type: none"> Incidence of adverse events up to 1 year post-injection 	Phase III; NCT04349956 Long-term follow-up study of patients with osteoarthritis of the knee treated with UBX0101 or placebo
ABT-263	<ul style="list-style-type: none"> Clear senescent cells Lower inflammation 	Women 18–55 years N = 12	Single dose	<ul style="list-style-type: none"> Plasma drug levels 	Phase I; NCT01053520 Bioavailability of a new ABT-263 formulation
<i>Stem cell exhaustion</i>					
Allogeneic human mesenchymal stem cells	<ul style="list-style-type: none"> Modulate immune response Tissue repair 	Frailty 60–95 years N = 65	1 month	<ul style="list-style-type: none"> Incident emergent serious adverse events 	Phase I/II; NCT02065245 hMSC in patients with aging frailty via intravenous delivery (CRATUS)
Longeveron mesenchymal stem cells	<ul style="list-style-type: none"> Modulate immune response Regenerative capacity 	Frailty, slow gait speed, and elevated TNF- α 70–85 years N = 150	180 days	<ul style="list-style-type: none"> 6-minute walk distance 	Phase IIb; NCT03169231 Phase IIb to evaluate longeveron mesenchymal stem cells to treat aging frailty
<i>Autophagy</i>					
High-dose trehalose	<ul style="list-style-type: none"> Activate autophagy 	Healthy 50–79 years N = 110	12 weeks	<ul style="list-style-type: none"> Arterial stiffness 	Phase N/A; NCT01575288 Oral trehalose therapy to reverse arterial aging
<i>Endocrine and cell-to-cell communication</i>					
Myostatin antibody LY2495655	<ul style="list-style-type: none"> Inhibit myostatin 	History of falls and muscle weakness > 75 years N = 201	24 weeks	<ul style="list-style-type: none"> Appendicular lean body mass 	Phase II; NCT01604408 A study in older participants who have fallen and have muscle weakness

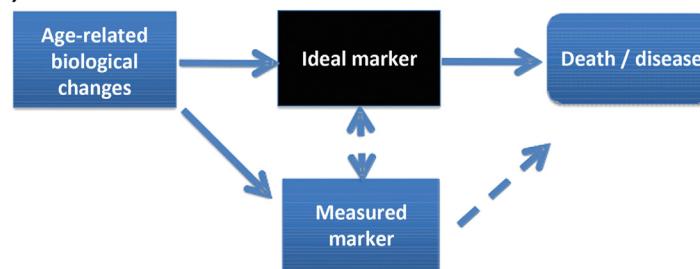
(Continued)

TABLE 30.1 (Continued)

Intervention	Mechanism or target	Population	Duration	Outcome	Trial phase and identifier
Myostatin antibody LY2495655	• Inhibit myostatin	Patients undergoing elective total hip arthroplasty > 50 years N = 400	12 weeks	• Appendicular lean body mass	Phase II; NCT01369511 A study of LY2495655 in older participants undergoing elective total hip replacement
Plasma from young donor	• Lower circulating growth factors, inflammation	Alzheimer's disease 50–90 years N = 18	9 weeks	• Adverse events • Feasibility	Phase?; NCT02256306 The PLasma for Alzheimer SymptoM Amelioration (PLASMA) Study (PLASMA)
Plasma from young donor	• Lower circulating growth factors, inflammation	> 35 years N = 200	1 month	• Blood biomarkers	Phase N/A; NCT02803554 Young donor plasma transfusion and age-related biomarkers
Plasma from young donor	• Lower circulating growth factors, inflammation	Parkinson's disease 50–80 years N = 16	8 weeks	• Adverse events	Phase I; NCT02968433 The Stanford Parkinson's Disease Plasma Study (SPDP)
Plasma form young donor	• Lower circulating growth factors (G-CSF), inflammation	Frailty 55–95 years N = 30	24 months	• Adverse events • Immune risk profile	Phase I/II; NCT03458429 Safety, efficacy of FFP from healthy donors to ameliorate frailty and enhance immune function in older individuals

(A)

FIGURE 30.3 Biomarkers are measurements used to substitute for an outcome that may be rare or take a long time to develop. Biomarkers may be (A) on the causal pathway or (B) correlated with a causal marker or the outcome itself.

(B)

blood-based biomarkers of aging identified from published reports and expert suggestions, only 86 were considered feasible and reliable for a large multicenter trial such as TAME (Justice et al., 2018a). This list was

refined to select candidate biomarkers emphasizing (1) relevance to aging, (2) robust and consistent ability to predict all-cause mortality and trial outcomes, and (3) responsiveness to intervention.

BOX 30.1 Criteria for biomarkers of aging.

An ideal longevity biomarker would:

1. *Correlate with chronological age.* If a marker does not change with age, it is unlikely to change in response to an intervention that affects the aging process.
2. *Have a strong and consistent relationship predicting long-term mortality across a variety of populations, disease conditions, and comorbidities independent of age.* Measures of acute physiological distress may predict short-term mortality but may not predict long-term survival; the relationship should be seen regardless of underlying disease-specific pathologies.
3. *Be responsive to change.* If the marker is unchangeable, it cannot be used to evaluate an intervention.

4. *Show a monotonic relationship with mortality.* For some physiological measures, the relationship with mortality is curvilinear. Pushing levels too low might lead to increased mortality (e.g., blood pressure).
5. *Be objectively measured.* Subjective measures can be powerful measures of outcome, but their interpretation across studies, populations, and cultures is problematic.
6. *Changes in the biomarker should be associated with differences in mortality risk.* This increases the confidence that changes caused by an intervention would be reflected in differences in long-term mortality.

The result was a short list of circulating biomarkers of biological aging that could be applied to clinical trials targeting age-related diseases or aging phenotypes:

- *Inflammation:* Interleukin-6 (IL-6) and tumor necrosis factor receptor are markers of proinflammatory cytokine dysregulation associated with mortality risk and mobility disability (Reuben et al., 2002; Penninx et al., 2004; Giovannini et al., 2011; Marti et al., 2014).
- *Stress response and mitochondria:* Growth-differentiating factor 15 is a member of the TGF- β superfamily recognized in mitochondrial dysfunction and aging stress response and is robustly associated with cardiovascular events, cognitive decline, and dementia (Wiklund et al., 2010; Jiang et al., 2016).
- *Cardiovascular:* N-terminal proB-type natriuretic peptide is typically used to screen and diagnose acute heart failure but also to predict nonvascular disease mortality (Lindholm et al., 2018) and physical decline in the absence of heart failure (Kuh et al., 2019).
- *Kidney aging:* Cystatin C is a marker of renal disease but is associated with mortality, multimorbidity, and physical and cognitive function in individuals without evidence of kidney disease (Shlipak et al., 2006; Sarnak et al., 2008; Hart et al., 2013; Newman et al., 2016; Hart et al., 2017).

Fasting insulin and insulin-like growth factor binding proteins and hemoglobin A1c are also strong candidate biomarkers of aging related to nutrient signaling and metabolism. These biomarkers are considered collectively to examine an intervention's effects on multiple aging pathways. Many options are possible to construct a multi-variable score of blood-based biomarkers of biological age, such as statistical modeling or principal component analyses or a simple rank-based biomarker index, as proposed for the TAME trial. For example, in the CHS an index of five circulating biomarkers was constructed; the

hazard ratio for mortality per point of the biomarker index was 1.30 (95% confidence interval: 1.25–1.34) and attenuated the association of age on mortality by 25% (Sanders et al., 2019). Investigations testing the utility of biomarker indices in clinical trials and association with outcomes such as multimorbidity and physiological function are underway.

Another class of biomarker strategy uses multisystem physiological, phenotypical, and clinical measures to calculate a composite score representing biological age that differs in older cohorts compared with a reference population. Examples include homeostatic dysregulation, phenotypic age, and Klemara-Doubal methods, which include measures that might not be associated with chronological age when considered independently but, when considered collectively in each of these models, may represent a meaningful biomarker of biological age (Klemara and Doubal, 2006; Li et al., 2015; Liu et al., 2018). For example, several groups have applied the Klemara-Doubal method, which takes biochemical measures and uses a reference population to calculate what an individual's predicted chronological age would be (Klemara and Doubal, 2006). This method was used in a post hoc analysis of the CALERIE trial (discussed later in the chapter), which showed that caloric restriction for 2 years reduced the advancement of physiological age compared to control participants (Belsky et al., 2017).

Horvath (2013) showed that aging is associated with distinct patterns of DNA methylation, and early epigenetic biomarkers took advantage of these chronological changes in methylation to calibrate “clocks” that could estimate age (Hannum et al., 2013; Horvath, 2013). Subsequently, many age estimators based on the levels of DNA methylation have been developed, and each “clock,” or DNA methylation biomarker, is unique to its calibration method (Levine et al., 2018; Lu et al., 2019; Belsky et al., 2020). These estimators can detect a myriad of

age-related diseases, can predict mortality and adverse health events, and can reliably identify individuals who appear physiologically older than their chronological age (Marioni et al., 2015; Quach et al., 2017; Horvath and Raj, 2018). A few small studies suggest that methylomic patterns can change in response to an intervention, but much more work needs to be done (Stubbs et al., 2017; Chen et al., 2019; Fahy et al., 2019; Justice and Kritchevsky, 2020).

Limitations for both animal and human models

Some issues of translational work in the field of aging are inadequately addressed in either current animal models or human studies. The usual investigative paradigm employed and rewarded by grant or manuscript reviewers is that of focusing on a single, isolated experimental variable (e.g., specific gene knock-outs) while controlling as many other potentially influencing factors as possible. This approach has been applied not only to mouse models, as outlined previously, but also to humans. We will again use immune senescence as an example. The SENIEUR criteria (Ligthart et al., 1984) were originally derived to highly select aged adults with little or no comorbid illness for comparison to young adults with no illness to isolate the factor of age. Only individuals who have aged successfully are enrolled in such studies, but these are not at all “typical” seniors; they are the “elite” aged. This point was made by several researchers who argue for a broader paradigm (Castle et al., 2001). The greatest value for human studies using criteria such as those of SENIEUR is when the “elite” group is compared to older adults with comorbidity (Trzonkowski et al., 2009). The same is true of mice purchased from aging colonies. If only the very aged are used in experiments and compared to young adults, the senior group represents only those animals that have aged in an elite fashion and survived to old age. The strategy of using an evolved, broader model for animal studies in which old “well” versus old “diseased” comparisons are made (rather than just old vs young comparisons) is virtually never employed. Further, assessment of physical and cognitive function in animal models that mimic human functional outcomes of interest (e.g., disability) are critical to develop further (e.g., Shively et al., 2012; Justice et al., 2016) if animal models are to translate to humans.

The explicit exclusion of animal and human studies that include comorbidity in order to keep the models simple is perhaps the biggest disconnect in the translational science of aging. In disease-focused research, single-hit animal models or predominant illness cohorts may be appropriate experimental models, but translational geriatrics demands that the investigative model embrace complexity as a foundational element. About half of all

Medicare patients have three or more chronic conditions (e.g., coexistent heart disease, diabetes, and arthritis). The effects of multimorbidity are not additive but multiplicative (Boyd et al., 2008). For example, the relative risk for disability is increased 2.3-fold in patients with heart disease, 4.3-fold for those with arthritis, but 13.6-fold for those with both (Boyd et al., 2008). It is impossible to ignore these issues in conducting translational geriatric research without almost ensuring failure when research from simple, age-focused animal or human models is applied to the vastly heterogeneous senior population.

Examples of experimental therapies in aging research

Caloric restriction and intermittent fasting

Since the 1930s, scientists have known that reducing caloric intake can have a profound effect on lifespan. This is among the most robust interventions in all of science, and the observation holds true in yeast, flies, worms, and rodents (Fontana et al., 2010). There is conflicting literature with regard to the effect of calorie restriction (CR) in nonhuman primates (NHPs) with increased median lifespan {25.9 years [Interquartile range (IQR): 7.10] for ad libitum-fed animals versus 28.3 years (IQR: 7.29) in calorie-restricted animals} in one study (Colman et al., 2014). Another study showed no difference in lifespan whether CR was initiated early or late in adulthood, but there was a trend in the CR group toward a lower incidence of age-related disease (cardiovascular, diabetes, neoplasia; $P = .06$) (Mattison et al., 2012). Extensive investigation of the mechanisms underlying this observation using both CR and mutational approaches has shown that conserved nutrient sensing—particularly IGF and target of rapamycin (Mirzaei et al., 2014)—and redox pathways (Walsh et al., 2014) are very likely implicated.

Of course, extreme CR, that is, starvation, has severe, lifespan-limiting effects, so optimally restricting calories in a “modest” range—25%–30%—seems to have the greatest effect in animal models (Fontana et al., 2010). Similarly, the timing of caloric restriction is critical, even with only modest CR. There are data suggesting that the earlier one starts CR, the more effective it is; however, there are significant negative effects of even moderate CR on reproductive capacity (Fontana et al., 2010). CR not only extends lifespan but also mitigates disease development (Anderson and Weindruch, 2012; Colman et al., 2014) and maintains physical function in rhesus macaques (Yamada et al., 2013), thus increasing the healthspan as well.

Small molecule CR mimetics may hold the best promise for human interventions, and once pathways were elucidated, at least one such compound, rapamycin, was

tested in the ITP and shown to extend median lifespan in both male (10%–23%) and female (18%–26%) mice (Miller et al., 2011, 2014) and to slow age-related declines in physical activity (Wilkinson et al., 2012). However, there do appear to be specific organ (testicular degeneration, cataracts) and molecular alterations (insulin sensitivity, IGF changes) that differ between CR and rapamycin (Miller et al., 2011; Wilkinson et al., 2012). Therefore, not surprisingly, the pleiotropic effects of CR are not completely recapitulated by a single small molecular mimetic.

Evaluating the effects of CR in humans is complex. Chronic CR as determined by low body mass index (BMI) is associated with poor health in humans. There are few “natural experiments” because most undernourished human populations are also typically impoverished with high burdens of communicable disease and high mortality rates. In developed countries, while there tends to be an excess of mortality in older adults with either very high or very low BMIs (weight/height²), there is little relationship between BMI and mortality across a wide range of intermediate BMIs. Very low BMIs in older individuals are usually a sign of poor health and ongoing unresolved health issues. These observations do not directly address the question of whether voluntary and nutritionally adequate caloric restriction has benefits in terms of either healthspan or lifespan in older adults. The question has been approached from a number of directions, building on the substantial knowledge base built upon CR animal model systems.

Mercken et al. (2013) identified 15 (BMI = $19.2 \pm 1.1 \text{ kg m}^{-2}$) middle-aged individuals who had voluntarily restricted their energy intake by 30% for an average of 9.6 years. Their muscle gene expression profile was compared to that of nonobese age-matched controls (BMI = $25.3 \pm 2.3 \text{ kg m}^{-2}$) consuming a typical Western diet. The transcriptional profile of the calorically restricted group showed downregulation of IGF-1/insulin-signaling pathways compared to the controls. The pattern of transcriptional changes was remarkably homologous to that seen within the muscles of calorically restricted rats, with downregulation of genes related to inflammation and an upregulation of genes associated with mitochondrial biogenesis, autophagy, and antioxidant enzymes. Investigators from the Pennington Biomedical Research Center evaluated three different strategies to achieve 10% weight loss in nonobese adults in a 6-month pilot study (Heilbronn et al., 2006). Compared to the control group, weight loss was associated with lower insulin levels but not lower glucose levels. DHEAS and protein carbonyl levels were not affected, but caloric restriction did significantly lower core body temperature and lean body mass-adjusted resting metabolic rate (RMR). Cells derived from humans after they have undergone a weight

loss intervention show an increase in heat stress resistance, Sirt1 protein levels, and PGS-1α mRNA levels compared to cells collected before the intervention (Allard et al., 2008). The CALERIE study explored disease risk factors and quality of life in nonobese humans aged 21–51 years. Participants were randomized to a 2-year intervention designed to achieve 25% CR or to an ad libitum (AL) diet. The primary outcomes were change from baseline RMR adjusted for weight change and core temperature. Secondary outcomes were plasma triiodothyronine (T3) and tumor necrosis factor alpha (TNF-α) (Ravussin et al., 2015). RMR decreased significantly more in the CR cohort than in the AL cohort at 12 months ($P = .04$) but not at 24 months. Core temperature differed little between the groups. T3 decreased more in the CR cohort at 12 and 24 months ($P < .001$), while TNF-α decreased significantly more only at 24 months ($P = .02$). A secondary analysis of the data by Belsky et al. (2017) suggested that the pace of physiological age was slowed by CR according to commonly obtained clinical measures of energy and lipid metabolism.

These studies do not target older populations and thus provide proof-of-principle information on the effect of CR on longevity-related pathways but would not provide strong evidence in support of the idea that CR would extend the lifespan or healthspan of older adults.

Using a biomarker approach, one can evaluate the likelihood that energy restriction would reduce mortality rates in older individuals. There have been a handful of clinical trials that evaluated weight reduction in older overweight and obese adults, defined as a mean baseline age of ≥ 65 years. These studies show that short-term (5–18 months) weight reduction significantly increases gait speed, increases peak VO₂, and reduces IL-6 levels and cystatin-c levels (Nicklas et al., 2004; Rejeski et al., 2011; Villareal et al., 2011; Shaver et al., 2019). While muscle mass is lost when total mass is reduced, it does not appear that weight loss interventions affect muscle strength, suggesting that weight loss improves muscle quality, defined as muscle force/muscle cross-sectional area (Villareal et al., 2011).

There have been some studies of the effect of CR on mortality in humans despite the challenges of doing so. The Swedish Obesity Study followed 2010 individuals who had received gastric banding or bypass surgery and compared their mortality experience to the experience of matched controls (Sjostrom et al., 2007). The surgical procedures generated a sustained weight loss of between 14% and 25% and were associated with a 24% reduction ($P = .04$) in all-cause mortality over 10.9 years of follow-up. However, as it was a nonrandomized study, it is unclear whether selection bias or confounding contributed to the observed mortality advantage. In addition, the study did not include older persons. The best evidence for a

benefit from CR comes from randomized trials evaluating weight loss interventions compared to control or non-weight loss interventions. There are three reports in the literature of randomized trials of weight loss that followed subjects for more than 5 years: the ADAPT study of obese and overweight older adults with knee osteoarthritis, the TONE study of overweight and obese older adults with mild hypertension, and the Look AHEAD trial (Look AHEAD Research Group et al., 2013; Shea et al., 2010, 2011). Only ADAPT and TONE targeted older adults with mean baseline ages older than 65. All three studies found that individuals who were randomized to weight reduction had lower all-cause mortality with follow-up times ranging from 8 to 10 years. The relative reductions in risk were 50% ($P = .01$), 12% ($P > .05$), and 15% ($P = .11$) for ADAPT, TONE, and Look AHEAD, respectively. In a metaanalysis of published literature, Kritchevsky and colleagues observed a significant 15% reduction in total mortality (Kritchevsky et al., 2015). The mortality benefits from the human studies may be due to the treatment of obesity rather than to biological changes associated with CR. The ADAPT study was an 18-month intervention, but the mortality advantage persisted throughout the follow-up period. The Look AHEAD and SOS study survival curves are instructive in that in both studies, the survival advantage for the weight-losing groups did not begin to manifest until 4–5 years after the initiation of weight loss. This delay in benefit provides a benchmark for understanding how long it may take for changes on the cellular level to play out as reductions in mortality risk. In contrast, beneficial changes in many biomarkers are evident after 6 months of intervention.

Experimental therapies targeting cellular senescence

Cellular senescence and its SASP are implicated in the pathogenesis of multiple, diverse age-related diseases that share few risk factors other than age, such as nonalcoholic steatohepatitis, idiopathic pulmonary fibrosis (IPF), cardiovascular diseases, Alzheimer's disease, and diabetic chronic kidney disease, among many others (Yanai and Fraifeld, 2018). Moreover, activating a suicide protein that clears *p16^{Ink4a}*⁺ cells in transgenic mice alleviates multiple aging phenotypes, including metabolic and vascular dysfunction, cataract development, lipodystrophy and adipose tissue dysfunction, and frailty, and it preserves muscle fiber diameter and prolongs time and distance achieved on a treadmill run to exhaustion (Baker et al., 2011; Xu et al., 2015; Roos et al., 2016; Schafer et al., 2017). This expansive plurality of effects indicates that cell senescence contributes to many age-related diseases, and therapeutically targeting these cells may

alleviate a broad range of potential disease indications that are common in older age.

Efforts are now underway to intervene in this process. The first senescent cell antiapoptotic pathways (SCAPs) were identified by Zhu et al. (2015) using a bioinformatics approach based on expression profiling of senescent versus nonsenescent human cells (Zhu et al., 2015). The discovery of SCAPs led to development of senolytic drug therapies. Senolytic agents selectively induce senescent cell apoptosis by transiently disabling the SCAPs that defend senescent cells against their own proapoptotic environment. Broader drug and treatment classes, termed senotherapeutics, include strategies to eliminate senescent cells (senolytics), shut down their secretory machinery (senomorphics or senostatics), or prevent or blunt the accumulation of senescent cells (e.g., untargeted approaches such as exercise and caloric restriction).

To date, approximately 20 agents tested show effectiveness in clearing senescent cells while largely sparing nonsenescent cells in vitro. A select few of these demonstrate effectiveness in vivo in mice and are being translated into early-stage clinical trials in humans (Table 30.2). Of these, the most well-established drugs in the translational testing pipeline are dasatinib and quercetin (D + Q), fisetin, and navitoclax/UBX0101.

Dasatinib + quercetin

D + Q were the first senolytic agents to be identified (Zhu et al., 2015). Dasatinib is currently indicated for use as a second-line chemotherapeutic agent for treatment of chronic myeloid leukemia that is resistant to another tyrosine kinase inhibitor imatinib and acts as an immunomodulatory agent in conditions other than cancer (Weisberg et al., 2007; Blake et al., 2008; Rivera-Torres and San Jose, 2019). Quercetin is a polyphenol derived from plants and is a nonspecific kinase inhibitor that targets B cell lymphoma-2 (BCL-2), insulin/IGF-1, and HIF-1 α SCAP network components and is senolytic, possibly as a consequence of its inhibitory effects on multiple antiapoptotic genes (PI3K and other kinases) (Zhu et al., 2015; Malavolta et al., 2016; Reyes-Farias and Carrasco-Pozo, 2019). The D + Q combination is effective in vitro, eliminating cultured senescent cells originating from several different types of human and mouse cells (Zhu et al., 2015; Roos et al., 2016; Farr et al., 2017; Kirkland et al., 2017; Ogrodnik et al., 2017; Schafer et al., 2017; Zhu et al., 2017; Musi et al., 2018; Tchkonia and Kirkland, 2018; Xu et al., 2018).

Converging evidence in vivo suggests that D + Q may alleviate a range of age- and senescence-related disorders in mice (Zhu et al., 2015; Roos et al., 2016; Farr et al., 2017; Ogrodnik et al., 2017; Schafer et al., 2017; Musi et al., 2018; Xu et al., 2018). For example, in a mouse

TABLE 30.2 Survey of senotherapeutics preclinical and clinical testing.

Basic research: cells and mouse models				
Study	Design	Model/population	Intervention	Key finding
Zhu et al. (2015)	(a) Transcript analysis and in vitro testing; (b) In vivo preclinical intervention testing	Aged, radiation-exposed, and progeroid Ercc1 mice	D + Q versus vehicle By oral gavage	Senolytic drugs that interfere with antiapoptotic mechanisms in senescent cells selectively eliminate these cells and extend healthy aging in mice
Fuhrmann-Stroissnigg et al. (2017)	(i) SA β -gal assay screening platform; (ii) Preclinical intervention testing in progeroid mice	Ercc1 murine embryonic fibroblasts and progeroid mice	HSP90 inhibitor 17-DMAG by oral gavage	SA- β -gal screening platform identified new senolytics, including HSP90 inhibitors, which were validated by in vivo testing for senolytic activity and healthspan
Roos et al. (2016)	Preclinical interventions testing in aged mice	24-month aged mice and ApoE-mice on Western diet	D + Q versus vehicle By oral gavage	Senolytic treatment alleviates established vasomotor dysfunction in aged mice and in atherosclerotic mice
Farr et al. (2017)	Preclinical intervention testing in aged mice with established bone loss	20- to 22-month-old naturally aged mice	D + Q or JNK inhibitor (ruxolitinib) versus vehicle By oral gavage	Senolytics improve bone mass and strength in older mice with established bone loss
Ogrodnik et al. (2017)	Preclinical interventions testing in aged mice	C57BL/6 male mice or INK-ATTAC transgenic mice	Genetic (INKATTAC) or D + Q versus vehicle By oral gavage	Cellular senescence results in impaired fat metabolism and removal of senescent cells diminishes liver steatosis
Zhu et al. (2017)	Cell culture: primary cells incubated with potentially senolytic agents	Primary human adipocytes, HUVEC, and IMR90 + 10 Gy radiation or sham	Fisetin or A1331852 or A1155463 versus vehicle	Fisetin, and BCL-2 inhibitors (A1331852, A1155463) selectively induce apoptosis in senescent cells in cell-type specific manner
Musi et al. (2018)	Preclinical intervention testing in transgenic AD models	Four AD transgenic mouse models with late-stage pathology	D + Q versus vehicle By oral gavage	Tau accumulation initiates a chronic degenerative process culminating in neuron loss and brain dysfunction that is alleviated by senolytic therapy
Schafer et al. (2017)	(i) Senescence biomarkers in IPF patients and mouse (ii) Preclinical intervention testing	Mice with bleomycin inhalation lung injury IPF model	D + Q versus vehicle By oral gavage	Fibrotic lung disease is partially mediated by senescent cells, but senolytic improves lung compliance and exercise capacity in a bleomycin mouse model
Xu et al. (2018)	Preclinical interventions testing in aged mice	20-month-old male mice	D + Q versus vehicle By oral gavage	Intermittent senolytics to senescent cell-transplanted mice alleviated physical dysfunction and increased posttreatment survival by 36%
Yousefzadeh et al. (2018)	Preclinical interventions testing in aged and progeroid mice	Progeroid Ercc1 and naturally aged mice	Fisetin 100 mg/kg versus control diet In diet	Flavonoid fisetin is senotherapeutic and increases lifespan in mice
Chang et al. (2016)	Preclinical interventions testing in aged mice	Sublethally irradiated or normally aged mice	ABT263 50 mg/kg/d versus vehicle by oral gavage	Senolytic ABT263 selectively kills senescent cells in culture in a cell type- and species-independent manner by inducing apoptosis

(Continued)

TABLE 30.2 (Continued)**Basic research: cells and mouse models**

Study	Design	Model/population	Intervention	Key finding
Childs et al. (2016)	Preclinical intervention testing in atherosclerosis-prone mice	High-fat fed or normal chow fed mice	Ganciclovir (GCV) or navitoclax versus vehicle	Senescent cells increase atheroma; senolytic agents perturb the proatherogenic microenvironment
Jeon et al. (2017)	Preclinical interventions testing in aged mice; cell culture of senescent cells incubated in senolytic compounds	Mice undergoing anterior cruciate ligament transection (ACLT) to model osteoarthritis	UBX0101 By six intraarticular injections versus vehicle	Senescent cells develop in the cartilage and synovium after articular joint injury, and senolytic reduced the development of posttraumatic OA
Clinical pilot studies: human disease				
Justice et al. (2019)	Single-arm open label pilot and feasibility clinical study of senolytics	Patients aged ≥ 50 years with stable idiopathic pulmonary fibrosis	D (100 mg/d) + Q (1250 mg/d), intermittent (3 days on, 4 days off)	Human open-label pilot supports study feasibility and provides initial evidence that senolytics may alleviate physical dysfunction in IPF
Hickson et al. (2019)	Open label phase I pilot study	Patients with diabetic kidney disease	D (100 mg/d) + Q (1000 mg/d) 3 days	Senolytic therapy significantly decreases senescent cell burden in CKD

model of pulmonary fibrosis, senescent cell clearance of approximately 30% via senolytic drug combination reduces relative expression of p16^{INK4a} and SASP factors, improves lung compliance, and extends distance run to exhaustion (Schafer et al., 2017). A single oral dose with this senolytic drug combination in old mice reduced adipose p16^{INK4a} expression and SASP within 5 days compared to vehicle-treated animals. In mice with mobility impaired by radiation exposure, a single senolytic course improved treadmill endurance within 4 days, and this improvement persisted for over 7 months (Zhu et al., 2015). Moreover, D + Q treatment can prevent the physical dysfunction, accelerated onset of age-related diseases, and early death caused by transplanting small numbers of senescent cells into young mice (Xu et al., 2018). Importantly, late-life D + Q treatment in aged wild-type mice delayed age-related diseases as a group, suppressed frailty, and extended median lifespan (Xu et al., 2018).

Fisetin

On the basis of the effectiveness of the flavanol quercetin, other natural flavonoids were tested for senotherapeutic (Zhu et al., 2017; Yousefzadeh et al., 2018). In vitro, fisetin is a potent senolytic that selectively reduces senescent cell viability and senescence markers in human adipose tissue (Yousefzadeh et al., 2018). Although fisetin is senolytic, it is thought to have potent senomorphic

activity. It inhibits the activity of several proinflammatory cytokines, including TNF- α , IL-6, and the transcription factor NF- κ B (Gupta et al., 2014), and it may act as an antioxidant via upregulated synthesis of glutathione (an endogenous antioxidant) (Khan et al., 2013; Gupta et al., 2014). Importantly, administration of fisetin in mice late in life reduces the expression of senescence markers in multiple tissues, restores tissue homeostasis, and extends healthspan and lifespan (Yousefzadeh et al., 2018). A key translational advantage of fisetin is its excellent anticipated safety profile. Flavonoids such as fisetin are members of a broadly distributed class of plant pigments that are regularly consumed in the human diet with no known toxicity. Although physiological benefit may require larger doses than would be achieved through a common Western diet, there are no known toxicities from fisetin intake either through dietary sources or as a nutraceutical.

BCL and MDM2 pathway inhibitors

Many senolytics that have been identified through screening target antiapoptosis factors overexpressed in senescent cells, such as the BCL-2 family of proteins (Zhu et al., 2015, 2017). Identifying novel inhibitors of the BCL-2 and BCL-XL pathway is an area of active research. For example, navitoclax (ABT263) is an inhibitor of BCL-2, an oral drug being tested in cancer that has senolytic activity (Zhu et al., 2016), although its significant adverse

effects impede the use of navitoclax as a chemotherapeutic or senolytic agent, leading to intense study for new and better BCL-2 family inhibitors. One such agent is UBX0101, a BCL-2 and MDM2 inhibitor that is under proprietary development by Unity Biotech. Intraarticular injection of UBX0101 cleared senescent cells induced by anterior cruciate ligament transection surgery in mice and by spontaneous osteoarthritis in aged mice (Jeon et al., 2017). In chondrocytes isolated from humans with osteoarthritis and cultured ex vivo, UBX0101 reduced markers of senescence while increasing markers of apoptosis. On the basis of these preclinical results, UBX0101 was advanced to phase II and phase III clinical trials for treatment of osteoarthritis, but lack of efficacy in phase III has led to uncertainty regarding future use or next-stage testing.

Senolytic drug trials

Based on the success of D + Q in mice, this drug combination is the first senolytic taken into trials in humans for disease conditions, including IPF (NCT02874989), chronic kidney disease (NCT02848131), hematopoietic stem cell transplant survivors (NCT02652052), and Alzheimer's disease (NCT04063124) (Hickson et al., 2019; Justice et al., 2019). Each study is evaluating short-term, intermittent treatment with oral administration of D + Q. Dose are based on the FDA-approved dose for inducing apoptosis in human cancer cells (dasatinib) or product labeling and extrapolation of in vivo evidence in animals (quercetin). Elimination half-lives are both less than 6 hours (Christopher et al., 2008; Moon et al., 2008). Senescent cell clearance occur within 18 hours of a brief exposure, and senolytics do not need to be present continuously to occupy a receptor or affect an enzyme (Kirkland et al., 2017; Schafer et al., 2017). Rather, they act in a hit-and-run manner, which supports the rationale behind intermittent treatment. Proof of concept for this hit-and-run dosing strategy was established in an open-label randomized trial in patients with diabetic chronic kidney disease. Three days of dosing with D + Q reduced biomarkers of cell senescence in adipose tissue; p16^{INK4A}- and p21CIP1-expressing cells were reduced by 35% and 17%, respectively, and cells with increased biomarker SA- β -gal were reduced by 62% (raw unadjusted data) (Hickson et al., 2019).

The first-in-human open-label clinical trial on senolytics was conducted in patients with stable, mild to severe IPF to evaluate the safety, feasibility, and potential impact of D + Q intermittent oral dosing over 3 weeks (Justice et al., 2019). Fourteen participants were enrolled, and 100% of them completed intermittent drug self-administration; no subjects withdrew or were otherwise lost to follow-up. Though not a primary aim of this study,

statistically significant (within-subject) and clinically meaningful improvements in physical function, including 6-minute walk distance, 4-m usual gait speed, chair-rise time, and a performance summary score, and the short physical performance battery were seen following D + Q treatment. These parameters were measured before and 5 days after the last D + Q dose, well beyond these drugs' elimination half-lives.

A key aim of the study was to track reported symptoms and adverse events (Justice et al., 2019). No changes in laboratory tests suggestive of hepatic or renal toxicity were found. Pulmonary function was unchanged, possibly owing to the advanced disease state, drug-dosing schedule, brief trial period, or insufficient target engagement. Adverse event reports and thorough symptom questionnaires revealed mostly mild to moderate side effects within 24 hours of dosing, although these were transient and deemed acceptable and consistent with the underlying IPF diagnosis or with study procedures or were known side effects of the drugs. Follow-up studies of D + Q or other senotherapeutics, such as fisetin, are aimed at varying the dosing schedule and agent to minimize potential side effects while maximizing senescent cell clearance and disease-specific effects. On the basis of these early results, and proof of concept in pilot studies in IPF and diabetic kidney disease, several senolytics are now being advanced to next-stage clinical trials.

Translational aging resources

Animals and animal tissues

There are important animal resources for aging research that have primarily been assembled by the (NIA Research Resources, 2021). A number of rodent resources are available, including mice and rats across the age spectrum of various strains, F1 crosses, and calorie-restricted animals. In addition, a tissue bank of specimens is available. All investigators with an aging-focused grant are eligible to access these resources, but pricing and access priority vary by specimen or strain and by funding source (NIA grantee vs other). Other options for rodents include retired breeders from several manufacturers; however, investigators are cautioned that retired males often fight when re-paired in housing and may require individual cages. Females that have produced multiple litters have substantial variation from nulliparous females for some biological systems (e.g., immunology).

NHP resources are also available (Primate Aging Database, 2021). When compared to rodents, NHPs better model a number of critical issues in aging research. NHPs have more human-like joints and movement that may better simulate arthritis, functional decline, and sarcopenia than rodent models. Recently, functional assessments that

mimic physical performance measures in humans have been demonstrated to decline with age in NHPs (Shively et al., 2012).

Cohorts and populations

The NIA and other institutes of the National Institutes of Health (NIH) have made decades-long investments in a number of large cohorts either directly to follow aging (e.g., BLSA, Health ABC), disease-focused in an aging-related area (e.g., MESA, CHS), or in specific groups that were initially middle aged but have now entered “geriatric” status (e.g., Women’s Health Initiative). Other important surveys provide critical data in aging cohorts (e.g., Health and Retirement Survey). These are summarized in a number of websites with access information and specimen repositories (NIA Aging Research Biobank, 2021; Resources, 2021). This includes a new NIA Aging Research Biobank, which serves as a critical resource for long-term biospecimen storage for NIA-funded investigators and provides access to data and samples obtained in aging cohort studies and clinical trials in older adults (NIA Aging Research Biobank, 2021).

Tools and toolboxes

The NIH Toolbox is a recently released, multidimensional set of brief measures assessing cognitive, emotional, motor, and sensory function across the lifespan (NIH Toolbox for the Assessment of Neurological and Behavioral Function, 2021). The NIH Toolbox was created to address the need for a standard set of measures that could be used across diverse study groups and designs in various settings to assess neurological and behavioral function, facilitating the study of changes across the lifespan. Many resources in the NIH Toolbox are free, but fees may apply for its use in projects that are not funded by the NIH.

The Patient Reported Outcomes Measurement Information System (PROMIS) is a free resource that was developed to provide validated patient-reported outcomes for clinical research and practice (Patient Reported Outcomes Measurement Information System, 2021). PROMIS uses computer-adaptive testing and traditional paper instruments in global health, physical function, fatigue, pain, sleep/wake function, emotional distress, and social health. PROMIS provides resources in four key areas: (1) comparability—measures standardized across common domains, metrics, and conditions; (2) reliability and validity—metrics for each domain have been rigorously reviewed and tested; (3) flexibility—can be administered in a variety of ways or formats; and (4) inclusiveness—designed to span all study populations regardless of literacy, language, physical function, or life course.

Conclusion

Aging is the most important risk factor for most adult diseases, functional decline, disability, and death. *Geroscience* is an approach that uses the broad biochemical pathways affected by aging as a tool to understand and intervene in these important health- and life-related outcomes. These pathways—the hallmarks or pillars of aging—are manifested during normal chronological aging, they accelerate aging under experimental aggravation, and their experimental amelioration retards normal aging increasing healthy lifespan. Animal interventions in the critical pathways that have been identified to date have demonstrated the potential to impede and even reverse some hallmarks or pillars of aging in rodents and extend their healthspan. In early human trials, biomarkers of senescence and other hallmarks have been reversed by short-term interventions, and a few clinically important outcomes have even improved (e.g., influenza vaccine response, walking speed). Many limitations and challenges still exist, and there are important ethical considerations, but geroscience has enormous potential to influence the development of many diseases and extend the human healthspan.

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Chapter 31

Translational medicine: the changing role of big pharma

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Abstract

The pharmaceutical industry has gone through dramatic changes since the birth of biotechnology. Pharmaceutical companies no longer have a monopoly on drug development, nor do they represent the primary source of biomedical innovation. Since biotech firms have emerged on the scene and academia has embraced translational research as a way to help advance their own discoveries, pharma companies have begun to foster relationships with those sectors in a strategy to externalize innovation. Public–private partnerships offer a low-risk way for pharmaceutical companies to tap into academic talent and stay abreast of cutting-edge research into new targets, technologies, and tools for advancing drug development. The COVID-19 pandemic broke many barriers, bringing fierce competitors together more closely than ever before via precompetitive collaborations to expedite the creation of countermeasures in response to the crisis. The question is whether this will trigger long-term change that can accelerate the translation of laboratory discoveries to new medicines and reduce their costs.

Keywords: Biotech; pharma; public–private partnership; pre-competitive consortia; innovation ecosystem; external innovation; COVID-19; academic translation

Introduction

The growth of translational research as a basis for creating medicines has coincided with ground-shifting changes in the landscape of drug development, as seen in new structures, relationships, and attitudes of the key players. Together, the stakeholders in this integrated ecosystem are advancing their ability to convert discovery biology into new therapies, involving multiple players along the way.

Thirty years ago, there was one key player: the pharmaceutical companies known colloquially as “Big Pharma.” Now big pharma sits alongside successful

biotechnology companies, startups, academic organizations, and venture capital (VC) firms, existing in an innovation ecosystem that includes multiple other service providers that play important roles in drug development.

This chapter will discuss how pharma has adapted to the changes around it and how it has moved to a model that is heavily dependent on translation of academic discoveries to fuel the pipeline of new therapies via a widespread strategy of external innovation.

History: how did we get here?

The oldest pharmaceutical companies date back to the mid-19th century, when they began to industrialize the generation of chemicals for medical use. The corporations that are currently recognized as Big Pharma have their roots in companies that were founded in the second half of the 19th century or the early 20th century.

Until the birth of biotechnology, pharmaceutical companies dominated the invention, manufacturing, and sale of new drugs, and the vast majority of the drugs were small molecules. The corporations were seen as houses of science and the primary if not sole source of bringing new medicines to the public.

However, these organizations became large and unwieldy, with campus-type properties removed from city centers. They relied on internal employees for new ideas, and their competition primarily came from the few other pharmaceutical companies that existed.

This model was disrupted by the launch of the biotech industry in the latter part of the 20th century, marked by the foundation of Genentech Inc. (now part of Roche) in 1976 and followed by early pioneers such as Hybritech Inc. in 1979 (later bought by Eli Lilly & Co. and then Beckman Coulter Inc.) and Amgen Inc. in 1980. Those companies were formed to capitalize on the newly

discovered DNA technology and provide an efficient means of making highly targeted protein-based drugs. The result was the creation of new recombinant protein drugs, including, significantly, antibody-based drugs that added a completely new therapeutic modality to address unmet medical needs.

Biotech: the big disruptor

The following decades saw the biotech sector grow as new companies were formed, with “bubbles” and “booms” of investor frenzy followed by market collapses and subsequent, sometimes slower, recoveries. But all the time, the biotech sector was marching forward with new technology, changing the landscape of drug development and triggering tectonic shifts in how drugs were made and by whom.

The growth was fueled by a convergence of advances in biology, computers, and software engineering and catalyzed by two landmark pieces of legislation.

The technology shifts opened up new possibilities for rapid data processing, compound generation, and gene manipulation, which led to the development of transformational tools such as polymerase chain reaction (PCR), sequencing, gene chips, high-throughput screening, and transgenic mice. Together, these advances created opportunities to address the causes of disease in ways that had been unimaginable a decade or two before. The new potential was epitomized by the human genome project, which itself sparked a “genomics bubble” in which investors and entrepreneurs looked to the power of genomics to unlock knowledge about diseases that had hitherto been black boxes to biologists and to the potential for population genomic studies to yield medicines tailored to individual patients (Collins, 1999).

None of this would have resulted in the emergence of the biotech sector without legislation that effectively turned translational research into a reality. The Bayh-Dole Act of 1980 and the Hatch-Waxman Act of 1984 effectively democratized innovation and opened the door for academics and other individuals outside of Big Pharma to pursue their own ideas of how to create new drugs and technologies and how to capitalize on their own ideas.

Bayh–Dole allowed universities, nonprofit organizations, and small businesses to patent and essentially own inventions arising from federally funded research (Markel, 2013). The aim of the act was to stimulate growth in the sluggish economy by allowing unexploited research discoveries from universities and hospitals to be commercialized.

Hatch-Waxman defined patent exclusivity terms in order to spur innovation for both generic and brand-name drugs (Kesselbaum, 2011). For innovators of new molecular entities (NMEs), one of the key provisions of the law

was the extension of a drug’s patent lifetime after US Food and Drug Administration (FDA) approval to compensate for some of the time lost during clinical testing and the FDA review period (Kesselbaum, 2011). By providing a period of patent exclusivity, the act aimed to enable drug developers to recover their investment and secure financial returns that justified their risk. In short, Hatch-Waxman provided a greater incentive for VC firms and other investors to promote entrepreneurship in drug discovery.

These developments also caused a shift in the outlook for scientists themselves and led to a marked increase in entrepreneurship among academics in the life sciences. Thus a PhD no longer suggested a choice between a path forward in academia or a career in a big corporation; instead, it opened doors to financial and intellectual independence with the option of founding one’s own company or joining a startup. At the same time, this diverted top talent away from pharma companies, which had once offered a stable and steady income, and created a marketplace of opportunity and ideas in which researchers could take control of their own careers.

Expansion of the biotech sector

While pharma companies sought to increase productivity and bolster pipelines through mergers and acquisitions (LaMatta, 2011), the biotech sector underwent rapid expansion as the VC community rushed to form new startups that created a significant market of small and medium-sized enterprises (SMEs) (Lähteenmäki and Lawrence, 2005; Hodgson, 2006). The new companies started to produce drug candidates that put them squarely in place as competitors—and partners—for Big Pharma.

During the 1980s and 1990s the number of small companies that produced NMEs doubled from 78 to 145 (Munos, 2009). Between 1998 and 2007, 25% of the innovative drugs approved by the FDA originated in biotechs and 31% in academia, while Big Pharma contributed 44%. (Innovative drugs were defined by Kneller (2010) as “scientifically novel” if they had a novel mechanism of action or were first in a distinct class of compounds at the time of approval.) In addition, three-quarters of the drugs from academia were first licensed to biotechs versus one-quarter to pharma companies (Kneller, 2010).

This scenario largely persists today. A U.S. Congressional Budget Report released in April, 2021 found that about one-third of drugs developed since 2009 came from companies with annual revenues under \$100 million, and companies with annual revenues less than \$500 million (i.e. biotechs) account for more than 70% of the nearly 3,000 drugs in Phase III clinical trials.

However, despite their decreasing contribution to innovative NMEs, pharma companies play a seminal role

in bringing most of these products to market, via deals covering specific compounds or platform technologies that could yield multiple compounds.

The majority of biotechs do not have the funds or the expertise to carry out large or complex clinical trials or to navigate their way through the regulatory process all the way to approval. In addition, most do not have a sales force to commercialize the assets.

These licensing deals give biotechs an often much-needed influx of money and a way to have their products reach patients, while they allow pharma companies to boost to their pipelines, bring significant revenue, and have to some degree solved the companies' declining ability to innovate.

Moreover, pharma companies have not been completely disintermediated from the innovation end of the spectrum. Several have created their own corporate VC firms. These operate independently from the company's business division, have separate investment funds, and have dedicated teams that scout the latest biotechnologies and fund startups ([Fletcher, 2001](#)).

Indeed, following the financial crash of 2008, corporate VC firms were largely credited for stepping into the chasm that was created when the financial markets dried up. Their involvement in new company formation grew; by 2016 pharma company VC firms were involved in about one-third of new companies raising startup money ([Martz, 2017](#)).

Academia and integrated discovery nexuses

Academia has now established itself as a core part of the biopharma ecosystem, responsible for a large proportion of the new companies that are formed and the innovations that enter pharma pipelines.

A BioCentury analysis found that 75% of new companies that raised seed or series A funding from 2017 to 2019 had technology roots in academia ([Durkin-Wolfe and Hansen, 2019](#)).

Translational research has become a central component of many universities and academic medical centers, which have embraced the concept first as a means to converting lab-based discoveries to tangible benefits for patients, and second as a means for bringing revenue beyond that obtained through grants and government or other funding.

In some cases, new academic organizations have been created that are dedicated to translational research. One example is the Francis Crick Institute in the United Kingdom, which opened in 2016 and was formed by six different institutions: the Medical Research Council, University College London, Imperial College London,

King's College London, the Wellcome Trust and Cancer Research UK. The Crick gives scientists funding to advance programs with translational potential and links scientists with clinicians and industry experts who can advise them ([Fishburn, 2016](#)).

In other cases, universities have created specific programs that operate as so-called integrated discovery nexuses, fostering innovation by bringing academic researchers together with experts from industry, VC communities, and contract research organizations ([Fishburn, 2013](#)) ([Fig. 31.1](#)).

One example is Stanford University's SPARK program, which provides funding of up to about \$50,000 to Stanford applicants to develop ideas with commercial potential to a point at which they can be licensed, partnered, or spun out or, in some cases, to other endpoints that could benefit patients. SPARK involves weekly meetings at which funded investigators meet with volunteer experts from the industry, who advise on strategic considerations for the translational programs ([Fishburn, 2014b](#)). The program, started in 2006, has been adopted in over 60 academic institutions in over 20 countries.

Other models, such as the California Institute of Quantitative Biosciences (QB3), involve multiple universities and are partly state-funded ([May, 2011](#)). QB3 is a nonprofit organization with a commercialization arm, the Innolab, that provides startup space at facilities in three University of California campuses—UC Berkeley, UC San Francisco, and UC Santa Cruz—and at additional properties in West Berkeley and Palo Alto, close to Stanford University.

Several state and local governments have created other types of science parks and biotech incubators that provide laboratory space and facilities to spur business creation in their communities ([Fishburn, 2013](#)).

The common elements of these structures are the integration of expertise and services across different subsectors of the biopharma ecosystem and the central mission of enabling and expediting the translation of discovery research into clinical programs.

Pharma meets academia: models of external innovation

Different pharma companies have adopted various formats for partnering with academic organizations, ranging from bricks-and-mortar institutions to umbrella licensing deals with specific universities to deals with individual research labs.

These partnerships provide pharma companies with access to new science at low risk and relatively low cost, while researchers and new entrepreneurs receive funding, expertise, and training to help advance their translational

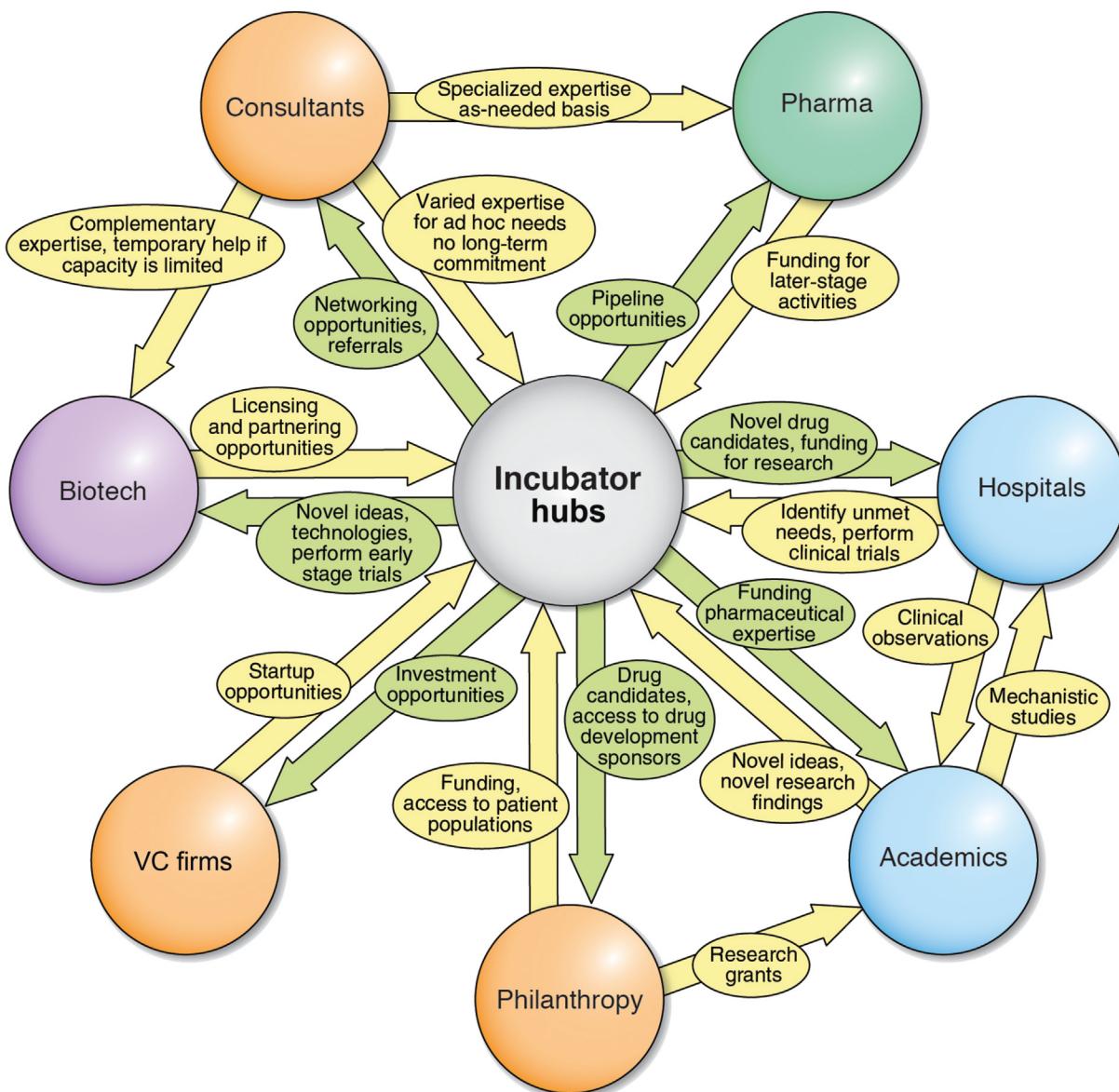


FIGURE 31.1 The integrated discovery nexus. Translational programs or centers, often based in universities or hospitals, serve as incubator hubs for helping to commercialize and advance laboratory discoveries. They create a network of interactions with participants from many parts of the drug discovery ecosystem, each of which has something to gain from its involvement in the network. Examples include Stanford University's SPARK program, the University of California's QB3 program, the CTSI program at the University of California, San Francisco (UCSF), and Johnson & Johnson's Janssen Labs. Reproduced from Fishburn, C.S. *Translational research: the changing landscape of drug discovery*, *Drug Discov. Today*, 18 (2013) 487–494 with permission from Elsevier.

projects. From the pharma companies' perspective, the partnerships solve the inherent problem of having limited internal innovation because of the companies' size, structure, and culture (Fishburn, 2013; Tralau-Stewart et al., 2009; Ratner, 2011). By forming ongoing partnerships rather than simply licensing the assets, the companies defray the risk, spend less money up front, and buy time to assess the molecules or technology or to select the optimal compounds before making a larger commitment of resources.

Perhaps more important, these models increase the probability of success for early-stage projects by providing expertise about the key factors to consider for translating drugs, an area in which academics frequently have little training. Such factors encompass the likely medical use of a compound in the relevant patient population in addition to what would be required for an FDA filing, such as dosing, route and frequency of administration, pharmacokinetics and pharmacodynamics, formulation, and appropriate toxicology assessment.

In some cases, pharma companies form straightforward partnerships with academic organizations. For example, GlaxoSmithKline PLC, AstraZeneca PLC, Merck & Co (Merck Sharpe and Dohme), and Bristol Myers Squibb Co. have all formed partnerships with the Francis Crick Institute.

However, almost all of the major pharma companies also have programs directed at external innovation that complement their standard business development outreach to academic organizations.

Pfizer Inc. launched its Global Centers for Therapeutic Innovation (CTI) in 2010 to create collaborations with academia and help to bridge the gap between discovery and the clinic. CTIs have been established in Cambridge, Massachusetts; New York; San Diego; and San Francisco. They cover four therapeutic areas: oncology, inflammation and immunology, rare diseases, and internal medicine. The CTIs have relationships with dozens of academic institutions, have started over 94 projects, and have advanced at least six drug candidates into clinical trials. Pfizer funds the preclinical and clinical development programs and provides access to compound libraries, screening capabilities, antibody development technology, and other expertise. The CTI model allows researchers to publish their work, and the intellectual property (IP) is jointly owned by the university and Pfizer. However, Pfizer has an exclusive commercial option to the compounds that progress to the clinic, and the university receives milestone payments if the option is exercised (Ratner, 2011; Cain, 2010).

Similarly, Johnson & Johnson (J&J) announced in 2012 that it would form four innovation centers in academic and biotech hot spots: Boston, California, London, and Shanghai. The goal is to form alliances and collaborations with academics and entrepreneurs that can take different forms, such as early-stage funding, creation of startups, or straightforward licensing deals. The innovation centers house J&J scientists mixed with corporate investors and business development executives (Senior, 2013).

J&J has a separate model in its innovation network: the JLabs incubators, which provide facilities, resources, and education to entrepreneurs in a no-strings-attached model that acts as a gateway for entrepreneurial scientists to advance the translation of discovery research or innovative concepts. As of 2020 JLabs had opened 13 sites in hotspots such as Boston, San Francisco, San Diego, and Shanghai as well as emerging hubs such as Toronto, Philadelphia, New York, Houston, and one outside the United States in Belgium. JLabs counts 676 companies among current startups and alumni, of which 463 are in the biopharmacology sector. JLabs also partnered with the US Biomedical Advanced Research Development Authority in a Blue Knight initiative to accelerate

innovation to global health security. In August 2020, during the COVID-19 pandemic, the Blue Knight initiative selected seven JLabs companies that could address perceived gaps in the COVID-19 pipeline.

Bayer AG created a similar system with its CoLaborator network, which offers incubator space to startups. The pharma provides lab space for rent, access to Bayer infrastructure and expertise, and networking throughout the local R&D community. Like JLabs, CoLaborators are often close to academic institutions, although they are more global, being located in San Francisco, Berlin, Moscow, and Kobe, Japan. Before establishing the CoLaborators, Bayer formed a master agreement with the University of California, San Francisco (UCSF), that covered central issues of IP and publication and gave Bayer access to discoveries from UCSF labs. Under the agreement, IP on a compound was owned by whichever side created it; UCSF researchers gained access to Bayer compounds but were required to gain consent before publishing. The academics also had access to Bayer's tool compounds, on which they could publish freely (Parmley, 2014).

Almost all the major pharma companies include deals with academic labs or organizations as a core component of their business development and licensing activity. In some cases this is through dedicated initiatives, such as Sanofi's Sunrise program (Martz, 2016); in others it is through routine partnering activities.

Precompetitive consortia

Despite the greater integration of pharma companies and academia, several roadblocks remain that hamper efficient translation. Precompetitive consortia have emerged as a structure that allows multiple pharma companies, academics, and other stakeholders to solve industry-wide problems collaboratively.

Some of these consortia address system-wide limitations caused by insufficient infrastructure. For example, real-world data are being recognized as an important tool that could advance drug development, but there are no common standards for collecting and annotating data, and there is little interoperability between the data collection systems that different organizations employ. In addition, clinical trial data are often not disclosed, in particular data for failed trials, which means that investigators and companies are not able to benefit from the full breadth of data that have been generated. Precompetitive consortia and data-sharing platforms such as Vivli and TransCelerate have been designed to address some of these issues. These platforms provide tools for sharing information and knowledge, much of which was previously held as proprietary and confidential, and many pharmas have actively participated. Vivli is run by the

Multi-Regional Clinical Trials Center of Brigham and Women's Hospital and Harvard; TransCelerate is run by TransCelerate BioPharma Inc.

In other cases, limitations in translation are due to technology issues that can be solved only by science, and precompetitive consortia have been created to address specific problems that are too large for any single organization to tackle or to pool libraries or data from multiple organizations (Denee et al., 2012; Mittelman et al., 2013).

For example, the Biomarkers Consortium (<http://www.biomarkersconsortium.org/>) was established by the Foundation for the National Institutes of Health (FNIH) to develop and validate biomarkers in multiple disease areas. Biomarkers represents a new area of vital importance to the entire industry, but there have been multiple parallel efforts to search for biomarkers for different diseases and no clear standards for validating them or obtaining regulatory approval. In 2018 the FDA qualified a group of kidney biomarkers from the BioMarkers Consortium and C-Path's Predictive Safety Testing Consortium, marking the first qualification of a clinical safety biomarker. The PTSC was formed in 2006 to identify new and improved safety-testing methods and to obtain regulatory approval for them.

Another success story for the Biomarkers Consortium was the identification of adiponectin as a predictor of metabolic response to PPAR (peroxisome proliferator-activated receptor) agonists in type II diabetes patients (Wagner et al., 2010).

Other types of consortia aim to improve the efficiency of drug development by improving the design of candidate molecules. The Structural Genomics Consortium (SGC)¹ generates three-dimensional protein structures and chemical tool compounds as probes to explore how subtle changes between proteins affects their functional properties. The consortium was established in 2003 and has 10 pharma partners and 3 partners from nonprofit and Canadian government organizations. The SGC has thus far produced structures for over 1500 proteins, which represent about 15% of the human proteome, and has made them publicly available.

Attitudes, ethics, and standards

The closer relationship among sectors in the biotech ecosystem, in part via the integrated discovery nexuses, has begun to chip away at the old view among academics that Big Pharma represents “the dark side.”

The concept of making money from discoveries was anathema for a long time in academia. However, it has become clear to many academics that without investment—and without the machinery for translating discoveries into products—their discoveries will go unused and patients will not benefit. There are few viable paths for nonprofit

enterprises to translate the wealth of academic discoveries into medicines, certainly not at scale. Instead, almost all paths to turning discoveries into products go through Big Pharma. Translational discoveries either are adopted early on by pharma companies via the external innovation structures described or are spun out into companies or licensed by SMEs. In the latter case, few companies are able to take programs to market without a pharma partner, and even when they do, they still do so for profit.

While academics have in many cases embraced the capitalist model of creating drugs and have engaged directly with the industry, there remains a large gap between pharma companies and academic labs in standards and what industry views as the quality or reliability of the research. The result is that academic labs have created a reproducibility crisis, in which an alarmingly low proportion of the results that are published can be consistently reproduced (Fishburn, 2014a).

Part of the reason is the lack of regulatory and commercial training for academic translational scientists. This is now being addressed via the external innovation arms and the discovery nexuses, which provide guidance on how to construct experiments and programs in accordance with a target product profile (TPP). The TPP represents a skeleton document laying out the value to the patient that the therapy represents: specifically, the disease case, how the therapy improves on the standard of care and competitive agents, and the preclinical and clinical standards that must be met to demonstrate that improvement, encompassing both safety and efficacy.

Coronavirus and the road ahead

The COVID-19 pandemic that began in 2020 has the potential to produce lasting changes in the biopharmaceutical industry, extending from how companies are viewed by the public to how they work together to solve industry-wide problems.

In the last two or more decades, the pharma industry has been held in low esteem among the general public, largely owing to its approach to drug pricing. Many companies continued to raise prices even for old drugs and have battled to extend patent lifetimes and prevent the entry of cheaper generics to the market. They gave the appearance of putting patients over profits and allowed competitive practices to slow down drug development, a process that takes over a decade and costs more than \$1 billion from discovery to market.

The COVID-19 crisis triggered a dramatic change. Decade-long development times were not an option for this pandemic. Pharma companies that are normally fierce competitors gathered together to share data as well as best

1. <http://www.thesgc.org/>.

practices, and suspended or slowed down many other activities to put all their efforts into creating therapies and vaccines to counter the novel coronavirus. Three major consortia were formed: the COVID R&D Alliance, composed of industry members; the ACTIV consortium, headed by the FNIH, containing pharma companies as well as the FDA, the European Medicines Agency, the Centers for Disease Control and Prevention, and the National Institutes of Health; and the COVID-19 Therapeutic Accelerator, headed by the Bill & Melinda Gates Foundation and Novartis AG. The three consortia had many overlapping members and coordinated to ensure that they did not duplicate activities ([Usdin, 2020](#)).

The pandemic created a new paradigm for the rapid translation of discovery research to create therapies, diagnostics, and vaccines, although how much will be sustainable in the post-COVID-19 era remains to be seen. The first publication of the sequence of the SARS-CoV-2 virus was rapidly employed by the international scientific community, from academia to pharma companies, to understand the biology of the virus and develop countermeasures. Academics at Oxford University created the RECOVERY trial, a master protocol adaptive platform trial that went from first draft protocol to first patient in under 10 days and produced actionable results within 100 days. Another group at Oxford University created the ChAdO1 nCoV-19 vaccine (AZD1222), which was

licensed by AstraZeneca PLC and became one of the front-runner vaccine candidates. Other candidate therapies and vaccines were rapidly launched into trials, and the close relationship that had formed over the last decade between the academic and pharmaceutical sectors served as a platform for accelerating the creation of countermeasures in the face of a crisis. Two of the most successful vaccines came from innovations in small companies that had been advancing the novel mRNA technology over the prior decade. The Comirnaty vaccine from Pfizer Inc. was created by its partner BioNTech SE, a German biotech founded by academic entrepreneurs Ugur Sahin and Özlem Türeci, from the Johannes Gutenberg University in Mainz, Germany. Moderna Inc., which created the second mRNA vaccine, was founded in 2010 as a VC-backed biotech with collaborators at Harvard University and the Massachusetts Institute of Technology.

Many norms of practice were shattered during the pandemic ([Fishburn and Usdin, 2020](#)). Sharing of clinical trial data, a practice that had been growing in the previous few years through various data-sharing platforms, became seen as essential for expediting decisions. Master protocol adaptive trial designs, which the industry has been slow to adopt, proliferated as drug developers saw them as the most efficient means to test and triage candidate therapies. The drug development process also took a big step toward harnessing the use of real-world data as well as

BOX 31.1 Pharma and biotech: What's in a name?

As the drug development landscape has grown and the organizations have changed, the names have stuck but the old definitions for a pharmaceutical company or a biotech company have started to lose their relevance. Traditionally, pharmaceutical companies produced small-molecule drugs and were fully integrated drug development companies with their own sales forces. Biotechs, by contrast, produced biologicals or therapeutics derived from living organisms, such as cell-based or DNA-based therapies, and were often barely bigger than an R&D organization, with no sales force. Pharma companies were conceived of as large, high market-cap organizations with a powerful political lobby. Biotech companies, by contrast, were thought of as small, nimble, generally lower market cap organizations that used innovative approaches to solve medical problems. While the term *Big Pharma* persists today, the line between pharmas and biotechs is hazy, and the term is losing relevance. As [Table 31.1](#) shows, at least 10 biotech companies have market caps in the range of—and sometimes greater than—traditional pharma companies. Some of the seminal biotechs that changed the field have been acquired by pharmas, though in some cases they maintain some independence of operations. Genentech is generally thought of as the first biotech, but it is now part of Roche. Genzyme, which

was acquired by Sanofi, changed the course of the industry, demonstrating that there is a powerful business model in pursuing rare diseases with very small patient populations. The top two takeovers of biotechs by pharmas—the \$74 billion acquisition of Celgene Corp. by Bristol Myers Squibb Co. and the \$62 billion deal for Shire PLC by Takeda Pharmaceutical Co. Ltd—put valuations of those biotechs in the range of some of the pharma–pharma mergers of the 1990s and 2000s. How many independent big biotechs there is room for in the market is an open question. But the distinction is melting away, with a tail of SME biotechs ranging from fully integrated public companies with full sales divisions to newly formed private companies with a handful of employees. According to Morrison and Lähteenmäki, there were 671 publicly listed biotechs in 2018 ([Morrison and Lähteenmäki, 2019](#)). Exact numbers for private companies are not available, but BioCentury's BCIQ database contains more than 2000 privately held active biotech companies. There is also no distinction in the type of therapy the companies create, with all types of companies engaging in traditional modalities—small molecules and monoclonal antibodies—as well as new modalities, such as RNAi, cell therapies, gene therapies, and other types of treatment.

TABLE 31.1 List of big pharma and big biotechs.

	Company name (earliest root company)	Year founded (year of earliest root)	Headquarters	Stock ticker(s)	Market cap (September 28, 2020)
Big Pharma	Abbott Laboratories	1900	Abbot Park, IL	NYSE: ABT	\$183.2B
	AbbVie Inc.* (<i>Abbott Labs</i>)	2013 (1900)	North Chicago, IL	NYSE: ABBV	\$152.2B
	AstraZeneca plc* (<i>Astra AB</i>)	1999 (1913)	Cambridge, United Kingdom	LSE: AZN NASDAQ:AZN	\$143.9B
	Baxter International Inc.	1931	Deerfield, IL	NYSE:BAX	\$40.3B
	Bayer AG	1863	Leverkusen, Germany	Xetra:BAYN	\$61.3B
	Boehringer Ingelheim GmbH	1885	Ingelheim, Germany	Private	Private
	Bristol Myers Squibb Co.	1887	New York, NY	NYSE:BMY	\$134.1B
	Eli Lilly and Co.	1876	Indianapolis, IN	NYSE:LLY	\$143.7B
	GlaxoSmithKline plc* (<i>The Beecham Group</i>)	2000 (1859)	London, United Kingdom	LSE:GSK NYSE:GSK	\$93.8B
	Johnson & Johnson	1887	New Brunswick, NJ	NYSE:JNJ	\$383.5B
	Merck & Co. Inc.	1891	Kenilworth, NJ	NYSE:MRK	\$209.8B
	Novartis AG* (<i>Sandoz</i>)	1996 (1886)	Basel, Switzerland	NYSE:NVS SWX:NOVN	\$192.1B
	Novo Nordisk A/S	1923	Basqvaerd, Denmark	CSE:NOVO B NYSE:NOVO	\$160.6B
	Pfizer Inc.	1849	New York, NY	NYSE:PFE	\$200.3B
	Roche	1896	Basel, Switzerland	SWX:ROG OCTQX:RHHBY	\$305.7B
Big Biotech	Sanofi (<i>Lavaz group</i>)	1973 (1947)	Paris, France	Euronext:SAN NASDAQ:SNY	\$127.1B
	Takeda Pharmaceutical Co. Ltd.	1781	Japan	TOKYO:4502 NYSE:TAK	\$58.2B
	UCB S.A.	1928	Brussels, Belgium	Euronext:UCB	\$21.6B
	Amgen Inc.	1980	Thousand Oaks, CA	NASDAQ:AMGN	\$142.8B
	Biogen Idec Inc.	1978	Cambridge, MA	NASDAQ:BIIB	\$43.2B
	Gilead Sciences Inc.	1987	Foster City, CA	NASDAQ:GILD	\$78B
	Regeneron Pharmaceuticals Inc.	1988	Tarrytown, CA	NASDAQ:REGN	\$61B
	Vertex Pharmaceuticals Inc.	1989	Boston, MA	NASDAQ:VRTX	\$69.8B
	Seattle Genetics Inc.	1998	Bothell, WA	NASDAQ:SGEN	\$32.2B
	Illumina Inc.	1998	San Diego, CA	NASDAQ:ILMN	\$43.9B
	Moderna Inc.	2010	Cambridge, MA	NASDAQ:MRNA	\$27.4B
	Beigene Ltd.	2010	Beijing, China	NASDAQ:BGNE HKEX:6160	\$25.2B
	Alexion Pharmaceuticals Inc.	1992	Boston, MA	NASDAQ:ALXN	\$25.1B

(Continued)

TABLE 31.1 (Continued)

	Company name (earliest root company)	Year founded (year of earliest root)	Headquarters	Acquired by (Year)	Market cap (September 28, 2020)
Acquired Big Biotechs	Celgene Corp.	1986	Summit, NJ	Bristol Myers Squibb (2019)	\$74B
	Genentech Inc.	1976	South San Francisco, CA	Roche (2009)	\$46.8B
	Genzyme Corp.	1981	Cambridge, MA	Sanofi (2010)	\$23.9B
	Shire plc	1986	Dublin, Ireland	Takeda Pharmaceutical Co. Ltd. (2019)	\$62B

The definition of pharma and biotech companies has changed over time (see Box 31.1). Today's big pharmaceutical companies trace their roots to the late 19th and early 20th centuries. Companies in italics are the earliest corporate root of the pharmas that exist today. AbbVie Inc. was formed from a split of Abbott Labs in 2013. Sanofi was created in 1973 when French oil company Elf Aquitaine acquired the Labaz group, a pharmaceutical company formed in 1947.

*Starred pharmas were formed in the years shown by mergers of older companies: Novartis AG (Ciba-Geigy Ltd. and Sandoz Ltd.); GlaxoSmithKline PLC (Glaxo Wellcome PLC and SmithKline Beecham); AstraZeneca PLC (Astra AB and Zeneca group). The biggest biotech companies included in the list are those with market cap values above \$25B as of September 27, 2020, which put them in the range of the big pharmas. Select major biotech deals involving big pharmas are also shown.

Source: BioCentury BCIQ database.

use of digital technologies to advance translation, although the limitations of those still-embryonic fields also became clear (Fishburn and Usdin, 2020).

For the first time in decades the public's view of pharma companies focused on their ability to create life-saving medicines and preventions. It remains to be seen how many of those practices will remain in the aftermath of the pandemic and how much the pharma industry, in partnership with academic labs, can continue to accelerate the translation of laboratory discoveries to new medicines (Box 31.1).

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Chapter 32

Translational science in medicine: putting the pieces together-biomarkers, early human trials, networking, and translatability assessment

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Abstract

The aim of this chapter is to integrate the main aspects constituting translational science in medicine that are described in detail in the preceding chapters. Four major tasks of a novel science are defined and established as the cornerstones of the emerging systematics: biomarkers, early clinical trials, translational planning and networking, and translatability assessment. The translational planning and networking theme is introduced here as a central prerequisite of translational success. Possible models of collaboration are detailed for the major players in the translational plot, mainly those from academia and the pharmaceutical industry. The chapter also defines and specifies the essential integrating role of a chair or institute for translational medicine and underlines the key role of translational plans as living documents that are updated at all milestone achievements.

Keywords: Translational planning; networking; translatability assessment; living document; chair of translational medicine

The preceding chapters of this book detail the manifold aspects of translational processes in biomedical research. As stated repeatedly, these toolboxes, algorithms, and networking aspects need to be integrated into a meaningful, comprehensive, and structured way. In this respect, it is important to note that what is frequently simply called *translational medicine* should be developed into a solid science; the title of this book emphasizes this by inclusion of the word *science* in the title: *Translational SCIENCE in Medicine* (a science that should also exist in, for instance, physics or chemistry).

This novel science needs to be developed similarly to biostatistics, which had to be developed in the 1970s to

reflect the rapidly growing demands in biomedical research. In particular, clinical studies were driving this development to generate what is now called evidence-based medicine. This evidence had to be derived from experimental (both preclinical and clinical) data by ever more sophisticated statistical analysis. A new science emerged, although its roots could be tracked back to the 17th century. Today, almost all medical faculties, all major pharmaceutical companies, and biomedical research institutions provide the required expertise and practical work in independent departments or institutes for biostatistics.

A similar path is envisioned for translational science in medicine, which has started to exist but still requires major developments of scientific content and institutional representation. Promising developments, mainly in the United States, are detailed in Chapter 1, Introduction and Definition.

While conceptually still in its infancy, this novel science requires major scientific input at both the conceptual and implementational levels, which is the major target of this book.

To more clearly structure the scope and key elements of translational science in medicine, four major tasks are proposed as cornerstone assets of this novel science:

Task 1: Need for rigorous science-based biomarker classification in regard to predictivity in translational processes

Task 2: Need for rigorous science-based design of early-stage, exploratory, or “smart” human trials

Task 3: Need for careful, foresighted planning and coordination of major players in translational processes, including academia-industry interactions and networking across boundaries

Task 4: Need for rigorous science-based predictivity scoring in translational processes to reduce or ameliorate weak projects to create thrust for promising ones

Details of three of these essential tasks are given in Chapter 12 (task 1), Chapters 17–21 (task 2), and Chapter 11 (task 4). Task 3 has not been explicitly elaborated yet but is as essential in that it creates the necessary planning and networking as an indispensable prerequisite of translational success. Because tasks 1, 2, and 4 may flourish independently in separate “silos” and thus might not be seen as integral components, their carefully planned integration may be considered the main challenge of translational processes.

A key element of bringing together all the different disciplines that are necessarily involved in translation (e.g., molecular biologists, pharmacologists, animal researchers, pharmacokinetics and pharmacodynamics experts, human trialists, clinicians) is foresighted planning. A document must be created on day one of a relevant discovery, for example, of a novel receptor or a novel function of a known receptor as a new drug target candidate. Translation will work only if both clinical researchers and preclinical scientists have an educated vision of how to translate early findings (mostly *in vitro* findings) into useful human medicines. They should have an idea about unmet medical needs (interest of patients otherwise not optimally treated) and about disease constituents, including the major biomarkers in the field. They should know about the obstacles and limitations of human experiments (e.g., “serial brain slicing” as only readout = no way into human development).

It is obvious that basic scientists need to do the following:

1. Develop interest, maybe driven by awards through funding restrictions if translation is not convincingly envisioned;

2. Obtain education in the major areas of translational science of medicine, as detailed by the four major tasks described above;

3. Seek and accept advice from translational scientists, mainly from later stages of development, typically clinicians if preclinical researchers are concerned.

In an ideal world, basic researchers would be able to set up a primordial translational plan from day one; as a living document, this plan requires updating at all major milestone achievements. It has to describe decision trees (if this happens, then this will be done next; if not, then that will be done), milestone achievements, and all major constituents of translational success. A template for an early translational plan is shown in Fig. 32.1. As can be seen, the plan should be short and concise, reflecting the best of current knowledge, and should be based on a minimum of speculations, all of which should be marked as such.

This translational planning has to embrace and integrate the potential collaborators and should be based on established translational networks. There are various models of the organization of such a translational network. Its major feature is the coordinating unit or chair functioning as network organizer and administrator. This capacity has to identify resources (potential network participants), identify gaps to be closed by scouting for suitable novel participants, connect distant players, and amalgamate interface resistances, in particular relating to the cooperation of industry and academia (public-private partnerships). It also has to provide support by legal expert, in particular, intellectual property specialists, and to organize administrative tasks to cope with, for example, variable compensation schemes in private and public entities. Obviously, even these central coordinating duties cannot be accomplished by a single person but require a group of specialized team players in the central organization or a university chair.

Use the translational medicine plan (TMP) as facilitator and guidance for improved networking at the various interfaces, (discovery, translational medicine, and clinical) to enable early input from clinical into discovery and vice versa

Create a concise, short document detailing the studies planned to identify and evaluate biomarkers and human exploratory studies

Content

1. Introduction

2. Plans to support

- a. Target identification and validation e.g., by human exploratory studies
- b. Diagnostics: Identification of targeted population
- c. Biomarkers/surrogates
 - i. Pharmacodynamic activity
 - ii. Safety

3. Project planning—timelines and resource needs (GANNT-chart, FTEs, \$)

FIGURE 32.1 Template for an early translational plan.

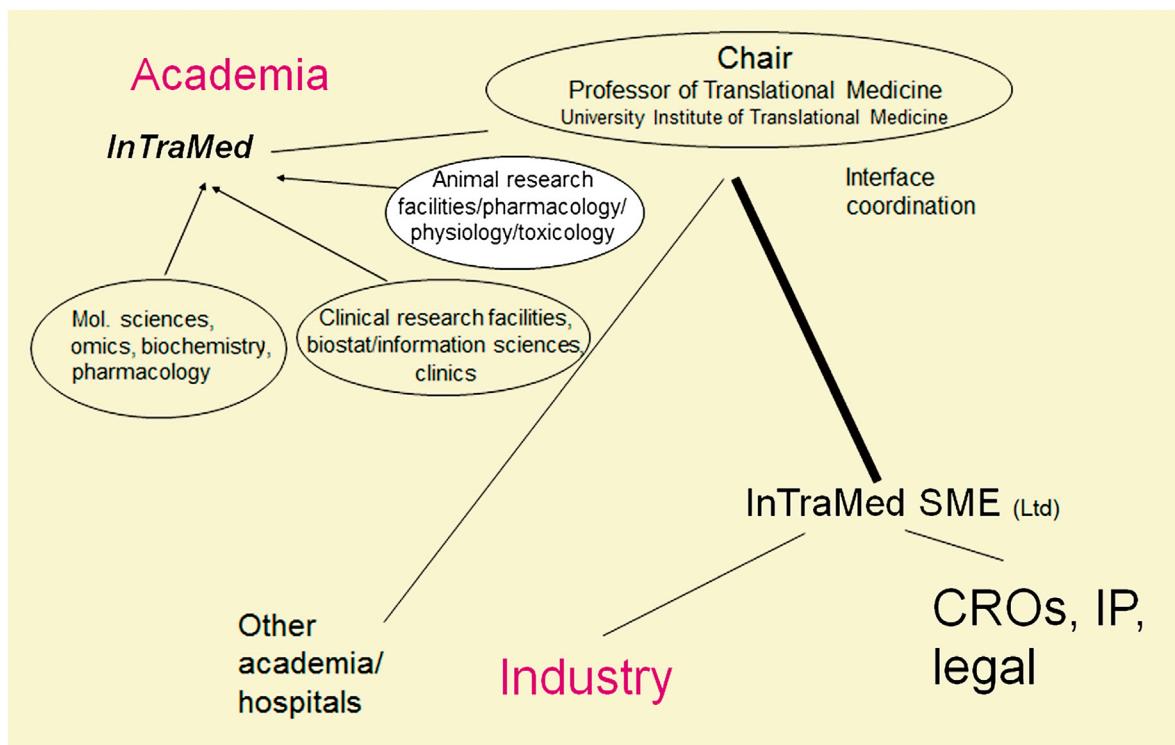


FIGURE 32.2 Sketch of a translational medicine network of public and private partners that have hands-on involvement in translational actions. It is based on the cooperative Institute of Translational Medicine (InTraMed), backed by a small to medium-sized enterprise (SME) limited company, to bring academic resources, contract research organizations (CROs), intellectual property (IP) management, and legal backup in contact with industry. From Wehling M. Drug development in the light of translational science: shine or shade? *Drug. Discov. Today* 16 (2011) 1076–1083, by kind permission.

A potential networking model is proposed in Fig. 32.2 (for comparison see Fig. 31.1, which is similar but more comprehensive). This is only one of many sketches, all aiming at connecting and intertwining often-distant players in the translational plot that is indispensable if translation should be successful.

An established networking structure is probably the single most important prerequisite of translational success; planning for translation will be done through a consortial and thus integrative approach. Those structured and educated approaches will aim to attract expertise and skills in addressing the other three major tasks.

It is important to note that during a typical translational development, public and private partners will have uneven shares of contribution. At certain stages, public partners lead; at other stages, private partners lead. Typically, academia is strong at the very early stages of target discovery and validation (the first highly visible paper in leading basic science journals such as *Nature* or *Science*), whereas lead optimization is better placed in the pharmaceutical industry with access to huge compound libraries and outstanding expertise in medicinal chemistry. Lead identification may be done by either player. This scheme may be different if biologicals are involved, which may be designed and even produced at a small scale by academia.

As development progresses, early human trials (phase 0, exploratory smart trials) are clearly a domain of public contributors, as they often require complex human instrumentation, especially imaging, which is typically available only in major hospitals. There is a large yet unexploited potential for academic hospitals to venture into this area, as industry attempts at establishing human imaging centers (e.g., by Glaxo in London from 2005) have largely failed. This is mainly due to the fact that only utilization of expensive human diagnostic machines by studies and regular patient care make the investment profitable. Academic institutions may progress their own drug developments at maximum to classic phase II trials and achieve proof of concept as a major value increment. Unfortunately, this highly recommended exit point is not often reached by academia, and industry has to take over or collaborate at earlier stages in many instances. Beyond this, most drug development will have to be passed on to industry because phase III trials are very large and expensive, therefore being almost excluded from public financing. In a recent overview on academic drug development in the United States, collaboration rates between academia and industry started at 32% for preclinical; rose to 55% in phase I, to 76% in phase II, and to 93% in phase III; and reached 100% at the new drug application/biologicals license

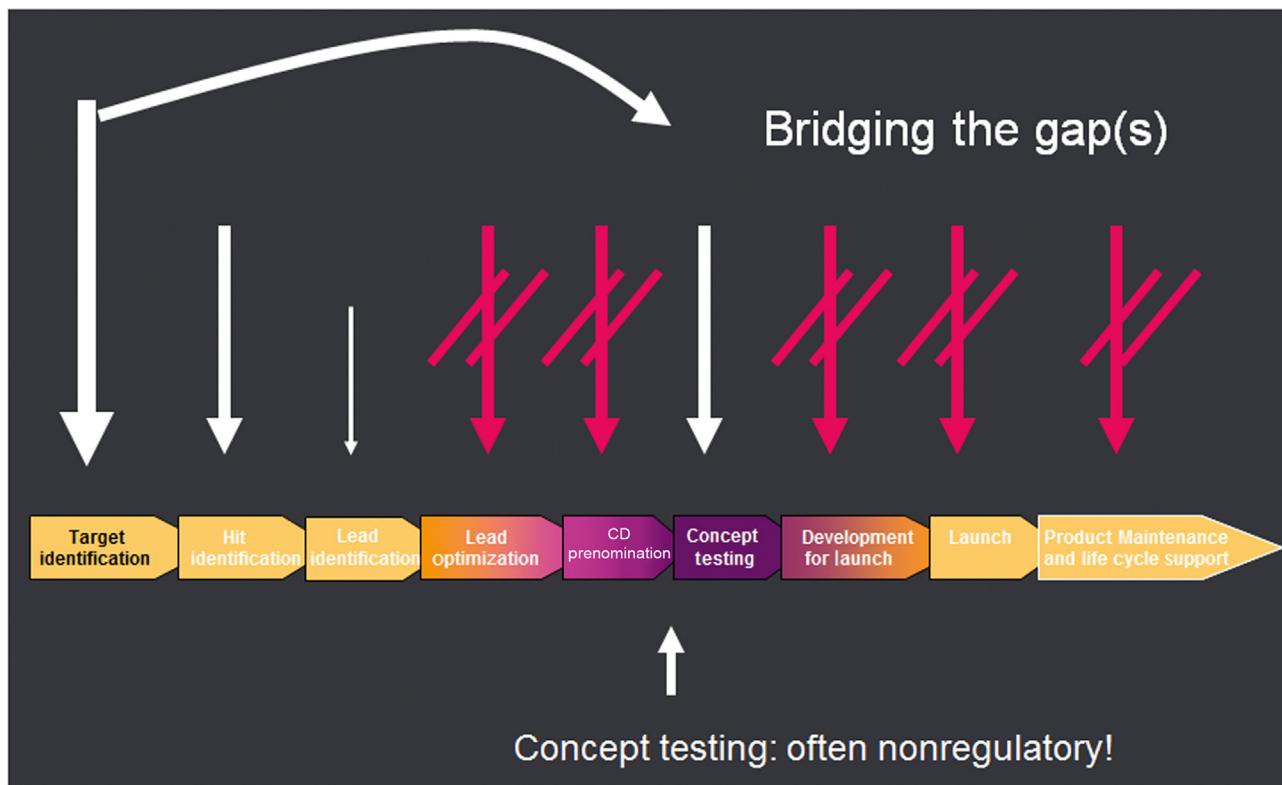


FIGURE 32.3 Strengths of academia versus industry in translational science. Academia is strong in target identification and/or validation, possibly lead identification, and early (“smart”) nonregulatory clinical trials (*white arrows*). By contrast, lead optimization, regulatory clinical trial development, particularly phase III trials, and launch and product maintenance need to be done by industry (*red arrows*). *From Wehling M. Drug development in the light of translational science: shine or shade? Drug. Discov. Today 16 (2011) 1076–1083, by kind permission.*

application phase (Takebe et al., 2018). Of course, collaboration does not mean complete takeover in a black-or-white mode, and codevelopment of these projects may continue with changing shares across the entire path of development. Nevertheless, these figures show that industry is often onboard as early as phases I and II and thus ahead of the most prominent value inflection point past phase II or proof of concept.

The preferences and strengths of private and public contributions are depicted in Fig. 32.3.

Only if properly organized and planned will translational science in medicine yield successfully translated projects. Task 4 (translational assessment) will guide researchers, investors, and funding agencies to the potentially promising projects and unravel weaknesses of others to be amended by efforts directed to those deficiencies.

This assessment is thus a central obligation and best placed in the core structures of the translational network, in most cases to be covered by the translational department or chair. This central institution is thus pivotal not only in organizing translation, but also in adjudicating funds and resources in a robust and transparent process of translatability assessment.

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Chapter 33

Learning by experience

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Abstract

The final chapter of this book demonstrates two case studies in which the essential messages from the preceding chapters are projected onto real-life cases. Both a successful and a failed translational project were chosen from the cardiovascular arena. Torcetrapib—a high-density lipoprotein (HDL) cholesterol—increasing drug—was developed despite a considerable translational risk because the main biomarker, HDL cholesterol concentration in human plasma, had never before been firmly established as a risk factor with therapeutic implications. The phase III pivotal trial not only failed but also produced excess mortality. The latter was likely attributable to off-target toxicity (an increase in blood pressure) as shown by the congener drug dalcetrapib. This drug was developed at an even higher translational risk because the main biomarker had been devaluated by the first drug in the field, but at least it proved to be nontoxic—although also not successful with regard to the reduction of cardiovascular endpoints. The failure of this second drug was clearly foreseeable. At the other end of the spectrum, varenicline was analyzed as a smoke cessation aid that scored high in translatability assessment. The main reasons for this were a clear biomarker indicating smoke cessation and a positive congener experience in the past.

Keywords: Torcetrapib; dalcetrapib; varenicline; translatability scoring; risk factor

Example of a smart, successful translational process

Smoking cessation is a powerful tool to prevent cardiovascular disease and a variety of malignancies. As a central task in overall patient management, smoking cessation is one of the most beneficial interventions possible. It has been shown to reduce the incidence of cardiovascular disease and lung and other cancers, and it significantly reduces overall mortality. Similar to one of the most successful innovations in cardiovascular treatment, the introduction of statins, it does not seem as much of a stretch to claim that aids to stop smoking represent cardiovascular

interventions. Therefore the example of varenicline, the best currently available drug aid to reduce the craving for and satisfaction of smoking, has been chosen here.

There have been various attempts to support people who are willing to stop smoking, and they have been accentuated as the harmful side effects of smoking have become more and more evident. In 2008 the Centers for Disease Control and Prevention reported that cigarette smoking and exposure to secondhand smoke resulted in an estimated 443,000 deaths per year in the United States ([Centers for Disease Control and Prevention, 2008](#)), and in 2019 the World Health Organization estimated that about 8 million deaths annually are caused by tobacco ([World Health Organization, 2019](#)). Quitting smoking is beneficial regardless of time point and age ([Rigotti and McDermott, 2019](#)). Therefore all methods for helping people quit have significant bearings on public health.

Until recently, the most commonly used pharmacological aids were nicotine itself and bupropion, a psychotropic drug. Nicotine is available in different preparations (tablets, ointment, lozenges, e-cigarettes, etc.); it occupies and stimulates central nervous system receptors ($\alpha 4\beta 2$ nicotinic receptors in the ventral tegmental area), whose stimulation results in dopamine release in the nucleus accumbens through the mesolimbic system. Because it is the natural stimulatory component of cigarette smoke, exogenous nicotine has the same effect as smoke-borne nicotine: It causes dopamine release at a site that is known to be the convergence site of almost all types of addiction. Dopamine release in the nucleus accumbens is induced by alcohol, cocaine, and amphetamines and also by food intake and nicotine. Addiction to those agents and habits thus is similar, and treatment strategies are also similar to a certain extent. Thus pharmaceutical strategies for helping patients quit smoking targeted a controlled reduction of dopamine release. This can be achieved principally through nicotine replacement therapy, in which “clean” nicotine substitutes for the cigarette-based nicotine supply, which is tied to the poisonous tar components

that cause most of the health problems related to smoking. Because the smoking habit itself will taper off if the act of smoking is not repeated frequently, the habit slowly disappears, and many components of craving for a cigarette fade away. However, it is obvious that replacing a poison with a different version of the same poison may not represent the smartest way to cure addiction, as the addictive agent with potentially noxious cardiovascular effects (hypertension, tachycardia) is still being supplied.

The next incremental gain was the introduction of bupropion, which is clinically used as an antidepressant. It was noted that dopamine release can be attenuated by this drug and that this could be beneficial in addiction treatment. Thus it gained market approval, among uses in other addiction treatments, as a smoking cessation drug. However, its chemical similarity to amphetamines may be the reason for a multitude of side effects, including sleeplessness and seizures, that are not trivial. Therefore there was a strong demand for more effective and safer drugs in this area. Another approach starting from compounds similar to nicotine has been in practice since the 1960s: Cytisine is a partial agonist at the nicotinic receptor and was tried as a smoking cessation aid in the 1960s and 1970s. To cut the long cytisine story short, it was not an international success because its deployment was locally restricted to Europe, and at that time, there were no clinical trials fulfilling current quality criteria. This drug, if profiled to be similarly effective as varenicline but much cheaper, could save many lives in developing countries and is still considered to be a valuable aid for smoking cessation ([Paduszyńska et al., 2018](#)).

Which features of the foregoing developments made translation of varenicline successful? Which biomarkers could be used?

The main translational discovery dates back to the early cytisine experiences in humans: Partial agonism at the nicotinic receptor (the particular type identified later as $\alpha 4\beta 2$) does produce the expected result. As receptors are occupied, the nicotine antagonistic properties are evident in that a new cigarette and its nicotine are ineffective, and the partial activation does release enough dopamine to prevent people from experiencing heavy craving. Cold turkey is avoided, and satisfaction after smoking a cigarette is absent.

The translational evidence was based on isolated receptor subtypes cloned from animals and humans and on valid animal models of nicotine dependence and nicotine side effects (e.g., hypothermia) that are absent in the limited ceiling effects of partial agonists. A congener compound had produced beneficial clinical effects, and biomarkers at the animal level (behavioral measures) and comparably simple clinical studies with smoking habits and psychometric scales for craving and satisfaction were established and validated at the time of varenicline translation. Thus

not only in vitro test systems to analyze binding and agonist or antagonist properties had been developed, but also animal models that were sensitive and predictive for both efficacy and safety aspects. These models seemed to reflect the benefits of partial agonism at the nicotine receptor. They clearly showed that unwanted nicotine effects were absent with varenicline and thus demonstrated the low ceiling effect of partial agonism as mentioned above. Thus in the predictivity scoring system for biomarkers described in this book, the preclinical biomarkers (in animal models) would have achieved comparably high scores, had they been retrospectively validated by the human experiences with cytosine and other similar compounds. The human biomarkers to measure craving and satisfaction had been validated by other model compounds (more by bupropion than cytisine, as the latter had probably been in clinical development when legal requirements were absent or low).

Thus this combination of human data for drugs aiming at both the same clinical entity (bupropion) and the same principle mechanism (cytosine and, for a second similar compound, lobeline), animal data from very predictive models, and in vitro receptor data made the tailored development of varenicline possible and is the base of its clinical success (for a review, see [Jordan and Xi, 2018](#)).

This example shows that experiences from suboptimal development processes (mostly dating back to preregulatory periods in which clinical development was random and possibly dangerous to the patients) are valuable tools for defining translational strategies and assessing the biomarkers to be trusted in present processes. Many of the approaches at translational promotion have been utilized in this way (using experiences obtained from model compounds, reverse pharmacology, and meticulously developed animal models). One potentially painful question remains: If cytisine had been developed in the same way as varenicline was, would varenicline be any better than cytisine? This question cannot be answered unless comparative trials with cytisine are performed. Under current conditions this is very unlikely, as cytisine has no genuine patent protection, and no one will ever invest in such a compound. There are publications on comparative study protocols (e.g., [Walker et al., 2019](#)) but no results to date. This irony—that the model compound has helped the expensive patent-protected compound to go through a successful translational program but has never proved its own strength—is among the seemingly inevitable strange or even bizarre features of drug development processes.

Example of a failed translational process

Let us consider the development of torcetrapib as a historical example of a failed translation process that still has very recent implications. The torcetrapib case had already

cost Pfizer about \$800 million since the placebo-controlled ILLUSTRATE trial showed excess mortality of high-risk cardiovascular patients, leading to the cessation of drug development (Barter et al., 2007). The secondary costs of this failure could even be counted in billions of dollars and could well affect the future well-being of the entire company. It was hoped that this compound would become a blockbuster and—as a combination product—prolong the life cycle of the company’s biggest seller at that time, atorvastatin, whose patent was due to expire in 2010 or 2011. Not only did mortality significantly increase; atherosclerosis progression under the drug was even worse than that under the placebo as measured by intima media thickness (Nissen et al., 2007). This biomarker represented a very reliable, high-scoring parameter at that time, an appreciation that has been partially lost meanwhile (see Chapters 11: Target Profiling in Terms of Translatability and Early Translation Planning and Chapter 14: Cardiovascular translational biomarkers: translational aspects of hypertension, atherosclerosis, and heart failure in drug development - in the digital era). Why were expectations running high, why was the disappointment tremendous, and what role did translational processes play?

Torcetrapib is a small molecule aiming at the inhibition of the cholesteryl ester transfer protein (CETP), which is known to be involved in cholesterol transport and therefore seems to be a key enzyme in the metabolism of cholesterol, which has been connected to cardiovascular disease, the number one cause of death worldwide. When the trial was designed, CETP inhibition was thought to result in increased concentrations of high-density lipoprotein (HDL) cholesterol (“good” cholesterol), which is normally degraded by transferring cholesterol esters into very low density lipoprotein (VLDL) or low-density lipoprotein (LDL) particles (Nissen et al., 2007). Increasing HDL cholesterol was seen to be an appropriate way to further reduce cardiovascular risk, which can be reduced by roughly 20%–30% by statins, for example. This shows that there is still an unmet medical need to further reduce premature cardiovascular deaths, despite the success story of statins and other cardiovascular preventive measures (e.g., acetylsalicylic acid). As early as the 1950s and 20 years later with the first publication of the famous Framingham study (Gordon et al., 1977; Nikkila, 1953), evidence of a strong negative correlation between cardiovascular morbidity and mortality and HDL cholesterol concentration was emerging, and epidemiological (i.e., noninterventional) trials always confirmed that relationship. There were interventions in which HDL cholesterol increases were associated with decreased cardiovascular endpoints, namely, by exercise and by drugs: nicotinic acid (Brown et al., 2001; Taylor et al., 2004) and gemfibrozil, a fibrate drug (Frick et al., 1987). This evidence was the basis of the generalizing

assumption that any intervention leading to increased HDL cholesterol values should be beneficial to patients.

In light of this background, CETP inhibitors appeared to be a promising principle; in various animal models and early human trials they did what they were expected to do: They increased HDL cholesterol levels (Brousseau et al., 2004), and they could even prevent diet-induced atherosclerosis in New Zealand White rabbits (Morehouse et al., 2007).

Up to this point, the reasoning behind the torcetrapib trials seemed sound. Yet the resultant big clinical trial led to an excess of 34 deaths in the torcetrapib group. Therefore a critical reappraisal is not only warranted but desperately needed to do justice to the patients who had to die in the context of drug development.

There were two major tracks of reasoning to explain the failure of torcetrapib that should be clearly separated, especially in regard to translational issues:

1. The drug did something else than just CETP inhibition, and this is the cause of failure—the so-called off-target effects of the drug.
2. CETP inhibition, as such, is unexpectedly harmful or at least not beneficial to humans.

The main off-target effect of torcetrapib was an elevation of blood pressure that amounted to 5.4 mmHg systolic in the ILLUSTRATE trial. CETP inhibition is not thought to be causal in this regard, as patients with genetic CETP deficiency do not show elevated blood pressure (Tall et al., 2007). In between, other congener compounds, for example, dalcetrapib, showed that this effect is not inherent to CETP inhibition (Schwartz et al., 2012).

Where could translational processes have intervened with regard to blood pressure? This effect was not apparent in animal experiments, as far as data in the public domain are concerned. It is not rare that side effects cannot be detected in animals, and translational toxicology is a major area that urgently needs substantial development. However, there will be gaps in our grasp of side effects, and surprises will be inevitable. In the torcetrapib case, one wonders how such an essential effect on blood pressure could have been overlooked or misinterpreted in the early human trials. In 2006 two similar trials were published (Davidson et al., 2006; McKenney et al., 2006) in which blood pressure effects were inconsistent. In the first trial, blood pressure values (systolic or diastolic, mmHg, group size around 30 patients) were 123.0/78.4, 118.7/76.6, 119.0/78.0, 120.0/77.6, and 120.4/76.5 for the placebo and featured 10, 30, 60, and 90 mg/day of torcetrapib. Corresponding figures for the second trial were 118.5/77.7, 120.7/76.9, 117.1/75.1, 119.4/79.4, and 121.4/78.8. At first glance, one would not suspect a substantial effect on blood pressure, with the second trial showing a mean increase of 2.9 mmHg systolic at the

highest dose. Apparently, the increase in blood pressure in the pivotal trial, the ILLUSTRATE trial, was much larger than that in phase II trials (Tall et al., 2007). Obviously, no plausible explanation for this discrepancy can be given. Small sample sizes with considerable scattering (as is visible in these two examples of blood pressure readings) may have contributed to the failure to detect an effect on blood pressure, but blood pressure may not have been a major readout, and its acquisition could have been less accurate than in the larger ILLUSTRATE trial. However, this remains speculative, although the description of blood pressure measurements is explicit in the ILLUSTRATE trial (Barter et al., 2007), whereas it is not mentioned in the phase II trials. This may indicate, although it does not prove, that blood pressure reading was not very standardized in these trials. In addition, patient populations were different, as were concomitant medications. From the translational perspective it might have been better to have looked at the very important, surrogate-type biomarker blood pressure in a more detailed way in the earlier trials. The significant blood pressure increase in ILLUSTRATE was unforeseeable on the basis of the data that had been obtained prior to this trial. The only translational shortcoming at this point may have been the undervaluation of this surrogate in the early trials, and drug development researchers should take this into account as a translational learning experience. Thus a rise in blood pressure that, at an average of 2 mmHg systolic, has been considered insignificant could still indicate a rise of 5 mmHg in the pivotal trial, and bad luck seems to have played a partial role in this context. On the other hand, there is a tendency to suppress unfavorable effects such as blood pressure increases if they are not dramatic enough to be unavoidably seen even in small trials. As calculated by Nissen et al. (2007), it is highly unlikely that the negative impact of the increase in blood pressure of systolic 5.4 mmHg not only balances a putative beneficial effect of CETP inhibition, but even outweighs it and causes an increase in mortality. Therefore there was good reason to assume that CETP inhibition per se may not keep up with the promises generated by in vitro and animal in vivo data, although it excessively increases HDL cholesterol levels in humans: 60 mg/day of torcetrapib raised HDL levels by 72% in the ILLUSTRATE trial (15,000 patients total, treatment for 12 months). Here is the true translational problem. The data summarized previously have clearly demonstrated that low HDL is associated with high cardiovascular risk and vice versa, but there has been comparably little evidence that interventional changes of this biomarker are associated with beneficial risk changes. According to the scientific systematic approach, HDL cholesterol so far has not been considered a risk factor, only a risk marker (see the previous discussion). One of the pitfalls were

those earlier positive data on nicotinic acid that could not be confirmed in a more recent trial (HPS2-THRIVE Collaborative Group et al., 2014).

In other words, HDL concentration had been established as a biomarker that seemed to be associated with disease but lacked the major components of being a surrogate, such as LDL cholesterol, in that interventional data have been sparse and as yet inconclusive. Thus it was not even close to qualifying as a surrogate marker and would score below 40 in the biomarker predictivity score described in Chapter 12. In major recommendations on cardiovascular prevention (e.g., in the 2019 ESC/EAS Guidelines for the Management of Dyslipidaemias; Mach et al., 2020), HDL cholesterol is still seen as a risk marker but not as a treatment goal. This formalistic approach is supported by several facts that need to be considered: HDL cholesterol is a major component of reverse cholesterol transport, which describes the removal of cholesterol from, for example, plaque macrophages through HDL down the fecal route. CETP inhibition blocks HDL cholesterol ester transport, and fractional HDL cholesterol clearance is diverted to the liver, but total clearance remains constant. Thus cholesterol excretion in feces is not increased. Whether HDL increases resulting from this mechanism are equivalent to those resulting from exercise or niacin is questionable. CETP inhibition may alter the important partial functions of HDL, namely, antioxidant and antithrombotic activities, which are known beneficial properties of “native” HDL. Obviously, HDL does not equal HDL, and HDL may become dysfunctional under CETP inhibition (Nissen et al., 2007). The situation would be different if the turnover of HDL is stimulated and serum levels are elevated or if HDL degradation is slowed and turnover is even reduced thereby. Further important doubts about CETP inhibition as a beneficial intervention are derived from human genetic data. In humans who are genetically deficient in CETP, no longevity is described (Hirano et al., 1997). In more recent evaluations, HDL particle size and oxidation status are seen to be more important than its plasma concentration (Wilkins and Seckler, 2019).

Which translational paradigm had been neglected in the translational process? The assessment of HDL cholesterol concentration as a biomarker was too positive. The truth that its interventional record was not nearly as good as that of LDL cholesterol was ignored. Even before statins had been introduced, the translational predictive value of LDL had been established beyond mere epidemiological evidence; this value further increased dramatically in light of all the positive interventional trials with statins, leading to LDL's status as a surrogate. It was highly speculative to assume that any intervention that raised HDL would be beneficial because this had been shown in only a few previous trials that, however, were confounded

by the fact that the agents that were tested (niacin and fibrates) affect not only HDL but also LDL, and a separation of the contribution of the two effects is difficult. In this situation it should have been accepted that this biomarker, as it was determined at that time (just concentration), did not warrant a pivotal mortality trial. At this stage, more reliable biomarkers should have been tested prior to the pivotal mortality trial in much smaller trials, putting fewer patients at risk. Imaging techniques—including intravascular ultrasound or magnetic resonance imaging techniques in groups of 100–200 patients—should have been applied in phase II trials to get enough evidence for atherosclerosis improvement to support a large mortality trial, a view that is largely shared by [Nissen et al. \(2007\)](#).

At present, there is enormous competitive and economical pressure in pharmaceutical companies, which may lead to “missed” phase II trials. These phase II trials—if carefully designed—would be essential for successful translation, and in the given example, powerful biomarkers for the proof-of-principle stage are available. For torcetrapib, they were either missed or explored in parallel to the

mortality trial. The sequential character of biomarker-driven intermediaries in novel areas without established surrogates needs to be reinstated to protect both patients and companies. If pharmaceutical companies continue to lose big bets such as this one—especially those whose risk could have been significantly reduced by 1–2 years of preparatory clinical trials—economic damage and public judgment of moral incompetence are inevitable.

Even worse, similar drugs have gone through the same process and little has been learned from the failed example. The results of the failed torcetrapib development should have been reflected in the development of congener compounds, such as dalcetrapib by Roche. If the translatability scoring instrument described in Chapter 11, Target Profiling in Terms of Translatability and Early Translation Planning, had been applied to assess translational risk at a fictive date before commencement of the pivotal phase III trials, torcetrapib would have been given a comparably low score (=high translational risk) of 1.95 compared to 4.14 for varenicline. The individual score items are shown in [Table 33.1A](#). For comparison the score is also shown for dalcetrapib, which, after the

TABLE 33.1 Translatability scores^a derived from specified items for torcetrapib, varenicline (part A),^b and dalcetrapib (part B).

Part (A)

	Torcetrapib		Varenicline	
	Score 1–5	\sum (score \times weight/100)	Score 1–5	\sum (score \times weight/100)
<i>Starting evidence</i>				
In vitro, including animal genetics	5	0.1	5	0.1
In vivo, including animal genetics (e.g., knock-out, overexpression models)	5	0.15	5	0.15
Animal disease model	4	0.12	5	0.15
Multiple species	1	0.03	5	0.15
<i>Human evidence</i>				
Genetics	1	0.05	1	0.05
Model compounds			5	0.65
Clinical trials	2	0.26	4	0.52
<i>Biomarkers for efficacy/safety prediction</i>				
Biomarker grading	2	0.48	5	1.2
Biomarker development	2	0.26	4	0.52
<i>Concept for proof-of-mechanism, proof-of-principle, and proof-of-concept testing</i>				
Biomarker strategy	2	0.1	5	0.25
Surrogate/endpoint strategy	2	0.16	4	0.32

(Continued)

TABLE 33.1 (Continued)**Part (A)**

	Torcetrapib		Varenicline	
	Score 1–5	$\sum (\text{score} \times \text{weight}/100)$	Score 1–5	$\sum (\text{score} \times \text{weight}/100)$
<i>Personalized medicine aspects</i>				
Disease classification, Responder concentration	3	0.09	1	0.03
Pharmacogenomics	3	0.15	1	0.05
<i>Sum</i>		1.95		4.14

Part (B)

	Dalcetrapib	
	Score 1–5	$\sum (\text{score} \times \text{weight}/100)$
<i>Starting evidence</i>		
In vitro, including animal genetics	5	0.1
In vivo, including animal genetics (e.g., knock-out, overexpression models)	5	0.15
Animal disease model	4	0.12
Multiple species	1	0.03
<i>Human evidence</i>		
Genetics	1	0.05
<i>Model compounds</i>		
Clinical trials	2	0
<i>Biomarkers for efficacy/safety prediction</i>		
Biomarker grading	2	0
Biomarker development	2	0.26
<i>Concept for proof-of-mechanism, proof-of-principle, and proof-of-concept testing</i>		
Biomarker strategy	2	0.1
Surrogate/endpoint strategy	2	0.16
<i>Personalized medicine aspects</i>		
Disease classification, Responder concentration	3	0.09
Pharmacogenomics	3	0.15
<i>Sum</i>		1.06

^aWehling (2009); Wendler and Wehling (2012).^bData in part (A) from Wehling M. Drug development in the light of translational science: shine or shade? *Drug. Discov. Today* 16 (2011) 1076–1083 with kind permission.

completion of the ILLUSTRATE trial, is even much lower (1.06) than that for torcetrapib, indicating the highest possible level of translational risk (Table 33.1B).

Coming back to the principles of translatability scoring, the score was already low and the risk high for torcetrapib. After the excess mortality in the ILLUMINATE trial and the insufficient explanation of beneficial effects

as being overcompensated by off-target effects, a congener based on the same principle had an even greater likelihood of failure. The score of 1.06 for dalcetrapib should have been seen as a stopper for consequent development—in particular for the initiation of the expensive phase III trial. This almost lowest possible score mostly reflected the devalidation of HDL concentration as a risk factor and

therapeutic target in the ILLUMINATE trial. The related items in the biomarker segment of the score panel were seen to be zero, reflecting this fact. Zero values should always be discussed as stoppers of a program in its present form. With this information, further investments should have been made in the development of prospectively useful biomarkers rather than a phase III trial. Coming as no surprise, Roche had to stop the development of dalcetrapib after the phase III trial (Schwartz et al., 2012) failed to show any beneficial effect of the drug on hard cardiovascular endpoints. However, the trial proved that the excess mortality seen with torcetrapib was likely to reflect only off-target effects of that particular compound.

Even that second failed CETP inhibitor did not prevent companies from developing further congeners. Anacetrapib by Merck had a small cardioprotective effect in a large clinical trial (HPS3/TIMI55-REVEAL Collaborative Group et al., 2017). However, the company stopped its further development, perhaps because a small effect on LDL cholesterol may have contributed to the very modest overall effect; therefore the arguments to convince health insurers to pay a decent prize were seen to be weak. The failure of yet another CETP inhibitor, evacetrapib (Lincoff et al., 2017; Menon et al., 2020), in high-risk cardiovascular patients, including diabetic patients, should stop this approach forever.

In summary, a very important, highly predictive biomarker with surrogate status (blood pressure) was undervalued, and a weak biomarker (HDL cholesterol) was not properly developed and originally was rated too high with no subsequent reflection of its further devalidation during the course of the development of CETP inhibitors.

This example shows the importance of appropriate evaluation of the predictive value of a given biomarker. It underpins the dramatic need for a systematic approach to assessing biomarkers objectively to avoid billions of wasted dollars but also to avoid unjustified exposure of patients to the inevitable risks of clinical trials. HDL functionality and particle size are now major biomarker development areas, and some time from now, a particular fraction or property of HDL may become a successful biomarker, leading to the development of novel therapies that will greatly benefit patients. It is certain that CETP inhibitors will not be among them.

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Note: Page numbers followed by “*f*,” “*t*,” and “*b*” refer to figures, tables, and boxes, respectively.

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