REVIEW ARTICLE

Biomarkers in Lung Cancer Screening: Achievements, Promises, and Challenges

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Received 13 September 2018; revised 20 November 2018; accepted 26 November 2018
Available online - 4 December 2018

ABSTRACT

The present review is an update of the research and development efforts regarding the use of molecular biomarkers in the lung cancer screening setting. The two main unmet clinical needs, namely, the refinement of risk to improve the selection of individuals undergoing screening and the characterization of undetermined nodules found during the computed tomography–based screening process are the object of the biomarkers described in the present review. We first propose some principles to optimize lung cancer biomarker discovery projects. Then, we summarize the discovery and developmental status of currently promising molecular candidates, such as autoantibodies, complement fragments, microRNAs, circulating tumor DNA, DNA methylation, blood protein profiling, or RNA airway or nasal signatures. We also mention other emerging biomarkers or new technologies to follow, such as exhaled breath biomarkers, metabolomics, sputum cell imaging, genetic predisposition studies, and the integration of next-generation sequencing into study of circulating DNA. We also underline the importance of integrating different

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Disclosure: Dr. Peled is an advisor for and has received honoraria from AstraZemeca, Bohringer Ingelheim, Bristol-Myers Squibb, Eli Lilly, Merck Sharp and Dohme, Novartis, Pfizer, Roche, NovellusDx, Foundation Medicine, Inc., and Gaurdant360; in addition, he is a coinventor in breath analysis in lung cancer. Dr. Ajona, Dr. Pio, and Dr. Montuenga are listed as coinventors of two patents related to the use of complement fragments as biomarkers. Dr. Sozzi and Dr. Boeri are coinventors of three patent applications regarding the microRNA signature MSC. These patents were licensed to a private company, Gensignia Life Science, under regulations of Fondazione IRCCS Istituto Nazionale dei Tumori of Milan. Dr. Zulueta is an employee and shareholder of VisionGate, Inc. Dr. Spira is an employee of Johnson and Johnson and has provided prior consultant to Veracyte, Inc. Dr. Mazzone has participated in advisory board discussions for Exact Sciences, SeerPharma, and Grail in the past 12 months. Dr. Seijo has received professional fees for speaking engagements regarding lung cancer screening and received an unrestricted grant from Menarini in support of the Jimenez Díaz Foundation's lung cancer screening program. The remaining authors declare no conflict of interest.

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ISSN: 1556-0864
https://doi.org/10.1016/j.jtho.2018.11.023

molecular technologies together with imaging, radiomics, and artificial intelligence. We list a number of completed, ongoing, or planned trials to show the clinical utility of molecular biomarkers. Finally, we comment on future research challenges in the field of biomarkers in the context of lung cancer screening and propose a design of a trial to test the clinical utility of one or several candidate biomarkers.

Keywords: Biomarkers; Lung cancer; Screening; Test validation; Clinical utility; Trial design

Lung Cancer Screening Challenges: Is Imaging Sufficient for Successful Screening? The Unmet Needs

Current evidence supports lung cancer screening of subjects who fit the National Cancer Institute–sponsored National Lung Screening Trial (NLST) criteria. The NLST compared annual low-dose computed tomography (LDCT) screening with conventional chest radiography and found that LDCT screening achieved a 20% reduction in lung cancer mortality after only three rounds of screening.[1] Prospective data accrued by the International Early Lung Cancer Action Program from thousands of screening rounds also support annual screening with LDCT,[2] whereas data from the European NELSON trial, a Dutch-Belgian initiative, are also favorable toward screening.[3] Results from the NELSON trial that were recently reported at the International Association for the Study of Lung Cancer meeting confirm the findings from the landmark NLST, showing that use of LDCT as a screening tool is effective, leading to a 26% (95% confidence interval: 9%–41%) reduction in lung cancer deaths in asymptomatic men at high risk for lung cancer after 10 years of follow-up. The reduction in mortality was even more impressive in women (OR for death = 0.39–0.61). The overall rate of adherence in that study, which enrolled almost 16,000 individuals, was 86%.[4] Consequently, recommendations from prominent scientific societies support screening with LDCT despite concerns regarding false-positive findings, risk of overdiagnosis, logistical challenges, and differences in selection criteria.[5–8] Chief among these concerns is the widespread adoption of the NLST age and tobacco exposure (≥30 pack-years) inclusion criteria by screening guidelines. Current evidence suggests that such criteria may preclude screening of many individuals who are at risk.[9–13] It is becoming clearer that a more sophisticated risk-based strategy, taking emphysema into account, for example, may be better than the current NLST criteria.[9,14–19]

The advantage of the LDCT-based protocol is its simplicity and its high sensitivity. Refined criteria defining positive findings that are largely based on nodule size and/or volume reduce false-positive rates. That notwithstanding, there is a need for evidence-based biomarkers to support pretest and posttest (LDCT) risk assessment.[20] Ideally, robust biomarkers would optimize image-based screening in two ways. First, they would allow further refinement of screening selection criteria, independent of age and tobacco exposure, to limit the costs of lung cancer screening. This risk management biomarker strategy would be a welcome addition to current screening practice. For example, a number of single-nucleotide polymorphisms have been proposed in this regard as potential biomarkers of constitutive genomic risk for a given individual (see the section Genetic Predisposition to Lung Cancer). Such biomarkers are the focus of ongoing research when integrated with current clinicoepidemiological risk models for lung cancer.[21] Second, a validated panel of biomarkers may provide a posttest risk assessment capable of informing clinical decision making in the management of indeterminate pulmonary nodules (IPNs). Current management of IPNs is largely based on watchful waiting and may imply a risk of dissemination. Nodules found on annual screening, which are often so small that they are out of reach of current biopsy techniques, may benefit from a biomarker-based risk assessment. In particular, biomarkers may be helpful in the case of patients with nodules that need closer surveillance or a decision regarding biopsy. Patients with multiple nodules or those subject to frequent interval scans during screening might also benefit. Finally, biomarkers might also inform decisions regarding screening intervals, personalized follow-up of survivors of screen-detected early-stage lung cancer, outcome prediction, or response to adjuvant therapy for those at high-risk of recurrence. In the present review we will refer only to the biomarkers intended for the first two unmet needs (risk management and IPN characterization). Other recent articles have dealt with early prognostic biomarkers of lung cancer.[22,23] In the current review, we will focus initially on biomarkers that are noninvasive, reproducible, and validated and conclude with other promising technologies that are being developed in the context of early detection.

What Is a Good Biomarker?

The National Institutes of Health define a biomarker 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."[24] A useful biomarker must influence clinical decision making in a manner that leads to improved patient care. The benefit of clinical decisions based on true test results must outweigh the harm of decisions based on false positives or false negatives. In the risk management setting, a biomarker should minimize harm and expense without leading to an increase in lung cancer deaths. When used for IPN characterization, a biomarker should anticipate the diagnosis of malignancy without substantially increasing the number of diagnostic procedures performed for benign nodules or delaying therapeutic procedures for malignant nodules.[25]

Optimizing Discovery of Lung Cancer Biomarkers

We believe that validation of a clinically useful biomarker should adhere to the following principles, which are summarized in Table 1.

Study Design. Much is gained from the careful selection of the molecular approach chosen and should be guided by the intended use, where the biomarker would find potential clinical utility.[26] All too often investigators focus on versatility, seeking biomarkers that address multiple clinical needs such as risk assessment, diagnosis, or response to therapy. Much merit lies in limiting scope by addressing specific clinical needs.

Biomarker Stability. Information about the stability of the analyte over time, including changes in temperature, pH, and enzymatic or oxidative stress is critical.[27]

Analytical Validation. Biomarker measurements should follow a well-defined strategy, and they should be accurate, precise, and robust. Validation should include testing of reproducibility against larger sources of variability such as biospecimen collection (e.g., sample processing, freeze-thaw cycles, duration of storage, etc.), operator characteristics, laboratory environment, and quality control (standard curves, standard operating procedures). Some variability is inherent to the technology itself (energy source, enzymatic activity, and temperature control).[28] Metrics of success include coefficient of variance, z statistic, limits of detection, and quantitation.

Clinical Validation. The ideal diagnostic biomarker is both sensitive and specific, with diagnostic likelihood ratios independent of known predictors of the disease (e.g., age, smoking history, or chronic obstructive pulmonary disease [COPD]). Validation should be performed in the clinical context of intended use. Case-control studies are discouraged; prospective cohort studies and observational registries are preferable. The biomarker will be tested in multiple cohorts with similar prevalence of disease. Biomarkers rarely perform well across a large range of disease prevalences, and their performance characteristics are often susceptible to changes in simple variables such as age or disease stage.

Clinical Utility. The biomarker should be tested for clinical utility in larger studies in a pragmatic setting that does not disrupt the clinical workflow. The goal of any biomarker is to achieve superior performance compared with standard of care–based approaches and eventually reduce the cost and harm of testing while limiting false-negative rates.[25] Study design is challenging owing to randomization and the need to affect clinical management (see in the section Future Research Challenges for a potential trial design). Ultimately, implementation of the biomarker in routine practice will determine its true value for clinical decision making.

The Metrics of Success

Biomarker performance and accuracy are dependent on the intended use and current alternatives. A successful biomarker must supersede the current standard of care,[27,29,30] be cost-effective, be welcomed by the community, and eventually demonstrate cancer control if early detection is the goal (Fig. 1) or promote personalized medicine by identifying candidates for targeted therapies. Understanding traditional metrics of success in this context is key. Sensitivity and specificity, for example, are often unstable over multiple variables such as age or

Table 1. Principles to Optimize the Research on Development of Lung Cancer Biomarkers

- Principle 1: Select the molecular approach guided by the intended use
- Principle 2: Look for stable analytes that are minimally dependent on storage time, temperature, pH, and enzymatic or oxidative stress
- Principle 3: The analyte should be measured with accuracy, precision, and robustness. Thoroughly test for reproducibility across sources of variability, laboratories, conditions, etc.
- Principle 4: Test the biomarker in multiple cohorts in the clinical context in which it will be considered for use (screening, nodule management). Case-control studies are discouraged, whereas prospective cohort studies and, eventually, observational registries are favored though less convenient
- Principle 5: Conduct the tests in larger cohorts to demonstrate superiority over standard of care, reduction of cost, and reduction of false-positive and false-negative rates

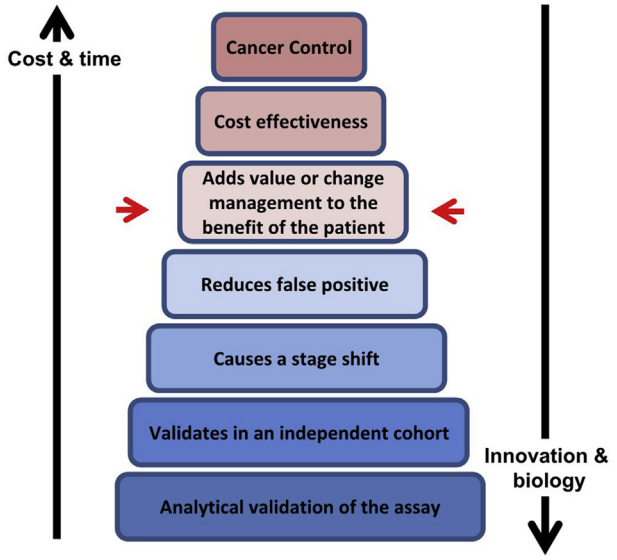

Figure 1. Criteria for clinical use of biomarkers.

disease stage. Positive predictive values and negative predictive values (NPVs) are dependent on the prevalence of disease. Receiver operating characteristic curves (i.e., true positive rates versus false-positive rates) are helpful, but they complicate decision making because of the need for dichotomous biomarker cutoff values. Reclassification indices have a role in testing a biomarker's ability to accurately reclassify cases and controls and therefore influence clinical decision making.

Current Promising Molecular Biomarkers

Molecular Biomarkers for Lung Cancer Screening

Blood is an obvious first choice as the source of biomarker candidates for lung cancer screening. Blood-based biomarkers provide an overview of the whole patient body, including the primary tumor, metastatic disease, immune response, and peritumoral stroma. However, sputum, bronchial lavage or aspirate samples, exhaled breath (EB), and airway epithelium sampling are unique to lung and other respiratory tract cancers as potential sources of alternative biomarkers. These may provide information regarding molecular changes that may be anatomically closer to the tumor cells and their microenvironment and therefore potentially more relevant to clinical decision making in screened patients with early-stage disease (Fig. 2). Urine and saliva have also been collected as potential sources of biomarkers. The former is particularly useful in a metabolomics-based approach.

A concise review of the most prominent molecular biomarkers for lung cancer screening includes examples of molecular candidates for both risk management and IPN characterization in diverse stages of validation. We have included those that we consider the most promising. We are well aware of the risk of omitting potential candidates. Figure 3 includes a list of biomarkers that have reached different levels of validation.

AAbs. Autoantibodies (AAbs) develop in response to an abnormal tumor antigen in some patients with lung cancer, often in the preclinical phase well before symptoms appear or imaging-based detection is possible. AAbs have been identified in all histological types and stages of lung cancer. They are usually absent or found in low titers not only in those without cancer but also in many patients with the disease. AAb panels are therefore likely to be specific but not sensitive. Use of a well-validated AAb panel has been studied in different screening cohorts as an approach to management of lung cancer risk.[31–38] In a clinical validation study including all lung cancer histological types and stages the panel performed well, with 93% specificity but only 40% sensitivity.[39] Similarly, a practice audit of 1699 patients (61 with lung cancer and one-third in stage I) found that the panel had high specificity (91%) but low sensitivity (37%).[35] AAbs may find a place in clinical practice by improving the overall test accuracy of hybrid panels featuring diverse biomarkers.[40]

Complement Fragments. Lung cancer can activate the complement cascade by the classical complement pathway.[41] Concentrations of a downstream split product of this pathway, C4d, are increased in biological fluids from patients with lung cancer. Plasma C4d levels have been linked to increased lung cancer risk in a cohort of 190 asymptomatic individuals, including 32 patients with screening-detected cancer who were enrolled in the International Early Lung Cancer Action Program cohort (OR = 4.38; 95% CI = 1.61–11.93). In that study, potential confounders such as emphysema and COPD did not appear to affect C4d plasma levels.[41] Unfortunately, its use as a marker for the selection of high-risk individuals could not be validated by using samples from the MILD computed tomography (CT) screening trial.[42] Nevertheless, the results on its use for the management of IPNs are more promising. Plasma samples from patients from two independent cohorts with malignant nodules presented significantly higher levels of C4d than did those from patients with benign nodules. In selected patients with intermediate-sized pulmonary nodules (8–30 mm), C4d plasma levels identified benign lung nodules with an 84% NPV and a 54% positive predictive value. Once again, the test enjoyed high specificity (89%) at the expense of low sensitivity (44%).[42]

miRNAs. Circulating microRNAs (miRNAs) reflecting tumor-host interactions have emerged as potential biomarkers for cancer diagnosis and prognosis irrespective of tumor stage and mutational bourden.[43] The role of miRNA-based liquid biopsies has been assessed in the

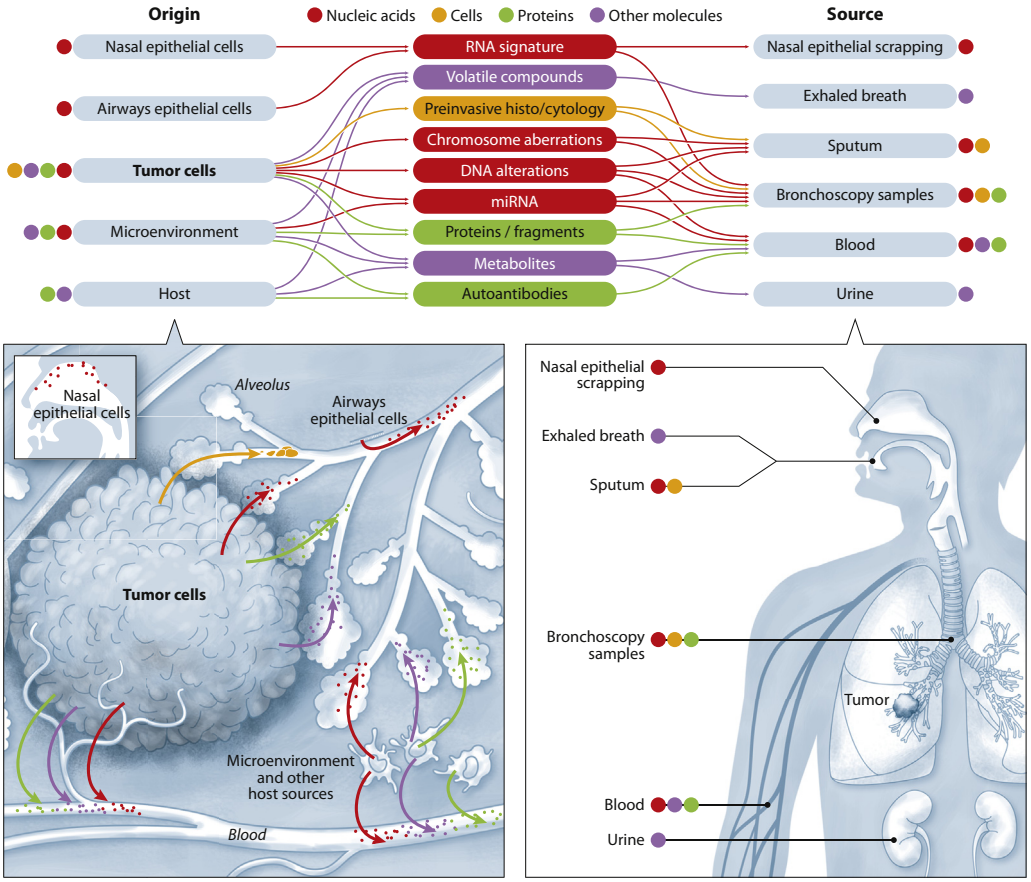

Figure 2. Currently explored biomarker candidates. miRNA, microRNA.

context of screening with LDCT in two large Italian retrospective validation studies.[44,45] Use of the miRNA signature classifier (MSC) and the miR-Test resulted in fivefold and fourfold reductions in the LDCT false-positive rate, respectively, with comparable specificity (81%–75%) and sensitivity (87%–78%). In postsurgical plasma samples, the MSC showed good performance in monitoring disease relapse.[46] The two tests are now undergoing prospective validation in three independent screening trials enrolling a total of 16,000 high-risk subjects.

ctDNA. The value of circulating tumor DNA (ctDNA) as a biomarker in advanced tumor stages is well established.[47,48] However, its role in early lung cancer detection is still uncertain.[49] Abbosh et al. reported 48% overall sensitivity, setting a threshold of two single-nucleotide variants in 96 patients with stage I to III NSCLC. Sensitivity ranged from 15% for stage I adenocarcinomas to 100% for stage II or III squamous cell carcinomas.[50] Current efforts to develop next-generation sequencing (NGS) technologies to study ctDNA in the context of early detection may improve sensitivity in this context. A recently reported test for pan-cancer early detection combined the NGS analysis of ctDNA in blood with a large panel of protein biomarkers in 1005 patients with stage I to III pan-cancer and 812 cancer-free controls.[51] Although specificity was higher than 99%, sensitivity ranged from 33% for breast cancer to 98% for ovarian cancer. The sensitivity for lung cancer was 59% in 104 patients.

DNA Methylation. Tumor tissue is characterized by a global DNA hypomethylation status together with hypermethylation of specific CpG islands in the promoter region of tumor suppressor genes.[52] Hypermethylation of at least one of four studied genes was detected 20 years ago in 15 of 22 NSCLC tumors (68%) but not in any paired normal lung tissue sample. In these primary tumors with methylation, 11 of 15 samples (73%) also had abnormal methylated DNA in the matched serum sample.[53] More recently, a 64-gene panel quantitative polymerase chain reaction assay was used to study 204 serum samples from 33 patients with lung cancer, 68 patients with fibrotic interstitial lung disease, 42 patients with COPD, and 61 healthy controls. The test had 88% sensitivity and 90% specificity when compared with controls, and 88% specificity when compared with patients with COPD and interstitial lung disease.[54] In 2017, Ooki et al. reported that a six-gene panel correctly

Candidates	Biomarker	Target	Phase 1 Discovery, prediction	Phase 2 Assay validation	Phase 3 Retrolongitudinal	Phase 4 Clinical validation*	Phase 5 Clinical utility	References	Trial
SERUM/PLASMA									
Specific proteins/autoantibodies	Three proteins (CEA, CA-125, and CYFRA 21–1) and 1 AAb (NY-ESO-1)	RMS						40	
	Two proteins (LG3BP and C163A) and clinical features	DIPN						59	NCT01752114
	Seven AAbs (p53, NY-ESO-1, CAGE, GBU4-5, SOX2, HuD, and MAGE A4)	RMS						33,34	NCT01700257
		DIPN						38	
	Six proteins (CEA, CA-125, SCC, CYFRA 21–1, NSE, and proGRP)	DIPN						116	
	Complement fragment C4d	RMS						41	
		DIPN						42	
MiRNA	Ratios among 24 miRNAs	RMS						44, 117	NCT02247453
		DIPN							
	Signature of 13 microRNA + 6 for normalization	RMS						69, 118, 119	COSMOS II trial
		DIPN							
	Signature of 2 microRNA	DIPN						120	
DNA methylation	SOX2 and PTGER4 methylation	DIPN						121	
Circulating tumor nucleic acids	Circulating tumor DNA; NGS technology	RMS						113	NCT02889978
	Circulating tumor DNA; NGS technology	DIPN						122	
	Circulating tumor DNA; Ion Torrent DNA Sequencing technology	DIPN						123	
	Circulating tumor DNA; TEC-Seq technology	RMS						124	
	Signature of 29 genes (RNA)	DIPN						125	
	ctDNA mutation and proteins (CA-125, CEA, CA19-9, PRL, HGF, OPN, MPO, and TIMP-1)	DIPN						51	
TUMOR/AIRWAY EPITHELIUM									
Chromosome aberrations	Chromosome regions copy number or fusions (FISH)	DIPN						126	
mRNA gene expression classifier	Twenty three gene classifier	DIPN							NCT01309087 NCT00746759
SNPs	20 SNPs for COPD and clinical features	RMS						127	
SPUTUM, BREATH AND URINE									
DNA methylation	SHOX2 and RASSF1A methylation	RMS						128	
MiRNA	Signature of 3 microRNA	DIPN						129	
Exhaled breath	VOC- Nanoparticle Biometric Tagging (NBT)	DIPN							
	VOC- Field Asymmetric Ion Mobility Spectrometry (FAIMS)								NCT02612532
Tumor cells	>700 morphological features (by Cell CT)	RMS							
		DIPN							
	Buccal nanocytology	RMS						130	
	Porphyrin differential uptake by tumor cells	RMS						131	
Unrine markers	Metabolites	RMS						84	

RMS: risk management in screening context; DIPN: diagnosis of indeterminate pulmonary nodules; *DECAMP-1 and DECAMP-2 trials (NCT01785342 and NCT02504697) are currently recruiting patients in order to test some of these biomarkersAAB Autoantibody.

Figure 3. Candidate biomarkers for lung cancer early detection and phase of development. AAb, autoantibody; CA-125, cancer antigen 125; CAGE, cancer-associated antigen gene; CEA, carcinoembryonic antigen; COPD, chronic obstructive pulmonary disease; CT, computed tomography; CYFRA, cytokeratin fragment; DIPN, diagnosis of indeterminate pulmonary nodules; FISH, fluorescence in situ hybridization; HGF, hepatocyte growth factor; HUD Hu antigen D, embryonic lLethal, abnormal vision, drosophila-like protein 4; LG3BP, galectin-3 recombinant protein; MAGE, melanoma-associated antigen; MIRNA, microRNA; MPO, myeloperoxidase; NBT, nanoparticle biometric tagging; NGS, next-generation sequencing; NSE, neuron-specific enolase; NY-ESO-1, New York esophageal squamous cell carcinoma 1; OPN, osteopontin; PRL, prolactin; PROGRP, progastrin-releasing peptide; PTGER4, prostaglandin E receptor 4; RASSF1A, an isoform of Ras association domain family member 1; RIMS, risk management screening context; SHOX2, short stature homeobox 2; SNP, single-nucleotide polymorphism; SOX2, SRY-box 2; TEC-Seq, targeted error correction sequencing; TIMP1, TIMP metallopeptidase inhibitor 1; VOC, volatile organic compound.

classified 43 stage IA and 42 control subjects with 72% sensitivity and 71% specificity.[55] Hulbert et al. recently described a three-gene model discriminating subjects with suspicious nodules on CT imaging (150 subjects with stage I or II NSCLC and 60 controls) with 98% sensitivity and 71% specificity in sputum and 93% sensitivity and 62% specificity in plasma samples.[56]

Blood Protein Profiling. Many studies have identified measurable serum antigens in patients with lung cancer. Panels of serum cancer antigens have been developed to improve diagnostic accuracy. One panel of three serum proteins (carcinoembryonic antigen, cancer antigen 125, cytokeratin fragment 21-1) and an AAb (New York esophageal squamous cell carcinoma 1) performed well in a high-risk cohort, with 71% sensitivity and 88% specificity for lung cancer.[40] Clinical validation was performed in a separate high-risk cohort (based on age and smoking history) with lower sensitivity (49%) but higher specificity (96%). The incorporation of clinical variables improved accuracy.[57] A different panel of cancer antigens (carcinoembryonic antigen, cancer antigen 15.3, squamous cell carcinoma, cytokeratin fragment 21-1, neuron-specific enolase, and progastrin-releasing peptide) increased the area under the curve of a clinical prediction model based on

nodule size, age, and smoking status from 0.85 to 0.93 in a series of 3144 symptomatic individuals, including 1828 with lung cancer (52% stage IV).[58] A two-protein biomarker ratio combined with a lung nodule clinical risk predictor had a sensitivity of 97% and a specificity of 44% for malignant disease in a series of 178 patients with suspicious lung nodules. The biomarker's performance was evaluated for nodules that had a calculated pretest risk of malignancy of 50% or less. This integrated classifier could have led to a 40% relative reduction in invasive testing for patients with benign nodules (10% absolute risk reduction) while potentially delaying the management of 3% of malignant nodules.[59]

RNA Airway and Nasal Signature. On the basis of the "field of injury" paradigm,[60] airway epithelial gene expression has been developed as a diagnostic biomarker for lung cancer. The initial studies focused on bronchial airway epithelial cells obtained by endobronchial brushings of the mainstem bronchus.[61,62] A 23-gene biomarker measured in bronchial epithelial cells has been tested as an adjuvant diagnostic biomarker for patients undergoing bronchoscopy for suspected lung cancer.[63] This biomarker underwent clinical validation in two independent prospective cohorts, demonstrating a sensitivity of 88% to 89% and a specificity of 48%. The biomarker was particularly helpful in patients with an intermediate (10%–60%) pretest risk of lung cancer (91% NPV). Patients with inconclusive bronchoscopy results could have benefited from the biomarker's negative predictive value by avoiding further invasive testing, suggesting such patients could be followed safely with serial imaging studies.[30] After analytic validation[64] and other clinical studies,[65,66] the test received a favorable Medicare coverage decision in 2017.

The same field of injury concept may be useful in samples of nasal epithelial cells. This approach has obvious advantages as a minimally invasive diagnostic alternative for those not undergoing bronchoscopy as part of their clinical work-up. A 30-gene nasal expression panel has been developed for diagnosing lung cancer among ever-smokers with suspected disease, demonstrating improvement in area under the curve, sensitivity, and NPV when combined with clinical risk models.[67] This is preliminary evidence in need of supporting studies.

Current Trials in Which Biomarkers Are Considered or Included

Clinical validation study results have been published for a handful of biomarkers. Other biomarkers linger at various stages of development, whereas a few have entered formal clinical testing. The aforementioned panel of AAbs[31] is currently being assessed as part of a Scottish National Health Service–funded randomized controlled screening study enrolling 12,000 subjects (the ECLS study [NCT01925625]). A bronchial gene expression classifier that could improve the diagnostic performance of bronchoscopy is being tested in a large registry. The combination of the plasma MSC with LDCT results informs screening intervals in 4119 at-risk subjects in the bioMILD screening trial (NCT02247453).[68] Plasma samples that were prospectively collected during the COSMOS II screening trial have been profiled to set up and validate the clinical utility of the microRNA-signature based miR-Test.[69] The DECAMP-1 and DECAMP-2 prospective observational trials (NCT01785342 and NCT02504697) have been designed to examine a variety of existing biomarkers for lung cancer diagnosis as well as new biomarkers discovered specifically in this clinical setting. DECAMP-1 seeks to improve follow-up of patients with IPNs by determining whether analyzed biomarkers are able to distinguish incidentally detected malignant from benign pulmonary nodules in high-risk smokers, whereas DECAMP-2 will test biomarkers of risk in screened asymptomatic high-risk individuals.

An exciting amount of high-quality discovery and clinical validation work is ongoing. Some companies are in the process of planning true clinical utility studies for lung nodule management. The lack of an established trade-off regarding the consequences of true and false biomarker results is a challenge that every biomarker developer will face. It would behoove the clinical community to provide guidance regarding acceptable trade-offs in both the screening and lung nodule management settings.[25]

Emerging Biomarkers, New Technologies to Follow, and Future Directions. The Power of Integration

The aforementioned biomarkers have been the object of intense research, and a number of them are being assessed in a risk management strategy to recommend screening or aiming to characterize IPNs. We will now discuss promising new technologies with potential, including integrated approaches to biomarker development in lung cancer screening.

EB Biomarkers

There is growing evidence to support the use of EB, including EB condensate (EBC) for cancer detection. The use of EBC, which includes cells and DNA fragments, may even support detection of EGFR-resistant clones.[70] The

volatile fragments of EB are sensitive biomarkers of lung cancer. Volatile organic compounds can be captured and analyzed by a wide range of technologies, including gas chromatography/mass spectrometry, nanosensors, colorimetric sensors, and other methods.[71,72] An artificially intelligent nanoarray sensor has been used in the diagnosis and classification of 17 different diseases from breath samples of 1404 subjects, providing 86% accuracy. Some studies suggest that such an array may discriminate benign from malignant pulmonary nodules[73] or predict response to therapy and recurrence.[74] It may also distinguish histological type[75] or predict molecular analysis results.[76] Interestingly, it apparently may also discriminate between different types of cancer (lung, breast, colorectal, and prostate).[77]

Sputum Cell-Based Image Analysis

Although cytologic examination of sputum has traditionally failed to yield either adequate or useful samples for lung cancer screening, the advent of *enhanced cytology*, in which sophisticated image analysis algorithms are combined with artificial intelligence, may yet prove sputum useful in this context. A newly developed test can identify abnormal cells in sputum samples of screened patients.[78] This test may be used as a primary screening modality with a reported sensitivity of 90% when at least 800 bronchial cells are available for analysis, or it may be integrated with LDCT in the context of a conventional CT-based screening program for IPN characterization.[79] In the latter case, fewer cells may be needed because the clinician can integrate clinical, molecular, or conventional sputum cytologic data with imaging results for greater diagnostic accuracy.

Metabolomics

A range of different analytical platforms and methodologies have been applied to identify metabolic biomarkers of lung cancer.[80] Metabolomics provides a direct functional readout of the phenotypic changes associated with the development of lung tumors and their microenvironment. Metabolomics has several advantages when compared with other "omics," including a reduced number of metabolites and a wide range of biological samples that can be tested. Changes in lung cancer metabolites include those involved in glycolysis, the citric acid cycle, amino acid metabolism, and cell membrane synthesis.[80,81] Metabolomics can differentiate between histological subtypes or genetic backgrounds.[82,83] A panel of metabolites excreted in the urine, namely, creatine riboside and *N*-acetyl neuraminic acid, have been associated with lung cancer risk before clinically detectable disease.[84,85] Panels as well as individual markers in blood, sputum, or EBC have also been proposed to identify high-risk candidates for screening or to discriminate between benign and malignant IPNs.[86–91] Finally, other omics, such as microbiomics, are providing us with novel diagnostic markers that merit a closer look.[92]

Genetic Predisposition to Lung Cancer

The advent of genomewide association studies may provide the lung cancer community with strong evidence of genetic susceptibility genes, which may be included in lung cancer risk prediction models.[14,15,93] Current evidence from a major review in 2017 of more than 1000 candidate association studies identified 22 variants in 21 genes that had strong cumulative epidemiological evidence of significant associations with lung cancer risk.[16] The OncoArray Consortium research program[21] has provided recent new insights and a new set of susceptibility genes.[94] However, the potential contribution of this new set of genes to risk prediction models used in lung cancer screening trials has yet to be demonstrated.[95] Clinical applications aside, these susceptibility genes do provide an insight into the biological process and association with specific diseases, which are relevant to lung cancer etiology.[96,97] Clearly, there is a wealth of information captured within the current genome-wide association studies' data sets. Taking advantage of it will depend on state-of-the-art mathematical and statistical approaches capable of incorporating large numbers of single-nucleotide polymorphisms into risk models, artificial intelligence, and supervised machine learning approaches.[98,99]

Integration of Molecular Biomarkers with Radiomics and Artificial Intelligence

The current scientific field of *radiomics*, a term first used by Dutch researcher Philippe Lambin in 2012, is a newcomer in search of biomarkers among the seemingly limitless supply of data related to lung cancer imaging–based phenotypes and tumor microenvironment.[100,101] The accumulation of detailed imaging data in the current era of artificial intelligence has set the stage for much progress in this field. Deep learning architectures, for example, can be useful in characterization of lung nodules.[102,103] Current research in the field is centered on robust identification of the region of interest in time; direct spatiotemporal phenotypic characterization of tumor microenvironments; integration of multiscale information at the local (nodule), regional (lobe), and organ levels; and integration of imaging, clinical, and omics data in end-to-end learning architectures.

The combination of imaging-based deep learning with molecular biomarkers may be very powerful in the characterization of IPNs. Radiomics can identify *EGFR*- and *KRAS*-mutated tumors.[104,105] Imaging signatures based on quantitative analysis of imaging data can also predict survival.[106] Some studies have shown that the integration of plasma biomarkers and radiological characteristics is a better predictor of lung cancer in patients with IPNs.[107] Prediction models integrating serum biomarkers with clinical characteristics and radiographic features of suspicious nodules correctly identified malignant nodules in several studies.[29,108] The integrated models outperform the use of serum biomarkers alone and overall represent a very promising approach for the future of early lung cancer detection, especially if artificial intelligence is incorporated.[109–111]

Integration of Multiple NGS Analysis in ctDNA

We have already alluded to the use of NGS of ctDNA as a promising strategy for early lung cancer detection. The biggest technical challenge is sensitivity. In an attempt to overcome this limitation, a recently reported test for pan-cancer early detection combined the NGS analysis of ctDNA in blood with a large panel of protein biomarkers[51] (see the section ctDNA). Although promising, the study had some important limitations, including the fact that most patients with cancer were symptomatic and the fact that control subjects had no comorbidities that could have acted as confounding variables.

The scientific community is also awaiting results of the Circulating Cell-free Genome Atlas Study for early cancer detection, enrolling 15,000 participants (including cancer-free controls) in the United States and Canada. Plasma samples collected at baseline and during 5 years of follow-up will be analyzed by whole genome sequencing (WGS) for copy number variation (CNV), targeted DNA sequencing (a 507-gene panel), and whole genome methylome profiling. Preliminary results in an observational case-control setting include 95% specificity, high sensitivity for advanced lung cancer in 54 patients (85% for targeted sequencing, 91% for CNV WGS, and 93% for methylome profiling), and modest sensitivity for 63 patients with stage I to III lung cancer (48% for targeted NGS, 54% for CNV WGS, and 56% for methylome profiling).[112] The generalizability of these findings to the screening setting is uncertain. A recent review by Aravanis et al.[113] addressed the challenges that NGS faces in early cancer detection. The authors suggested that a successful pan-cancer screening NGS-based blood test would have to test up to 1000 genes, and the ctDNA limit of detection would have to improve 10-fold from the current 0.1% to less than 0.01%. More importantly, a validation trial would have to enroll between 10,000 and 100,000 individuals. Despite these seemingly long odds, an observational trial (NCT02889978) investigating the discriminating power of the Circulating Cell-free Genome Atlas Study test is already under way.

Future Research Challenges

Table 2 summarizes the research challenges faced by biomarker development in the context of lung cancer screening. The interaction between genetics and environment is multidimensional and hard to control. Samples need to be carefully collected, processed by using standard operating procedures, and annotated by using clinical variables reliably collected from patients and/or electronic medical records.[114] Informed consent is essential to preserve certificate of confidentiality. Researchers with a certificate of confidentiality may only disclose identifiable, sensitive information if the subject consents, whereas anyone conducting research as a subawardee or receiving a copy of identifiable sensitive information must also comply with and understand disclosure restrictions. Even though samples may be anonymized, genetic fingerprints may reveal a subject's

Table 2. Challenges Faced by Research on Development of Lung Cancer Biomarkers

• Challenge 1: Need for deeper knowledge of lung carcinogenesis, the tumor molecular and cellular landscape, the gene-environment relationship, etc.
• Challenge 2: Need for careful sample collection, processing using standard operating procedures, and properly annotated clinical data in the intended use type of patient (screening cohorts)
• Challenge 3: Need to obtain the samples from individuals after informed consent and comply with all rules, regulations, and policies regarding research involving human subjects
• Challenge 4: Need to establish robust consensus criteria for the selection of the single or integrated combined biomarkers to be tested
• Challenge 5: Need to design and approve new mechanisms to show clinical utility of care, reduction of cost, reduction of false-positive and false-negative rates, and acceptable ratios of true and false results
• Challenge 6: Need to further convince stakeholders and research promoters and funders of the relevance of developing single and integrated biomarkers to optimize the efficacy of current lung cancer screening protocols
• Challenge 7: Need to analyze, determine the causes of, and try to overcome potentially unnecessary hurdles to approval even after utility testing is complete

identity, rendering us vulnerable to the misuse of our most personal information. On the other hand, genetic privacy acts can hinder progress in this field.[115]

Because so many biomarkers are approaching clinical validation, the field is in great need of standardized metrics of clinical utility. In the context of lung cancer screening, we can envision a study design (Fig. 4) that would test the clinical utility of a biomarker-based risk assessment strategy. Because many patients with lung cancer do not meet NLST or other ($PLCO_{2012}$, LLP_{v2}) proposed screening criteria, the study would test the value of a candidate biomarker as a predictor of risk independent of age and tobacco exposure and therefore justify annual screening with LDCT for 5 years (the duration of the trial). Conceivably, patients not meeting the U.S. Preventive Services Task Force or other formally accepted screening criteria could be prospectively enrolled on the basis of modeling outcomes. Indeed, the selection criteria could also include those used in other settings, such as the PanCAN and UKLS, which used risk-based prediction models.[21,94] The study would perform biomarker testing by using a clinically validated biomarker(s) and assign patients to management strategies based on the results of combined testing. Those identified by the biomarker as having a lung cancer risk akin to those meeting U.S. Preventive Services Task Force, $PLCO_{2012}$, LLP_{v2}, or other accepted criteria would be offered LDCT. Those identified as having a lower risk profile would be followed without LDCT. All subjects would sign an informed consent and undergo biomarker testing (or a series of tests). The primary outcome of this hypothetical trial would be risk prediction accuracy. Nodule management would follow current clinical guidelines. Biomarker test results would be shared with the patient and his or her provider, who would in turn decide in light of the results whether LDCT is warranted. We would recommend testing patient-reported outcomes based on expected risks and benefits of getting tested, the way in which the test results are communicated, anxiety related to test results, smoking habits, and willingness to undergo further testing based on biomarker results. We would also recommend determining the accuracy of the risk assessment before and after biomarker testing as well as outcome values. The best sequence (annual versus biannual) and combination of tests to offer should also be tested. Such a study would pave the way for a lung cancer screening strategy driven or at least reinforced by biomarkers of risk. An alternative trial designed to validate the clinical utility of diagnostic biomarkers in the context of IPNs that are found incidentally could also be undertaken.

The Future of Molecular Biomarkers in the Context of Lung Cancer Screening

Despite the vast potential of existing candidates and methodologies, no single molecular biomarker of lung cancer is currently being used in routine clinical practice. The clinical validation and utility steps are critical, but much more demanding, resource intensive, and time-consuming than the initial discovery and retrospective validation. That notwithstanding, the unmet clinical needs remain. Individual risk needs to be refined, and screening criteria need to be modified to have an impact on lung cancer–related mortality. Orphan images of IPNs stand to improve our success differentiating benign from malignant with a robust biomarker at our disposal. There is

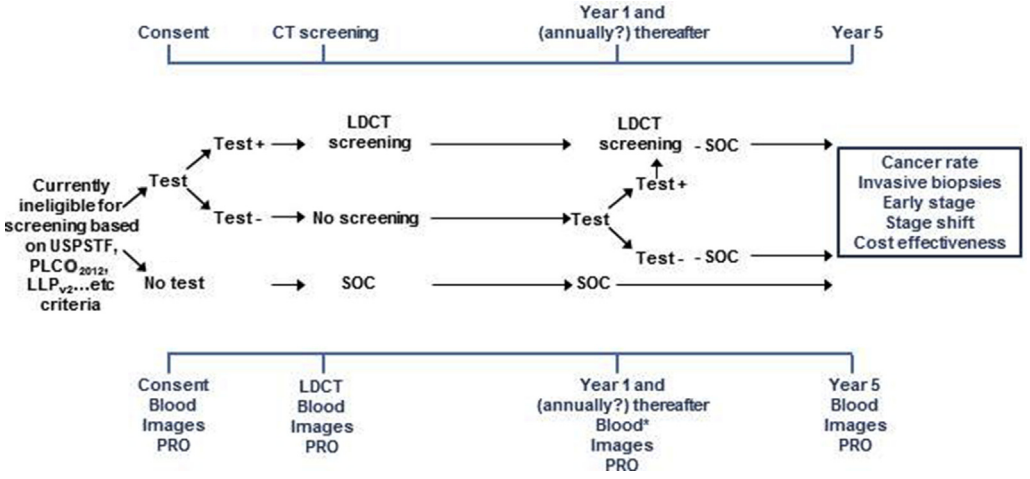

Figure 4. Design of a trial to test the clinical utility of one or several candidate biomarkers. CT, computed tomography; LDCT, low-dose computed tomography; SOC, standard of care; USPSTF, U.S. Preventive Services Task Force; PRO, patient-reported outcomes.

also a clear unmet need for prognostic molecular and clinical markers for patients with screening-detected early-stage tumors. Although some believe that testing a new biomarker would be comparable to the gargantuan effort embodied by the NLST, we believe that less complex and more affordable validation is possible in the setting of established lung cancer screening programs.

There is plenty of room for improvement. We need to promote studies integrating promising candidate biomarkers, including molecular and image-based biomarkers, and the use of artificial intelligence technologies to help in selection of the most appropriate combinations. Head-to-head comparisons of biomarkers in specific clinical scenarios would also be welcome. Deep mining of the troves of data provided by ongoing screening efforts with new mathematical and computational models based on machine learning will surely help. This will require a systematic collection of patient samples in the context of screening. Finally, ways to prove the cost-effectiveness of the new tests, as well as to overcome the potential hurdles to get the approval by regulatory agencies, need to be considered in the list of challenges that we face ahead in the development of molecular biomarkers in screening. Although the logistics and expense of such an effort may seem daunting at first, we believe that the long-term outcome may prove highly efficient.

Acknowledgments

The work of Dr. Montuenga and Dr. Pio work is funded by Foundation for Medical Research (FIMA), the Spanish Ministry of Economy and Innovation, and Health Research Fund (FIS)/European Regional Development Fund (FEDER) (FIS projects PI16/01821, PI17/00411, and CIBERONC), Spanish Association Against Cancer (AECC) Scientific Foundation (GCB14-2170 and PROYE16091), and the Juan Serrano and Ramon Areces Foundation. Dr. Sozzi's work is funded by investigator grant 18812 and 5×1000 grant 12162 from the Italian Association for Cancer Research. Dr. Boeri was supported by a Fondazione Pezcoller Fellowship. Dr. Massion's work was supported by National Cancer Institute grant A152662. We thank Heber Longas (a medical and scientific illustrator from Fundamentium) for his contribution to the graphics of the present review, in particular Figure 2.

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