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Genomic Profiling for Clinical Decision Making in Myeloid Neoplasms and Acute Leukemia

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Abstract:
Myeloid neoplasms and acute leukemias derive from the clonal expansion of hematopoietic cells driven by somatic gene mutations. While assessment of morphology plays a crucial role in the diagnostic evaluation of patients with these malignancies, genomic characterization has become increasingly important for accurate diagnosis, risk assessment, and therapeutic decision making. Conventional cytogenetics, a comprehensive and unbiased method for assessing chromosomal abnormalities, has been the mainstay of genomic testing over the last several decades and remains relevant today. However, more recent advances in sequencing technology have increased our ability to detect somatic mutations through the use of targeted gene panels, whole exome sequencing (WES), whole genome sequencing (WGS), and whole transcriptome sequencing (WTS) or RNAseq. In patients with myeloid neoplasms, whole-genome sequencing represents a potential replacement for both conventional cytogenetic and sequencing approaches, providing rapid and accurate comprehensive genomic profiling. DNA sequencing methods are employed not only for detecting somatically acquired gene mutations, but also for identifying germline gene mutations associated with inherited predisposition to hematologic neoplasms. The 2022 International Consensus Classification (ICC) of myeloid neoplasms and acute leukemias makes extensive use of genomic data. This report aims to help physicians and laboratorians implement genomic testing for diagnosis, risk stratification, and clinical decision making and illustrates the potential of genomic profiling for enabling personalized medicine in patients with these hematologic neoplasms.

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Abstract

Myeloid neoplasms and acute leukemias derive from the clonal expansion of hematopoietic cells driven by somatic gene mutations. While assessment of morphology plays a crucial role in the diagnostic evaluation of patients with these malignancies, genomic characterization has become increasingly important for accurate diagnosis, risk assessment, and therapeutic decision making. Conventional cytogenetics, a comprehensive and unbiased method for assessing chromosomal abnormalities, has been the mainstay of genomic testing over the last several decades and remains relevant today. However, more recent advances in sequencing technology have increased our ability to detect somatic mutations through the use of targeted gene panels, whole exome sequencing (WES), whole genome sequencing (WGS), and whole transcriptome sequencing (WTS) or RNAseq. In patients with myeloid neoplasms, whole-genome sequencing represents a potential replacement for both conventional cytogenetic and sequencing approaches, providing rapid and accurate comprehensive genomic profiling. DNA sequencing methods are employed not only for detecting somatically acquired gene mutations, but also for identifying germline gene mutations associated with inherited predisposition to hematologic neoplasms. The 2022 International Consensus Classification (ICC) of myeloid neoplasms and acute leukemias makes extensive use of genomic data. This report aims to help physicians and laboratorians implement genomic testing for diagnosis, risk stratification, and clinical decision making and illustrates the potential of genomic profiling for enabling personalized medicine in patients with these hematologic neoplasms.

Introduction

Genomic characterization is essential for the management of myeloid neoplasms and acute leukemia, providing critical information for diagnosis, risk-assessment, therapeutic decisions, residual disease monitoring, progression, and treatment resistance (**Figure 1**). Chromosome banding analysis complemented by a variety of molecular studies are a central facet of evaluation, with new genomic techniques increasingly being used to improve characterization as described below and in **Table 1**. This manuscript is meant to be a practical guide for the application of genomic methods in the clinical evaluation of myeloid neoplasms and acute leukemia.

Conventional Methods

Chromosome banding analysis: Karyotyping remains the most widely used and unbiased method for assessing chromosomal abnormalities including numerical (amplifications and losses) and structural (translocations, deletions, and inversions) abnormalities[1-3]. The main limitations are the requirement for live culturable cells, low resolution (5-10 Mbases), and low sensitivity (abnormalities present in 5-10% of cells or an analytical sensitivity of ~10^{-1}). Turnaround times are generally between 2-21 days and may vary considerably between laboratories.

Fluorescence in situ hybridization (FISH): FISH is often used to complement chromosome banding analysis and can be performed on both cultured dividing cells (metaphase) and fixed or non-dividing cells (interphase). FISH probes can only identify genomic events at specific targeted regions, but is more sensitive than cytogenetics (abnormalities in 1-5% of cells or an analytical sensitivity of ~10^{-2}) and can detect cytogenetically cryptic abnormalities.[4,5] Turnaround times are generally 1-3 days.

Chromosomal microarrays (CMA): CMAs are typically used to identify small, unbalanced abnormalities or cryptic copy number alterations, but do not detect balanced rearrangements and, unlike karyotype, cannot distinguish changes occurring in separate clones. In addition, CMAs including single nucleotide polymorphism probes (SNP-arrays) can detect loss of heterozygosity (LOH) and facilitate the determination of chromosomal ploidy.[6] Unlike FISH, CMAs are unbiased and can detect abnormalities genome-wide. CMAs are run from tumor DNA without requiring live cells and can detect small abnormalities (20-100 Kb) present in 20-30% of tumor cells (or an analytical sensitivity of $>10^{-1}$). Turnaround times are generally between 3-14 days. While not 'array-based', multiplex ligation-dependent probe amplification (MLPA) can also be used to detect specific copy number alterations (including single exon events) through the use of multiple sequence-specific probes spanning a specific region which are then amplified to determine DNA copy state.[7]

Optical Genome Mapping (OGM): Optical genome mapping methods are an unbiased approach that use genome-wide high-resolution enzymatic restriction digests of high molecular weight genomic DNA to identify structural variants such as translocations, inversions, and copy number alterations.[8] While not widely used in the clinical laboratory today, turnaround times are typically 4-7 days with maximum sensitivity of ~5%.[9]

Polymerase chain reaction (PCR): PCR is a technique based on the enzymatic replication of DNA (or cDNA complementary DNA from reverse transcribed RNA) and can generate tens of billions of copies of a particular small DNA or cDNA fragment (the sequence of interest), allowing gene mutations to be detected by various methods. Most clinical PCR applications use allele-specific PCR (AS-PCR) with primers for a specific mutation that only produce a PCR product when the mutation is present. Quantitative real-time PCR (qPCR), can rapidly quantify specific fragments containing a sequence alteration and is used to detect specific single gene

mutations or gene rearrangements. Digital PCR (dPCR) technologies enable absolute quantification through partitioning the reaction into thousands of independent PCR reactions to achieve high levels of sensitivity (one mutation in 10,000 normal cells or a sensitivity of 10^{-4}). qPCR and dPCR methods are generally suited to detect specific recurrent genetic alterations such as single gene mutations (e.g. *JAK2* p.V617F, *KIT* p.D816V) and distinct fusions (e.g. *BCR::ABL1*) for diagnosis and disease monitoring. Turnaround times are generally 2-5 days.

Sanger sequencing: Sanger sequencing detects small gene-level DNA variation from PCR-amplified DNA fragments (<1 kb). Individual DNA bases are detected by electrophoresis due to the random incorporation of fluorescently labeled chain-terminating dideoxynucleotides by DNA polymerase during in vitro DNA replication. Sanger sequencing is generally used to detect gene mutations confined to single exons (e.g. *CEBPA*, *CALR*) and has a relatively low analytic sensitivity of approximately 20% ($>10^{-1}$), but can be turned around more rapidly than next generation sequencing (NGS) based methods.

Next Generation Sequencing (NGS)-based methods

General Concepts: Building upon the previously described 'conventional' genomic methods, next generation sequencing (NGS), massive parallel sequencing (MPS) or high throughput sequencing (HTS) uses millions or billions of parallel sequencing reactions to identify genomic abnormalities. NGS is highly scalable and can be coupled with enrichment technologies to interrogate a small subset of key genes (targeted gene panels) up to thousands of genes or genomic regions (whole-exome sequencing [WES]) or can be used without enrichment to detect full genome (whole genome sequencing [WGS]) or transcriptome-wide (whole transcriptome [WTS or RNAseq]) genomic abnormalities. Depending on the design of the assay, NGS can be used to study the full range of genomic variation, including single nucleotide variants (SNVs), small insertions/deletions (indels), structural changes (copy number alterations; CNAs), gene

fusions or chromosomal translocations, gene expression, and DNA methylation, and may be used for initial diagnosis as well as monitoring.

The abundance of a detected variant is generally represented as a variant allele frequency (VAF, **Figure 2A**), which represents the ratio of sequencing reads that contain a variant at a given position in the genome divided by the total number of reads at the position. VAFs are considered a 'semi-quantitative' measure, as the exact methods used to calculate VAFs, VAF precision, and VAF accuracy will differ slightly between laboratories. Additionally, chromosomal aneuploidy, LOH, or gene amplification/deletion can skew both inherited and somatic variant VAFs either higher or lower. The methods used to separate somatic variants (those present in cancer cells) from 'germline' variants (polymorphisms or pathogenic alterations in germline DNA) may also differ between laboratories. While the gold standard for establishing somatic status of a variant involves sequencing both tumor and paired non-neoplastic tissue DNA, this approach is expensive and impractical for most clinical testing. Instead, many laboratories often infer variants with VAFs near 50% (in regions without copy number alterations) as germline; the use of population databases (1000 Genomes[10], gnomAD[11], dbSNP[12], etc) to filter out known polymorphisms is highly suggested to separate germline variants from somatic mutations. The exact methods used to filter polymorphisms varies widely between laboratories[13] and may give rise to both false positive somatic calls, especially in genes with low probability of loss intolerance (pLI)[14] scores like *TET2*[15], and false negative calls, often resulting from the presence of clonal hematopoiesis of indeterminate potential (CHIP) variants (i.e. *DNMT3A* p.R882) coded as polymorphisms in 'normal' population databases.[16,17] The limit of detection for standard NGS assays is generally determined by the sequencing coverage depth and typically ranges from 2-5% VAF (figure 2B); reliable detection of variants below this level typically requires error-correction methods (described below).

In addition to inter-laboratory differences in VAFs and assignment of somatic status to variants, variant classification and annotation, including assigning variants to different 'tiers' based on pathogenicity or clinical significance, may differ between laboratories. In general, it is advised that interpretations follow professional guidelines such as the Association for Molecular Pathology/American Society for Clinical Oncology/College of American Pathologists (AMP/ASCO/CAP) guidelines[13], or disease-specific National Comprehensive Cancer Network (NCCN) guidelines, or World Health Organization (WHO) guidelines where possible. Variant annotations should also be considered in the context of the patient's disease and genomic data should be interpreted in conjunction with blood and bone marrow morphology, flow cytometry, and relevant clinical data.

Targeted Gene Panels: Driver mutations (recurrent somatic mutations known to be involved in disease pathogenesis) in specific myeloid neoplasms and acute leukemias tend to occur in a core group of 20-50 genes and are ideally suited to detection by small gene panels.[18,19] Targeted panels, used most frequently in clinical laboratories, direct sequencing to specific genes or genetic regions that have defined clinical relevance and dictate clinical management.[20,21] Key genes that should be included in sequencing panels for different diagnostic entities are summarized in **Table 2**. Enrichment strategies (**Figure 2C**) include the capture of genetic regions of interest though hybridization (hybrid capture using DNA or RNA probes) or PCR amplification (amplicon enrichment). This provides critical benefits over broad exome (WES) and genome (WGS) sequencing by increasing sensitivity in clinically relevant regions and by decreasing sequencing cost. Depending on the design, targeted panels may be able to detect copy number alterations (CNAs) and chromosomal translocations in addition to SNVs and indels. It should be noted that the detection of larger insertions including *FLT3* internal tandem duplications (ITDs) and *KMT2A* (*MLL*) partial tandem duplications (PTDs) generally require specialized informatics approaches and may not be detected by all panels.[22,23]

Targeted sequencing methods may also be applied to RNA via capture or PCR amplification of cDNA, techniques used primarily to detect recurrent chromosomal translocations in hematologic malignancies Turnaround times for targeted gene panels are generally 5-14 days.

Genomic sequencing (WES and WGS) and transcriptomic sequencing (WTS or RNASeq): Broad genomic sequencing assays allow for the detection of genomic alterations anywhere in the coding genome (WES whole exome sequencing, interrogating 1-2% of the whole genome) or entire genome (WGS-whole genome sequencing).[24,25] WGS and WES entail higher sequencing costs and more extensive data analysis pipelines, and do not typically achieve the same level of coverage depth as targeted gene panels, resulting in lower analytic sensitivity. Most WES applications are primarily limited to the research setting. In comparison, WGS, which can detect a full range of genomic alterations including copy number alterations and chromosomal rearrangements, has shown promise as a clinical application especially in cases with unsuccessful conventional cytogenetics.[26] Whole transcriptome sequencing (WTS or RNAseq) detects both chromosomal rearrangements and changes in messenger RNA (mRNA) and microRNA (miRNA) expression and is primarily limited to research and discovery[27]. In acute lymphoblastic leukemia (ALL), WTS has led to identification of unique B-ALL subtypes and development of targeted panels for clinical use.[28] Decreasing cost, increasing wider availability, and evidence for clinical utility will likely foster the integration of genomic sequencing technologies into routine clinical testing.

Molecular measurable residual disease (MRD) methods

qPCR and FISH: The oldest and most established method for monitoring MRD in hematopoietic neoplasms rely on the detection of previously identified translocations (*PML::RARA, BCR::ABL1, RUNX1::RUNX1T1*) or recurrent insertions/deletion (*NPM1* exon 11 mutation (ENST00000296930); the same mutation may also be annotated in exon 12 depending on the

transcript used by the laboratory). Recurrent translocations may be detected either by FISH or more sensitive qPCR from RNA, with sensitivities of 10^{-2} and 10^{-6}, respectively.[29] In AML high sensitivity monitoring for highly recurrent *NPM1* exon 11 gene mutation can be accomplished by qPCR with a sensitivity of ~10^{-3} or lower.[30]

High sensitivity sequencing for somatic variants: The sensitivity and specificity of NGS can be improved with unique molecular identifers (UMIs) to tag individual DNA templates on single or dual strands of the target and can increase the analytic detection sensitivity up to 10^{-6}.[31-33] For detecting low levels of molecular disease after treatment in AML, a sensitivity of at least 10^{-3} is recommended.[34] UMI's are used to computationally "collapse" DNA sequence information into "consensus" reads, allowing removal of PCR or sequencing errors absent in identically-tagged templates (**Figure 2D**).[35-38] UMI-based sequencing can be coupled with DNA enrichment to create generalized MRD panels or to monitor previously detected mutations in a patient-specific manner.

T-cell receptor (TR) and Immunoglobulin (IG) NGS-based MRD approaches: NGS of the hypervariable regions of *IG (IGH, IGK,* or *IGL*) and/or TR (*TRB, TRG)* can be used to measure MRD in B or T-cell lymphoblastic leukemias. Although IGH/TR rearrangements are reasonably specific for identifying a patient's neoplastic clone, importantly, these sequences may rarely occur as part of the normal immune repertoire at <10^{-4} and conversely, clonal sequences may continue to change through ongoing VJ recombination potentially resulting in false negative calls. Thus, caution should be exercised in interpreting very low levels of an IG/TR clone that is identical to the patient's ALL clone.[39-42] It is also recommended that laboratories follow more than one IG/TR clone (when possible) to reduce the chance of false negative MRD errors.

Appropriate genomic testing depends on the clinical scenario or diagnostic disease category. The sections below provide recommendations for genomic testing in specific clinical contexts and myeloid neoplasm subgroups, for diagnosis, classification, prognosis, and disease monitoring after therapy. Suggested genes to be tested in specific diagnostic entities are summarized in **Table 2**.

Myeloid neoplasms and Inherited/germline disorders

With the advent of NGS, individuals are increasingly recognized as having potentially deleterious germline variants that predispose to hematologic neoplasms, especially myeloid neoplasms (**Table 3**).[43-49] Most of these are inherited, but some can occur *de novo* and are newly acquired in that individual's germline, and as such, can be inherited by that individual's descendants. Current indications for germline genetic testing include patients with two or more cancers, one of which is a hematologic neoplasm,[50] and those with a hematologic neoplasm and a positive family history. Although germline genetic testing historically has been performed mainly on patients with myeloid neoplasms diagnosed under the age of 40-50, it is now recognized that young age at diagnosis or positive family history are not required to justify genetic testing.[51,52] Thus, germline predisposition risk should be considered for all patients diagnosed with a myeloid neoplasm regardless of age, since some germline predisposition alleles, like those in *DDX41*, present at older ages.[43,48,53-55] In patients with mutations detected on sequencing panels that could represent pathogenic germline variants (*e.g., CEBPA*, *DDX41*, *GATA2*, *RUNX1*, or *TP53* mutations, among others) and occurring at approximately 50% variant allele frequency, germline predisposition testing should be considered particularly if they persist in remission.[56] Genetic counselors and health care providers should be familiar with testing options, including optimal sample types (*e.g.,* cultured skin fibroblasts to ensure exclusion of somatic mutations present in hematopoietic cells) and other types of tissues accepted by some

laboratories (including hair follicles or skin biopsies washed to remove blood), as well as available testing platforms.[57] Challenges to clinical testing for these disorders include the lack of training for most clinicians regarding these conditions, the rapid increase in genes under consideration, the high proportion of variants of uncertain significance (VUSs) in less well-studied genes, the need to distinguish germline from somatic mutations, and a lack of standardization in the field regarding which patients and which genes should be tested.[57]

Germline variants are categorized into 5 tiers according to the American College of Medical Genetics and Genomics/AMP as pathogenic, likely pathogenic, VUS, likely benign, and benign;[58] but only pathogenic and likely pathogenic variants are considered disease-causing and followed clinically. Germline variant classifications can be found in ClinVar (https://www.ncbi.nlm.nih.gov/clinvar). Gene-specific guidelines are available for *RUNX1* and are under development for other myeloid predisposition genes.[59] Importantly, as additional information regarding gene/allele function and additional patient cases (both unrelated cases and segregation data from known families) accrue, gene variant classification can change over time, and VUSs can be re-classified as likely pathogenic/pathogenic, complicating individual and family counseling. Recognition of hereditary myeloid neoplasms may alter patient management, especially regarding the consideration of allogeneic hematopoietic stem cell transplant (allo-HSCT) using a related donor, as well as health surveillance strategies for the patient and relatives who share the deleterious variant.[60-63] Testing for germline risk alleles should be performed as early as possible during clinical management to facilitate treatment plans that may include allo-HSCT.

Certain germline disorders may be associated with additional clinical features, like those associated with quantitative and qualitative platelet defects: *ANKRD26*, *ETV6*, and *RUNX1;* and those variably associated with additional organ dysfunction, for example: *GATA2* with immunodeficiency; Shwachman Diamond Syndrome with exocrine pancreatic insufficiency and skeletal dysplasia; Fanconi anemia (FA) with congenital anomalies, squamous cell carcinomas,

and liver tumors; and dyskeratosis congenita with pulmonary fibrosis, liver cirrhosis, and vascular anomalies; among others. Some genes, like *CEBPA*, confer germline risk only to myeloid neoplasms, whereas other genes may confer risk to a variety of hematologic neoplasms and solid tumors. Additional testing can be a helpful complement to germline genetic testing. For example, telomere flow FISH can identify patients with short telomere syndromes, about 30% of whom will not have a gene variant identified on panel testing.[64] FA chromosomal breakage studies by diepoxybutane/mitomycin-C analysis are useful because of the challenges of FA genetic testing, including common VUSs in FA genes, deletions, distinguishing *cis* versus *trans* arrangements of FA gene mutations, treatment effects, and somatic mosaicism which may mask the diagnosis. Of note, the tumor spectrum associated with each disorder may expand over time as longer follow-up of additional individuals and families becomes available. Additionally, germline predisposition to lymphoid malignancies is emerging in importance and often overlaps with myeloid neoplasm risk genes. Future work will reveal a more comprehensive list of hematologic neoplasm predisposition genes and will influence how broadly to offer predisposition testing among patients with established myeloid neoplasms as well as those with sustained cytopenias.

Cytopenic patients with a suspicion for MDS

A key diagnostic challenge for hematologists is determining whether persistent cytopenia reflects myelodysplastic syndrome (MDS) or other causes. Increasingly, gene-panel sequencing is being used in this population to aid diagnosis. The absence of clonal driver mutations in a patient with unexplained cytopenia, referred to as idiopathic cytopenia of undetermined significance (ICUS), has high negative predictive value (NPV) for MDS.[65] Conversely, the presence of mutations in this scenario suggests either clonal cytopenia of undetermined significance (CCUS) or MDS, necessitating further workup in most cases. Mutant hematopoietic

cell clones may also be found in individuals with normal blood counts and no evidence of hematologic neoplasm, referred to as clonal hematopoiesis of indeterminate potential (CHIP) or age-related clonal hematopoiesis (ARCH).[66] Although CHIP is associated with increased risk of developing hematological[67-70], cardiovascular disease[71,72], and all-cause mortality[70,73], testing in non-cytopenic patients is not currently recommended due to lack of evidence that intervention is indicated. In cases where CHIP is identified incidentally, patients may be seen by clinicians who can provide reassurance that the clinical course is generally benign, with suggestions for lifestyle modifications to lower cardiovascular disease risk.[74] For those with CHIP or CCUS with higher risk features (discussed further below), more frequent surveillance of blood counts may be warranted to assess for early signs of malignant transformation, as discussed below. Features of CHIP, ICUS, CCUS, and MDS are summarized in **Table 4**.

The decision to perform gene-panel sequencing in cytopenic patients is dependent on the pre-test probability of a myeloid neoplasm, such as MDS, which is in turn informed by the clinical context and additional laboratory testing. Initial work-up should include examination of a peripheral blood (PB) smear and lineage-appropriate studies, including nutritional deficiencies (iron, B12, folate, copper), or toxicity evaluation for hemolysis, renal failure, liver disease, splenomegaly or monoclonal gammopathy, and reconciliation with medications. Cytopenia(s) identified below certain thresholds (hemoglobin <10 g/dL, platelets <100 K/μL, absolute neutrophil count <1.8 K/μL) increases the pre-test probability of MDS[75]; however, MDS is not confined to these defined thresholds and gene-panel sequencing may be considered even in patients with mild cytopenia depending on clinical suspicion of MDS. While at least 6 months of unexplained cytopenia has been suggested to establish its chronicity for some MDS subtypes[75], gene-panel sequencing might be considered earlier, particularly in those with severe cytopenia or where clinical suspicion is high. Clinical factors that positively influence the pre-test probability of MDS include 1) a history of prior chemotherapy or radiation, particularly with

delayed count recovery on therapy and 2) family history of a hematologic neoplasm, in which case germline testing is also indicated.

There is high concordance in mutation detection between peripheral blood (PB) and bone marrow (BM) and testing from PB can be used to predict the likelihood of a myeloid neoplasm in the BM.[76,77] The high negative predictive value of 95% from targeted sequencing of PB in unselected cytopenic patients suggests that gene-panel sequencing can identify those with a very low risk of a myeloid neoplasm who may not require an invasive and costly BM assessment.[65] However, BM sampling is the only means for assessing BM morphology and is required to diagnose and distinguish between MDS, other clonal cytopenias (such as CCUS), and unexpected alternative malignancies which may be missed with PB-only screening. In addition, BM sampling ensures accurate disease classification, enables conventional cytogenetics and provides key information for treatment decisions or clinical trial eligibility. The detection of somatic driver mutations (especially high-risk ones, see below) in the PB warrants a BM biopsy for further evaluation, although it is not necessary to repeat gene-panel sequencing on the concurrent BM sample.[76-78]

In the absence of a morphological diagnosis of MDS, the presence of a driver mutation in a cytopenic patient (i.e., CCUS) is strongly predictive of a subsequent myeloid neoplasm. There are typical high-risk patterns of mutations which further increase this likelihood, including the number of detected driver mutations and a higher VAF. A 10% VAF threshold is predictive of progression[79]; however, higher thresholds (e.g., 20%) may be more applicable in unselected cytopenic patient's populations in whom other etiologies for the cytopenia have not already been excluded[65]. Mutations in spliceosome genes and/or co-mutation patterns of epigenetic genes *DNMT3A*, *TET2*, and *ASXL1* are highly predictive of progression to a myeloid malignancy with co-occurring mutations in *RUNX1, EZH2, CBL, BCOR, CUX1, TP53,* or *IDH1/IDH2* being most specific for progression to a myeloid neoplasm with MDS.[79,80] Although (with the exception of multi-hit *TP53* mutation) these high-risk mutational patterns are not currently part of the

diagnostic criteria for MDS, studies have demonstrated that high-risk CCUS has comparable clinical outcomes to low-risk MDS.[79] By contrast, solitary *DNMT3A* mutations, even in the context of cytopenias, may have an indolent course mimicking CHIP.[80] Multi-hit *TP53* variants (>1 mutation or mutation plus loss of the alternate allele) are associated with genomic instability and high risk disease and are considered to be diagnostic of a myeloid neoplasm with mutated *TP53* in the current International Consensus Classification.[81,82] In the post-chemotherapy or radiation therapy patient, the identification of pathogenic variants in *TP53*, *PPM1D* and *CHEK2* indicate high risk for developing a therapy-related myeloid neoplasm.[83]

MDS and MDS/MPN

The National Comprehensive Cancer Network (NCCN) recommends incorporation of somatic gene mutation testing in PB or BM for MDS patients, given the correlation of somatic mutations with disease risk in MDS and the potential to use targeted therapies like IDH inhibitors.[77,84] While somatic gene mutations do not replace morphologic assessment, they are relevant to categorize MDS. The ICC guidelines recognize the mutation-defined entities MDS with mutated *SF3B1*, MDS and MDS/AML with mutated *TP53*, and MDS/MPN with thrombocytosis and *SF3B1* mutation.[82] In addition, specific somatic mutations represent exclusion criteria (e.g. *BCR::ABL1* rearrangement) for these and other entities, further supporting the need for genetic testing for accurate class assignment. NGS also significantly aids risk assessment and clinical decision-making in IPSS-R intermediate risk MDS patients who are potential candidates for allo-HSCT.[85]

In the MDS/MPN entity chronic myelomonocytic leukemia (CMML) clonal driver mutations can be detected in over 90% of cases, with the combination of *TET2* (especially biallelic variants) and *SRSF2* being highly specific for a myelomonocytic phenotype.[86] Consequently, genomic

profiling can provide supportive evidence for a diagnosis of CMML and focused gene panel testing is recommended.[87] In patients presenting with a monocytosis, the absence of a clonal driver mutation in the PB has a very high negative predictive value for CMML while a demonstrable mutation is not only strongly predictive of a neoplastic diagnosis in the BM but also significantly impacts overall survival even in those without a confirmed morphological diagnosis (clonal monocytosis of undetermined significance, CMUS).[88]

The integration of somatic mutations into prognostic scoring systems provides more accurate risk stratification of individual patients.[89,90] Within the International Working Group for Prognosis in MDS, a clinical-molecular IPSS model (IPSS-M) has been recently developed and validated for MDS (https://mds-risk-model.com).[91] In the MDS/MPN entity CMML, somatic mutations are integrated in clinical/molecular prognostic systems resulting in improved risk stratification.[92] This includes the analysis of *ASXL1*, *NRAS*, *RUNX1*, and *SETBP1* with sequencing of these genes being strongly recommended in patients eligible for transplantion.[87] Analysis of a minimum of 20 genes is recommended for CMML patients being considered for active treatment.[87] Somatic mutations may also predict response and/or outcome after selected treatments in MDS and MDS/MPN, for example *TP53* mutations are consistently associated with shorter survival after allo-SCT[93,94] and somatic mutations in *TP53* also predict increased response to hypomethylating agents (HMA).[95] As with other disease-related variables, somatic mutations may require reassessment to update individual risk in case of significant clinical changes or before disease-modifying treatments. It must be noted that, although combining genomic profiling with hematologic and cytogenetic variables improves risk assessment, prospective real-world data and clinical trials are required to translate this improved stratification into evidence-based recommendations for clinical decision-making.

In patients undergoing disease-modifying therapies, genomic profiling is potentially instrumental to measure response and MRD in MDS and CMML. Persistent disease-associated mutations after allo-SCT are associated with a higher risk of progression.[96] Molecular monitoring of MRD has also been applied after HMA therapy[97]; however, additional studies are warranted to confirm its clinical value. In patients with CMML, response to HMA has been associated with changes in DNA methylation, without decrease in mutation allele burden, arguing for a predominantly epigenetic effect[98]. However, to date, somatic mutation analysis has not been incorporated in consensus-based response measurement in MDS or MDS/MPN, while evidence of molecular clonal evolution (i.e. the acquisition of new pathogenic mutations and/or cytogenetic aberrations) has been proposed as a criterion for disease progression in adult MDS/MPN.[99]

Finally, compounds targeting proteins or signaling pathways disrupted by recurrently mutated genes have been tested in recent clinical trials in MDS and MDS/MPN, and the number of compounds entering clinical investigated will likely increase in the future.[100] Although at present few agents besides lenalidomide for MDS with del(5q) have been licensed for clinical use in MDS or MDS/MPN, genomic profiling is instrumental to give patients access to these targeted therapies within the context of clinical trials.

Inclusion of probes for copy-number detection or use of SNP array-based karyotyping is highly recommended to capture chromosomal abnormalities, especially copy-neutral loss-of-heterozygosity (CN-LOH).[26,93] Detection of CN-LOH is particularly important to capture multi-hit *TP53* lesions. In fact, while most of the multi-hit *TP53* lesions can be detected on the basis of variant allele frequency (VAF) higher than 50% or the presence of -17 / del(17p), as many as 6% of these lesions display <50% VAF, thus potentially escaping from detection based on these criteria.[81] The use of FISH 'panels' to detect recurrent cytogenetic abnormalities (-5, del(5q), -7,

etc) is not required in the setting of adequate metaphase cytogenetic studies and most studies has not increased diagnostic yield.[101-103]

Myeloproliferative, Mast Cell Neoplasms, and Eosinophilic Neoplasms

Classical* myeloproliferative neoplasms *(MPN). Screening for mutations in the known driver genes *JAK2*, *CALR* and *MPL* is mandatory for establishing MPN diagnosis.[75,104] Panel-based NGS may not be required to establish the initial diagnosis, but is recommended in driver mutation-negative subjects to identify uncommon somatic variants (e.g. indels in *JAK2* exon 12 in PV, non-canonical somatic mutations in *JAK2* and *MPL*)[105], as well as rare germline variants in *JAK2*/*MPL* that cause hereditary thrombocytosis or erythrocytosis and mimic MPN.[106] It is recommended that *JAK2* p.V617F VAF be obtained in DNA from PB samples or purified granulocytes. Since the advent of disease-modifying agents (e.g., interferon)[107] requires serial measurements to inform treatment, quantitative mutation abundance should be reported. An increasing VAF from baseline has been associated with disease progression to post-PV/ET MF.[108,109] NGS for testing for variants in other myeloid neoplasm-associated genes also has prognostic value: *ASXL1, EZH2, SRSF2, IDH1*,*IDH2, and U2AF1*, are considered "high molecular risk" (HMR) mutations in PMF[110] and are included in current risk classification schemes[111,112], while the clinical value of additional mutations in ET and PV is still uncertain. Although at present there is no general consensus on how extensive the search for additional mutations by NGS should be[113], inclusion of *TP53*, *NRAS/KRAS*, and *RUNX1* may be worthwhile since these mutations are likely to have an impact on outcome and/or resistance to treatment.[114,115] Similarly CSF3R mutation status should be evaluated as truncating mutations occurring outside of the proximal membrane region have shown sensitivity to dasatinib.[116,117] Persistence of MPN-associated variants 3-6 months post allo-HSCT associates with an increased risk of relapse; the use of highly sensitive *JAK2* p.V617F PCR-tests (sensitivity of at

least 0.01%) may be particularly useful in this scenario.[118] Post-allo-HSCT monitoring of variants after confirmation of molecular remission is not routinely performed, but may be useful in case of suspected relapse (*e.g.* falling donor chimerism).

Chronic neutrophilic leukemia (CNL). Variants in *CSF3R*, which encodes the G-CSF receptor, are found in 60-80% of individuals with this rare MPN.[116,119,120] Identification of the commonest activating mutation, p.T618I, has therapeutic significance since responses to ruxolitinib have been reported.[116,121] Although *CSF3R* variants are most prevalent in CNL, they are not specific to this diagnosis and can also be seen in other myeloid disorders, particularly in atypical CML and CMML.

Systemic mastocytosis (SM). In SM, the *KIT p.*D816V mutation is identified in >90% of patients at diagnosis.[122,123] A BM sample should be used for greater sensitivity, however PB positivity for *KIT* mutation indicates multilineage involvement and suggests an associated hematological neoplasm (AHN)) and establishes a B-finding for diagnosis of smoldering SM when present with a ≥ 10% VAF.[124,125] High sensitivity assays such as allele-specific PCR (from RNA or DNA) or dPCR are recommend over standard NGS for the identification of *KIT* p.D816V. The VAF (abundance) of *KIT* p.D816V should ideally be reported, which is more easily calculated from DNA rather than RNA. With the advent of KIT inhibitors (midostaurin, avapritinib)[126,127], measurement of changes of *KIT* p.D816V VAF in PB, if positive at baseline, might have important value for monitoring and prognostication, although this is still exploratory.[127,128] In *KIT* D816V-negative cases, sequencing rarely detects other *KIT* mutations at position 816 (e.g. p.D816H/N/Y) or 822 (p.N822K) in exon 17 or in the extracellular domains.[125] In advanced SM, prognosis is adversely affected by additional somatic mutations, e.g. in *SRSF2, ASXL1, RUNX1* (so-called S/A/R mutations), identified by NGS.[129] In chemotherapy- or tyrosine kinase inhibitor (TKI)-resistant/refractory patients, an NGS myeloid

panel might identify the emergence or expansion of clones with new (e.g. K*RAS/NRAS* or *TP53*) or pre-existing mutations, but at present it is not mandatory for patient management. Cytogenetics may reveal prognostically negative abnormalities (e.g. -5, -7, complex karyotype), and should be performed at diagnosis and at progression or relapse.[130,131]

Neoplasms with Eosinophilia. Eosinophilia is most often reactive, and efforts should be made to identify secondary reactive etiologies before embarking on costly and unnecessary molecular tests.[132] The two most frequent molecular abnormalities to search for, concurrently or stepwise, are *FIP1L1::PDGFRA* (which can be detected by RT-PCR, FISH or NGS assays) and *KIT* D816V (which points to SM as the underlying cause for the eosinophilia).[133] Cytogenetic analysis on a BM aspirate identifies reciprocal translocations indicating rearrangement of the tyrosine kinases (TK) *PDGFRA* (4q12), *PDGFRB* (5q31-33), *FGFR1* (8p11), *FLT3* (13q12), and *JAK2* (9p24), associated with myeloid/lymphoid neoplasms with eosinophilia and tyrosine kinase gene fusions (MLN-Eos) . Other cytogenetic abnormalities, e.g. deletions, monosomies, or complex karyotype, as well as presence of somatic mutations may help to classify eosinophilia as clonal. FISH analysis is used primarily to identify the specific TK gene rearrangement; however, the specific fusion may also be confirmed by RT-PCR or DNA/RNA NGS fusion detection and NGS methods may be particularly useful in identifying patients with cryptic fusions. The monitoring of *PDGFRA/B* rearrangement by FISH or fusion transcripts by RT-PCR is commonly used in patients treated with TKI, although there is no standardization equivalent to the *BCR*::*ABL1* International Scale for CML. Some patients treated with TKIs have achieved undetectable *FIP1L1::PDGFRA* fusion transcripts and have been able to discontinue treatment and remain in molecular remission.[134]

Apart from MLN-Eos with tyrosine kinase fusion genes, MPN-U with eosinophilia and chronic eosinophilic leukemia are rarely associated with mutations in *STAT5B* (p.N642H)[135], *JAK2*

(p.V617F, ex13InsDel)[136] and *JAK1 (*p.R629_S632delinsSA*).*[137] Additional mutations in *ETNK1, RUNX1, ASXL1* or *EZH2* may be prognostically informative. T-cell clonality (detected by PCR or NGS) is found in both reactive and clonal eosinophilia, and may point to the lymphocytic HES variant.

Chronic myeloid leukemia (CML). CML is a model of molecularly based diagnosis and monitoring since all patients have the causal *BCR*::*ABL1* fusion, which can be detected by FISH or RT-PCR in PB samples. The *BCR*::*ABL1* transcript type should be characterized at diagnosis. For the ~97% of patients with the common e13a2/e14a2 types, *BCR*::*ABL1* transcript levels measured on an international reporting scale determine treatment response and guide therapeutic decisions.[138,139] A rise during treatment can signal drug resistance and should trigger *BCR*::*ABL1* kinase domain mutation analysis, as these mutations are the major known resistance mechanism. Emerging data suggest that mutations in other cancer-related genes are implicated in drug resistance.[140] These include those of *RUNX1* and *ASXL1* mutations*,* and *IKZF1* deletions*.* The NCCN suggests myeloid mutation panel testing for patients diagnosed with accelerated or blast phase or to identify *BCR*::*ABL1*-independent resistance mutations in patients without kinase domain mutations.[139] However, *BCR*::*ABL1* mutations mostly co-occur with other mutated genes[141,142] and myeloid mutation panels will not detect all relevant variants associated with lymphoid blast phase*.*

AML

The diagnostic work-up of AML includes annotation of cytogenetic and molecular aberrations in the setting of morphologic assessment to confirm the diagnosis; in particular, the blast count defining AML is lower in the setting of some recurrent genetic abnormalities as described in the ICC (**Supplemental Table 1**). As the knowledge of genomic abnormalities is paramount to

AML treatment decisions, we recommend reporting results of mutations associated with diagnostic classification within 5 days when possible; in most cases elaboration of a therapeutic plan can safely await these results.[143]

Complete genomic evaluation including cytogenetics, NGS panel, *FLT3*-ITD testing, and FISH/CMA (if needed to confirm cytogenetic findings or to provide more rapid results, in the case of FISH compared to metaphase cytogenetics)[144] should be performed to identify genomic abnormalities that define specific AML subtypes, as well as for abnormalities within the 2022 European Leukemia Network (ELN) risk classification to determine prognosis in patients treated with standard intensive chemotherapy and to inform consolidation treatment choice, especially pertaining to the role of allo-HSCT in first remission**.** Important updates in the 2022 ELN risk classification[145] include the categorization of all *FLT3*-ITD mutations within the intermediate-risk group regardless of *FLT3*-ITD allelic ratio or *NPM1* co-mutation, and the addition of AML with MDS-related mutations in the adverse-risk group. Favorable-risk disease associated with *CEBPA* mutations has been revised to specify bZIP in-frame mutations of *CEBPA*, regardless of mono- or biallelic status.[146,147]

With over a dozen genes incorporated within current AML classification and risk stratification systems, the use of gene-panel testing provides the most cost-effective testing approach. Due to limitations with most NGS-based assays, *FLT3*-ITD and *FLT3* TKD determination is often performed separately by PCR and capillary electrophoresis. Rapid annotation of additional mutations of therapeutic relevance such as *IDH1, IDH2, FLT3-ITD* and *FLT3*-TKD, is necessary to determine best treatment approaches given the availability of targeted mutant-specific inhibitors. Immunohistochemistry (IHC) can rapidly detect abnormal cytoplasmic expression of mutant NPM1 in formalin-fixed paraffin-embedded samples or cell blocks, providing utility in situations of myeloid sarcomas, *NPM1* mutations outside exon 11, or in resource-limited

settings where molecular techniques are not available.[148,149] Hot-spot mutations in *IDH1* and *IDH2* can also be rapidly evaluated by immunohistochemistry, and p53 protein accumulation or null-pattern expression correlates with the presence of *TP53* mutations in most cases of AML.[150-155] *TP53* mutation present at a variant allelic frequency (VAF) ≥10% now defines the new category of AML with mutated *TP53*. For other class-defining or risk-defining mutations, the VAF cutoff has not been established.

Conventional karyotyping at diagnosis can be aided by rapid testing for gene fusions (either by RT-qPCR, FISH or NGS based fusion panels), for example *PML::RARA*, *RUNX1::RUNX1T1* and *CBFB::MYH11*. "Myeloid FISH panels" to test for common MDS-associated chromosomal aberrations associated with adverse risk can be useful, particularly in settings where metaphase cytogenetics are not available.[144] Any clonal karyotype or FISH positivity present above the validated lab threshold should be considered a positive result. In the setting of non-evaluable cytogenetics, CMA can also be a useful adjunct to identify unbalanced abnormalities as well as cryptic copy number alterations. More recently WGS has been proposed as a single comprehensive assay for the evaluation of AML.[26]

Once in remission, monitoring of MRD by molecular methods (RT-qPCR, dPCR, NGS) and multi-parameter flow cytometry (MFC) allows ongoing refinement of relapse risk estimations, providing the opportunity to identify impending relapse and possibly allow for early intervention or modified treatment approaches, such as consideration of allo-HSCT in favorable-risk patients who retain detectable MRD by qPCR after completion of planned consolidation therapy. The importance of MRD in AML was confirmed in a meta-analysis of over 80 publications with >10,000 patients: the estimated 5-yr OS was 68% vs 34% in patients in AML remission with MRD- vs MRD+ status.[156] While proven interventions to eradicate MRD are currently lacking, the detection of persistent MRD after completion of consolidation, or MRD "relapse" correlates

with inferior outcomes including increased risk of relapse and decreased overall survival. Current guidelines recommend MRD assessments after 2 cycles of standard therapy, at the end of treatment, and then evaluation every 3 months (if BM) or every 4-6 weeks (if PB) for 24 months.[34] Recommended time points for MRD assessment in patients receiving less intensive treatment regimens are not yet established.

MRD monitoring by molecular methods and MFC may provide complementary data.[157] If an RT-qPCR assay (e.g. *NPM1*, core-binding factor (CBF) fusion) is available, this is considered the preferred method for MRD evaluation, and should be performed in the diagnostic sample to allow for estimation of the kinetics of response during treatment.[158] Both peripheral blood (PB) and bone marrow (BM) may be used for MRD evaluation, but detection sensitivity in PB may be lower.

Outside of RT-PCR and MFC, NGS is an alternative method for MRD assessment which can provide useful information about emerging mutations not present at AML diagnosis **(Figure 3)**. However, NGS MRD analysis may be performed in conjunction with FC as some AMLs will be more amenable to detection by one method or the other, depending on the mutations present and phenotype, respectively.[157] Note that the sensitivity of most routine NGS panels is ~2% VAF, however, the ELN recommends error-corrected NGS with a minimum sensitivity of ~0.1% VAF.[34] While driver alterations such as *NPM1* and CBF-fusions (*RUNX1::RUNX1T1* and *CBFB::MYH11*) are typically present in the founding clone and retained at relapse, mutations of signaling genes (i.e. *FLT3*-ITD, *NRAS/KRAS*) are often sub-clonal and may vary in their presence over time and during treatment, with low negative predictive value. This is especially true in patients with *FLT3*-mutated AML after receipt of FLT3-directed treatments, as ~40% of patients can relapse with *FLT3*-wild type clones.[159] Caution must be taken in the interpretation of residual epigenetic mutations, including “DTA” (*DNMT3A*, *TET2*, *ASXL1*), which represent

pre-leukemic clones and are not predictive of relapse.[156,160] Residual *SRSF2* and *IDH1/2* mutations may be similarly non-informative for MRD assessment.[161]

ALL

Genomic studies have led to the identification of new ALL entities [28,162,163] of prognostic and therapeutic significance[164,165], even in the context of MRD-based risk-adapted therapy (**Supplemental Table 1**). These optimally require sequencing-based approaches to identify all genomic features of clinical importance. However, the choice of diagnostic approach depends in part on how genomic information will be used to guide management, and the availability of genomic and conventional diagnostic assays in individual laboratories.

Routine diagnostic approaches

Chromosome banding analysis and FISH are widely used for identification of aneuploidy (hyperdiploidy and hypodiploidy) and subtype-defining chromosomal alterations (e.g. *BCR::ABL1*, *ETV6::RUNX1*, *KMT2A::AFF1*, *TCF3::PBX1,* iAMP21, etc.) many of which are used for risk assignment and treatment stratification. FISH assays may be used for rapid identification of translocations and gene fusions (**Figure 4**), including those in *BCR::ABL1*-like B-ALL for which targeted therapies are currently available (e.g., *ABL*-family kinase genes, *JAK2*, *CRLF2* and *NTRK3*). However, these assays do not detect all clinically relevant alterations, for example focal insertions of *EPOR* into immunoglobulin loci[166] and sequence mutations (e.g. *JAK1/JAK2/JAK3*) and deletions (e.g. *SH2B3*) that also drive kinase signaling[167]. PCR assays can identify subtypes defined by gene fusions and point mutations (e.g. *PAX5* p.P80R and *IKZF1* p.N159Y). Quantitative RT-PCR may be used to identify the gene expression profile of *BCR::ABL1*-like ALL[168], but subsequent testing (e.g. FISH, targeted or transcriptome sequencing) is required to identify the driver kinase-activating alterations. Quantitative RT-PCR

can also be used to identify deregulated gene expression characteristic of recently identified entities (*DUX4*, *EPOR, NUTM1*, and *CDX2/UBTF*)[163,169] but alteration-specific confirmatory diagnostic approaches are desirable.

Several entities may benefit from flow cytometry analysis of subtype-defining antigen expression patterns, such as CD371 in *DUX4*-rearranged ALL and surface expression of TSLPR (encoded by *CRLF2*), a sensitive and specific indicator of *CRLF2*-rearrangement (**Figure 4**). A major advantage of flow cytometry-based evaluation is rapid turnaround time within 1-2 days.

Capture-based sequencing approaches

The diverse genomic landscape of some subtypes, in particular *BCR::ABL1*-like B-ALL, can make diagnosis challenging. Capture-based approaches [170] or amplicon-based sequencing can detect most common chimeric fusions in B-ALL simultaneously, and are well-suited to identify the wide spectrum of rearrangements in *BCR::ABL1*-like B-ALL; however they may fail to detect complex rearrangements, like *EPOR* in *BCR::ABL1*-like B-ALL and some fusions are difficult to capture if breakpoints involve regions in the introns which may not be feasible to comprehensively tile. DNA-based NGS panels are commonly used to detect common secondary mutations, such as mutations in Jak and Ras pathway signaling genes and mutations associated with relapsed ALL (e.g. *TP53, CREBBP,* and *NT5C2*).

Multiplex ligation-dependent probe amplification (MLPA) assays are widely used to identify focal DNA copy number alterations in single genes and the "*IKZF1*^{plus}" composite genotype[171] (defined as *IKZF1* deletions co-occurring with deletions in *CDKN2A/B*, *PAX5*, or in the pseudoautosomal region 1, PAR1, in the absence of *ERG* deletion), which has been associated with high risk features. However, MLPA cannot be used to identify all relevant alterations: PAR1 deletions accompany *P2RY8::CRLF2* but not *IGH::CRLF2* rearrangements,

and *ERG* is deleted in only ~50% of *DUX4*-rearranged ALL. Thus, *PAR1* and *ERG* deletions identify only a subset of *CRLF2*- and *DUX4*-rearranged ALL, respectively.

Transcriptomic and genomic sequencing

In contrast to targeted approaches, genome-wide sequencing can identify the full spectrum of alterations in a single approach, virtually diagnosing all different entities.

Transcriptome sequencing provides comprehensive characterization of fusion transcript chimeras (**Figure 4**), mutant allele expression (**Figure 4**), gene expression profiling and ploidy[169,172] to identify subgroups and several phenocopies (e.g., *BCR::ABL1*-like ALL, *ETV6-RUNX1*-like, *KMT2A*-like and *ZNF384/362*-like)[169 173]. The availability of a reference dataset of leukemia transcriptomes, such as the St. Jude Cloud (https://www.stjude.cloud)[174] allows analysis and classification of individual samples against a reference dataset, without the need for an extensive local cohort. Limitations of WTS include poor sensitivity to detect rearrangements that involve complex/repetitive regions (e.g. involving antigen receptor loci and *DUX4*)[175,176] or those that do not generate a chimeric transcript, and limited sensitivity for sequence variants that are not expressed or result in nonsense-mediated decay, however these alterations can be detected by WGS.

T-ALL subgroups are mostly defined by deregulation of T-lineage transcription factors. These are highly diverse in terms of the genes involved and the genomic drivers of deregulation, challenging to identify comprehensively, and are not consistently associated with outcome, thus are not typically identified in current diagnostic workflows[28,162]. WGS can detect the diverse genomic alterations that more commonly involve intergenic regions in T-ALL than B-ALL (e.g. *TLX3,* T cell receptor gene loci) that deregulate oncogenes, and non-coding sequence mutations that generate neo-enhancers (e.g. *TAL1* and *LMO1/2*)[28,162]. One entity of clinical relevance is early T-cell precursor ALL (ETP ALL), a high-risk subset of early T-lineage and stem cell leukemias most commonly identified by immunophenotyping ($CD7^+$ and typically

cytoplasmic $CD3^+/CD2^+$; $CD1a^-$, $CD8^-$; myeloperoxidase negative but positive for at least one stem cell/myeloid marker)[177]. ETP ALL is genetically diverse, but one third of ETP ALL and T/myeloid mixed phenotype acute leukemia cases have structural variants deregulating *BCL11B* which may be detected by WGS (**Figure 4**) or for the majority by FISH to detect disruption of the *BCL11B* locus[178].

Molecular quantitation of measurable/minimal residual disease (MRD)

Early MRD monitoring at the end of induction and consolidation phases of therapy has important prognostic and, subsequently, therapeutic implications[179-181]. Conventional approaches include multi-parametric flow cytometry and allele-specific PCR for *IG*/*TR* gene rearrangements[180,182]. To be clinically relevant, MRD analysis needs to be accurate and sensitive (at least $\leq 10^{-4}$). Recently, high-throughput NGS (HTS) of *IG/TR* rearrangements[182], which can reach a sensitivity of 10^{-6}, is becoming more widely used.

General Conclusions and Future Directions

Myeloid neoplasms and acute leukemias are characterized by a complex coexistence of multiple clones that evolve over time.[183] Historically, these have been studied in bulk samples, precluding a more direct understanding of the clinicopathologic effect of such clonal complexity. Recent studies of clonal architecture at a single cell level, offer unique insights into the interaction of clones, suggesting that the presence of distinct clones may potentially affect the growth and fitness of the others.[184-186] At present the use of single cell sequencing remains confined to research setting, however, in the future such assays could help predict disease progression and may guide therapeutic strategies to intercept clonal evolution and allow for individual targeting of clones in multi-clonal disease. The field will continued to be shaped by advanced methods such as proteomics and Cellular Indexing of Transcriptomes and Epitopes

sequencing (CITE-Seq) which can identify potential therapeutic targets and characterize simultaneous gene and protein expression at the single cell level[187,188], and computation artificial intelligence approaches that can identify new relationships in complex data sets.[189,190] In the nearer term, it is expected that the continued decline in sequencing costs will drive further adoption of more frequent panel-based and MRD testing for disease monitoring and broader genomic methods such as WGS for comprehensive genomic evaluation.

It is important to note that these recommendations reflect current practice, based on current treatments and disease classification. As the classification of myeloid neoplasms and their treatments evolve and as genomic testing methods continue to advance, the recommendations for testing will inevitably change and require updating.

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Table 1. General advantages, limitations, and clinical applications of comprehensive genomic methods

Technique	CG	FISH	CMA	OGM	Targeted	Exome	WGS	RNAseq
Viable cells	Yes	No	No	No	No	No	No	No
Resolution	~5 MB	100-200 kb	20-100 kb	5-50 kb	1 bp	1 bp	1 bp	1 bp
Coverage	Genome	Targeted	Genome	Genome	Targeted	Exome	Genome	Genome, Targeted
Alterations	CNV, SV	CNV, SV	CNV, LOH	CNV, SV	← SNV, Indel, CNV, SV, LOH →			Gene expression, SV
Sensitivity (VAF)	5-10%	1-5%	30%	5%	2%	5-10%	10%	5%
TAT (days)*	2-21	1-3	3-14	4-7	5-14	5-14	3-14	5-14
Cost*	$	$	$$	$$$	$$-$$$	$$$	$$$$	$$-$$$
Worldwide use*	High	High	Low	Low	Medium	Low	Low	Low
Used in:								
MDS and MDS/MPN	D, FU	D, FU	D, R	D, R	D, MRD†	D	D	ND
MPN	D	D	D	D	D, MRD†	D	D	ND
AML	D, R	D	D	D, R	D, MRD†	D	D	D
ALL	D, R	D	D, R	D, R	D	D	D	D

CG: cytogenetics, CMA: chromosomal microarray, CNV: copy number variations, D: diagnosis, ES: exome sequencing, FU: follow-up, Indel: small insertion-deletions, LOH: loss of heterozygosity, MRD: minimal/measurable residual disease, ND: not done, OGM: optical genome mapping, R: Relapse; SNV: single nucleotide variants, SV: structural variants, TAT: turnaround time, WGS: whole genome sequencing.

* TATs, Cost, and Use approximated. Actual TATs, Cost, and Use vary significantly by region and laboratory

† When used in conjunction with high coverage sequencing and error correction methods for increased sensitivity/specificity for low abundance mutations.

Table 2. Gene Mutations in Myeloid Neoplasms and Leukemia Indicated for Clinical Testing

Indication	Single Gene Mutations	Structural Variants*
MDS, MDS/MPN, Cytopenia	*ASXL1, BCOR, BCORL1, CBL, CEBPA, CSF3R, DDX41, DMNT3A, ETV6, ETNK1, EZH2, FLT3-ITD, FLT3-TKD, GATA2, GNB1, IDH1, IDH2, JAK2, KIT, KRAS, KMT2A-PTD, NF1, NPM1, NRAS, PHF6, PPM1D, PRPF8, PTPN11, RAD21, RUNX1, SAMD9†, SAMD9L†, SETBP1, SF3B1, SRSF2, STAG2, TET2, TP53, U2AF1, UBA1, WT1, ZRSR2*	
MPN and Mastocytosis‡	*ASXL1, CALR, CBL, CSF3R, DNMT3A, EZH2, IDH1, IDH2 JAK2§, KIT, KRAS, MPL, NRAS, PTPN11, RUNX1, SETBP1, SF3B1, SH2B3, SRSF2, TET2, U2AF1, ZRSR2*	*BCR::ABL1§*
Eosinophilia	*ASXL1, CBL, DNMT3A, EZH2, KRAS, NRAS, RUNX1, SF3B1, SRSF2, STAT5B, TET2, U2AF1*	*BCR::ABL1§, FGFR1::R, FLT3::R, JAK2::R, PDGFRA::R, PDGFRB::R*
AML	**Genes required for diagnosis and risk-stratification:** *ASXL1, BCOR, CEBPA, DDX41, EZH2, FLT3-ITD§, FLT3-TKD§, IDH1, IDH2§, NPM1, RUNX1, SF3B1, SRSF2, STAG2, TP53, U2AF1, ZRSR2* **Additional genes recommended to test for at diagnosis and for use in disease monitoring:** *ANKRD26, BCORL1, BRAF, CBL, CSF3R, CUX1, DNMT3A, ETV6, GATA2, JAK2, KIT, KMT2A-PTD, KRAS, NRAS, NF1, PHF6, PPM1D, PTPN11, RAD21, SETBP1, TET2, WT1*	*BCR::ABL1§, CBFB::MYH11, DEK::NUP214 MECOM::R, KMT2A::R, NUP98::R, RUNX1::RUNX1T1,PML::RARA§*
ALL B	*CREBBP, CRLF2, FLT3, IDH1, IDH2, IKZF1, IL7R, JAK1, JAK2, JAK3, KMT2D, KRAS, NF1, NRAS, PAX5, PTPN11, SETD2, SH2B3, TP53*	*ABL1::R§, ABL2::R, CRLF2::R, CSF1R::R, DUX4::R, EPOR::R, ETV6::R, JAK2::R, KMT2A::R, MEF2D::R, NUTM1::R, PAX5::R, PDGFRA::R, PDGFRB::R, TCF3::R, ZNF384::R*
ALL T	*DNMT3A, ETV6, EZH2, FBXW7, FLT3, IDH1, IDH2, IL7R, JAK1, JAK3, KRAS, MSH2, NOTCH1, NRAS, PHF6, PTEN, U2AF1, WT1*	*BCL11B::R, LMO2::R, MYB::R, NUP::ABL1, NUP214::R, STIL::R, TAL::R, TLX1::R, TLX3::R*

* Conventional karyotype should be performed on all cases at diagnosis. Specific FISH, RT-PCR, or gene fusion NGS assays (targeted DNA/RNA or WGS) may be included depending on clinical context and results of other clinical studies

† Pediatric patients

‡ Mast cell disease with suspicion of associated hematologic neoplasm (AHN)

§ FDA-approved targeted therapy

Table 3. Clinical Considerations for Germline Predisposition Testing

Clinical Considerations Regarding Germline Predisposition Testing		
WHO?	Individual with two or more cancers, one of which is a HM	
	OR	
	Individual with a history of a HN AND	
		a relative within two generations diagnosed with a HN, OR
		a relative within two generations diagnosed with a solid tumor at age 50 or younger, OR
		a relative within two generations diagnosed with another hematopoietic abnormality
	OR	
	Individual whose tumor-based molecular profiling identified a deleterious variant with a VAF consistent with germline status*	
	OR	
	HM diagnosis at a much younger age than is typical	
IDEAL AGE for testing?	Individuals of all ages should be considered for germline predisposition testing, since some gene variants drive myeloid malignancies even at advanced ages (*e.g., DDX41*)	
WHAT SAMPLE?	ideal:	gold standard- cultured skin fibroblasts [some clinical laboratories also accept bone marrow-derived mesenchymal stromal cells]
	possible:	skin biopsy, with washout of peripheral blood
		hair follicles [may not yield sufficient DNA for comprehensive testing]
	not recommended:	saliva/buccal swab [highly contaminated with peripheral blood, especially lymphocytes]
		fingernails [may be contaminated with monocytes]
WHAT TEST?[t]	whole exome sequencing augmented with spike-in probes for non-coding regions known to contain predisposition loci followed by analysis of gene groups	
	whole genome sequencing (if available), with a virtual panel of appropriate genes, including non-coding regions and copy number variation studies	
	panel-based next generation sequencing	
COMPLEMENTARY testing	telomere flow-FISH can identify individuals with short-telomere syndromes, although interpretation can be confounded by active disease and/or treatment	
	diepoxybutane (DEB) and mitomycin C (MMC) analyses identify excessive chromosome breakage and assist in the diagnosis of Fanconi anemia	

HOW can you tell if a variant is germline?	variant is present in DNA derived from a preferred tissue source (see above) at a VAF consistent with germline status* OR
	variant is present in the index patient plus one other relative at a VAF consistent with germline*
WHEN?	at HN diagnosis
	at recognition of a potential germline allele from tumor or other screening, including somatic variants suggestive of an underlying germline variant (*e.g.,* R525H-encoding variant in *DDX41*)
	prior to hematopoietic stem cell transplant using a relative as a donor
WHY?	plan surveillance for other cancers or organ dysfunction
	plan hematopoietic stem cell transplant using a related donor
	allow pre-implantation genetic testing
	cascade testing throughout the family

Abbreviations: HN, hematopoietic neoplasm; VAF, variant allele frequency

* Generally considered to be a VAF between 30-60% when tested on an appropriate sample type.

ł Genes curated as those in which deleterious variants confer risk for hematopoietic malignancies are increasing in number. Resources that delineate up to date genes to consider for testing include: https://dnatesting.uchicago.edu/ and https://panelapp.genomicsengland.co.uk/panels/59/. Several biases regarding testing need to be kept in mind and considered. There may be ascertainment bias in some publications, with gene variants described in a cancer cohort but not in a control, non-cancer cohort, resulting in a study that lacks a comparison of an observed variant frequency versus an expected variant frequency. Confounding factors, such as age, prior genotoxic therapies, and other familial factors, may contribute to cancer risk along with that conferred by the germline variant. Pathologic classifications of myeloid malignancies, including myelodysplastic syndromes, clonal cytopenias, and clonal hematopoiesis, shift over time and may complicate interpretations of individual and family histories.

Table 4. Features of CHIP, ICUS, CCUS, and MDS

	Cytopenia/ Dysplasia	VAF cutoff	Commonly mutated driver genes	Higher risk features
CHIP	No/Minimal (<10%) to None	>2%	*DNMT3A*, *TET2*, *ASXL1*, *PPM1D, JAK2, ZBTB33, ZNF318, TP53, CBL, GNB1, SF3B1, SRSF2,* loss of Y chromosome	Mutations in *TP53*, *ASXL1*, *JAK2*, *SF3B1*, *SRSF2*, *U2AF1*, or *IDH1*/*IDH2*. >1 driver mutations, VAF>10%
ICUS	Yes/Minimal (<10%) to None	None	None	None
CCUS	Yes/Minimal (<10%) to None	>2%	*TET2, DNMT3A, ASXL1, SRSF2, ZRSR2, SF3B1, U2AF1, IDH1/2, RUNX1, EZH2, JAK2, CBL, KRAS, CUX1*, *TP53*	Spliceosome gene mutations *DNMT3A, ASXL1, TET2* in co-mutational patterns (*RUNX1*, *EZH2*, *CBL*, *BCOR*, *CUX1*, *TP53,* or *IDH1/IDH2* most specific*),* >1 driver mutation, VAF>10%
MDS	Yes/Yes	None	See text	See text

Figure Legends

Figure 1. How molecular profiling can inform clinical decision making in MDS. Professional illustration by Patrick Lane, ScEYEnce Studios.

Figure 2. Key concepts in sequencing-based diagnostics. A. Variant Allele Frequency (VAF) represents the ratio of sequencing reads that contain a variant divided by the total number of reads at that position. As most somatic mutations are heterozygous, doubling the VAF generally indicates the fraction of cells with the mutation (except when the mutation occurs in a region of copy number alteration). B. Coverage represents the number of sequencing reads (red and blue indicating forward and reverse reads) that span a particular region. Approximate coverage levels for different sequencing approaches are compared. Higher coverage (or more independent observations) generally yields more sensitive sequencing. Shown on the right is the coverage depth required to detect mutations at various VAFs. Binomial sampling probability for detection of variants with VAFs of 50% (typical inherited variants; black), 2% (general sensitivity for targeted panels; red), and 0.1% (MRD assays; blue) assuming each variant must be seen at least twice. C. DNA sequencing methods. In whole genome sequencing libraries are created by ligating sequencing adapters (grey and orange) to the 3' and 5' ends of short genomic DNA fragments called 'inserts'. Gene panels or exome sequencing enriches DNA of interest form a library using anti-sense capture probes (green) labeled with biotin which are then hybridized to DNA inserts from the sequencing and then physically enriched using streptavidin-coated magnetic beads. D. High sensitivity sequencing for measurable residual disease (MRD) detection requires error correction in order to reliably identify mutations below the intrinsic error rate of the sequencer and to account for PCR errors. Error-corrected deep sequencing reduces false positive calls for low VAF variants by tagging individual DNA molecular with unique molecular identifiers (UMIs). In this example a 'true' mutation 'T' is present in a single DNA

molecule which labeled with a UMI (green). Library amplification and sequencing will result in duplicate DNA molecules each labeled with the same UMI. Randomly accumulated sequencing and PCR errors (orange) will be present in only a subset of reads with a particular UMI (green, purple, red). During sequencing analysis, variants present on only a subset of reads from a particular 'read family' with the same UMI will be discarded as errors; true mutations present in the original DNA molecule will be detected in all reads within a read family with the same UMI. UMI methods can be further improved by tracking both DNA strands using 'duplex sequencing' which can yield sensitivities of 10^{-6}.[31] Professional illustration by Patrick Lane, ScEYEnce Studios.

Figure 3. Sequencing based tumor burden monitoring in myeloid neoplasms. (A) In this AML example, sequencing identifies *NPM1* and *DNMT3A* mutations at diagnosis with the *NPM1* mutation representing the founding clone (green-based on higher mutation VAF) and *DNMT3A* representing a subclone (gray--based on lower mutation VAF). Estimated sensitivity to detect mutations for different sequencing approaches is shown below. As the patient enters remission the *NPM1*-mutated clone is partially cleared, becoming undetectable by panel-based sequencing and WGS, but remains detectable by high sensitivity measurable/minimal residual disease (MRD) sequencing. In this example the *DNMT3A*-mutated clone remains without significant change in VAF, consistent with persistent clonal hematopoiesis. Finally, the patient relapses with the same *NPM1*-mutated founding clone plus a newly acquired *NRAS* mutation. (B) A comparison of sequencing methods for MRD monitoring. While WGS offers the greatest sequencing breadth and is capable of detecting structural variants such as copy number alterations and chromosomal translocations, standard coverage is generally only ~60x, limiting detection to mutations with VAFs >10%. Targeted sequencing is generally limited to 50-500 genes providing minimum sequencing breadth but can achieve high coverages (1000x) at a lower cost than WGS and provides adequate sensitivity (2% VAF) for initial diagnostic

evaluation. MRD panels are similar to targeted panels but employ much higher sequencing depths and use unique molecular identifiers (UMIs) to achieve sensitivities of ~0.1% VAF allowing for MRD monitoring. MRD panels are generally easy to implement but may be of limited clinical utility for patients with few mutations covered by the panel. In patient-specific MRD sequencing, mutations are identified at diagnosis using broad methods such as exome sequencing or WGS ensuring an adequate number of mutations to track. These mutations are then individually targeted using custom probes at subsequent time points. By focusing sequencing on known mutations, patient-specific MRD can achieve extremely high detection sensitivities for nearly all patients. Patient-specific MRD, however, can be logistically challenging and expensive to implement as it requires custom probe designs and validation for every patient. Patient-specific method also cannot detect newly acquired mutations that were not targeted by probes at diagnosis. Professional illustration by Patrick Lane, ScEYEnce Studios.

Figure 4. Identification of distinct subtypes of ALL through gene expression profiling.

Representative breakapart fluorescence in situ hybridization for *KMT2A::AFF1* fusion. Th upper panel shows a cell with DNA FISH for *KMT2A 5´* and *3´* showing one intact allele and one disrupted allele. The lower panel shows a second hybridization added on top of the first with *AFF1 3´* which confirms disruption of *KMT2A* and fusion to *AFF1 3*'. **B)** Cartoon showing overexpression of CRLF2 and detection by flow cytometry. The image was created in Biorender (https://biorender.com/). **C)** Schematic representation of *NUTM1*-rearrangements with multiple fusion partners and multiple breakpoints detected by whole transcriptome sequencing (WTS) and visualized in ProteinPaint (https://proteinpaint.stjude.org/). Ex, exon. The approach in parenthesis (whole genome sequencing, WGS) is alternative to WTS. **D)** Integrative Genomics Viewer visualization of *BCL11B* Enhancer Tandem Amplification (BETA) **E)** t-distributed stochastic neighbor embedding (t-SNE) representation from WTS data of B-All subtypes

highlighted in different colors. Each dot represents a sample (total samples = 2,004). Image is from Kimura *et al* [Blood. 2022 Feb 22;blood.2022015444]. Professional illustration by Patrick Lane, ScEYEnce Studios.

Data Sharing Statement

This review manuscript contains no original data.

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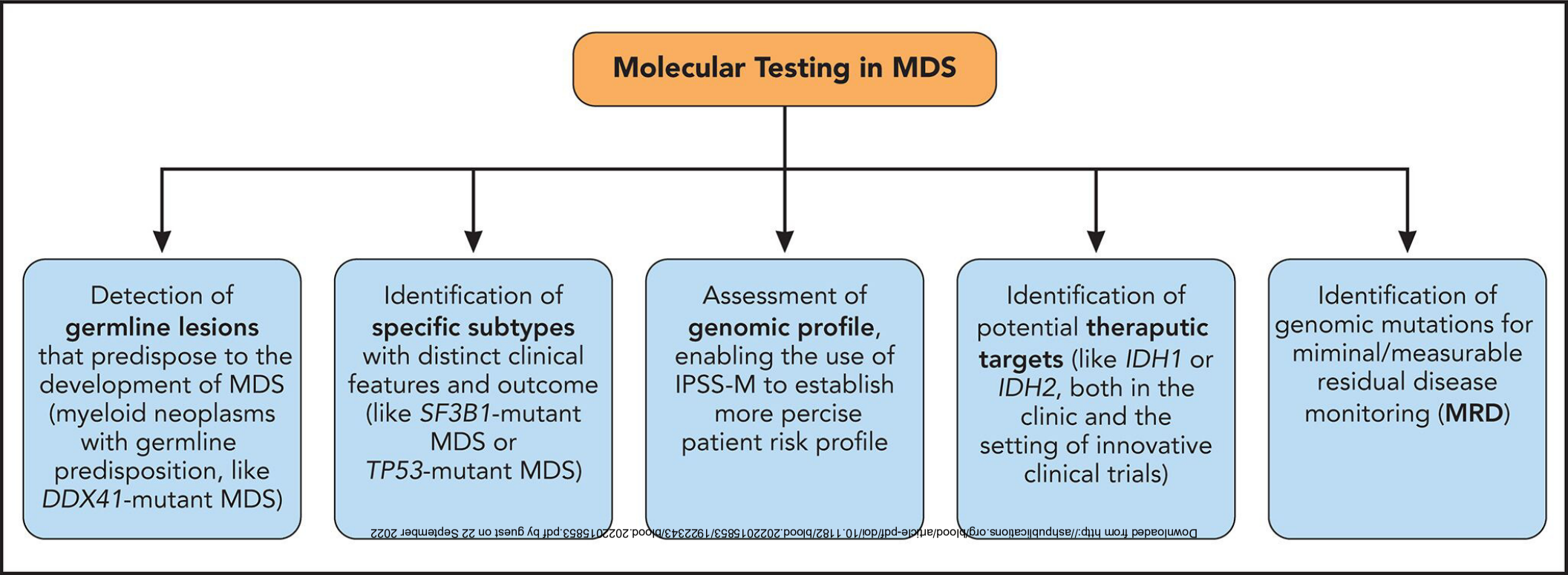
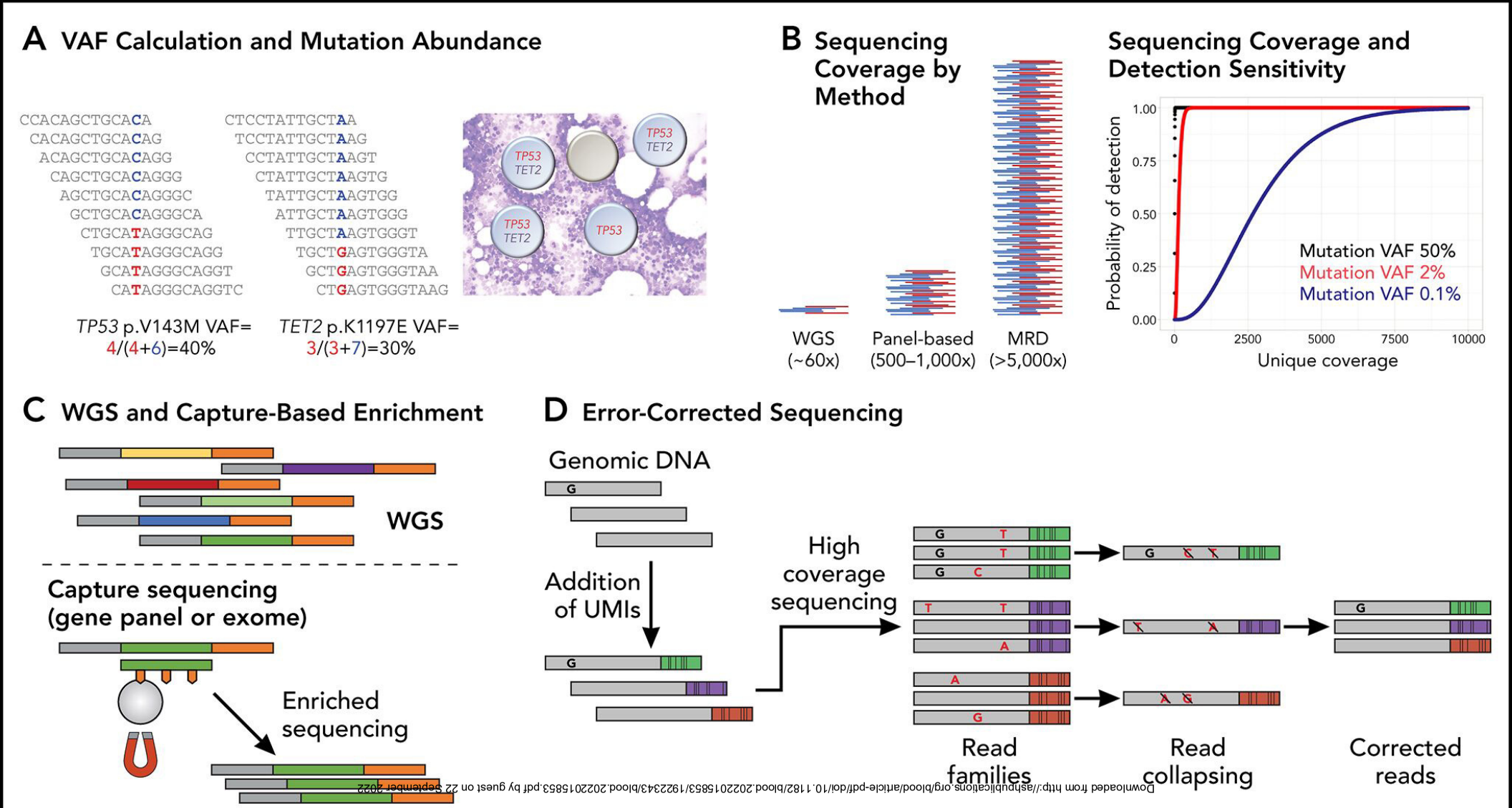
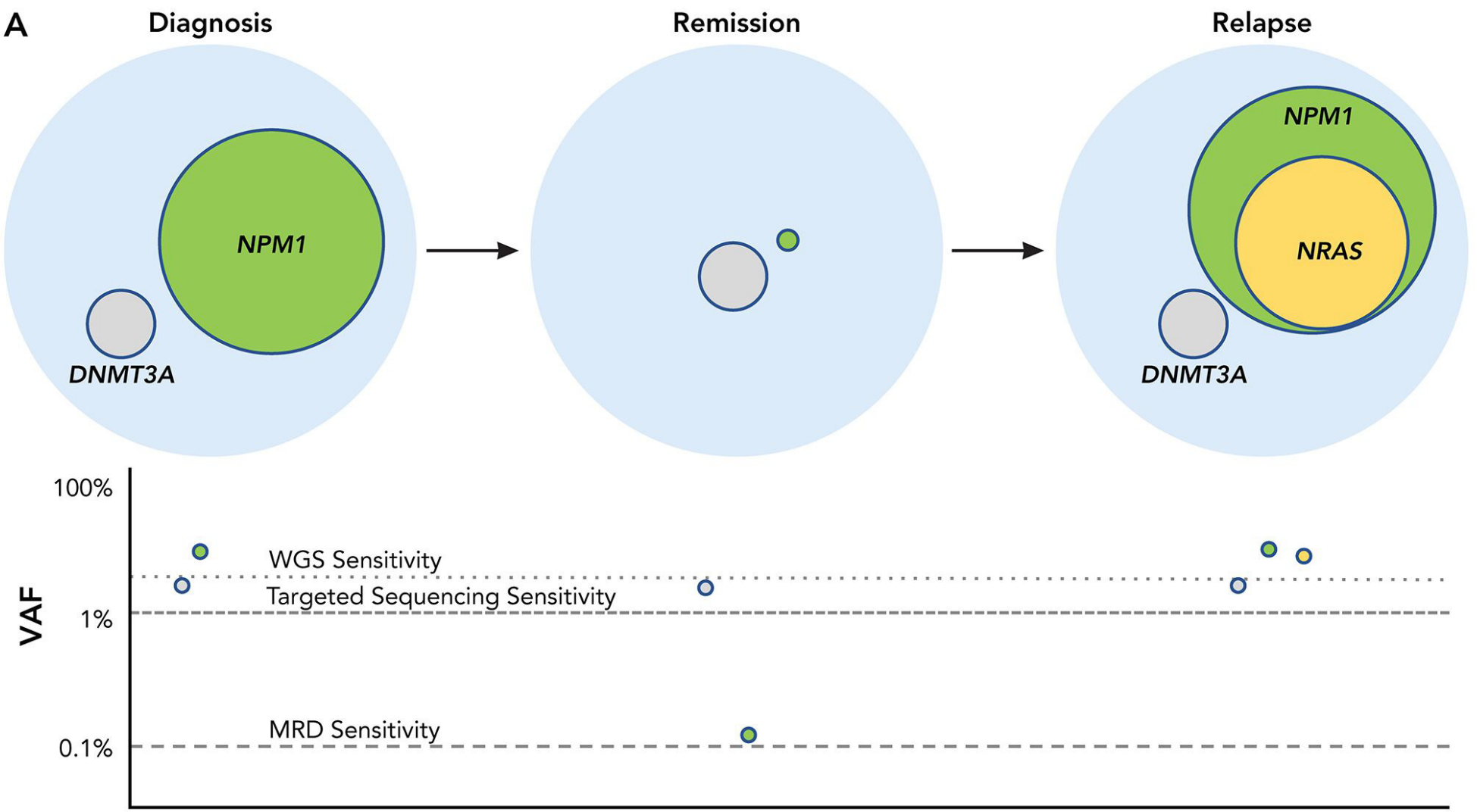




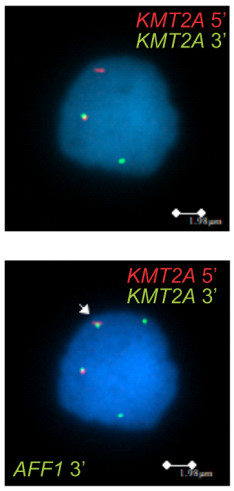


B

	Cost	Breadth	Depth	Sensitivity	Emerging Mutations	Structural Variants
WGS	$$$$			10%	+	+
Gene panel	$$			2%	+	+/–
MRD panel	$$$			~0.1%	+	–
Patient-specific MRD	$$$$			<0.1%	–	–

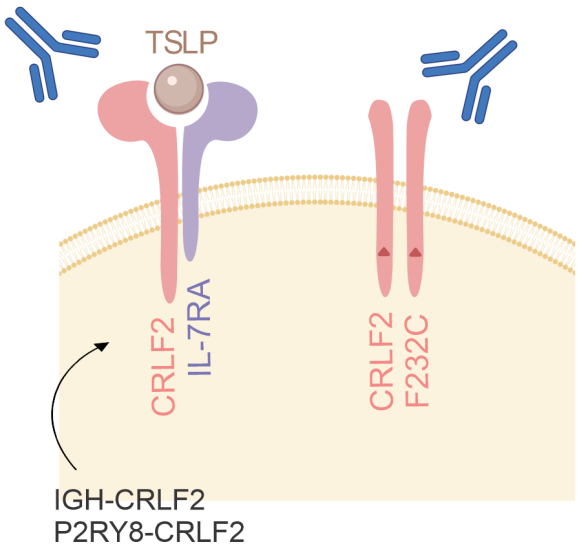
A

Breakapart FISH:
KMT2A::AFF1



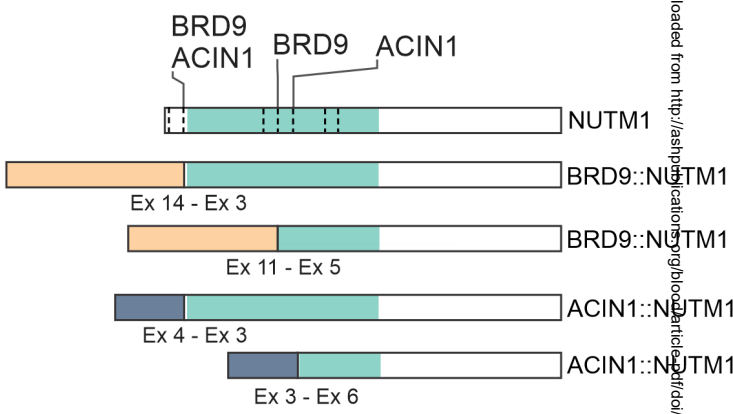
B

Flow cytometry:
CRLF2 overexpression



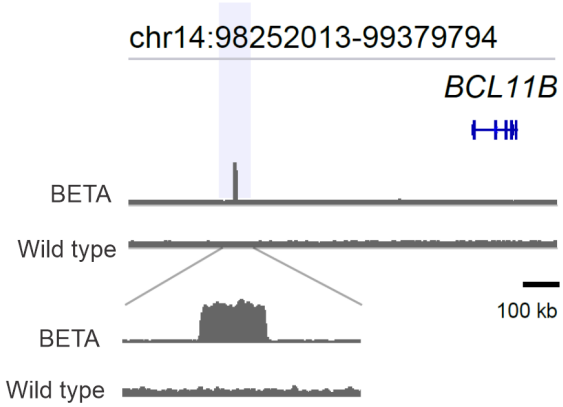
C

WTS (WGS): chimeric gene fusion detection



D

WGS: focal amplification (BETA)



E

WTS: B-ALL subtyping