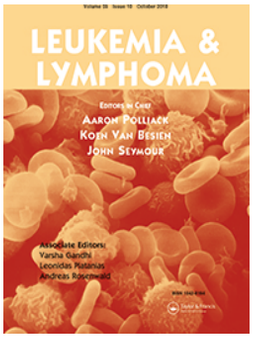

Leukemia & Lymphoma

ISSN: 1042-8194 (Print) 1029-2403 (Online) Journal homepage: https://www.tandfonline.com/loi/ilal20

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To cite this article: Mrinal M. Patnaik (2018) The importance of *FLT3* mutational analysis in acute myeloid leukemia, Leukemia & Lymphoma, 59:10, 2273-2286, DOI: 10.1080/10428194.2017.1399312

To link to this article: https://doi.org/10.1080/10428194.2017.1399312

LEUKEMIA & LYMPHOMA
2018, VOL. 59, NO. 10, 2273–2286
https://doi.org/10.1080/10428194.2017.1399312

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REVIEW

The importance of *FLT3* mutational analysis in acute myeloid leukemia

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ABSTRACT

Activating mutations in FMS-like tyrosine kinase 3 (*FLT3*), including internal tandem duplications (ITDs) and tyrosine kinase domain (TKD) mutations, are common in patients with acute myeloid leukemia (AML). *FLT3*-ITD is a negative prognostic factor that remains prognostically relevant even after intensive chemotherapy and/or stem cell transplant. *FLT3* testing was historically viewed as being purely prognostic; however, with the advent of *FLT3* inhibitors, it will likely be seen as both prognostic and predictive. The multikinase inhibitor midostaurin, in combination with chemotherapy, is the first targeted agent to significantly prolong survival in patients with newly diagnosed *FLT3*-mutated AML and was recently approved by health authorities. Recently, the European LeukemiaNet recommended *FLT3* testing (both TKD and ITD) for all patients with AML, with results required within 3 days. The need for optimized, multigene platform testing incorporating *FLT3* mutations will increase as knowledge of interactions between *FLT3* and other myeloid-relevant mutations grows.

ARTICLE HISTORY
Received 19 July 2017
Revised 29 September 2017
Accepted 24 October 2017

KEYWORDS
AML; *FLT3* testing; prognostic; predictive marker; *FLT3* tyrosine kinase inhibitor

FLT3 mutations in AML

FMS-like tyrosine kinase 3 (FLT3), a member of the type III receptor tyrosine kinase family [1,2], is expressed in ≈90% of leukemic blasts of patients with acute myeloid leukemia (AML) [3,4]. *FLT3* mutations occur in approximately one-third of patients with AML (Figure 1) [5–13]. In-frame duplications of 3 to >400 base pairs (bp), known as internal tandem duplications (ITDs), are the most common, occurring in up to 30% of adult patients with de novo AML [5,6,14]. However, *FLT3*-ITD is not expressed equally among patients with *FLT3*-ITD–positive (*FLT3*-ITD+) disease [15]. Differences in expression levels, measured using the *FLT3*-ITD-to-wild-type (WT) allelic ratio, impact prognosis [16]. This ratio is a measure of the relative signal intensity derived from the fluorescently labeled products amplified from the *FLT3*-ITD and *FLT3*-WT alleles using a polymerase chain reaction (PCR) assay [17,18]. Consensus is that a high *FLT3*-ITD-to-WT allelic ratio is a negative prognostic factor [17,19–25]; however, until recently, no standard definition existed as to what cut-off distinguished a low vs high allelic ratio. The 2017 European LeukemiaNet (ELN) guidelines defined 0.5 as the cutoff between low (*FLT3*-ITD^{low}; <0.5) and high (*FLT3*-ITD^{high}; ≥0.5) allelic ratios [16].

Mutations within the tyrosine kinase domain (TKD) are the second most common type of *FLT3* mutation in AML (occurring in up to 14% of adult patients with AML) [13,17,26]. Mutations within the TKD are primarily point mutations within the activation loop (e.g. residues D835, I836, and Y842) of the TKD2 [6,13,18,27] and, to a lesser extent, within the TKD1 (e.g. residues N676 and F691) [12,27]. Other point mutations and smaller insertions/deletions have also been identified within the TKD and other domains (e.g. extracellular and juxtamembrane domains [occurring in ≈2% of patients with AML]) [8,9,11,13]. The prognostic significance of *FLT3*-TKD mutations in the overall AML population and the impact of the *FLT3*-TKD allelic ratio are still debatable and may depend on additional mutations as well as the cytogenetic background [13,24].

Both *FLT3*-ITD and *FLT3*-TKD mutations are common in patients with AML with normal karyotype (30–39% and 6–14%, respectively), but they are also associated with karyotypic abnormalities, such as t(15;17)/PML-RARA (30–39% and 8–9%, respectively) and core binding factor AML (5–8% and 4–14%, respectively) [13,17,26,28,29]. *FLT3*-ITD is also frequently associated with t(6;9) [*DEK-NUP214*] abnormalities (in up to 90% of patients) [17,30,31]. Importantly, the prognostic impact of *FLT3* mutations can vary by cytogenetic group. For example, in patients with t(15;17) abnormalities, there was no difference in outcome between those with and without *FLT3*-ITD mutations; however,

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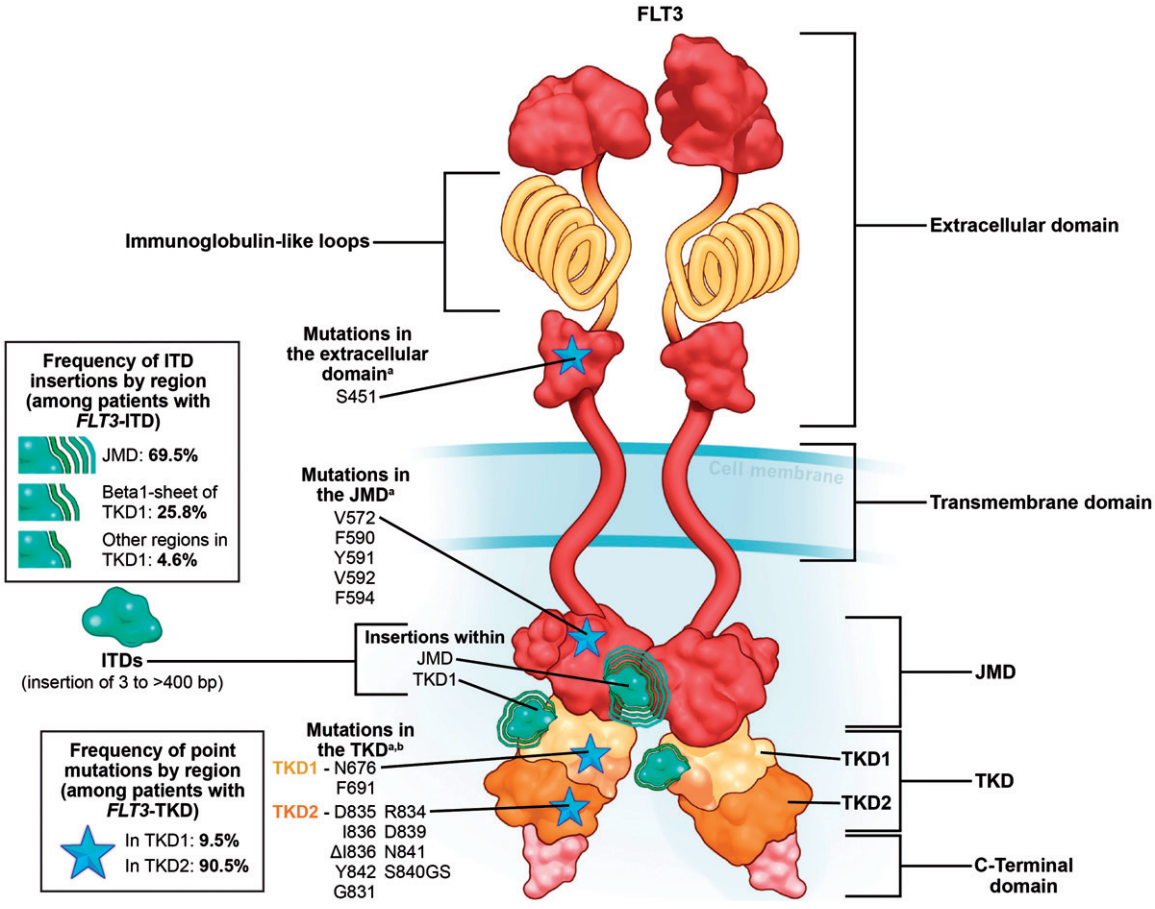

Figure 1. FMS-like tyrosine kinase 3 (*FLT3*) contains 5 functional domains: an immunoglobulin-like extracellular domain, a transmembrane domain, a juxtamembrane domain (JMD), an interrupted tyrosine kinase domain (TKD), and a small C-terminal domain. Internal tandem duplications (ITDs), insertions of 3 to >400 base pairs (bp), are the most common mutations in *FLT3*. ITDs occur in up to 30% of patients with acute myeloid leukemia (AML); of these, 69.5% are located in the JMD and 30.5% are located in the TKD (25.8% in the beta1-sheet and 4.6% in other regions). Activating mutations within the TKD occur in up to 14% of patients with AML; of these, 90.5% are located within the activation loop of the TKD2 and 9.5% are located within the TKD1. Additional activating mutations have been identified at the very low frequency within the extracellular domain (<1% of cases) and the JMD (<1–2% of cases) [5–13]. [a]Additional point mutations that have been identified in patients with AML – but have not been found to be activating mutations *in vitro* – include mutations within the extracellular domain (e.g. T167, V194, D324, Y364, and V491), transmembrane domain (e.g. I548 and V557), JMD (e.g. V579 and E598), TKD1 (e.g. A680 and M737), and TKD2 (e.g. V816, A814, and T784) [6,8,9]. [b]The majority of mutations within the TKD are point mutations that result in amino acid changes; however, activating mutations caused by insertions (e.g. insertion of glycine and serine between residues S840 and N841 [S840GS]) and deletions (e.g. ΔI836 and ΔE598/Y599) have also been identified in the TKD [6,10,11,13].

patients with *FLT3*-TKD had significantly worse outcomes (compared with those with *FLT3*-WT) [13,17,32]. Furthermore, recent advances indicate that the prognosis for patients with *FLT3* mutations can be affected by the presence or absence of additional mutations [14,16,33]. For example, patients who are *FLT3*-ITD negative (*FLT3*-ITD−) or *FLT3*-ITD^{low} and positive for nucleophosmin 1 mutations (*NPM1*+) have a favorable prognosis, whereas those who are *FLT3*-ITD − or *FLT3*-ITD^{low} with *NPM1*-WT or *FLT3*-ITD + and *NPM1*+ have an intermediate prognosis. Patients who are *FLT3*-ITD^{high} with *NPM1*-WT have a poor prognosis [16] and are less likely to achieve complete remission (CR) with induction chemotherapy than patients with other *FLT3*/*NPM1* combinations ($p < .005$) [34].

FLT3 testing: a prognostic marker

Current FLT3 testing landscape

Historically, patients with AML were stratified into risk groups based on age, performance status, white blood cell count, and cytogenetics [35]. Subsequently, gene mutations (e.g. *NPM1*, *FLT3*, *TP53*, and *CEBPA*) were recognized as important prognostic factors and thus included in testing recommendations in the United States and Europe [16,36]. Until recently, *FLT3* testing

was recommended as a prognostic marker only in patients with cytogenetically normal AML. However, new recommendations for *FLT3* testing in all patients with AML are a result of the approval of the first *FLT3*-targeted therapy, midostaurin, and the recognition that *FLT3* is a negative prognostic marker, regardless of cytogenetics [16,36–37]. Importantly, results of *FLT3* testing should be made available within 48–72 h after the initial diagnosis of AML so that targeted therapy can be initiated in a timely manner [16].

Little information exists on the real-world *FLT3* testing rates in patients with AML, but a retrospective chart review suggests that despite the recommendations, *FLT3* testing is not always performed, even in patients with cytogenetically normal AML. According to a retrospective registry review of molecular marker testing performed at a single referral center between 2010 and 2012, only 77% of patients with cytogenetically normal AML were routinely tested for *FLT3* [38]. Furthermore, there is a gap in molecular testing rates (including *FLT3*) between academic centers and community referral sites, as suggested by the results of a single-institution retrospective chart review that analyzed molecular testing rates over time (2008–2012). Despite an increase in testing over time, testing rates were significantly higher at academic centers than at community sites (93% vs 41%; $p < .001$) [39]. Routine testing for *FLT3* in patients with cytogenetically normal AML had been recommended since at least 2010 [40], which corresponds to the time at which molecular testing was routinely performed in 100% of patients at academic centers but not at community sites [39]. This suggests that there is a lack of awareness or knowledge about the importance of molecular testing at community sites. More recently (2015), 294 members of professional societies in the United States and Europe were surveyed about their testing practices. Among responders, 51 and 46% indicated that they tested for *FLT3*-ITD in all patients and selected patients, respectively [41]. This survey was intended to provide a baseline for testing prior to the release of the diagnostic workup guidelines jointly issued by the College of American Pathologists and the American Society of Hematology in 2017 [36]. It would be expected that testing rates, particularly those for *FLT3*, will soon increase given that *FLT3*-targeted therapies are entering the market. One potential hurdle to widespread *FLT3* testing in the past was the lack of commercially available tests. It will be interesting to see whether testing rates at community sites will catch up to those at academic centers – especially now that commercially developed *FLT3* testing assays are routinely incorporated into clinical trials and are beginning to hit the market [42,43].

Methods for testing FLT3

The first method for the prognostic identification of *FLT3*-ITD mutations involved PCR amplification and subsequent sequencing of the juxtamembrane domain region within the *FLT3* gene [44]. Since then, several methods have been developed or adapted for identifying mutations and aberrant karyotypes (Table 1) [45–51]. These methods vary in their sensitivity, turnaround time, and development stage [52]. Some methods have been used in the clinic for >10 years, while others are still being validated.

The first method to be readily adopted and widely used in clinical trials is a modified PCR technique that uses capillary electrophoresis to resolve fluorescently labeled PCR products and can measure the *FLT3*-ITD-to-WT allelic ratio [18]. Subsequently, a multiplex PCR assay was developed that uses two sets of fluorescently labeled primers to simultaneously amplify the ITD and D835 mutant regions [50]. The resulting PCR products are then digested with EcoRV restriction endonuclease and resolved using capillary electrophoresis. *FLT3*-ITD mutations are identified by comparing the size of the amplification products (the reference WT product is 330 bp; ITDs are >330 bp). Mutations in D835 and I836 remove a naturally occurring EcoRV restriction endonuclease site in the WT amplification product, resulting in a larger fluorescently labeled fragment (129 bp; the WT product is 80 bp). Real-time

Table 1. Comparison between *FLT3* testing methods.

FLT3 testing technique	Specificity for *FLT3* mutants	Sensitivity[a]	Turnaround time
Fluorescence-labeled polymerase chain reaction [50,51]	Highly specific (>99%); detects mutations only within amplified region	5%	3 d
Whole-genome sequencing [46,49]	Unbiased approach; detects *FLT3*-ITD and other *FLT3* mutants	>20%	7–12 d
Whole-exome sequencing [46]	Unbiased approach; detects *FLT3*-ITD and other *FLT3* mutants	>5%	Not reported; faster than whole-genome sequencing
Multiplex-targeted next-generation sequencing [46–48]	Unbiased approach; 99–100% detection of *FLT3* mutants	1–2%	3–20 d
Karyogene [45]	Highly specific (100%); samples are enriched for *FLT3* exons	>5%	<14 d[b]

FLT3: FMS-like tyrosine kinase 3; ITD: internal tandem duplication
[a]Detection of mutant allele variants per fraction of total cells.
[b]For samples run once weekly; turnaround time can be <10 d for samples run twice weekly.

quantitative PCR (RT-qPCR)–based tests have been proposed as alternatives for detecting *FLT3*-ITD, *FLT3*-TKD, and other point mutations [53,54] and can also be used for monitoring disease progression (see Role in detection of minimal residual disease). PCR-based methods have short turnaround times [50,51] and are highly selective. Their major limitation is that very few *FLT3*-TKD point mutations can be detected unless the PCR products are sequenced.

More recently, next-generation sequencing (NGS) approaches have been developed that are capable of screening many molecular markers. These NGS approaches can be broadly divided into two large groups: whole-genome sequencing, which captures the entire genome; and whole-exome sequencing, which selects for protein coding regions within the genome [46]. Despite their tremendous potential, NGS approaches are currently not suitable for the clinic: they generate large amounts of data that can be overwhelming for hematologists and may not provide additional value for the diagnosis and treatment of patients with AML. They also have long turnaround times. Additionally, *FLT3*-ITD is inherently difficult to detect using NGS approaches [46,47,55].

Multiplex-targeted NGS approaches, also known as gene panels, are more suitable for the clinic because they have rapid turnaround times and are highly sensitive for detecting variant alleles [46]. Using a recently validated 54-gene panel, researchers identified *FLT3*-ITDs of varying lengths and insertion sites at lower thresholds than conventional methods could detect [48]. Karyogene, a recently developed diagnostic tool that uses DNA capture to enrich for specific genes and cytogenetic abnormalities sequenced by high-throughput sequencing and analyzed with open-source software, was able to detect 49 predefined recurrent gene mutations, four chromosomal rearrangements, and several copy number aberrations in 62 samples from patients with AML [45]. Adopting a technology such as Karyogene has its advantages (e.g. it integrates cytogenetic and molecular diagnosis into a single method and has a relatively short turnaround time [$<$10 d]) [45] and disadvantages (e.g. it requires specialized high-throughput sequencing equipment and technical knowledge and skills).

Similarly, the use of gene panels for *FLT3* testing has both advantages and disadvantages. An advantage is that this technology can detect rare mutations and could aid in enrolling patient subgroups into clinical trials to better understand the impact of such mutations. For example, this technology would be useful to determine the prognostic and therapeutic impact of the recently identified, rare N767 mutation that confers resistance to certain *FLT3* inhibitors *in vitro* [12,56]. A potential disadvantage is that gene panel testing can have longer turnaround times (3–20 d) [46] than conventional PCR-based methods (48–72 h) currently used to screen patients in clinical trials [16,57,58].

Treatment for patients with *FLT3*-mutated AML

Until recently, the standard of care for patients with AML – induction and consolidation chemotherapy – remained unchanged for $>$25 years [59,60]. Outside the context of a clinical trial, therapy for patients with newly diagnosed AML depends on age, fitness level, and eligibility to receive intensive induction chemotherapy [16,59]. Most fit patients generally receive intensive anthracycline- and cytarabine-based induction chemotherapy, whereas older or unfit patients may receive lower-intensity induction chemotherapies (e.g. low-dose cytarabine or hypomethylating agents). For patients who achieve CR, the choice of consolidation therapy depends on their risk stratification group (i.e. favorable, intermediate, or unfavorable): patients with favorable risk receive high-dose cytarabine, whereas patients with intermediate or unfavorable risk in first complete remission (CR1) often undergo allogeneic hematopoietic stem cell transplant (alloHSCT), if eligible [16,59]. Before now, no targeted therapies were approved for patients with *FLT3*-mutated AML [37,61]. Despite this, outcomes in patients with *FLT3* mutations have improved over the past 15 years [62]. In a retrospective study of patients with AML evaluated at a single institution from 2000 to 2014, an increasing number underwent HSCT over time; and those who underwent HSCT, particularly in CR1, had improved survival compared with patients who did not receive an HSCT. In the study, a trend toward better response rates was seen in patients who received first-line chemotherapy in combination with *FLT3* inhibitors (mostly in the setting of a clinical trial) compared with those who did not [62].

Role of alloHSCT therapy

Transplant rates have increased significantly over the past 20 years, accompanied by increasing survival rates in patients with AML [62,63]. AlloHSCT is usually recommended for patients with *FLT3*-ITD mutations in CR1 who are eligible for transplant therapy and have a suitable donor [16,59]. These recommendations are supported by data from retrospective analyses

[19,64–70] but have yet to be validated in prospective trials.

Among patients with *FLT3*-ITD mutations in CR1, those who undergo alloHSCT have significantly better outcomes (e.g. prolonged survival and decreased risk of relapse) than those who receive chemotherapy alone [65]. Patients who have *FLT3*-ITD^{high} [19,64,70] or *FLT3*-ITD^{low} with *NPM1*-WT derive the most benefit from alloHSCT [19,64]. Despite this, *FLT3*-ITD remains a poor prognostic factor following alloHSCT [63,71]. Results from early-phase and retrospective studies suggest that patients with *FLT3*-ITD AML may benefit from the use of FLT3 tyrosine kinase inhibitors (TKIs) as maintenance therapy to prevent relapse following alloHSCT [19,72–75] – a hypothesis currently being investigated in clinical trials [76–80]. In the United States, the TKI sorafenib is often used off-label as post-transplant maintenance therapy [81].

Importantly, an analysis of alloHSCT rates outside the clinical trial setting revealed that less than half of patients who achieved CR went on to receive alloHSCT in CR1 (49.1%) [82], suggesting that real-world transplant strategies need streamlining.

FLT3 inhibitors

Multiple small-molecule TKIs that target FLT3 are in development for the treatment of patients with AML (Table 2) and have demonstrated clinical activity as a single agent or in combination with chemotherapy [19,27,58, 72–75,83–98]. Several FLT3 TKIs – including the multikinase inhibitors midostaurin and sorafenib and the more-selective FLT3 inhibitors crenolanib, gilteritinib, and quizartinib – are currently being evaluated or have completed evaluation in phase 3 clinical trials (Table 3) [58,78,80,99–108]. Each of these FLT3 TKIs has advantages and disadvantages. It has recently been proposed that multikinase inhibitors, such as midostaurin and sorafenib, are better suited as first-line therapy because of the polyclonal nature of AML, whereas more-selective agents, such as crenolanib, gilteritinib, and quizartinib, are more appropriate in the relapsed/refractory (R/R) setting [81]. Furthermore, even though all FLT3 TKIs have demonstrated inhibitory activity against ITD mutations, not all of them target important TKD mutations, such as the F691L 'gatekeeper' resistance mutation [27,84–86,91, 92,95,109].

In the Randomized AML Trial in *FLT3* patients <60 Years old (RATIFY), the largest study conducted to date in adult patients (aged 18 to <60 years) with newly diagnosed AML with *FLT3* mutations (ITD and TKD), midostaurin in combination with intensive induction and consolidation chemotherapy and as single-agent maintenance therapy reduced the risk of death compared with placebo by 22% and improved event-free survival (EFS) and disease-free survival [58]. The benefit in overall survival (OS) and EFS was independent of HSCT and FLT3 mutation status (*FLT3*-ITD^{high} [≥0.7], *FLT3*-ITD^{low} [<0.7], or *FLT3*-TKD). Grade 3/4 adverse events were comparable between the two arms except for rash, which was more common in the midostaurin arm. Midostaurin, in combination with induction and consolidation chemotherapy, became the first FLT3 TKI approved in the United States [37] and is listed as a potential therapy for patients with *FLT3*-mutated AML beginning in version 2 of the National Comprehensive Cancer Network guidelines [59] and the 2017 ELN recommendations [16]. Additional ongoing studies are evaluating midostaurin as frontline treatment for *FLT3*-ITD + AML (patients aged 18–70 years) in combination with lower-intensity therapies and as maintenance therapy following HSCT [76,79,110,111].

Sorafenib, in combination with standard chemotherapy, was evaluated in adults (aged 18–60 years) with newly diagnosed AML in the randomized, placebo-controlled, phase 2 Sorafenib in AML in patients ≤60 years (SORAML) trial [112]. Sorafenib demonstrated significant improvement compared with placebo in EFS ($p = .013$) and relapse-free survival ($p = .017$) but not OS ($p = .382$) in all patients; a similar trend in improvement, albeit not significant, was observed in patients with *FLT3*-ITD mutations (only 17% of patients had *FLT3*-ITD mutations). Sorafenib was associated with an increased risk of bleeding, fever, and hand-foot syndrome [112]. Addition of sorafenib to intensive chemotherapy did not result in clinical benefit (no significant improvements were observed in EFS or OS compared with placebo, and there was an increased rate of early death compared with placebo) in older patients (aged 61–80 years) [113]. Sorafenib was the first agent to demonstrate single-agent activity as maintenance therapy following HSCT. Promising results from several phase 1 and retrospective studies in patients with *FLT3*-ITD + AML [19,72–75,83] have led to a flurry of new studies evaluating *FLT3* TKIs in this setting (Table 3). Sorafenib showed promising activity in combination with azacitidine and decitabine (phase 2 and single-institution retrospective studies, respectively) in patients with *FLT3*-ITD + R/R AML [114,115]. Sorafenib is currently being investigated as frontline treatment for AML in combination with azacitidine in patients not eligible for standard chemotherapy and as single-agent maintenance therapy following alloHSCT [80,107,116]. In the United States, sorafenib is routinely

Table 2. Comparison of FLT3 inhibitors in late-phase clinical trials.

FLT3 inhibitor	Cell proliferation[a] Mutation	IC_{50}, nM	Other targets	Efficacy and safety[b]
Crenolanib (CP-868-596) [95–98]	ITD	9	PDGFR	• Single-agent activity (CR/CRi: 22%) in *FLT3*-mutated R/R AML[c] • High response rates (CR/CRi: 81%) in combination with intensive induction chemotherapy • Most common any-grade AEs: nausea, vomiting, diarrhea, infections, and rash • Most common grade 3/4 AEs: infections, rash, and nausea
	D835Y	5		
	ITD/D835Y	12		
	ITD/F691L	55		
Gilteritinib (ASP2215) [85,94]	ITD	2	AXL, LTK	• Single-agent activity (CR/CRi: 35%) in *FLT3*-mutated R/R AML • Most common any-grade AEs: diarrhea, fatigue, and abnormal liver function • Most common grade ≥3 AEs: febrile neutropenia, infection, and pneumonia
	D835Y	2		
	ITD/D835Y	2		
	ITD/F691L	22		
Midostaurin (PKC412) [58,86,91–93]	ITD	8	KIT, PDGFR, PKC, VEGFR2	• Limited single-agent activity • First agent to demonstrate significant survival benefit (vs placebo) in combination with chemotherapy • Most common any-grade AEs: nausea, vomiting, diarrhea, and fatigue • Most common grade 3/4 AEs: febrile neutropenia and infections • Midostaurin was associated with a significantly higher risk of rash (vs placebo) when administered in combination with chemotherapy
	D835Y	<10		
	ITD/D835Y	15		
	ITD/F691L	10		
Quizartinib (AC220) [27,85–90]	ITD	<1	KIT, PDGFR	• Single-agent activity (CR/CRi: 47%) in *FLT3*-mutated R/R AML • Initial QT prolongation concerns no longer an issue with lower doses (efficacy comparable to that of higher doses) • Promising preliminary efficacy in combination with chemotherapy • Most common any-grade AEs: nausea, vomiting, diarrhea, pyrexia, and fatigue • Most common grade 3/4 AEs: febrile neutropenia and nausea
	D835Y	6		
	ITD/D835Y	23–35		
	ITD/F691L	128		
Sorafenib [19,27,72–75,83,84]	ITD	1–2	KIT, PDGFR, RAF, VEGFR2/3	• Activity as single-agent maintenance therapy following HSCT and in combination with AZA as salvage therapy • In combination with chemotherapy, did not show a significant OS benefit in younger (aged 18–60 years) or older (aged 61–80 years) adult patients • Most common any-grade AEs: diarrhea, rash, nausea, and fatigue • Most common grade 3/4 AEs: rash, abdominal pain, and weight loss
	D835Y	>1500		
	ITD/D835Y	>2000		
	ITD/F691L	>2300		

AE: adverse event; AML: acute myeloid leukemia; AZA: azacitidine; CR: complete response; CRi: CR with incomplete blood count recovery; FLT3: FMS-like tyrosine kinase 3; IC_{50}: 50% inhibitory concentration; HSCT: hematopoietic stem cell transplant; ITD: internal tandem duplication; OS: overall survival; PDGFR: platelet-derived growth factor receptor; PKC: protein kinase C; R/R: relapsed/refractory; VEGFR: vascular endothelial growth factor receptor

[a]Measured in Ba/F3 cells transformed with plasmids carrying the indicated *FLT3* mutations.

[b]Only the most common nonhematologic AEs are listed.

used off-label as single-agent maintenance therapy following transplant [81] and in combination with hypomethylating agents as salvage therapy in patients with *FLT3*-mutated R/R AML [59,81].

Crenolanib, gilteritinib, and quizartinib have demonstrated single-agent activity in patients with R/R AML with *FLT3* mutations [89,94,98]. Among these agents, quizartinib is the most selective *FLT3*-ITD inhibitor and has shown the strongest single-agent activity in this patient population (Table 2). Despite initial safety concerns about QT prolongation with quizartinib in early studies, it has not been an issue in subsequent studies evaluating lower doses in which high response rates have been maintained [89]. Quizartinib is being evaluated in a phase 3, randomized study compared with salvage chemotherapy in patients with *FLT3*-ITD + R/R AML [102] and as frontline treatment for patients with *FLT3*-ITD + AML [106].

Crenolanib is currently being investigated in combination with salvage chemotherapy in two randomized, placebo-controlled, phase 3 studies in patients with R/R AML [100,103]. Crenolanib has shown

Table 3. Ongoing phase 3 clinical studies evaluating FLT3 inhibitors in patients with AML.

FLT3 inhibitor, development phase (study ID), study design (expected accrual)	Study status and enrollment (*expected primary completion date*)
Patients with newly diagnosed AML [58,106–108]	
Crenolanib, phase 3 (ARO-021; NCT03258931) Randomized, double-blind study of crenolanib or midostaurin in combination with induction and consolidation chemotherapy in patients (aged 18–60 years) with newly diagnosed AML with *FLT3* mutations ($N = 510$)	Not yet recruiting; anticipated start date, Nov 2017 (*Nov 2022*)
Midostaurin, phase 3 (RATIFY; CALGB 10603; NCT00651261) Randomized, double-blind, placebo-controlled study of midostaurin in combination with intensive induction and consolidation chemotherapy and as single-agent maintenance therapy in patients (aged 18–60 years) with newly diagnosed AML with *FLT3* mutations ($N = 717$)	Midostaurin significantly improved OS and EFS across all *FLT3* subgroups tested (high and low ITD-to-WT allelic ratio and TKD); benefit remained after censoring for HSCT. Ongoing, but not recruiting; primary data collection has been completed[a]
Quizartinib, phase 3 (QuANTUM-First; NCT02668653) Randomized, double-blind, placebo-controlled study of quizartinib in combination with intensive induction and consolidation chemotherapy and as single-agent maintenance therapy in patients (aged 18–75 years) with newly diagnosed *FLT3*-ITD + AML ($N = 536$)	Recruiting; accrual of patients began in May 2016 (*Nov 2020*)
Sorafenib, phase 3 (NCT01371981) Randomized, open-label study of bortezomib or sorafenib in combination with chemotherapy vs chemotherapy alone in patients with newly diagnosed AML ($N = 1750$)	Recruiting; accrual of patients began in Jan 2011 (*Sep 2018*)
Patients not eligible for intensive chemotherapy [105]	
Gilteritinib, phase 2/3 (LACEWING; NCT02752035) Randomized, open-label, three-arm study of gilteritinib alone or in combination with AZA vs AZA alone in patients (aged ≥18 years) with newly diagnosed AML with *FLT3* mutations who are not eligible to receive intensive induction chemotherapy ($N = 540$)	Recruiting; accrual of patients began in Jun 2016 (*May 2020*)
Patients with R/R AML [100–104]	
Crenolanib, phase 3 (ARO-007; NCT02298166)[b] Randomized, double-blind, placebo-controlled study of crenolanib in combination with salvage chemotherapy in patients (aged ≥18 years) with *FLT3* mutation–positive R/R AML ($N = 276$)	Trial was registered in Nov 2014 but has yet to commence enrollment (*Apr 2022*)
Crenolanib, phase 3 (ARO-013; NCT03250338) Randomized, double-blind, placebo-controlled study of crenolanib in combination with salvage chemotherapy in patients (aged 18–75 years) with *FLT3* mutation–positive R/R AML ($N = 322$)	Not yet recruiting; anticipated start date, Oct 2017 (*Oct 2020*)
Gilteritinib, phase 3 (ADMIRAL; NCT02421939) Randomized, open-label study of gilteritinib monotherapy vs salvage therapy (LDAC, MEC, AZA, or FLAG-IDA) in patients (aged ≥18 years) with R/R AML with *FLT3* mutations ($N = 369$)	Recruiting; accrual of patients began in Oct 2015 (*Jun 2018*)
Gilteritinib, phase 3 (NCT03182244) Randomized, open-label study of gilteritinib monotherapy vs salvage therapy (LDAC, MEC, or FLAG-IDA) in patients (aged ≥18 years) with R/R AML with *FLT3* mutations ($N = 318$)	Not yet recruiting; anticipated start date, Sep 2017 (*Mar 2020*)
Quizartinib, phase 3 (QuANTUM-R; NCT02039726) Randomized, open-label study of quizartinib monotherapy vs salvage therapy (LDAC, MEC, or FLAG) in patients (aged ≥18 years) with *FLT3*-ITD + R/R AML ($N = 363$)	Recruiting; accrual of patients began in Apr 2014 (*Feb 2018*)
Posttransplant maintenance [78,80]	
Gilteritinib, phase 3 (MORPHO; NCT02997202) Randomized, double-blind, placebo-controlled study of gilteritinib as maintenance therapy following alloHSCT in adult patients (aged ≥18 years) with *FLT3*-ITD + AML ($N = 346$)	Recruiting; accrual of patients began in Jun 2017 (*Aug 2024*)
Sorafenib, phase 4 (NCT02474290) Single-arm, open-label study of sorafenib maintenance therapy in patients (aged 18–60 years) with *FLT3*-ITD + AML who received alloHSCT ($N = 200$)	Recruiting; accrual of patients began in Jun 2015 (*May 2018*)
Maintenance following chemotherapy [99]	
Gilteritinib, phase 3 (GOSSAMER; NCT02927262) Randomized, double-blind, placebo-controlled study of gilteritinib as maintenance therapy following induction/consolidation therapy in patients with *FLT3*-ITD + AML in CR1 ($N = 354$)	Recruiting; accrual of patients began in Jan 2017 (*Mar 2024*)

alloHSCT: allogeneic hematopoietic stem cell transplant; AML: acute myeloid leukemia; AZA: azacitidine; CR1: first complete remission; EFS: event-free survival; FLAG-IDA: fludarabine + cytarabine + granulocyte colony-stimulating factor + idarubicin; FLT3: FMS-like tyrosine kinase 3; ID: identifier; ITD: internal tandem duplication; LDAC: low-dose cytarabine; MEC: mitoxantrone + etoposide + cytarabine; OS: overall survival; R/R: relapsed/refractory; TKD: tyrosine kinase domain; WT: wild type.

[a]The primary endpoint data for the RATIFY study were recently published [58]. However, Stone et al. [58] indicated that a supportive analysis for the OS endpoint will be reported at a later date and with a longer follow-up.

[b]A phase 1 study is also evaluating crenolanib in combination with sorafenib, a FLT3 inhibitor, in patients with R/R hematologic malignancies (NCT02270788).

promising activity in combination with intensive induction (cytarabine + daunorubicin or idarubicin) and consolidation chemotherapy in newly diagnosed *FLT3*-mutated AML, as demonstrated by the high overall response rates (CR/CR with incomplete blood count recovery: 88%) observed in preliminary analyses of an ongoing phase 2 study [97]. Crenolanib is also being evaluated in a phase 3 study (compared with midostaurin) in combination with induction and consolidation chemotherapy in newly diagnosed *FLT3*-mutated AML [108].

Gilteritinib, a highly selective FLT3-mutant inhibitor (including F691L), has undergone rapid development after initial promising single-agent activity [94]; there are currently five ongoing phase 3 trials. The first two trials are evaluating gilteritinib compared with salvage chemotherapy in patients with R/R AML [101,104]. A third trial is evaluating gilteritinib alone or in combination with azacitidine compared with azacitidine alone in patients with newly diagnosed AML who are not eligible for intensive chemotherapy [105]. Two additional trials are evaluating single-agent gilteritinib maintenance therapy following either alloHSCT or induction/consolidation chemotherapy in patients with *FLT3*-ITD + AML [78,99].

Additional studies of FLT3 inhibitors include early-phase trials of single-agent FLX925 (NCT02335814) and TAK-659 (NCT02323113) in R/R AML. A study of E6201 in patients with *FLT3*-mutated R/R AML or older patients (aged ≥60 years) with newly diagnosed AML who are not eligible for standard chemotherapy (NCT02418000) was recently terminated. Ponatinib, originally developed as a BCR-ABL1 inhibitor, has shown preclinical activity in *FLT3*-mutated AML models *in vitro* [117]; ongoing early-phase trials are evaluating ponatinib as frontline treatment for AML (NCT02779283) and as maintenance therapy in patients with *FLT3*-ITD AML in CR1 (NCT02428543).

New role of *FLT3* testing: diagnostic marker that drives therapy

Given the increasing knowledge of AML pathobiology and advances in *FLT3* testing methods, the current *FLT3* testing paradigm is likely to evolve. New risk-stratification models have been proposed that integrate the identification of additional molecular markers into the routine diagnostic workup [14,118]. More-radical proposals forgo cytogenetic testing and suggest implementing molecular markers as the sole determinant of risk stratification [119]. Adopting such a model would increase *FLT3* testing rates. Currently, some guidelines recommend *FLT3* testing for patients with normal cytogenetics only [120], but patients with abnormal cytogenetics also harbor *FLT3* mutations [6].

FLT3 testing will continue to be an important prognostic determinant and can guide therapeutic decisions [16,37]; thus, demand for rapid *FLT3* testing will likely increase in the future. There are three major areas that are critical to ensure that *FLT3* testing is clinically relevant: (1) universal adoption, (2) rapid turnaround times, and (3) harmonization. First, barriers to adoption can be overcome by increasing awareness about and access to *FLT3* testing. As previously mentioned, *FLT3* testing is now recommended for all patients with AML, and commercial kits are now available. Second, rapid turnaround times (<8 d) are required for patients with newly diagnosed AML to be able to receive midostaurin (the only approved FLT3 inhibitor to date) in combination with chemotherapy [37,61]. Current recommendations requiring *FLT3* testing results within 72 h [16] are well within these rapid turnaround times. However, it is not clear whether this benchmark will be met in the real-world setting. Third, given that the *FLT3*-ITD-to-WT allelic ratio is a determinant of risk stratification [16], harmonization of *FLT3* testing will be important to ensure that comparable results are achieved regardless of measurement procedure, time, or location of testing [121,122]. Currently, harmonization of *FLT3* testing will likely focus on PCR-based methods; however, in the future, NGS approaches that incorporate multigene panels could be the norm.

Role in detection of minimal residual disease

The term 'minimal (or measurable) residual disease' (MRD) is used to define the low levels of leukemic clones that may persist in patients who achieve a morphological CR and have a higher risk of relapse. These leukemic clones are not detectable by conventional microscopy but can be detected by more-sensitive techniques, including RT-qPCR, multiparameter flow cytometry, and even NGS [123,124]. Despite its importance as a prognostic marker, *FLT3*-ITD was long seen as an unsuitable marker for MRD monitoring because of patient-to-patient heterogeneity (e.g. length, insertion site, and allelic ratio) and inherent instability during the course of the disease [125–129]. However, more-sensitive PCR- and NGS-based techniques have recently been developed [43,130–132] and are becoming commercially available [133]. Nevertheless, the clinical application of these techniques needs to be validated in randomized clinical trials, as suggested by current recommendations [16]. Several ongoing, phase

3 clinical trials evaluating FLT3 TKIs now include MRD as an endpoint [78,99,103].

NPM1 has also emerged as a reliable marker for MRD monitoring because (1) *NPM1* levels remain stable throughout disease progression and (2) *NPM1* MRD levels have been clinically shown to correlate with therapeutic response. However, MRD monitoring has not yet been incorporated into AML disease management [59], given that no standard methods or definitive markers for MRD monitoring have been established [16]. The ELN is working on developing recommendations for MRD monitoring, which will likely include a combination of multiparameter flow cytometry and molecular-based assays.

Conclusion

Because of the recent results observed with FLT3-targeted therapies, the *FLT3* testing paradigm may shift from *FLT3* being regarded as a prognostic marker to being viewed as a diagnostic marker that can guide therapy choice. *FLT3* testing guidelines are beginning to change, including requirements for faster turnaround times (48–72 h), testing for both ITD and TKD mutations, and testing regardless of karyotype [16]. These changes will likely be adopted in the United States, requiring a shift in the order in which *FLT3* testing is performed. Currently, in many centers, cytogenetic and *FLT3* testing is done sequentially (i.e. *FLT3* testing follows cytogenetic testing); in the future, *FLT3* testing should be done in parallel with cytogenetic testing, as recommended in current diagnostic guidelines. This parallel approach will require education on the importance of *FLT3* testing, particularly in community oncology centers, to ensure widespread and timely testing. As we gain more insight into the prognostic impact of complex gene-gene interactions and molecular-cytogenetic abnormalities – and as new targeted therapies potentially become available – the diagnostic and therapeutic landscape of AML is likely to see major changes. Additional challenges in *FLT3* testing will include the need for harmonization of screening and MRD assays. Nevertheless, it is exciting to know that these changes and challenges are driven by gains in the development of therapeutic agents (evidenced by the large number of phase 3 trials evaluating FLT3 TKIs) for this high–unmet need patient population.

Acknowledgments

Editorial assistance was provided by Katherine Mills-Lujan, PhD, CMPP, and Pamela Tuttle, PhD, CMPP, of ArticulateScience LLC, and was funded by Novartis Pharmaceuticals Corporation. This work was supported by the National Center for Advancing Translational Sciences under CTSA Grant Number KL2 TR000136. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health.

Potential conflict of interest: Disclosure forms provided by the author is available with the full text of this article online at https://doi.org/10.1080/10428194.2017.1399312.

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