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Review

FLT3 mutational analysis in acute myeloid leukemia: Advantages and pitfalls with different approaches

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ABSTRACT

FMS-like tyrosine kinase 3 (*FLT3*) is one of the most closely studied genes in blood diseases. Numerous methods have been adopted for analyses, mainly in acute myeloid leukemia (AML) diagnostic work-up. According to international recommendations, the current gold standard approach allows *FLT3* canonical mutations to be investigated, providing the main information for risk assessment and treatment choice. However, the technological improvements of the last decade have permitted "black side" gene exploration, revealing numerous hidden aspects of its role in leukemogenesis.

The advent of the next-generation sequencing era emphasizes lights and shadows of *FLT3* conventional mutational analysis, highlighting the need for a more comprehensive study of the gene. However, more extensive analysis is opening new, unexplored questions whose impact on clinical outcomes is still unknown.

The present work is focused on the main topics regarding *FLT3* mutational analysis in AML, debating the strengths and weaknesses of the current gold standard approach. The rights and wrongs of NGS introduction in clinical practice will be discussed, showing that a more extensive knowledge of *FLT3* mutational status could lead to reconsidering its role in AML management.

1. Introduction

Identified for the first time thirty years ago, *FMS-like tyrosine kinase 3* (*FLT3*) is one of the most closely studied genes in hematopoietic malignancies [1,2]. In the context of acute myeloid leukemia (AML), *FLT3* is the most frequently mutated gene (~30% of adult newly diagnosed AML), together with *nucleophosmin-1* (*NPM1*) [3,4].

In the last decades, several methods, ranging from polymerase chain reaction (PCR) to next-generation sequencing (NGS), have been developed or adapted to identify *FLT3* mutations in AML [3]. Its prognostic role has been widely assessed, and two generations of therapeutic inhibitors have been developed and tested [5,6]. These methodological approaches vary in terms of sensitivity, turnaround time, costs, and development stage; some have mainly been used in clinical practice, while others are still being validated [3]. *FLT3* routine testing has been recommended in patients with cytogenetically normal AML (CN-AML) since at least 2010 [7]. In 2017 the importance of *FLT3* mutational analysis was recognized in the United States and Europe, and so included in testing recommendations [7,8].

The present work aims to address the main topics concerning *FLT3* mutational analysis in AML patients, focusing on the strengths and weaknesses of the current methodological approach. Some cases are reported, underlining certain critical aspects of the diagnostic work-up in light of technological improvements.

2. *FLT3* in AML: one gene, many faces

FLT3 is a type 3 receptor tyrosine kinase with a key role in expanding hematopoietic stem cells (HSCs) within the bone marrow (BM) [9]. It is encoded by the *FLT3* gene, mapped on chromosome 13 (13q12.2), approximately 97 Kbps long, made up of 24 exons. Structurally, the receptor consists of four regions: an N-terminal extracellular domain (ED) of 541aa with five immunoglobulin-like domains; a transmembrane portion (21aa); a juxtamembrane domain (JMD); an

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intracellular C-terminal region (431aa) with a tyrosine kinase domain (TKD). The two substructures of this domain are called N-lobe (TKD1) and C-lobe (TKD2) and are connected by an interkinase domain (Fig. 1). The extracellular region contains a binding domain with a high affinity for its ligand: the FLT3 ligand (FL) [9].

FLT3 is physiologically expressed by HSCs, common lymphoid and myeloid progenitors, and mature dendritic cells [10,11]. Binding to FL leads to homodimerization and autophosphorylation of FLT3, resulting in the transduction of pro-survival and proliferative signals through the RAS/MAPK, JAK/STAT, and PI3K/AKT pathways [12]. While *FLT3* mutations may be detected in almost any AML subtype, they are rarely observed in acute lymphoblastic leukemia (ALL) [13–16].

As previously pointed out, approximately 30% of adult de novo AML patients (it is estimated about 18% of patients older than 60 years) harbour an *FLT3* mutation with an aberrant activation of downstream pathways [13,14]. *FLT3* mutations typically occur in CN-AML but may also appear in the setting of AML with inv.(16), t(8;21), and t(15,17) [13]. Moreover, they frequently co-occur with other driver gene mutations such as *DNMT3A*, *NPM1*, and *IDH1/2* [13,14,17]. The two canonical mutations involved in AML are internal tandem duplications (ITDs), which occur in JMD or TKD1 (exons 14–15) and affect nearly 25% of AML patients, and point mutations in TKD2 (exon 20), which occur in almost 10% of AML patients [3]. Among the TKD alterations, codons D835 and I836 are the most frequently mutated, while no hotspot-ITDs have been identified since the variants are very heterogeneous (3 to >400 bps) (Fig. 1) [3]. The simultaneous presence of both mutations is defined as *FLT3* dual mutations and is found in a tiny subset of AML patients [18].

Since the advent of the NGS era, several non-canonical activating mutations have already been detected both at diagnosis and in the setting of refractory/relapsed patients [19,20]. Rare mutations may occur in ED, in JMD, in the ATP binding site of TKD1 or the activation loop of TKD2 (Fig. 1) [21–23]. Furthermore, wild-type (WT) *FLT3* is overexpressed in 93% of AML cases, almost 100% of B-ALL and 87% of T-ALL [24]. Therefore, *FLT3* upregulation may have a role in leukemogenesis, allowing the survival and proliferation of the leukemic clone [14,25].

3. *FLT3* diagnostic work-up, amid lights and shadows

3.1. *Fragment analysis (FA): is this enough?*

3.1.1. *Case #1*

A 68-year-old woman was diagnosed with AML with NPM1. Standard *FLT3* evaluation by FA analysis revealed the presence of an ITD [allelic ratio (AR) = 1] (Fig. 2A). She started induction therapy with cytarabine and daunorubicin ("7 + 3" regimen), and midostaurin, achieving complete remission (CR) without minimal residual disease (MRD) at the end of consolidation. Routine MRD assessment showed molecular relapse six months later, and the *FLT3* evaluation demonstrated the persistence of the same ITD with an AR = 14 (Fig. 2B). She started gilteritinib therapy, achieving a partial remission that is still lasting.

To extend knowledge of the mutational landscape at the basis of the observed AML clonal evolution, the anomalous AR value registered at the time of relapse was better investigated. What is the molecular mechanism generating such high ITD AR? Does a copy number variation (CNV) increase the number of the mutated allele or cause the loss of the WT? Is it a matter of copy neutral loss of heterozygosity (CN-LOH) due to somatic uniparental disomy (UPD) (Fig. 2C)? No indication can be obtained from FA in this regard. To clarify this aspect, a droplet digital PCR (ddPCR) CNV assay was performed to make an absolute quantification of the *FLT3* copy number at disease onset (ITD AR = 1) and at the time of relapse (ITD AR = 14). The assay excluded the occurrence of a CNV in both the evaluations performed (Fig. 2D). Based on these observations, the sole mechanism permitting the relevant ITD AR increase from diagnosis to relapse was a CN-LOH event due to somatic UPD; this event may occur in 7.5% of newly diagnosed CN-AML [26], but also in relapsed patients. Therefore, sometimes, conventional *FLT3* mutational analysis cannot be fully informative. The case presented proposes a condition (AR > 1) in which FA suggests the occurrence of a further event in the AML clonal evolution process, but all cases showing an AR ≤ 1 could hide more alterations (i.e.: CNV, UPD) not detectable with this approach. Notably, CN-AML carrying 13q UPD had a worse clinical outcome with shorter relapse-free survival (RFS) and overall survival (OS) [26,27]. Therefore, these subtypes could benefit from UPD

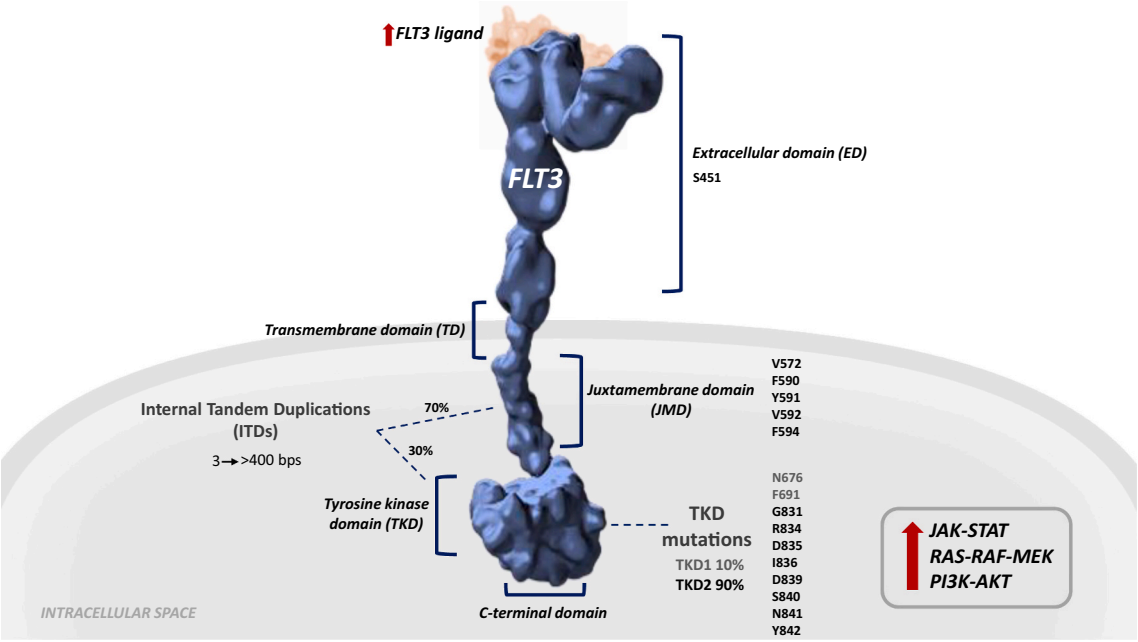

Fig. 1. FLT3 receptor schematization.
FLT3 functional domains are described with more frequent (ITDs and D835/I836) and non-canonical variants. All mutations reported cause aberrant activation of downstream pathways (RAS/MAPK, JAK/STAT, and PI3K/AKT), resulting in the transduction of pro-survival and proliferative signals for the leukemic clone.

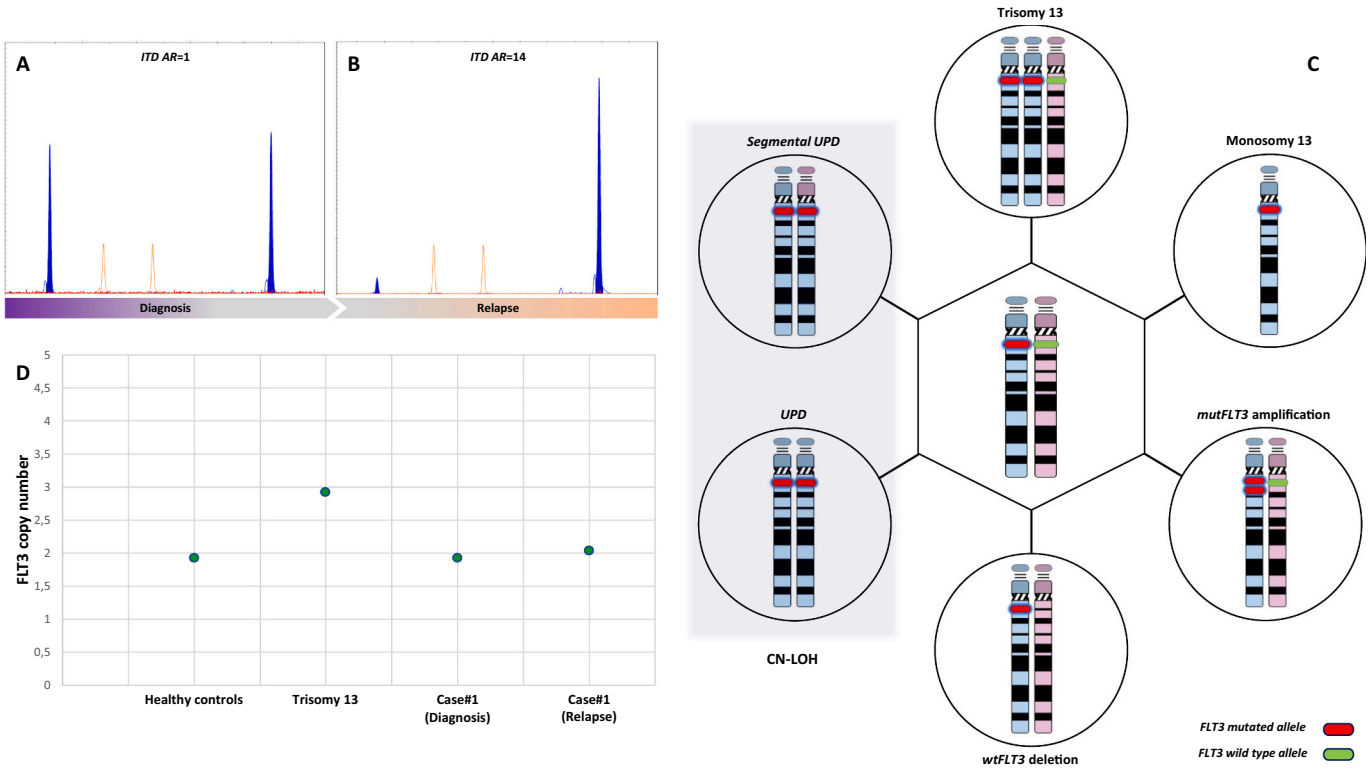

Fig. 2. Case#1.
FLT3 ITD fragment analysis at diagnosis (A) and relapse (B). (C) All possible somatic events causing an AR > 1. (D) ddPCR FLT3 copy number assessment; case#1 shows two FLT3 copies at diagnosis and relapse.

detection at the disease onset risk assessment.

According to the 2017 ELN recommendations, *FLT3* diagnostic work-up in AML should include screening for ITD-mutant to WT AR and TKD mutations at codons D835 and I836 [7]. International approval systems categorize the prognostic impact of *FLT3*-ITD according to its AR, distinguishing *FLT3*-ITDlow (AR < 0.5) from *FLT3*-ITDhigh (AR ≥ 0.5) cases. Conversely, the role of TKD mutations allelic burden has not yet been defined in AML management [7].

The current gold standard approach for *FLT3* study is FA by capillary electrophoresis (CE) [2,28]. The technology permits the detection of ITD and TKD (D835 and I836) mutations, allowing the quantification of ITD AR. Exons 14–15 (for ITD) and exon 20 (for D835/I836) are amplified by PCR using fluorescently labelled primers [2,28]. CE analyzes the PCR for ITD detection, and the ITD AR is evaluated as the ratio of the area under the curve (or of peak height) of mutant and WT *FLT3* alleles. The *Eco*RV restriction enzyme digests the PCR for TKD variants; the presence of a variant in one of the two codons codifying for D835 and I836 aminoacids deletes the EcoRV restriction site (5′-GAT/ATC-3′), and CE allows detection of the undigested product.

The strategy is rapid (2 days from DNA extraction to data analysis), easy (PCR, enzymatic digestion, FA), and affordable (<50 euro per sample) but provides only few usable data for patient management. Firstly, FA provides information only on the ITD burden, size and number, not data on the ITD sequence and localization (JMD or TKD1), whose prognostic impact will be discussed further. Secondly, FA is never a faithful reproduction of *FLT3* allelic mutational status and ITD burden; in fact, it is unable to distinguish heterozygous from homozygous mutations (due to loss of the WT allele: LOH) or CN-LOH events due to somatic UPD [26,27]. Furthermore, discrepancies between the AR measurement on genomic DNA or complementary DNA may affect the prediction of the outcome [29].

As to the detection of *FLT3* TKD mutations, FA allows only the recognition of the most frequent TKD alterations (D835 and I836), without distinguishing D835 from I836, nor providing information about the specific aminoacid change or loss and not allowing the investigation of the presence of other less frequent TKD mutations [30,31]. The impact of all these additional data on disease management will be discussed below.

3.2. NGS in FLT3 mutational analysis: the upside of the coin

3.2.1. Case #2

A 65-year-old man was referred to our institution for pancytopenia [white blood cell (WBC) count 1.4 × 109/L, hemoglobin (Hb) 9.8 g/dL, platelets (PLTS) 60 × 109/L]. The hematological work-up led to a diagnosis of myelodysplastic syndrome with excess blasts-2 (MDS-EB2). The patient underwent 26 cycles of azacytidine, achieving hematologic recovery. Immediately after, his blood counts worsened, and a BM examination revealed progression to AML with 30% blasts.

A baseline FA revealed the presence of *FLT3* ITD. NGS analysis identified the following variants: *DNMT3A* p.Lys589Ter (VAF: 34.2%), *IDH1* p.Arg132Cys (VAF: 14.4%), *RUNX1* p.Arg107Pro (VAF: 27.6%), *U2AF1* p.Ser34Phe (VAF:27.7%). No variants affecting *FLT3* were detected (Fig. 3A). Considering the discrepancy between FA and NGS, the long-read sequencing (LRS) (Oxford Nanopore Technologies) approach was performed for *FLT3* analysis on the same sample. The assay confirmed the presence of an ITD, 165 bps long (Fig. 3B). At the time of diagnosis, midostaurin was not yet approved for induction therapy. Accordingly, the patient was treated with cytarabine and daunorubicin ("7 + 3" regimen) but failed to respond. He was then enrolled in a trial with quizartinib as a single agent, achieving partial remission with incomplete hematologic recovery, but died a few months later of intracranial hemorrhage.

NGS is becoming ever more widespread in blood diseases research and diagnostic laboratories in the last decade, significantly increasing our genomic knowledge of hematopoietic disorders [32]. All the FA

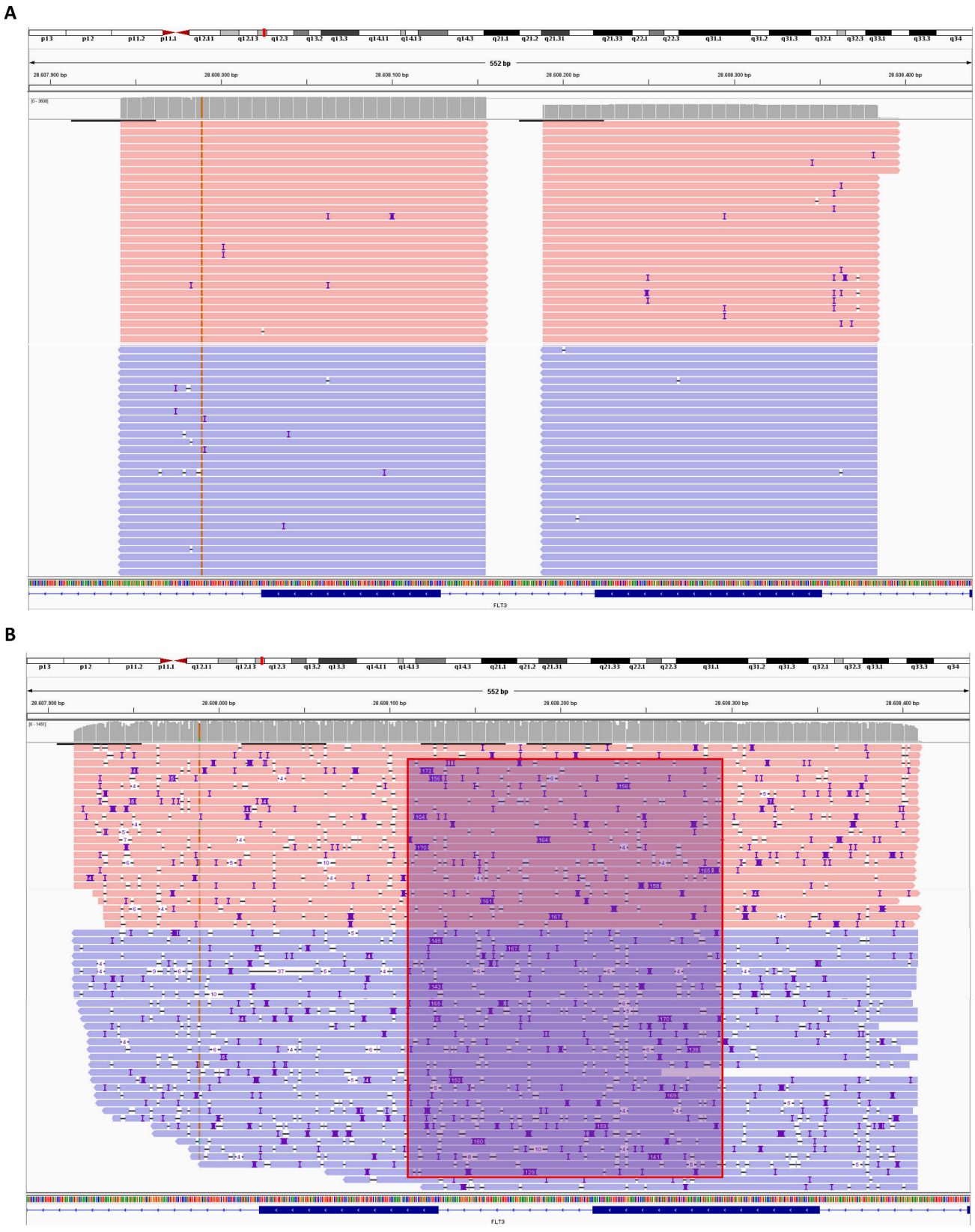

Fig. 3. Case#2.
IGV visualization of *FLT3* (exons 14–15) sequencing by a "short-reads" NGS approach (A) and LRS (B). The red square highlights the genomic region in which the "long-ITD" detected by LRS is mapped. With the IGV display options, insertions are flagged in blue. In the LRS pipeline, variant calling and annotation allowed determining the exact ITD site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

above-mentioned limits could be overcome by NGS introduction in *FLT3* diagnostic work-up [33], but despite its numerous advantages it has some weaknesses. In fact, NGS is a more laborious (up to five days from DNA extraction to data analysis) and expensive (hundreds of euros per sample) approach, demanding more sophisticated technologies and expertise. On the other hand, the NGS strategy permits analysis of the entire *FLT3* gene, thereby identifying ITDs and TKD mutations (differentiating D835 from I836, and providing specific information about the aminoacidic variation[31]) and non-canonical variants [23,31]. Several single nucleotide variants (SNV) and insertion/deletions (INDEL) are described, affecting JMD, the ATP binding pocket of TKD1, the activation loop of TKD2, but also ED [22]. Many of them cause sensitivity or resistance to FLT3 inhibitors, emphasizing the need for their detection [22].

Furthermore, NGS identifies the ITD sequence, allowing duplication in JMD or TKD1 to be localized: both these data influence the prognostic value of the alterations observed. The nucleotide sequence can classify ITDs as "typical" or "atypical", two newly defined categories [34]. The sequence of a typical ITD completely matches the WT *FLT3,* whereas an atypical ITD insert contains nucleotides exogenous to the WT *FLT3*. AML patients carrying typical ITDs benefit significantly more from FLT3 inhibitors and induction chemotherapy treatments than patients with atypical ITDs [34]. Moreover, NGS analysis could distinguish ITDs affecting JMD from ITDs affecting TKD1 [35]. This opportunity is of particular significance considering the recent data from the RATIFY study that categorize AML patients as having a JMD single mutation (55%), JMD and TKD1 mutations (26%) and TKD1 single mutations (19%). Intriguingly, midostaurin treatment is effective only for patients with a JMD single mutation, while patients with ITDs in TKD1 have the worst prognosis [35].

In light of these recent observations, the role of NGS in the *FLT3* diagnostic work-up becomes highly relevant. The technology allows a more sensitive detection, as recently demonstrated, but NGS, too, is affected by other weaknesses of both a technical and operative nature. First of all, ITDs are difficult-to-detect entities by NGS, given its heterogeneity in size (from 3 to >400 bp); conventional NGS platforms are "short-reads" technologies with a low capability to identify large insertions [36]. For this reason, the longest ITDs could be not identified [37], raising the risk of producing false-negative evaluations and considering *FLT3* WT patients who carry a "long-ITD", as in the clinical case presented above. This circumstance becomes relevant as the worst outcome is associated with ITD length. In fact, it was recently shown that in CN-AML patients without the *NPM1* mutation, increasing ITD length (≥60 bp) was associated with decreasing OS, shorter RFS, and a higher relapse risk than the short one (<60 bp) [38]. Several bioinformatic attempts have been made to overcome this limit [37,39], but the struggle must reckon with the unsolvable limit of the short-reads sequencing.

The other crucial NGS weakness posing an obstacle to introducing the technology in clinical practice and, in particular, in *FLT3* mutational analysis, is the low system scalability. All current NGS platforms available in clinical practice are built to produce large amounts of data; in fact, they are high throughput systems, inappropriate to study just one target or few target genes in a single patient [32]. In other words, in clinical practice, it is not easy to perform an NGS run for each newly diagnosed AML; on the other hand, the diagnostic timeliness required for *FLT3* diagnostic work-up (3–5 days) does not allow for multiplex analysis.

The emergence of a new generation platform based on "long-reads" sequencing (LRS) and characterized by high scalability yet tested in the study of hematological malignancies [40], could solve both the above weaknesses [39,41]. As recently demonstrated, these emerging technologies could offer a valid alternative, even if their use in clinical practice needs appropriate validation procedures [41,42].

3.3. More extensive analysis, more open questions

3.3.1. Case #3

A 36-year-old man was diagnosed with high-risk (WBC count: 49 × 109/L) acute promyelocytic leukemia (APL) with the PML-RARA rearrangement. A baseline *FLT3* mutation analysis (by FA) revealed the presence of two ITDs: ITD1 (60 bps long, AR: 0.15) and ITD2 (75 bps long, AR: 0.37) (Fig. 4A). The patient underwent induction therapy with all-trans retinoic acid (ATRA) and anthracycline-based chemotherapy (AIDA regimen), achieving CR without MRD at the end of consolidation. A few months after the completion of maintenance therapy, routine MRD assessment showed molecular relapse. The *FLT3* mutational status was reassessed. The analysis confirmed the persistence of only ITD1 (Fig. 4C-D). The patient underwent salvage therapy with ATRA and arsenic trioxide (ATO) but failed to achieve MRD negativity. He developed neurological symptoms (headache, confusion); computed axial tomography was performed and showed no active bleeding. A liquor sample analysis revealed a central nervous system APL localization. The patient died a few weeks later due to infectious complications.

A recent literature meta-analysis showed that *FLT3* mutations occur in approximately 27% of APL patients and are correlated with a higher WBC count and poorer prognosis [43]. The detection of multiple ITDs in an APL case with a molecular marker of disease such as PML-RARA, can allow us to observe and discuss the role of *FLT3* in MRD molecular monitoring (Fig. 4E). Notably, in the case presented, between the two ITD mutated clones detected at the disease onset, the smallest subclone (AR: 0.15) carrying the shortest ITD expanded in the relapse (Fig. 4F).

The first attempts, in the last decade, to apply a more sensitive strategy like NGS for *FLT3* analysis offered new unexplored observations about the role of gene mutations in AML. An attractive example is the recent data from the RATIFY study, demonstrating for the first time that over 50% of *FLT3*-ITD mutated AML patients have a polyclonal molecular pattern (multiple ITDs) [35]. The authors attribute the possibility of making this observation to the greater sensitivity of the method adopted (ie. NGS) compared to the standard techniques that had so far identified such clonal heterogeneity only in 14% of *FLT3*-ITD mutated cases [37].

Their data show that multiple ITDs constitute a negative factor influencing the response to induction therapy, together with the number of WBCs [35]. The *FLT3* tendency to present, in most cases, as a polyclonal molecular ITD pattern (up to nine co-occurring ITDs), prompts reflection about the role of gene mutational analysis in AML prognostic stratification, in therapeutic choices, and in molecular monitoring (Fig. 5).

But how could the awareness of the *FLT3*-ITD clonal heterogeneity existence impact the management of this aspects, in light of the 2017 ELN recommendations [7]?

First of all, in determining the *FLT3*-ITD AR, is it correct to consider the contribution of the prevailing ITD exclusively (an indirect measure of the most common disease clone), or would it be better to generate the AR as the sum of the multiple ITDs identified? With the AR, could the number of ITDs detected at diagnosis also play a prognostic role? No indication is available to date in this regard. Pursuing one path rather than the other could, in some cases and especially in patients with a borderline AR, alter the prognosis and, therefore, the therapeutic choice.

The results from Rucker et al. document the unfavourable impact of multiple ITDs on the response to induction therapy [35]. But what could be the molecular mechanism behind the worse complete remission rate observed in this subgroup? The subclones presence already at the disease onset almost certainly represents an evolutionary strategy of the disease that probably enhances treatment resistance, as observed in the context of other diseases [44]. A paradigmatic example is that of *ABL1* kinase domain mutations in chronic myeloid leukemia [45]. On this model, the pretreatment *FLT3* NGS analysis in AML patients resistant to midostaurin could reveal the association between the treatment failure and the presence of multiple ITDs, allowing us to decide, in a near future, on the most appropriate use of *FLT3* inhibitors.

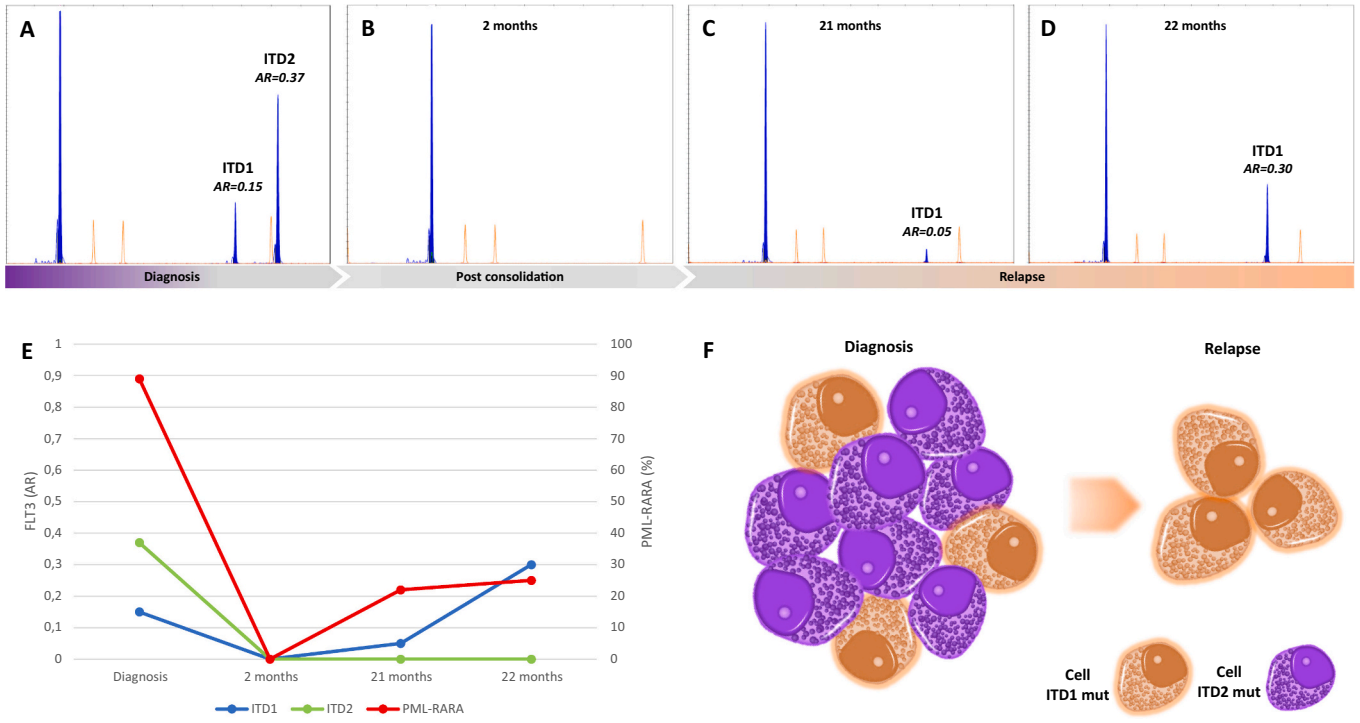

Fig. 4. Case#3.
FLT3 ITD fragment analysis at diagnosis (A), post-consolidation (B) and relapse (C—D). (E) Graphic visualization of ITD1, ITD2 and *PML-RARA* kinetics. (F) A simplification of the mutational landscape from diagnosis to disease relapse.

On the basis of these observations, a final aspect to consider is the much-debated role of *FLT3* in MRD molecular monitoring [46]. First of all, the data presented suggest we should definitively exclude, for this purpose, any method other than NGS [37,47]. Secondly, the high incidence of multiple ITDs at diagnosis, as in the clinical case presented, requires a reassessment of the previously discussed role of *FLT3* as a measurable disease marker [46] and imposes the need to shed light on the circumstances of the gene mutational state and the phasing of its mutations [42]. Data from the RATIFY do not establish with certainty whether multiple ITDs are attributable to different disease clones or may involve a single clone, as recently investigated for *BCR-ABL1* compound mutations in Philadelphia chromosome-positive leukemia [48]. The new generation of sequencing platforms [41], together with the most sophisticated single-cell sequencing technologies, will improve our understanding of drug resistance mechanisms linked to *FLT3* alterations.

4. Future considerations

During the last years, the expanding knowledge of the genomic landscape in AML has significantly changed our comprehension of the biology and the clinical course of the disease. Even if the backbone of treatment is still chemotherapy, many new agents have been developed based on specific molecular targets (i.e.: IDH1, IDH2, CD33), expanding the therapeutic armamentarium. In this context, targeting FLT3 has been an important goal; several efforts led to the approval of a multikinase inhibitor, midostaurin, associated with the "7 + 3" regimen, as induction therapy for AML patients harbouring an *FLT3* mutation, either ITD or TKD. Nowadays, first- and second-generation inhibitors are further classified as type 1 or 2 based on their specificity and activity on the active or inactive conformation of the FLT3 receptor, respectively. New molecules are constantly being developed and tested [6,49,50]. Nevertheless, the availability of "weapons" demands a precise comprehension of the multi-faced *FLT3* aberration in AML and the translation of this knowledge in clinical practice to engage a "sniper" against the disease.

None of the technologies available to study the *FLT3* mutational status can produce all-encompassing information; each shows peculiar strengths but hides several weaknesses (Table 1). On the other hand, we are not yet aware of the meaning of all this information we could receive, and not all of it has an established clinical impact. Ideally, the widespread diffusion of new generation technologies and their integration with conventional methods could better define the *FLT3*-mutated disease.

The clinical cases presented exemplify certain critical aspects of this scenario. In case #1, FA failed to detect an UPD event responsible for a "high ITD AR"; a somatic event conferring a worse prognosis in CN-AML and contributing to AML relapse. Case #2 presents a common problem with a debated solution. NGS analysis produced a false negative evaluation in a patient carrying a "long-ITD", the Achilles tendon of short-reads sequencing application to *FLT3* analysis. Is the quest for a more comprehensive description of *FLT3* mutational status worth missing a "long-ITD" with an established worse impact on the prognosis? Again, this is an example of how integrating distinct approaches may offer a solution to a well-known problem. Finally, case #3, showing multiple ITDs at the disease onset, is a paradigmatic example of why *FLT3* is not a "bona-fide" marker of MRD. Expect the unexpected!

To conclude, the *FLT3* biology and mutations are among the most closely studied topics in AML and perhaps the best-defined therapeutic targets, with an expanding pool of molecules, both approved and currently being tested. Strikingly, this broad knowledge is not yet coupled with diagnostic perfection in clinical practice. Probably, the standard work-up for the study of *FLT3* is no longer enough. A refined diagnostic strategy integrating classic technologies with NGS, single-cell analysis and new emerging approaches would provide a more detailed description of *FLT3* aberrations in AML and finally help the "sniper" to put it in his crosshairs.

Practice points

- Standard *FLT3* mutational analysis is probably no longer enough in AML.

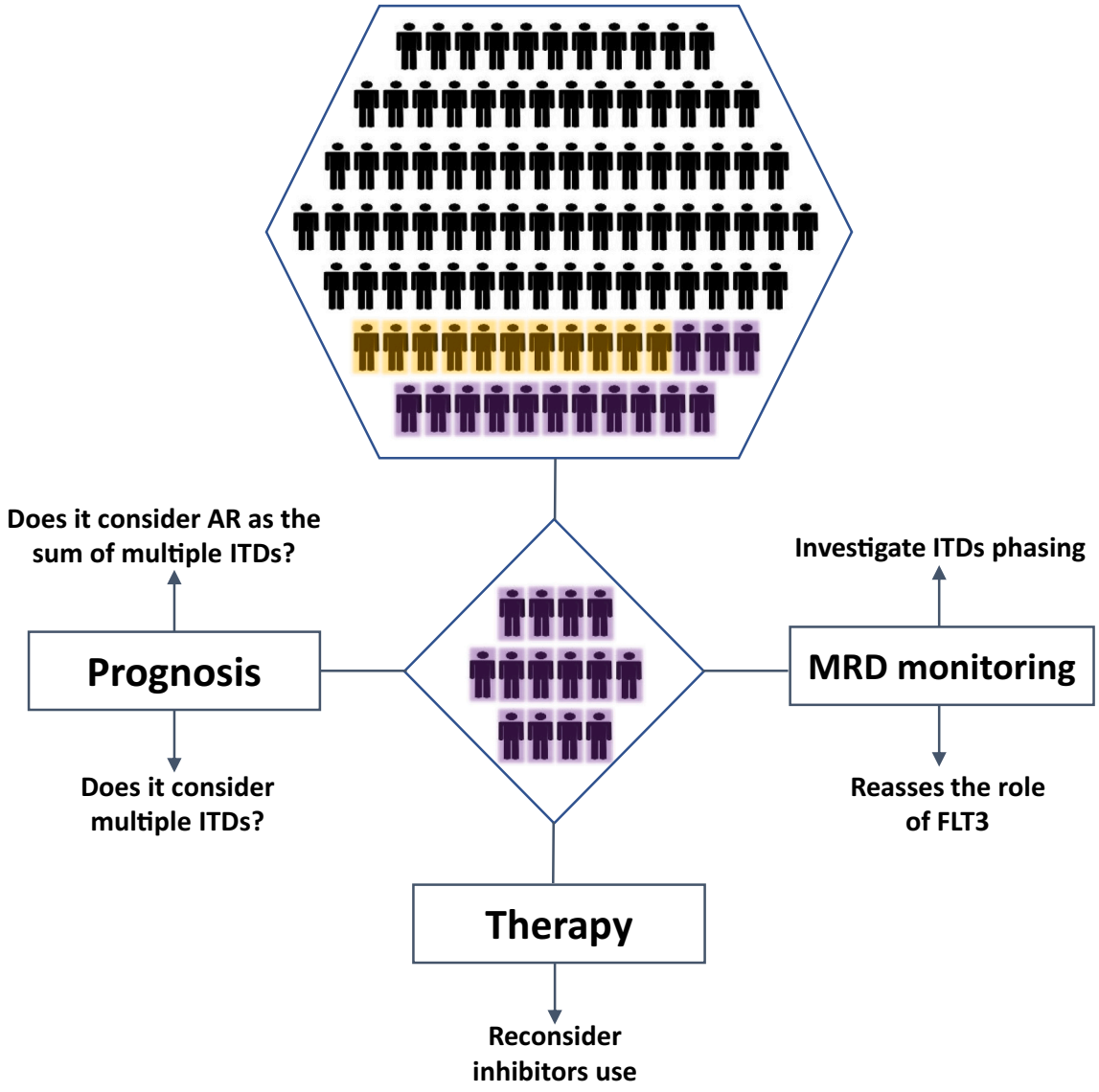

Fig. 5. Multiple *FLT3*-ITDs in AML management.
FLT3-ITD occurs in ~ 25% of newly diagnosed AML (highlighted in yellow and violet). Recent data show that only 46.5% of them (yellow) are single ITD, while the remaining 53.5% (violet) present multiple ITDs (from 2 to 9). This new observation requires a reconsideration of the role of *FLT3* in prognostic stratification, therapeutic decision making and molecular MRD monitoring of AML patients. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

- NGS approach shows peculiar strengths but hides several weaknesses.
- A *FLT3* more extensive analysis prompts reflection about the role of the gene in clinical practice.

Research agenda

- Consider the advantages of a multi-strategy approach for the *FLT3* diagnostic work-up.
- Test emerging technologies in the *FLT3* mutational analysis.
- *Re*-evaluate the impact of *FLT3* alterations in AML patients.

Ethics approval and consent to participate

The local ethics committee approved the study. Informed consent was obtained from all patients before study inclusion, in accordance with the Declaration of Helsinki. Patients' records/information were anonymized and de-identified before analysis.

Consent for publication

Consent for publication was obtained from patients before their enrolment in the present study.

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CRediT authorship contribution statement

Cosimo Cumbo: Conceptualization, Writing – original draft, Writing – review & editing. **Francesco Tarantini:** Conceptualization, Writing – original draft, Writing – review & editing. **Luisa Anelli:** Writing – review & editing. **Antonella Zagaria:** Writing – review & editing. **Giorgina Specchia:** Writing – review & editing. **Pellegrino Musto:** Writing – review & editing. **Francesco Albano:** Conceptualization, Writing –

Table 1
Strengths and weaknesses of standard and main emerging methodological approaches for *FLT3* mutational analysis in AML. FA: fragment analysis, NGS: next-generation sequencing, LRS: long-reads sequencing, UPD: uniparental disomy. *The development and diffusion of new benchtop platforms are redefining these features.

	FA	NGS	LRS
ITD detection (burden, size and number)	Yes	Yes	Yes
Long-ITD identification	Yes	No	Yes
Typical/atypical ITD identification	No	Yes	Yes
ITD localization (JMD/TKD1)	No	Yes	Yes
D835/I836 detection	Yes	Yes	Yes
Distinguish between D835 and I836	No	Yes	Yes
Determine the specific aminoacidic change (at 835/836 codons)	No	Yes	Yes
Non-canonical mutations detection	No	Yes	Yes
UPD identification	No	Yes	Yes
Mutation phasing	No	No	Yes
High sensitivity	No	Yes	No
Rapidity	Yes	No*	Yes
Cheapness	Yes	No*	Yes
Good scalability	Yes	No*	Yes

original draft, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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