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Pharmacological Impact of FLT3 Mutations on Receptor Activity and Responsiveness to Tyrosine Kinase Inhibitors

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Highlights

- **Structural aspects of the FLT3 receptor in signalling**
- **Clonal evolution of FLT3 mutants**
- **Recurrent mutations and its regulation of FLT3 signalling cascade and drug therapy**

Abstract

Acute myelogenous leukaemia (AML) is an aggressive blood cancer characterized by the rapid proliferation of immature myeloid blast cells, resulting in a high mortality rate. The 5-year overall survival rate for AML patients is approximately 25%. Circa 35% of all patients carry a mutation in the *FLT3* gene which have a poor prognosis. Targeting FLT3 receptor tyrosine kinase has become a treatment strategy in AML patients possessing *FLT3* mutations. The most common mutations are internal tandem duplications (ITD) within exon 14 and a single nucleotide polymorphism (SNP) that leads to a point mutation in the D835 of the tyrosine kinase domain (TKD). Variations in the ITD sequence and the occurrence of other point mutations that lead to ligand-independent FLT3 receptor activation create difficulties in developing personalized therapeutic strategies to overcome observed mutation-driven drug resistance. Midostaurin and quizartinib are tyrosine kinase inhibitors (TKIs) with inhibitory efficacy against FLT3-ITD, but exhibit limited clinical impact. In this review, we focus on the structural aspects of the FLT3 receptor and correlate those mutations with receptor activation

and the consequences for molecular and clinical responsiveness towards therapies targeting FLT3-ITD positive AML.

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1. Introduction

Acute myelogenous leukaemia (AML) is a heterogeneous blood cell cancer. It evolves from a clonal population with acquired mutations or chromosomal aberrations. These changes disturb the physiology of myeloid lineage cells, and causes uncontrolled proliferation and decreased differentiation of immature blast cells in the bone marrow and blood [1]. The occurrence and accumulation of acquired mutations are associated with the initiation of leukaemogenesis and predict prognosis. The most common acquired mutations in AML disease occur in the CCAAT/enhancer binding protein alpha (*CEBP-alpha* or *CEBPA*) and runt-related (*RUNX*) transcription factors; DNA methyltransferase 3 (*DNMT3*), ten-eleven translocation 2 (*TET2*) and isocitrate dehydrogenase (*NADP(+) 1 and 2* (*IDH1/2*) epigenetic regulators; additional sex comb-like 1 (*ASXL1*) chromatin modifiers; tumour protein p53 (*TP53*) tumour suppressor; *N-RAS*, mast/stem cell growth factor receptor Kit (*c-KIT*) and protein tyrosine phosphatase, non-receptor type 11 (*PTPN11*), feline McDonough sarcoma (FMS)-like (*FLT3*), transduction genes and nucleophosmin 1 (*NPM1*) [2]. In humans, the *FLT3* gene encodes for the FLT3 tyrosine kinase type III receptor protein, also known as cluster of differentiation antigen 135 (CD135) receptor. It belongs to the class III family of receptor tyrosine kinase (RTK). Under physiological conditions, FLT3 is highly expressed in human granulocyte-monocyte progenitor (GMP) and common lymphoid progenitor (CLP) cells [3,4]. Unlike humans, mice express FLT3 in CLPs but not GMPs, suggesting differences in the physiological function of FLT3 in their haematopoietic system [4]. FLT3 is also overexpressed in lymphoid and myeloid leukaemia, such as B-lineage acute lymphoblastic leukaemia (ALL), and adult and paediatric AML [3,5,6]. Most AML patients overexpress wild-type FLT3 receptor in their blast cells. *FLT3* gene mutation is found in about 35% of the patients. It is one of the most frequent genetic alterations found in AML[7].

The current identification and association of recurrent co-occurring mutations and karyotypes with clinical outcome serves as the base for the new AML classifications. The most common genotypes for AML with *FLT3* mutation (*FLT3*⁺) are: AML with *NPM1* mutation, AML with mutated chromatin, RNA-splicing genes, or both, AML with t(15;17)(q22;q12); PML–RARA, AML with t(6;9)(p23;q34); DEK–NUP214 and AML with driver mutations but no detected class-defining lesions[2,8]. *FLT3* mutation can also occur at diagnosis in AML patients presenting with a normal karyotype [2]. In addition, AML patients can be stratified in risk groups depending on the FLT3 status. A favourable outcome includes the presence of *NPM1* mutation in the absence of *FLT3* mutation. Conversely, *FLT3* mutation in the presence of normal *NPM1* predicts a poor outcome. Detailed risk stratification for AML have been

summarized elsewhere [8,9]. Classifying the patients according to groups is important to determine the best therapy, as it is common to see variation in the response to treatment among AML patients due to differences in karyotype and genotype [10,11]. Understanding structural features of the FLT3 receptor and frequent recurrent mutations provide insight in the clonal evolution and therapeutic response. This review seeks to highlight these main clinical points relating to the FLT3 mutations that are found in AML with a view to understanding their relevance to cellular functioning of FLT3, the evolving impact of these adaptations on the clinical landscape, and the avenues for more targeted therapeutic interventions with current and potential FLT3-directed drugs.

1.2 Structural aspects of FLT3 receptor activation

The *FLT3* gene is located on chromosome 13q12. 24 exons encode a 993 amino acid protein with a calculated molecular weight of 112,903 Daltons. FLT3 has a single transmembrane domain and is inactive as a monomer [12]. The ectodomain of FLT3 receptor comprises amino acids 27 to 543. It has five immunoglobulin-like (Ig-like) domains denominated D1, D2, D3, D4, D5, where D1 is *NH*₂-terminal and D5 is *COOH*-terminal, proximal to the plasma membrane. These domains are required for cell surface recognition, FLT3/FLT3 ligand interaction and consequent receptor dimerization. Dimerization of the receptor occurs when FLT3 ligand (FLT3LG) binds bivalently to the D3 IgG-like domain of two monomers, forming a bridge-like structure between the two monomers. Receptor dimerization causes conformational changes that allow juxtapositioning of the cytosolic domain. It exposes phosphorylation sites resulting in receptor activation. The affinity of the FLT3LG to the FLT3 receptor is not affected by deletion of D4 and D5 domain, which are required for ligand-induced dimerization of other structurally similar receptors [13,14]. The role of the transmembrane domains of RTK have been previously shown [15]. However, to our knowledge, no significant study has been published on FLT3 transmembrane domain.

The general structure of tyrosine kinases is composed of a *NH*₂- and *COOH*-lobe with a buried ATP binding domain and an activation loop that determines when the enzyme is active/inactive [16]. The juxtamembrane domain (JMD) of FLT3 is located between lobes and comprises a β -stand that follows the transmembrane sequence. The JMD can be divided into three regions; a binding motif (Y572 to M578), a switch motif (V579 to V592) and linker peptide (D593 to W603). The JMD is juxtapositioned over the kinase domains, preventing enzymatic activity. Conformational changes in the JMD caused by ligand binding expose the residues Y572, S574, Y589, Y591, and Y599, previously identified as autophosphorylation

sites [17]. It has been proposed that phosphorylation of either Y589 or Y591 in the switch domain of the JMD prevents its folding onto *COOH*-lobe, maintaining it exposed for further receptor phosphorylation. Dephosphorylation of these residues immediately cause JMD to lay back over the *COOH*-lobe to cover the kinase domain preventing further catalytic activation. Therefore, Y589 and Y591 can act as a negative regulator of the kinase [14].

The tyrosine kinase domain (TKD) is the catalytic domain of the RTK. It catalyses the transfer of a $(\text{PO}_4)^{3-}$ group from the adenosine triphosphate (ATP) to the targeted protein. This site is buried in a deep cleft and its access is controlled by the activation loop [18]. Kinases have distinct binding affinity towards different substrates to confer selectivity to its binding partners, even though they share fairly similar structures [19]. FLT3 has two TKD domains, TKD1 and TKD2, which are separated by a flexible and hydrophilic region denominated kinase insert. A hinged region is critical for full activation of the receptor. The activation loop is composed of an Asp-Phe-Gly (DFG) motif, where the orientation of the Asp side chain classifies the receptor in 'DFG-in' or 'DFG-out'. As 'DFG-in', the Asp side chain faces inwards and interacts with Mg^{2+} to open up for ATP binding. Conformational modification or mutations can cause mispositioning of DFG to create a 'DFG-out' conformation. In this case Asp side chain faces outward and the interface between lobes is affected and the receptor stays inactive. The activation loop needs to be open (DFG-in) to leave space for ATP binding and hydrolysis [16]. Understanding the structural coordination of these residues is important to study tyrosine kinase inhibitors.

1.3 Mutations in the FLT3 receptor of significance in AML

Expression of the FLT3 receptor in AML is an indicator of poor prognosis per se [20,21], and mutations in this gene are associated with a poor prognosis, increased relapse risk and a lower overall five-year survival rate [22–24]. ITD and point mutations in the TKD are the most common type of genetic alterations of the *FLT3* gene. Either mutation leads to the constitutive activation of the receptor and constant activation of the downstream signalling cascades. The most frequent amino acid mutation in the TKD domain of FLT3 is the substitution of the Asp (D) at position 835 within the activation loop. This residue corresponds to the D816 in the c-KIT receptor, which is structurally similar to FLT3 receptor and is also mutated in AML and extensively studied [25]. Mutation of D835V, D835Y, D839G and I867S in TKD were previously identified and characterized. All lead to receptor auto-activation [26]. Although less frequent, a N676K mutation in the ATP binding domain of FLT3 receptor has been identified in the core-binding factor subgroup of AML and it was shown to confer resistance to the

tyrosine kinase inhibitors (TKI) midostaurin (PKC412) and quizartinib (AC220) when tested in Ba/F3 cells overexpressing FLT3 [27]. Another study suggests that high frequency of TKD mutations is associated with inv(16), a common chromosomal translocation in the core-binding factor subgroup of AML [28].

Tandem exon duplications are an evolutionary feature of the eukaryotic genome alternative to splicing and results in new protein function [29]. About 25% of AML patients develop an ITD in the *FLT3* gene. Tandem duplications are most commonly developed in exons 14 and 15, which are part of the juxtamembrane domain (JMD) [30]. ITDs in the TKD1 domain have also been reported [31], they vary in length and amino acid sequence, although the FREYE sequence in exon 14 seems to be a common feature of the ITD [32]. In a cohort of 3365 AML patients, 689 FLT3-ITD positive patients were identified and the ITDs range from 3 to over 151 nucleotides with a median distribution of 51 additional nucleotides. Moreover, 32.4% of the *FLT3* gene analysed in this work contained an insertion of extra nucleotides in the ITD. Exon duplication within, or in close proximity to, the TKD1 domain are predicted to be poorer prognostic indicators when compared to FLT3 wild-type (WT), possibly because of conformational changes in the cytosolic domain. Such poorer prognoses may likely be caused by greater constitutive receptor activity due to diminution in autoinhibition[33]. This same study predicted that the length of the duplication has no influence on prognosis. However, there are divergent predictions regarding the phenotype and prognosis of the ITD⁺ AML when analysing its length. A different study associate poor prognosis with ITD length [34,35]⁴⁶; co-occurrence of long ITDs ($\geq 70\%$) and high allelic burden ($\geq 50\%$) indicated that patients from this subgroup possess the poorest prognosis [34,35]. Moreover, ITD-positive AMLs not only have a worse prognosis but also present with inferior relapse-free survival rates when Y591-Y597 in the JMD is duplicated. These Y591-Y597 tyrosine residues can be auto-phosphorylated, becoming sites by which the signal transducer and activator of transcription 5 (STAT5) binds through their SH2 domains to tyrosine-phosphorylated FLT3 and become activated [17].

1.4 Other frequent mutations of the *FLT3* gene

FTL3-ITD in the JMD and *FLT3-D835* are the most frequent mutations found in the *FLT3* gene; however, other less frequent mutations (but no less important) can occur (**Figure 1**). A N841I substitution in the activation loop of the FLT3 receptor confers conformational changes in its structure, altering the functioning of its activation loop. This results in ligand-independent

FLT3-mediated constitutive activation and STAT5 phosphorylation (P-STAT5), without any interference on receptor trafficking to the plasma membrane [36] .

ITD is not the sole mutation occurring in the juxtamembrane domain of FLT3; point mutations have also been detected (V592A, V579A, F594L and F590/GY591D) that also lead to autophosphorylation and constitutive activation of the receptor, albeit to a lesser extent than TKD and ITD mutation [37]. 672 patients from the AMLCG-99 trial were analysed and a V592 mutation discovered within the JMD was found to co-present with ITD mutations in patients at relapse. The same study analysed the development of ITD in FLT3-positive patients and 9% of them developed further mutations after relapsing (i.e. not present or undetectable at diagnosis); Those relapse-observable mutations include TKD, ITD, point mutations, or a combination of these [26].

Other alterations of FLT3 are observed in AML patients. For example, an ITD of 6-bp followed by a 10-bp deletion within the TKD has also been detected and resulted in replacement of the D835/I836 for a valine-isoleucine-proline-threonine (VIPT) amino acid sequence. D324N mutations in the third immunoglobulin-like domain were detected in patients at low frequency (1.5%) and no phenotype was found when FLT3-D324N was expressed in IL-3 dependent-Ba/F3 cells. FLT3-D324N mutation is also present in chronic myeloid leukaemia (CML) and acute lymphoblastic leukaemia (ALL) at higher frequency when compared to AML [38]. A substitution of the positively charged arginine by a less reactive glutamine (K663Q) in the TKD1 has also been found and it results in a gain-of-function phenotype, to activate downstream STAT5 and AKT signalling. Another AML patient had a N841H mutation in TKD2, which is considered homologous to the N822H mutation in the c-KIT receptor [39]. Finally, recurrent p.Q569Vfs*2 deletion results in a truncated receptor that traffics to the plasma membrane and loses kinase activity [40]. The occurrence of all these non-D835 or 'non-classic' ITD mutations suggest that working towards personalized therapy would benefit from establishing broader screening of *FLT3* mutations for clinical application.

1.5 Clonal evolution and heterogeneity in AML

In the heterogeneous environment of AML, dominant clones evolve and expand by acquiring genetic mutations. Clonal evolution of AML blasts relies on the progression of mutations that occur almost as a chain reaction. AML cells first acquire and accumulate driver mutations that prepare the cells for acquisition of additional genomic changes [41]. Mutation in genes that promote proliferation, such as those associated to the *FLT3* gene, are considered to be later events in AML evolution. Moreover, it has been shown that ITD is present in leukaemic stem

cells (LSC) at diagnosis[42]. It appears that FLT3-TKD is a later event in AML when compared to FLT3-ITD. Single-cell RNA sequencing analysis of bone marrow aspirates from AML patients identified *FLT3-TKD* as a characteristic of differentiated cells whereas, *FLT3-ITD* was present in undifferentiated cells with haematopoietic stem cell-like characteristics [43]. This finding suggests that the poor prognosis of *FLT3-ITD* patients is associated with the differentiation block conferred by this gene.

Comparing clonality of AML cells from patients at diagnosis with those at relapse has revealed two type of clones that survived therapy; one that evolved to relapse and another subclone that acquired new mutations and expanded to relapse [44]. To understand how heterogeneity evolves, a follow-up study using whole-exome sequencing of immunodeficient mice transplanted with AML cells from 26 different patients harbouring recurrent mutations were analysed at the time of xenotransplantation and followed up until AML was developed. In this cohort, most AMLs had changes in clonal composition and 83% resulted in clonal expansion when compared to the diagnostic sample, indicating increased clonal heterogeneity. In some cases, the original dominant *FLT3* mutant clone was lost, and some lost RAS mutant clones, suggesting that these mutations were present and required for initial expansion but not for the maintenance or survival of the relapsed clone. However, this model could not predict relapse when comparing transplanted clones with later xenografts [45]. *In vitro* studies tracking clonal evolution also shows high heterogeneity in the progenitor cells, proposed as the main cause of differences in the therapeutic response of patients [46,47]. Some co-occurring somatic mutations can co-operate with each other and cause a synergistic effect, also known as an epistatic effect. [41,47,48]. Co-occurrence of *Npm1* and *flt3-itd* mutation (*Npm1*^{TCTG/TCTG}; *Flt3*^{+/ITD}; *Npm1*^{TCTG/TCTG}; *Flt3*^{ITD/ITD}) caused lethal AML, which was not evident with a single mutation. Moreover, mice with cumulative allelic burden for homozygous mutation (*Npm1*^{TCTG/TCTG}; *Flt3*^{+/ITD} < *Npm1*^{+/TCTG}; *Flt3*^{ITD/ITD}) developed AML faster than heterozygous mutants [49]. All studies agree that a high mutant allelic burden predicts poor prognosis, because overall survival decreases as the ratio FLT3-ITD/FLT3-WT increases. ITD mutations detected in patient blood and bone marrow can be heterozygous or homozygous, the latter via loss of heterozygosity (LOH). Patients carrying the FLT3-LOH-ITD genotype have worse prognosis when compared to the heterozygous ITD [50]. However, low FLT3-ITD/FLT3-WT ratio could still represent a disposition to relapse due to minimal residual leukaemia, where a FLT3-ITD clone can survive and outgrow the FLT3-WT population [51]. LOH is likely to be an event that occurs after the development of the ITD in the gene [52]. Analysis of bone marrow

from AML patients at relapse demonstrate FLT3-TKD can be either loss or gained in relapse [53].

Together, these data demonstrate that relapse is associated with additional acquired mutations and *FLT3-ITD* seems to be one, but not the sole critical element for proliferation and survival of the malignant clone. To date, there is no conclusive understanding for the clonal combination predominant in the heterogeneous environment of different AML patients. Understanding the evolutionary patterns and the most frequent combination of mutations would support either personalisation or standardization of the new therapies.

1.6 Regulation of FLT3 signalling by the FLT3 ligand

Understanding FLT3LG physiology is an important aspect to understand the regulation of the FLT3 receptor itself. Full length FLT3LG is composed of 235 amino acids. It has a signal peptide in the *NH*₂-terminal domain for translocation to the endoplasmic reticulum (ER), where it is folded and glycosylated for correct trafficking to the cell surface, either as a transmembrane protein or as a cleavage product that is released as a soluble form [54,55]. *In vitro* experiments suggest FLT3LG expression is controlled by translation rather than transcription because of the high amount of mRNA compared to the low protein expression.

The murine *flt3lg* cDNA was used to clone the human *FLT3LG* and their amino acid sequences share 72% identity [56]. According to the literature, up to five murine-derived *flt3lg* splicing variant mRNAs were first detected. The *NH*₂-terminus is quite conserved among these variants and the human FLT3LG, whereas they differ in their *COOH*-terminal sequence [56,57]. A pre-clinical model of *flt3*^{-/-} mice demonstrate the importance of *flt3lg* in the expansion of progenitor cells and generation of dendritic and natural killer cells [58]. Proliferative activity of the FLT3LG is optimal when in synergy with other ligands, such as SDF-1 and c-KIT ligand [59,60]. Three murine *flt3lg* transcripts have been characterized. The most active transcript generates a transmembrane protein containing 21 amino acids in the cytosolic domain. The calculated molecular weight is 26,14 kDa. Heterologous expression in COS cells identified a 36 kDa protein, suggesting this transcript is highly glycosylated. The cleavage form of the ligand results in a 30 kDa protein. This isoform lacks exon 6, which seems to be responsible for the generation of a soluble form. Another transcript has 57 hydrophobic amino acids, suggesting it has a transmembrane domain more complex than a single transmembrane domain, which is predicted to be composed of approximately 20 amino acids. This transcript generates a protein no bigger than 30 kDa and is not detected in the supernatant. A third transcript has lower expression and contains an 83 bp insertion containing stop codons

that result in a truncated form with 9 extra amino acids. It generates a 24 kDa protein, therefore, likely not glycosylated. It is not clear how this transcript is processed in the secretory pathway. The data collected suggests the lack of a signal peptide for ER insertion and further secretion [57].

In humans, the predominant transcript results in a biologically active FLT3LG, which associates with the plasma membrane by a transmembrane domain. A fraction of this variant undergoes cleavage to generate a biologically active soluble protein, suggesting the ratio of soluble over membrane bound could have a physiological role [56,57]. TNF- α converting enzyme (TACE) is a metalloprotease that cleaves the pro-tumour necrosis factor alpha (pro-TNF- α) and was found to be the enzyme most likely to be responsible for the cleavage of the plasma membrane FLT3LG/flt3lg in humans and mice, giving rise to the soluble form [61,62]. TACE could have an important role in controlling FLT3LG/FLT3 receptor interaction and defining whether it would occur via endocrine, autocrine and paracrine signalling.

1.7 Regulation of FLT3 receptor and signal transduction

Conformational changes caused by FLT3LG binding to FLT3 initiates phosphoinositide 3 kinase (PI3K) and mitogen-activated protein kinase (MAPK) signalling cascades. Binding of the adaptor protein growth factor receptor bound protein 2 (GRB2) to FLT3 receptor cytosolic domain leads to extracellular signal regulated kinase (ERK) phosphorylation and regulation of downstream signalling. ERK is a key component of the Ras-Raf-MEK-ERK pathway and responds to many external stimuli that regulates cell proliferation and death. ERK activation is one of the mechanisms by which FLT3 induces cell proliferation and survival. Knockdown of FLT3 in MV4-11 and MOLM14 AML cells was shown to prevent phosphorylation of ERK1/2 (P-ERK1/2) [63]. The same effect is seen when FLT3 is treated with inhibitors, such as sorafenib, which reactivates ERK phosphorylation after 24 h treatment, in MV4-11 cell lines and patient blasts. This effect is reversed with low dose of mitogen-activated protein kinase (MEK) inhibition [64,65]. The mechanism by which FLT3 activates PI3K is not fully understood [66]. PI3K isoforms are signal transducer enzymes that catalyse the phosphorylation of phosphatidylinositol-3,4,5-trisphosphate (PIP3), a fatty acid strategically localized in the inner leaflet of the plasma membrane. Therefore, signalling through the PI3K is highly associated with the subcellular localization of interacting proteins.

PI3K pathway is active (or it can be activated) in either FLT3-WT, FLT3-TKD or FLT3-ITD, and it has been suggested that PI3K and ERK are less active in FLT3-ITD than FLT3-WT [63]. It is not clear which conditions MAPK pathway, PI3K pathways or both

together control FLT3-mediated signalling cascades, and further understanding of this would provide important insights into the whole spatial-temporal process between cascades. It is important to take in account that PI3K can be activated by Ras itself, by interaction with the p110 catalytic subunit of PI3K [67]. Therefore, it would be interesting to know if PI3K can be simultaneously activated by Ras and FLT3 receptor. Elucidation of the dynamics of these pathways in the presence of FLT3 could explain why it is active even in the mislocalized FLT3-ITD. Interestingly, clathrin-coated internalization of RTK induced by receptor binding leads to an increase of PIP3 in endomembranes [68]. It suggests that this could be another mechanism by which FLT3-ITD activates PI3K pathway, even when it is retained in the secretory pathway.

It is known that PI3K activates a wide range of proteins. However, it is not completely understood which of those would be active in FLT3-WT, FLT3-TKD or FLT3-ITD AML. PI3K activation by FLT3 leads to protein kinase B (PKB/AKT) phosphorylation (P-AKT) and inhibition of FOXO3A. Restoration of FOXO3A expression prevents FLT3-ITD-dependent cell growth in 32D cells [69]. PI3K also controls the MDM2-p53 axis via AKT. *TP53* gene protects the genome from mutations; it is able promote cell cycle arrest and apoptosis when the DNA repair machinery fails. Overexpression of MDM2, frequently found in AML, could promote tumour progression because it is a negative regulator of p53 [70]. Likewise, MDM2-p53 has a central role in checking for DNA failure sending deficient cells to death, suggesting that in addition to its oncogenic function, FLT3-PI3K-MDM2-p53 could play an important role in the clonal evolution of AML. Idasanutlin, a MDM2 inhibitor, is currently being tested in a multicenter, double-blinded, randomized, placebo-controlled, Phase III clinical trial to be used as combination therapy in relapse and refractory AML (NCT02545283) and may be a promising drug to be prescribed either itself alone or in combination with other TKI therapies.

Overexpression of anti-apoptotic proteins has been implicated in drug resistance in AML. AML cells expressing FLT3-WT receptor are resistant to apoptosis upon activation of PI3K and RAS by activating proteins of the anti-apoptotic pathway[71,72]. PI3K activation leads to pro-apoptotic protein B-cell lymphoma 2 (Bcl-2) - associated death promoter (BAD) phosphorylation by AKT. FLT3 receptor activation decreases expression of Bax, a pro-apoptotic BH3 protein, without affecting Bcl-2 expression in patient samples, suggesting that Bax is a major regulator of resistance to apoptosis in AML [73]. In addition, FLT3 was shown to prevent apoptosis in progenitor lymphoid and myeloid cells via myeloid leukaemia cell differentiation protein Mcl-1 (MCL-1)[3]. MCL-1 is overexpressed in MV411 (FLT3-ITD⁺), 32D cells heterologously expressing FLT3-ITD and all patients harbouring FLT3-ITD [74],

suggesting that MCL-1 prevents apoptosis via activation of the STAT5 pathway by FLT3 harbouring an ITD mutation.

A common feature of this family of proteins is the presence of a BH3 domain [75]. The use of BH3 mimetics to target FLT3-expressing AML has been a promising strategy to treat this pathology and overcome resistance. Venetoclax is a BH3-peptide mimetic that binds selectively to the hydrophobic BH3 binding domain groove of BCL-2 to promote programmed cell death by releasing pro-apoptotic proteins, such as BAX, BAK and BIM [76]. It was approved by the FDA in 2018 to treat newly diagnosed AML based on the study NCT02287233 and NCT02203773 [77]. The simultaneous use of BH3-mimetics to target Bcl-2 and MCL-1 has been shown to be very effective in preventing disease progression in mice engrafted with patient-derived leukaemia without affecting normal haematopoiesis [78]. Activation of STAT5 in FLT3-ITD-AML prevents PI3K-induced apoptosis, by upregulation of MCL-1 [79]. MCL-1 was shown to be required for AML growth and development in mice. Bone marrow-derived hematopoietic stem/progenitor cells transduced with MLL-ENL, MLL-AF9, or HOXA9 developed immature myeloid cells, in which MCL-1 was downregulated and BIM but not BID or PUMA was upregulated [80]. Targeting MCL-1 is therefore an attractive therapeutic target. S64315 is a selective inhibitor of MCL-1 and is currently in phase I clinical trial for AML and myelodysplastic diseases (NCT02979366). An AZD5991 study is currently recruiting for a 3-part trial Phase 1/1b/2a, as monotherapy dose escalation (part 1), relapsed and refractory AML (part 2), and a sequential dose escalation study in combination with venetoclax for refractory and relapse AML (NCT03218683). Therefore, controlling the apoptotic pathway in AML with *FLT3* mutations seems to be another efficient strategy to overcome this pathology and a succinct schematic is shown here (**Figure 2**).

The JMD structure is highly conserved amongst type III protein tyrosine kinases FLT3, platelet derived growth factor receptors (PDGFR), c-KIT and is important for auto-inhibition of this class of receptors. FLT3 is the only type III tyrosine kinase that develops ITD in this region, occurring often in the linker peptide, close to the hinge region [14]. This gain-of-function mutation causes constitutive activation and mis-trafficking of the receptor [81] and it seems to switch signalling from the MAPK and PI3K pathway to STAT5 [79]. Most literature describes STAT5 as a pathway specifically activated by FLT3-ITD²². Overexpression of FLT3 mutated to a FLT3-containing a perinuclear ER-retention sequence in cell lines prevents receptor trafficking to the plasma membrane and inducing mislocalization to the perinuclear region. This change in subcellular localization leads to a sustained STAT5 activation and a decrease ERK and AKT activation. It also causes to STAT3 phosphorylation [81]. FLT3-ITD

but not FLT3-TKD activates STAT5 because, unlike the ITD, TKD mutations have no effect in receptor trafficking to the membrane. Therefore, it is likely that different therapeutic strategies are required among patients [82]. STAT5 is a transcription factor regulated by the Janus kinase (JAK) pathway. Although much is known about STAT5 pathway, the mechanism by which it is activated in FLT3-ITD⁺ AML is not clear. Strong evidence suggests that STAT5 is directly phosphorylated by FLT3 instead of being phosphorylated by JAK, thus, bypassing JAK activation [83]. Mice transplanted with FLT3-ITD-Y589/591F did not develop a myeloproliferative phenotype typical of the FLT3-ITD⁺ AML, supporting the *in vitro* evidence for phosphorylation of Y589 and Y591 in the JMD of FLT3 as a requirement for STAT5 activation and disease progression [84]. Lestaurtinib (CEO-701) is an indolocarbazole considered a potential drug to target FLT3-ITD signalling in AML because of its selectivity towards JAK2. It has been suggested that treating leukaemia cell lines with lestaurtinib before standard induction therapy, such as cytarabine, reduces the efficiency of the therapy [85,86]. However, no benefit of using lestaurtinib in newly diagnosed patients has been seen in Phase III clinical trials [87]. It further suggests that STAT5 activation bypasses JAK activation.

STAT5 itself is a potential drug target in FLT3-ITD⁺ AML. In addition to MCL-1, it activates c-Myc, BCL-XL, the proto-oncogene serine/threonine-protein kinase 1 and 2 (PIM1/PIM2), cyclins, among others [88–92]. Interestingly, FLT3-ITD increases PIM1, MYC and B-cell lymphoma 2 (BCL-2) mRNA levels whereas C/EBP α and TNF2 are downregulated [93–95]. AC-4-130 is a STAT5 inhibitor that specifically binds to the SH2 domain of STAT5 causing downregulation of PIM1 and c-MYC mRNA and upregulation of TRAF1 and colony stimulating factor 1 (CSF-1) in FLT3-ITD⁺ AML cell lines MV4-11 (LOH-FLT3-ITD⁺) and MOLM-13 (heterozygous FLT3-ITD⁺) [83]. Together, these data suggest that FLT3-ITD phenotype is highly dependent on STAT5 signalling and targeting it in FLT3-ITD⁺ AML is a promising strategy (**Figure 2**).

1.8 The role of Y589, Y591 and Y599 on the JMD in FLT3 function

Structural studies identified tyrosine residues within the JMD as phosphorylation sites important for FLT3 signalling pathway activation in FLT3-ITD [14,84] Y589 and Y591, located within the β -strand 2 (BJ2) of the JMD, have their chains adjacent to each other, pointing directly to the COOH-lobe to promote auto-inhibition (**Figure 3**). It has been proposed that phosphorylation of these sites block interaction between BJ2 and the COOH-lobe to promote constitutive auto-activation[84,96]. While phosphorylation of Y589 and Y591 activates STAT5, phosphorylation of Y589 and Y599 (P-Y589 and P-Y599, respectively) regulate ERK

activation by FLT3-WT. A Y589F mutation on FLT3-WT enhances ERK-activation, whereas Y599F mutation decreased FLT3-WT activation, proliferation and cell survival in 32D cells. Y589 and Y599 are also binding sites for the proto-oncogene tyrosine-protein kinase (Src) [96]. The Src protein kinase is composed of nine proteins including tyrosine-protein kinase (Lyn), Gardner-Rasheed feline sarcoma viral (Fgr), or tyrosine-protein kinase HCK (Hck) and harbour a Src homology domain 2 (SH2) domain, which is known to bind to phosphorylated tyrosine residues within the consensus sequence (P-YEEI) [97,98]. Src kinases were found to interact with P-Y589 and P-Y599 of FLT3-WT when stimulated by FLT3LG, even if Y599 lacks the canonical YEEI motif. Interestingly, Y599 seems to be the phosphorylation site most responsive to FLT3LG-dependent receptor activation [96]. Patients harbouring an ITD with a duplication of the Y591 residue have increased BCL-2 expression, and it has been proposed that it blocks the p53 apoptotic pathway [99]. Together, these data suggest that Y591 duplication can be another strategy by which FLT3 mutated AML uses to evade cell death. There are other mechanisms by which FLT3 is regulated. Suppressor of cytokine signalling proteins (SOCS) contains a SH2 domain, a SOCS-box motif and its activity is associated to the JAK/STAT5 pathway. It has been shown that SOCS6 is recruited to phosphorylated FLT3 and binds to the phosphorylated Y591 (P-Y591) and Y919 (P-Y919) for receptor ubiquitination and internalization, suggesting that SOCS6 is a negative regulator of FLT3 [100]. The lymphocyte adapter protein (LNK) is highly expressed in haematopoietic cells and interacts with many kinases to control multiple cell functions. Phosphorylated Y572 (P-Y572), Y591 (P-Y591), and Y919 (P-Y919) in FLT3-WT and FLT3-ITD was shown to be a LNK binding site that causes suppression of FLT3-WT and FLT3-ITD signalling cascade [101]. The turnover of kinase receptor activation is controlled by the cleavage of the phosphate groups by protein phosphatases. To contribute to FLT3 receptor turnover, protein-tyrosine phosphatase density-enhanced phosphatase 1 (DEP-1) controls FLT3 dephosphorylation to regulate FLT3 activation. DEP-1 dephosphorylates Y589 and Y591 tyrosine motifs, in addition to the Y842 in the TKD domain [102]. Therefore, tyrosine motifs required for STAT5 signalling seem to be required for interaction with other proteins. In addition, these amino acids are often duplicated in FLT3-ITD, suggesting that further studies on how cells orchestrate these interactions would be beneficial to understand the mechanism FLT3 signal in different clones.

Moreover, DEP-1 activity is sensitive to oxidative stress. Excessive production of reactive oxygen species (ROS) oxidizes the catalytic Cys residue required for DEP-1 activity [102]. Just like DEP-1, most of tyrosine phosphatases are sensitive to these free radicals, because their activity relies on a catalytic Cys residues [103]. Intracellular levels of reactive

oxygen species (ROS) are increased in AML and it has been shown that an increase in ROS levels are induced by phosphorylation of STAT5 in FLT3-ITD⁺ AML to promote FLT3 signalling [104,105]. To further support this data, it has been shown that the transcription factor c-Jun and its c-Jun kinase (JNK), which act as oxidative stress sensors, are phosphorylated and active in AML. Moreover, they are part of the FLT3 signalling axis in AML [106,107]. The FLT3 receptor contains eight cytosolic cysteines (Cys681, Cys694, Cys695, Cys790, Cys807, Cys828, Cys925, Cys945). It is unlikely that these cysteines form disulfide bridges in the reducing environment of the cytosol, unless they are protected in a hydrophobic pocket [108]. However, their thiol side chain can be a target for biologically important post-translational modifications, such as *S*-glutathionylation, *S*-sulphenylation, lipidation (*S*-palmitoylation and prenylation in eukaryotic cells), *S*-nitrosylation and modifications by metabolic molecules, such as, retinoic acid (RA) and 4-hydroxy-nonenal (HNE), a product of lipid peroxidation [109]. These modifications could be implicated in conformational changes that serve as a switch for the signalling cascade of the receptor. Individual cysteine to serine mutations demonstrate that all of these residues are required for correct glycosylation, except for Cys694. C694S and C925S reduce kinase activity, whereas, C790S and C828S enhance activity but only C790S enhances STAT5 activation [110]. Therefore, oxidative stress and ROS in combination with post-translational modifications are possible ways that cells control FLT3 receptor signalling and hyperactivate signalling cascades required for AML progression.

1.9 Comparison of human and murine FLT3 sequence in the exon 14.

In mice, *flt3* receptor is encoded by the *fetal liver kinase 2 (flk2)* gene and it is physiologically expressed in lymphoid progenitor cells only [3]. The murine *flt3* shares 85.5% homology with human *FLT3*. Sequence alignment of exon 14 and exon 20, where ITD and TKD mutation occur, respectively, shows differences in the amino acid composition, which could imply differences in signalling and drug response. The aspartic acid (D) in the F R D Y E Y sequence, often duplicated in FLT3-ITD, is substituted by glutamic acid (E) in humans. In addition, proline 584 and leucine 585 in the mouse sequence are replaced by a serine in humans, suggesting that a phosphorylation site is being created in the human orthologue (**Figure 4**). *flt3*^{-/-}; *flt3*^{-/+} knockout alone does not cause significant changes to mice physiology, except for reduction in primitive B lymphoid progenitor cells in the bone marrow. Simultaneous knockout of *flt3* and *c-kit* cause severe haematopoietic deficiency in mice [111], suggesting that FLT3 plays an important role in the murine haematopoiesis. These clear species differences between

humans and mice obviously limit the usefulness of mice models for targeting FLT3 therapies in AML and any myeloid malignancies.

2. Tyrosine kinase inhibitors targeting FLT3-ITD

The standard AML treatment starts with induction therapy, usually a 3+7 regimen of daunorubicin and cytarabine followed by consolidation therapy, where lower doses are given to prevent relapse [112]. Patients harbouring FLT3-ITD mutation at diagnosis are more likely to relapse after standard therapy [113]. Development of tyrosine kinase inhibitors (TKI) for use as personalized therapy upfront, where a patient is treated according to the risk stratification, is a major advance in AML therapy recently [8,114]. Targeted therapy in general aims to reduce side effects and increase the chance of remission, variables that improve survival. Several TKIs targeting FLT3-ITD are being tested and some have been recently approved to be used in AML. TKI can be classified according to where it binds in the proteins [114]. Type I and II TKIs interact directly to the ATP binding pocket and are primarily classified according to the conformational state of the activation loop. Type I (first generation) binds to the active DFG-D-in where D is positioned inward, whereas and type II (second generation) binds to the inactive DFG-out conformation. Therefore, they are less specific and target the ATP binding site. Moreover, the binding affinity and selectivity of type I TKIs needs to be strong enough to overcome competition with endogenous ATP. Therefore, the effective concentration required to achieve 50% of the effect (EC50%) can be affected by the intracellular concentration of ATP [115]. Midostaurin and gilteritinib are type I inhibitors and were recently approved to treat FLT3-ITD⁺ AML. Sorafenib and quizartinib are type II inhibitors, which are more selective to FLT3-ITD⁺. These drugs are among the most promising therapeutic TKIs targeting FLT3-ITD⁺-AML to date and are still being investigated (**Table 1**).

Midostaurin (PKC-412) was the first kinase inhibitor approved for AML [116], it was derived from an alkaloid extracted from the *Streptomyces staurosporeus*. It was originally developed as a protein kinase C (PKC) inhibitor; however, it has a broad activity and inhibits multiple kinases at different binding affinities. It is an ATP analogue, composed of an indolocarbazole lactam rings. As a typical type I TKI, it binds to the kinase domain when the protein is in a DFG-in conformation. In a small trial, midostaurin was shown to be efficient to 70% of the FLT3-ITD⁺ AML cohort reducing circulating blast counts in 50% [117]. Midostaurin was then approved in 2017 for newly diagnosed patients harbouring FLT3-ITD⁺ AML, to be used in conjunction with cytarabine and daunorubicin. It is set to be used in combination with chemotherapy and as maintenance therapy for FLT3-ITD⁺ patients[116,118].

However, midostaurin is also known to benefit FLT3-WT patients, even if FLT3-ITD⁺ patients are more sensitive. This is likely due to its ability to bind multiple kinases, in particular c-KIT, which is known to have a synergistic effect with FLT3 in AML progression[59]. Upregulation of MCL-1 in 32D, MV4-11 and RS411 cells is efficiently reduced by midostaurin [74]. FLT3-TKD was shown to be more sensitive to midostaurin than FLT3-ITD when compared to FLT3-WT in 32D cells. Resistance to TKIs like midostaurin is a great concern and most of the patients develop acquired mutations in amino acids required for adequate binding of the drug to the receptor [114]. Indeed, G697R mutation, near the adenine pocket site of FLT3 confers resistance to midostaurin in K562 cell lines, suggesting that this residue is important for midostaurin binding. Meanwhile, FLT3-N841I activating mutation was shown to increase sensitivity of FLT3 to midostaurin [119,120]. Mutation on the asparagine 676 in the TKD (N676K) was present in blasts from patients resistant to midostaurin and was further confirmed to be the sole cause of resistance when FLT3-N676K was compared to FLT3-WT in 32D transfected cells [121]. Transcription analysis of a patient enrolled in a phase 2 clinical trial (NCT00045942) who presented two ITD mutations (ITD1 and ITD2) and treated with midostaurin developed resistance. It was associated with loss of ITD2 and acquisition of a mutation within ITD1. The resulting mutant, denominated FLT3-ITD627-E, was tested in 32D cells and presented increased MCL1 expression and STAT3 phosphorylation [122].

Gilteritinib is a pyrazinecarboxamide derivative classified as a type I TKI. It effectively induces apoptosis in cells harbouring FLT3-ITD and FLT3-D835Y. Docking simulation predicts the binding of Gilteritinib to the ATP binding site and not to the activation loop, allowing inhibition of FLT3-ITD and D835Y. It interacts with F691, a gatekeeper amino acid frequently mutated in quizartinib-relapsed AML patients, without losing effect [123,124]. Gilteritinib was approved by the FDA in 2018 for relapse and refractory patients with FLT3 mutations [125]. It is highly selective to FLT3 and is also a potent inhibitor of the tyrosine kinase AXL, which was shown to diminish FLT3-ITD phosphorylation, suggesting that it prevents receptor activation [123]. A phase III clinical trial (NCT02014558) tested Gilteritinib in refractory and relapsed AML and 3 out of 30 patients developed a *FLT3-F691L* mutation. Patterns of resistance were also identified; one patient lost the *FLT3-ITD* and acquired *NRAS* and *IDH2* mutations and the second harboured a *FLT3-TKD-D835Y* and a clone evolved by developing further *NRAS* mutation [126]. Interestingly, the National Institutes of Health (NIH) in US is currently recruiting patients to compare the benefits of midostaurin versus gilteritinib, the two type I (and, therefore, less specific to FLT3) inhibitors approved to be used in AML

patients (NCT03836209) and may answer more of these questions regarding mutation development in resistant patients.

Crenolanib is a potent benzamidazole that targets type I TKI with high selectivity to FLT3. It also has strong activity in the PDGFR alpha and beta [127]. It is a type I TKI with activity against AML-expressing FLT3-WT and FLT3 mutants [128,129]. Resistance to crenolanib has already been reported and investigated. Whole exome sequence identified mutation in the *N-RAS*, *IDH1*, *IDH2* and the *TET2* genes. Concomitant mutation of the *TP53* with *FLT3* is associated with resistance. Secondary mutations in the FLT3 gene were not frequent. Patients that responded poorly to crenolanib had pre-existing mutations in non-TKI genes [130].

Sorafenib is a biaryl-ureia that binds to the DGF-out conformation, therefore a type II inhibitor. It was developed to target the Raf-1 proto-oncogene, a serine/threonine kinase with high affinity to (vascular endothelial growth factor receptor) VEGF receptor [131]. It is a multikinase TKI, and it binds to FLT3. Combination of sorafenib with daunorubicin or cytarabine improves the efficacy of the therapy based on the overall survival analysis in a phase II clinical trial, although it also increased side-effects [132]. In other studies, FLT3-ITD AML patients responded rapidly and efficient to sorafenib. However, relapse onset was also developed [133]. 3 out of 6 children with AML harbouring a *FLT3-ITD* treated with sorafenib relapsed and acquired a *FLT3-D835H* mutation. One of the patients developed *FLT3-D835H-F691L* mutations concomitantly [134].

Quizartinib (AC220) is a type II tyrosine kinase inhibitor, a diaryl urea molecule containing a morpholinoethoxy group that improves solubility. The urea moiety interacts *via* hydrogen bond with C828 on FLT3 backbone [135]. Quizartinib is selective to FLT3-ITD. It decreases Pim-1 expression and inhibits STAT5 phosphorylation [136]. Unlike midostaurin, quizartinib binds specifically to the DFG-out conformation. Therefore, quizartinib does not bind to the FLT3 receptor that harbours the highly frequent D835 TKD mutation. Docking analysis suggested it functions by conformational modifications to the binding site, transforming the DFG-out into a DFG-in structure. Genomic DNA FLT3-ITD⁺ AML patients enrolled in a phase II clinical trial (NCT00989261), who relapsed quizartinib treatment were screened for TKD mutations and eight patients harboured TKD mutations D835Y, D835V, D835F, either solely or co-occurring with the gatekeeper mutation F691L [124]. D835 acquired mutations are one of the mechanisms of resistance to quizartinib and sorafenib, and possibly all type II TKI [137,138].

The use of allosteric inhibitors that bind irreversibly to RTK is an alternative to inhibitors that interact with active sites because of their increased selectivity. These are classified as type III TKI, because they do not bind to the ATP-binding site and cause allosteric changes that prevent the ATP binding [139], therefore, tend to be more specific and reduce side effects. Ibrutinib is an allosteric inhibitor of the Burton's tyrosine kinase (BTK) and forms an irreversible bond with BTK Cys481. Ibrutinib has been approved by the FDA and is the first line drug for chronic lymphocytic leukaemia (CLL) and mantle cell lymphoma (MCL) [140]. BTK is part of the TEC family and the Cys481 is quite conserved, therefore ibrutinib is a multitarget kinase inhibitor that binds to all TEC proteins [141]. Ibrutinib was shown to reduce proliferation in FLT3-ITD AML cell lines and primary cells but not FLT3-WT-expressing cells [142]. Whether this is achieved by directly or indirectly interacting with FLT3, has yet to be investigated. These data suggest that ibrutinib could be used in combination with standard therapies to target FLT3-ITD⁺ AML. Moreover, other inhibitors such as the pan-BTK/FLT3 inhibitor CG-806 are showing encouraging clinical usefulness in AML and lymphoid malignancies.

To overcome drug resistance developed by the use of TKIs that target FLT3-ITD⁺ AML (such as quizartinib), FF-10101, a next-generation TKI, has been developed. It is an allosteric and specific inhibitor of the FLT3 receptor that covalently modifies Cys695, which is distal to the DFG domain. FF-10101 is, therefore, a type III TKI shown to be a potent anti-proliferative drug in AML cells harbouring FLT3-ITD, D835, Y842, F691 and combination of those [143]. A Phase 1/2a dose escalation and dose ranging clinical trial for FF-10101-01 is currently recruiting patients with relapse and refractory AML, a great hope in overcoming FLT3 mutations-derived drug resistance (NCT03194685).

Mutations that lead to changes in the DFG conformation are the most rational explanation for type II TKI drug resistance, even if co-occurrence of FLT3-ITD and FLT3-TKD is not always reported as a frequent event [144]. Other mechanisms of resistance to quizartinib have been reported. A patient harbouring mutation in the gatekeeper residue Phe691 (F691L), normal karyotype, 86-bp mutation and NPM1 mutation relapsed following quizartinib treatment [145]. In addition, blast cells co-cultured with stromal cells resisted quizartinib-induced cell death and shown persistent activation of ERK [146]. In addition, regulation of FLT3 activation and the development of resistance could be related to the amount of FLT3LG that the blasts are exposed to. *In vitro* and *in vivo* data have shown that quizartinib and midostaurin treatment also increase the expression of FLT3LG, suggesting that increases in FLT3LG expression could be associated with the development of resistance to the treatment

[147–149]. Plasma concentration of FLT3LG increased in relapsed patients when compared with newly diagnosed patients [147]. Overall, it seems that relapsed patients are more sensitive to quizartinib and midostaurin than newly diagnosed patients due to the presence of acquired mutations on *FLT3*. A summary of selected TKIs targeting FLT3 is shown in **Table 1**.

Other TKIs were also designed or being repurposed to target FLT3 positive AML. For instance, G-749 a TKI that targets FLT3 WT, FLT3-ITD and FLT3 harbouring TKD mutations was shown to be a potent anti-leukaemic activity [150]. Dual targeting of FLT3-ITD and Pim-1, which is directly regulated by STAT5, with the selective inhibitor SEL24-B489 is being successfully tested in pre-clinical models [151].

We have seen that AML is characterized by clonal evolution, in other words, constant adaptation of the cancer cells by means of developing new mutations to adapt to changes in the surrounding environment. Therefore, while the use of monotherapy targeting a protein that control a vital pathway is shown to be effective, especially for patients that have been screened for specific mutation, such as FLT3-ITD. Clones have consistently and constantly evolving, to develop resistance to the targeted therapy leading to relapse [152]. Based on the literature reviewed so far, it is likely that clones precisely evolve by changing nucleotide sequences in their gene that will alter the mutational status, causing a relatively dynamic shift in the pathways required for survival and proliferation. Moreover, single cell studies have shown that clonal population can be heterogenic, which raise an addition concern in deciding the optimal therapy for each individual patient [153].

To overcome the challenges this type of cancer presents, even when such precise and effective therapies are available, it has been suggested that the combination of a TKI with other therapies would function as trap and prevent the cells to shift from one survival strategy to another and improve patient outcome and overall survival, for instance, midostaurin or gilteritinib with daunorubicin and cytarabine (NCT00651261, NCT04240002).

Moreover, diagnosis seems to be crucial, patients would benefit from having a periodical screen of key genes to detect changes in mutation patterns occurring from diagnosis to relapse, combining with a stablished characterization of the best therapy for combined mutations and chromosomal aberrations. For that to be achieved, establishment of combination therapy associated to certain genes in combination of an optimal screen of patient mutation and gene expression in the clinic would be ideal because patient prognosis is strongly relying in these events. A recent study has recently published an attempt to provide a platform for clinical applications of whole transcriptome RNA sequencing, bringing advancement for AML therapy [154].

Monoclonal antibody therapy targeting FLT3 receptor

Engineering of humanized antibodies developed to specifically target cell surface receptors with very low immunogenic response are an attractive strategy to treat many types of cancer, including AML [155]. Gemtuzumab Ozogamicin is an immunoconjugate of recombinant monoclonal antibody crosslinked with calicheamicin (N-acetyl- γ -calicheamicin), a cytotoxic drug that causes double strand breaks in the DNA [156]. After controversies regarding the safety of this product. It was approved, in 2017, by the FDA to treat adult and paediatric CD33-positive AML [157]. Interestingly, a clinical trial combining midostaurin and has been launched this year as a strategy to combat AML and prevent relapse (NCT04385290)

AML does not have specific surface marker, which makes it challenging to develop a specific antigen. Bispecific monoclonal antibodies were developed to bring T-cells in close proximity to the cancer cells by generating a monoclonal antibody co-expressing a T-cell epitope and cell-specific tumour epitope. Engineering of a monoclonal antibody expressing CD3 (T-cell epitope) and CD33, which targets myeloid derived suppressor cells (MDSCs) where shown to be present some anti-leukaemic effect. MDSCs appear high in AML patients and promote disease progress [158,159]. Chimeric antigen receptor T (CAR-T) cells have also been engineered to target AML cells expressing CD123 and CD33 receptors [160] .

Due to the considerable selective expression of FLT3 receptor in AML and progenitor cells, it is a candidate target for antibody therapy in AML. FLT3 receptor is quite unique. Unlike others class III RTK, it does not require homotypic interactions for activation relying only in the ligand binding to the domain III. Moreover, mutations in the extracellular domain of FLT3 that could lead to drug resistance is uncommon. Multiple studies, including clinical trials using monoclonal antibody therapy to target the FLT3 receptor are being proposed as a selective way of blocking FLT3-WT and FLT3 harbouring mutations that do not affect subcellular trafficking, for instance FLT3-TKD mutation [161,162] (ref).

Naive phage antibodies developed to antagonize FLT3-FL binding proofed to trigger receptor-mediated endocytosis while preventing ligand-induced cellular proliferation [163,164]. This data suggests that the binding of the antibody to the receptors could be leading to rapid internalization followed by lysosome degradation. To our knowledge, no study has shown the phosphorylation status of FLT3 receptor and its downstream elements upon antibody binding. However, many engineered antibodies targeting FTL3 to date lacked efficacy, as it is the case IMC-EB10 (NCT00887926). Perhaps, development of a recombinant antibody targeting FLT3 crosslinked with a TKI such as type I, assuming the small molecule would have access to the cytosolic domain of FLT3 after receptor-mediated endocytosis, would have a

significant effect in combating not only AML expressing surface-localized, but also FLT3-ITD.

3. Conclusions

AML is characterized by the accumulation of heterogeneous clones that develop different sets of mutations. Identifying the mutational profile allows each individual patient to be classified according to their risk stratification. Similarly, we would wish to predict stratified therapies that may be vital towards combating any relapse or development of drug-resistance. Here, we have focused on identifying those most frequent mutations of the *FLT3* gene observed clinically, so as to understand the cellular mechanisms of FLT3 protein kinase responses in survival and proliferation of that mutant clone. The presence of an ITD has been shown to be an earlier event compared to development of further TKD mutations. FLT3-ITD was shown to cause oncogenic addiction in AML cells through ERK, PI3K, STAT5 and anti-apoptotic BCL-2 family signalling. Switching off these signal streams seems to be the way to overcome aberrant signalling and resistance towards apoptosis in AML treatment. These pathways are also regulated by other kinases and the spatial-temporal activation of specific proteins in these signalling cascades depend on their level of expression, cellular environment and on the structural form of the receptor itself.

Although mutations are thought to be an evolutionarily random process, FLT3-ITD positive AML seems to develop gains and losses of its mutations in a strategic way, to guarantee clonal survival and proliferation over other clones. Tandem duplications of the JMD may create additional phosphorylation sites to enhance signalling. Fortunately, therapies are rapidly being developed to specifically target frequent re-occurring mutations in the *FLT3* gene. Co-expression of certain proteins or co-occurring mutations have an important role in the pathogenicity of AML. As outlined, the expression of adaptive mutations of the FLT3 receptor can confer resistance to apoptosis, and changes in subcellular localization can serve as a switch in signalling cascades. Likewise, regulation of signalling cascades by elevated concentrations of ROS in AML and any potential post-translational modifications of FLT3 are cellular adaptations that can further favour survival and proliferation of the leukemic clones.

The data published to date suggest that FLT3LG activates receptor signalling in a paracrine, autocrine and endocrine manner due to the multiple transcriptional and post-translational variants present. Mouse models are excellent to understand the physiological phenotype of mutations. FLT3 biology differs slightly between humans and mice, because of

the variable abundance in different progenitor cell types. Moreover, auto-phosphorylation sites often duplicated in FLT3-ITD of the JMD (Ser583 and Ser584) are not present in mice, and these sites are located near important tyrosine residues required for human STAT5 signalling (Tyr589 and Tyr591). Together it describes the versatility in signalling that this JMD site promotes.

Development of TKIs has emerged as a tool to combat resistance in AML by targeting oncogenic genes such as FLT3 in AML. Type I inhibitors are more promiscuous for having multiple targets. Type II are more selective to FLT3-ITD; however, mutations can occur as result of exposure to the drug. Indeed, mutation in the TKD domain of FLT3-ITD cause conformational changes that lead to resistance to type II treatment. Therefore, the alternative is to target motifs distinct from FLT3's ATP binding site. For this reason, the type III FF-10101 was developed as an alternative treatment. Interestingly, this allosteric inhibitor covalently binds to Cys695, which is shown to be sensitive to ROS, suggesting that the efficacy of the drug will depend on the REDOX status of the cytosolic environment and may be susceptible to post-translational modifications on this residue. Alternatively, targeting directly anti-apoptotic and signalling proteins using BH3-mimetics in combination with TKI could also offer promising options to achieve clinical remission.

Both FLT3 receptor and FLT3LG can function in synergy with other pathways. It means that activation of PI3K and ERK/MAPK pathways are more complex than what is currently appreciated. Understanding when and why cross-activation occurs would be of great clinical significance in characterising and classifying AML's ability to respond to targeted therapies. A better understanding of the impact of different ITD mutations and other related FLT3 mutations would support and guide current therapeutic strategies for AML, with the further aim of facilitating predictive therapeutic approaches tailored to the individual patient.

Figure legends:

Figure 1. Schematic design of the clinically relevant FLT3 mutations based on its primary structure.

FLT3 mutations are highlighted (cyan) in the schematic of FLT3 receptor primary structure (grey). Signal peptide (SP – light grey), TM (dark grey), JMD (light purple), TKD1 (dark green) and TKD2 (light green) are highlighted. Region where ITD mutations can occur is underscored and overlaps JMD and TKD1.

Figure 2. Summarized overview of the apoptotic pathway regulation in FLT3-ITD AML.

Autophosphorylated FLT3 ITD activates PI3K and STAT5 pathway and regulates apoptosis. In FLT3-ITD the STAT5 pathway prevails. The feedback loop created by Pim-1/STAT5 signalling is indicated by the dashed arrows. Ras/ERK pathway is also active.

Figure 3. Structural orientation of Y589 and Y591 in the juxtamembrane domain. Crystal structure of FLT3 cytosolic domain (PDB.1RJ8) illustrates the spatial orientation of Y589 (red) and Y591 (blue) in the JDM (wheat) (A). Measurement shows a 3.9 Angstrom distance between Y589 and Y591 (B). Visualized in Pymol©

Figure 4. Peptide sequence alignment of exon 14 and exon 20 of human (*FLT3*) and mouse (*flt3*). Exon 14 and exon 20 from mouse *flt3* (ENSMUST00000049324.12) and human *FLT3* (ENST00000241453.11) were aligned and the mismatching amino acids highlighted (blue). Exon sequence frequently duplicated in human FLT3-ITD is overlined (red).

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