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DOCTORAL SCHOOL
MEDICINE

DEVELOPMENT OF THE MOLECULAR DIAGNOSTIC PROGRAM AND PROGNOSTIC STRATIFICATION IN ACUTE MYELOID LEUKEMIA

STUDY OF *FLT3*-ITD ACUTE LEUKEMIAS

ABSTRACT OF Ph.D. THESIS

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PUBLISHED WORKS

1. **Dan S Soare**, Eugen Radu, Ion Dumitru, Aurora Arghir, Cristina Enache, Horia Bumbea, Ana M Vlădăreanu – FLT3-ITD DNA allelic burden, but not mRNA levels, influences the biological characteristics of AML patients. Revista Română de Medicină de Laborator, 2021;29(1):33-41. Impact factor (Clarivate) 2023: 0.5. doi: 10.2478/rrml-2021-004. https://rrml.ro/articole/download.php?ID=606 . (**Chapter 6**)

2. **Dan-Sebastian Soare**, Eugen Radu, Ion Dumitru, Viola Maria Popov, Horia Bumbea, Ana Maria Vlădăreanu - FLT3-ITD DNA and mRNA levels in AML do not correlate with CD7, CD33 and CD123 expression. Journal of Cellular and Molecular Medicine, 2020 Iulie; 24(13):7675-7679. Impact factor (Clarivate) 2023: 4.3. doi: 10.1111/jcmm.15255. (**Chapter 7**)

Chapter 1 – Acute myeloid leukemia: diagnostic, classification

1.1 Introduction

Acute myeloid leukemia (AML) is a clonal malignant condition characterized by the proliferation and accumulation of leukemic blasts in the hematopoietic bone marrow. The primary consequence of the proliferation and accumulation of blasts is the alteration of the bone marrow microenvironment and suppression of normal hematopoiesis [1–3]. As a result, patients may present with various symptoms or complications due to secondary bone marrow failure, including those related to anemia, thrombocytopenia, decreased immunity, and/or associated coagulopathy.

1.2 Classification and prognostic evaluation

Classically, AML is defined by the presence of blast cells exceeding 20% of the total nucleated cells in bone marrow aspirate samples and/or peripheral blood.

In 2022, new diagnostic criteria for myeloid neoplasms were published: the 5th Edition of the World Health Organization (WHO) Classification of Myeloid and Dendritic Neoplasms [4], and the International Consensus Classification of Myeloid Neoplasms and Acute Leukemias [5]. In these classifications, the threshold of 20% myeloid blasts required for AML diagnosis has been revised: in the presence of recurrent driver mutations of AML, the threshold required for AML diagnosis has been lowered below 20% [4,5]. For AML cases that are negative for any of the recurrent driver mutations, the threshold remains at a minimum of 20% leukemic blasts for the diagnosis of AML.

Additionally, a new entity, MDS/AML (Myelodysplastic Syndrome/Acute Myeloid Leukemia), was proposed by the 2022 International Consensus Classification of Myeloid Neoplasms and Acute Leukemias [5]. The MDS/AML condition is defined by the absence of specific recurrent driver mutations of AML and presents a blast cell percentage between 10 and 19% [4,5].

The list of driver mutations is presented comparatively between the two articles [4,5], in **Table 1.1**. Exceptions to the driver mutations from these new classifications are:

(i) AML cases with *BCR::ABL1*, where the threshold remains at a minimum of 20% to differentiate between cases of Chronic Myeloid Leukemia in the accelerated myeloid phase (10-19% blasts) and the myeloid blast phase (≥20% blasts) [4,5]

(ii) According to the 5th WHO Classification 2022 [4], AML with CEBPA mutations requires a 20% myeloid blast percentage for diagnosis.

Table 1.1 Comparative Presentation of the New Classifications of Acute Myeloid Leukemias According to Recurrent Mutations	
WHO Classification of Myeloid and Dendritic Neoplasms, 5th Edition 2022[4]	**International Consensus Classification of Myeloid Neoplasms and Acute Leukemias 2022**[5]
APL with *PML::RARA* translocation, <20% blasts	APL with t(15;17)(q24.1;q21.2)/*PML::RARA* ≥10% blasts
	APL with other *RARA* rearrangement ≥10% blasts
AML with *RUNX1::RUNX1T1* translocation, <20% blasts	AML with t(8;21)(q22;q22.1) / *RUNX1::RUNX1T1* ≥10% blasts
AML with *CBFB::MYH11* translocation, <20% blasts	AML with inv(16)(p13.1q22) / t(16;16)(p13.1;q22) / *CBFB::MYH11*, ≥10% blasts
AML with *DEK::NUP214* translocation, <20% blasts	AML with t(6;9)(p22.3;q34.1)/*DEK::NUP214*, ≥ 10% blasts
LAM with *KMT2A* rearrangements, <20% blasts	AML with t(9;11)(p21.3;q23.3)/*MLLT3::KMT2A* ≥10% blasts
	AML with other *KMT2A* rearrangements ≥10% blasts
AML with *MECOM* rearrangements, <20% blasts	AML with inv(3)(q21.3q26.2) / t(3;3)(q21.3;q26.2)/*GATA2::MECOM* ≥10% blasts
	AML with other *MECOM* rearrangements≥10% blasts
AML with *BCR::ABL1* translocation, ≥20% blasts	AML with t(9;22)(q34.1;q11.2)/*BCR::ABL1* ≥20% blasts
AML with *RBM15::MRTFA* translocation, <20% blasts	AML with other rare translocations ≥10% blasts
AML with *NUP98* rearrangements, <20% blasts	
AML with other driver mutations	
AML with *NPM1* mutations, irrespective of blast percentage	AML with *NPM1* mutations ≥10% blasts
AML with *CEBPA* mutation, ≥20% Blasts	AML with in-frame *CEBPA* bZIP ≥10% blasts
N/A	AML with mutated *TP53*, ≥20% blasts MDS/AML with mutated *TP53*, 10-19% blasts

AMl with MDS-associated mutations, ≥20% blasts
• Defining cytogenetic changes
  ○ complex karyotype (≥3 alterations)
  ○ Deletion of 5q or loss of 5q arm due to an unbalanced translocation
  ○ Monosomy 7, deletion of 7q, or loss of 7q arm due to an unbalanced translocation
  ○ Deletion of 12p or loss of 12p arm due to an unbalanced translocation
  ○ Monosomy 13 or 13q deletion
  ○ Deletion of 17p or loss of 17p arm due to an unbalanced translocation
  ○ Isochromosome 17q
  ○ Idic(X)(q13)
• Defining somatic mutations: *ASXL1, BCOR, EZH2, SF3B1, SRSF2, STAG2, U2AF1, ZRSR2*
	AML with MDS-associated mutations ≥20% blaști
	MDS/AML with MDS-associated mutations 10-19% blasts
	MDS-associated mutations: *ASXL1, BCOR, EZH2, RUNX1, SF3B1, SRSF2, STAG2, U2AF1* or *ZRSR2*
	
	AML with MDS-associated chromosomal modifications ≥20% blasts
	MDS/AML with MDS-associated chromosomal modifications 10-19% blasts
	Defined by the detection of:
	• Complex karyotype (≥ 3 alterations in the absence of other defining events)
	• del(5q)/t(5q)/add(5q)
	• -7/del(7q)
	• +8
	• del(12p)/t(12p)/add(12p)
	• i(17q)
	• -17/add(17p) or del(17p)
	• del(20q)
	idic(X)(q13)

APL – acute promyelocytic leukemia, AML – acute myeloid leukemia, WHO – World Health Organization, MDS – myelodysplastic syndrome, N/A – Not Applicable

Chapter 2 –*FLT3*-ITD and *NPM1* mutations

2.1 Introduction

FLT3-ITD and *NPM1* mutations are frequently associated and constitute one of the most common subtypes of AML. For this reason, this chapter will provide a detailed presentation of both FLT3 and NPM1 genes, their physiological roles, the most common mutations observed, and their role in the development of acute leukemias

2.2 *FLT3*-ITD mutation

The most common mutations found in the *FLT3* gene are in-frame insertions (which maintain the reading frame of the gene's codons) through tandem duplications in exon 14 or 15. Exon 14 encodes the juxtamembrane portion of the receptor, while exon 15 encodes tyrosine kinase domain 1. The reported insertions are highly heterogeneous, with variable lengths ranging from 6 to 281 base pairs (bp) and variable insertions randomly located within the amino acid range 567-670 [6–13].

2.3 *NPM1* mutation

The most frequent mutation in AML is the NPM1 mutation, found in approximately 30% of cases [14,15]. The described mutations are frame-shift insertion mutations found in exon 12. The most common mutations are 4 bp insertions between nucleotides 960 and 961, types A, B, D [16–20]. The main consequence is the predominant cytoplasmic localization of the NPM1 protein [16,21,22].

The sequence alterations induced by *NPM1* mutations are [16–19,23]: (i) alteration of tryptophan (W) residues at positions 288 and/or 290, which are necessary for the nucleolar localization signal; and (ii) the creation of a new nuclear export sequence (VSLRK). The combination of these two changes is required for the predominant cytoplasmic localization of the mutated NPM1 protein [24].

2.5 *FLT3*-ITD/*NPM1* prognostic evaluation

According to the ELN 2017 and ELN 2022 guidelines for the management of AML patients [15,25] all patients should be evaluated for the presence of *NPM1* and *FLT3*-ITD mutations to determine prognosis. The ELN 2017 model [15] for establishing genetic

prognosis in AML with *NPM1* and *FLT3*-ITD mutations is based on a series of retrospective studies [26–30], where the semi-quantitative evaluation of *FLT3*-ITD mutations, by determining the allelic ratio between *FLT3*-ITD/*FLT3*-WT, correlated with the presence or absence of the *NPM1* mutation, allows for prognosis adjustment.

Thus, *NPM1* positive/*FLT3*-ITD positive patients with a *FLT3*-ITD/*FLT3*-WT allelic ratio of <0.5 (*FLT3*-ITD low) have a relatively similar outcome to *NPM1* positive/*FLT3*-WT patients, with both groups being included in the favorable risk category [27–29]. Due to the favorable outcomes of these patients, consolidation with allogeneic hematopoietic stem cell transplantation (allo-HSCT) in first remission is not recommended [15,30].

Regarding *NPM1* positive/*FLT3*-ITD positive patients with a high *FLT3*-ITD/*FLT3*-WT allelic ratio of ≥0.5 (*FLT3*-ITD high) and *NPM1* negative/*FLT3*-ITD low patients, they are included in the intermediate genetic risk category [27–29].

Patients who are *NPM1* negative/*FLT3*-ITD high have the most aggressive course [26], and are included in the adverse risk category. These patients also derive the greatest benefit from consolidation with allo-HSCT in CR1 [30].

However, with the publication of the new ELN 2022 guidelines [25], the prognostic evaluation of *FLT3*-ITD cases based on the allelic ratio (AR) has been discontinued. The reasons for discontinuing prognostic stratification based on AR were: (i) lack of standardization of the AR evaluation method, and (ii) data published by K. Döhner *et al* [31], which additionally showed that the addition of the TKI midostaurin significantly improves prognosis, especially in patients with a *FLT3*-ITD/*FLT3*-WT AR ≥0.5.

2.6 Immunophenotype of *FLT3*-ITD and *NPM1* Leukemic Blasts

Although AML with *FLT3*-ITD is not considered a distinct entity in the 2016 WHO classification of myeloid neoplasms [32], patients with *FLT3*-ITD AML exhibit a specific immunophenotype characterized by high expression of CD33 and CD123 proteins [33–38]. Additionally, one study showed a direct correlation between the expression levels of CD33 and CD123 and the *FLT3*-ITD/*FLT3*-WT allelic ratio [34]. Other surface markers associated with *FLT3*-ITD AML include increased expression of CD7 [10,39–41].

NPM1 AMLs are considered a specific subtype of AML with recurrent mutations and most commonly present in two morphological forms at diagnosis [42]: (i) AML without differentiation or with minimal differentiation, corresponding to French-American-British (FAB) subtypes M0 and M1, characterized by CD117+, CD33+, CD13+/− expression; or (ii) myelomonocytic or monoblastic/monocytic AML, characterized by CD14+, CD64+

expression. Regardless of the morphological subtype, the immunophenotype of *NPM1*-positive acute leukemias is characterized by high/very high expression of CD33 and CD123 markers [33–38]. Another characteristic is the absence or low expression of the CD34 marker [16,43,44] in FAB M0 or M1 leukemias. The minority of *NPM1*-positive AML cases that express CD34 are associated with a worse prognosis compared to CD34-negative cases [45,46].

II. PERSONAL CONTRIBUTIONS

Chapter 3 – Hypothesis and objectives

3.1 Hypothesis

Hypothesis I – The implementation of molecular diagnostics for all AML cases can significantly improve patient management by:

i. confirming the immunophenotypic diagnosis;

ii. genetic prognostic stratification

- administration of targeted treatment—tyrosine kinase inhibitors with specific blocking action for the BCR::ABL1 or *FLT3*-ITD mutation;
- allows for the optimal selection of consolidation treatment plans: high-dose chemotherapy or consolidation with allogeneic stem cell transplantation;

iii. enabling the study of AML cases and the formation of study cohorts for the generation of original data, thus leading to a better understanding of the local AML patient population compared to data published from other countries.

Hypothesis II - Recurrent *FLT3*-ITD mutations are among the most common mutations found in AML, and positive patients exhibit certain distinct biological and clinical characteristics. Studying this mutation may allow:

i. the first-time clinical and biological characterization of a population of patients from Romania who are positive for the *FLT3*-ITD mutation;

ii. the description of specific or new biological characteristics of this *FLT3*-ITD-positive population;

iii. the potential improvement of *FLT3*-ITD case management: if the aspects mentioned above are confirmed, they will allow, besides a better understanding of this pathology, the generation of new research directions that may lead to improved management of these patients.

3.2 Main objectives

Based on the working hypotheses, the main objectives of the doctoral thesis were:

1. **Improvement of AML Diagnosis:** achieved through the implementation of molecular diagnostics and the evaluation of the presence of recurrent mutations in all AML cases. This objective will allow us to confirm the

immunophenotypic diagnosis, perform genetic prognostic stratification, and thus optimize treatment plans.

2. **Formation of Study Cohorts:** the creation of study cohorts of AML patients based on the implementation of molecular diagnostics. These cohorts can be used for research purposes and the generation of original data.
3. **Study of AML Cases Diagnosed at UEHB:** description of the AML patient population in Romania and comparison of the results with data published from other countries.
4. **Characterization of FLT3-ITD Mutations:** description and characterization of Romanian patients positive for the FLT3-ITD mutation in terms of clinical and biological data. This includes identifying specific features and potential new clinical or biological characteristics of this population.

Chapter 4 – General Research Methodology

4.1 Introduction

The initial diagnostic protocol for all cases of acute leukemia, including acute myeloid leukemias [4,15,25,47], is based on a well-established order of tests, which include: (i) morphological evaluation – including specific staining, (ii) immunophenotyping, (iii) cytogenetic evaluation, and (iv) molecular biology tests.

4.2 Study cohorts

All patients benefited from a complete diagnosis of acute leukemias through the Acute Leukemia Diagnostic Subprogram. All patients expressed their consent for diagnostic evaluations and for the use of their data, and patient data were used in accordance with Good Clinical Practice (GCP) guidelines and the Declaration of Helsinki.

The main study population from **Chapter 5 – subchapter 5.5 (Epidemiology of recurrent mutations in the cohort of patients diagnostically evaluated at the UEHB)** is represented by a cohort of 336 patients diagnosed at SUUB with AML who had validated results for all six recurrent mutations investigated.

The second study population from **Chapter 5 – subchapter 5.6 (Description and prognostic stratification of AML patients from the UEHB hematology department),** consisted of 108 patients from the UEHB Hematology Department who had validated results for all six recurrent mutations investigated and for whom **cytogenetic investigation data were available**.

The study population for **Chapter 6:** 117 patients diagnosed at the UEHB with AML and for whom **complete paraclinical data** were available, of which 32 patients were diagnosed with the *FLT3*-ITD mutation and a control group of 83 patients.

The study population for **Chapter 7:** 146 patients diagnosed at the UEHB with AML and for whom **immunophenotyping data were available**, 42 patients were diagnosed with the FLT3-ITD mutation and a control group of 104 patients.

4.3 *FLT3*-ITD assay

For the *FLT3*-ITD analysis, amplification was performed using hot start polymerase chain reaction (PCR) (with initiation at high temperature) from gDNA and cDNA samples with fluorescently labeled primers, followed by capillary electrophoresis and fragment analysis.

FLT3-ITD mutations were considered present if amplicons longer than the FLT3-WT products were detected, both for determinations from DNA and mRNA (cDNA) on the CEQ8000 genetic analysis system (Beckman Coulter, High Wycombe, United Kingdom).

The *FLT3*-ITD allelic ratio (AR) was calculated from gDNA samples as the ratio of *FLT3*-ITD/*FLT3*-WT. For patients who presented multiple *FLT3*-ITD populations (≥2 FLT3-ITD amplicons), the allelic ratio was calculated by summing the area uder the peak of each *FLT3*-ITD population and then dividing by area under the peak *FLT3*-WT. Quantification of the *FLT3*-ITD mRNA level was determined as an mRNA ratio (RR), calculated similarly to the AR.

Specific to **Chapter 7**, to test the influence of *FLT3*-ITD mutation expression on surface antigen expression, a third parameter was calculated, the ratio between RR (mRNA expression ratio) and AR (allelic ratio). This determined parameter represents the relative abundance of FLT3-ITD mRNA to gDNA, calculated as the RR/AR ratio.

4.4 *NPM1* assay

For the *NPM1* analysis, a hot start PCR reaction was performed from gDNA samples with fluorescently labeled primers. The *NPM1* primers used covered the region including intron 10 and exon 11 of the *NPM1* gene, according to [26].

NPM1 mutations were considered present if amplicons longer than the *NPM1*-WT product were detected, using capillary electrophoresis and fragment analysis on the CEQ8000 genetic analysis system (Beckman Coulter, High Wycombe, United Kingdom).

4.5 Statistical analysis

The student's t-test was used to compare datasets with normal distribution. For datasets with non-normal distribution, the Mann-Whitney U test was used, including for comparing MFI values (mean fluorescent index – geometric mean of fluorescence intensity values). Spearman correlation was used to evaluate the relationship between different continuous variables. Categorical variables were analyzed using Pearson's chi-square test or Fisher's exact test where applicable. P-values <0.05 were considered statistically significant, and only two-sided P-values were used.

Chapter 5 – Implementation of AML Molecular Testing and Epidemiology of Recurrent Mutations in Patients Diagnosed with AML

5.1 Introduction

The diagnostic protocol for acute leukemias [4,15,25,47], is based on a well-established order of tests: (i) morphological evaluation, (ii) immunophenotyping, (iii) cytogenetic evaluation, and (iv) molecular biology tests. The implemented methods were:

- Testing for recurrent fusion genes in AML – *CBFB::MYH11*; *RUNX1::RUNX1T1*; and *PML::RARA* by 2-step PCR followed by microcapillary electrophoresis.
- Subsequent testing of recurrent fusion genes using a one-step real-time multiplex PCR

5.2 Epidemiology of Recurrent Mutations in the Cohort of Patients Diagnostically Evaluated at the UEHB

Between February 2015 and February 2023, a total of 440 samples were validated for fusion genes, 466 samples for the *NPM1* mutation, and 501 samples for the *FLT3*-ITD mutation. Of the 440 validated results for the identification of fusion genes, the following were positive: (i) 13/440 for *CBFB::MYH11* (2.95%); (ii) 18/440 for *RUNX1::RUNX1T1* (4.09%); (iii) 32/440 for *PML::RARA* (7.27%); and (iv) 9/440 for *BCR::ABL1* (2.04%).

For the correct analysis of the frequency of the six mutations investigated at the UEHB, only patients who had validated results for all the recurrent mutations investigated were selected. Thus, a total of 336 patients were included. Of all the six evaluated mutations, only five mutations (*CBFBF::MYH11*, *RUNX1::RUNX1T1*, *PML::RARA*, *BCR::ABL1*, and *NPM1*) are considered driver mutations [15,48,49].

A total of 145/336 (43.15%) of patients were positive for at least one of the six mutations evaluated. Compared to previously published data [15,48,49], the frequency of recurrent driver mutations in our population is generally lower, but the proportions are maintained: the most frequent mutation is *NPM1*, and *PML::RARA* is the most frequent fusion gene (**Figure 5.1**).

In **Figure 5.2**, the distribution by counties of the 336 cases that benefited from complete molecular evaluation in our center is presented. The most frequent cases were from

Bucharest – 121 cases, Dolj County – 30 cases, Argeș County – 29 cases, and Teleorman County – 16 cases.

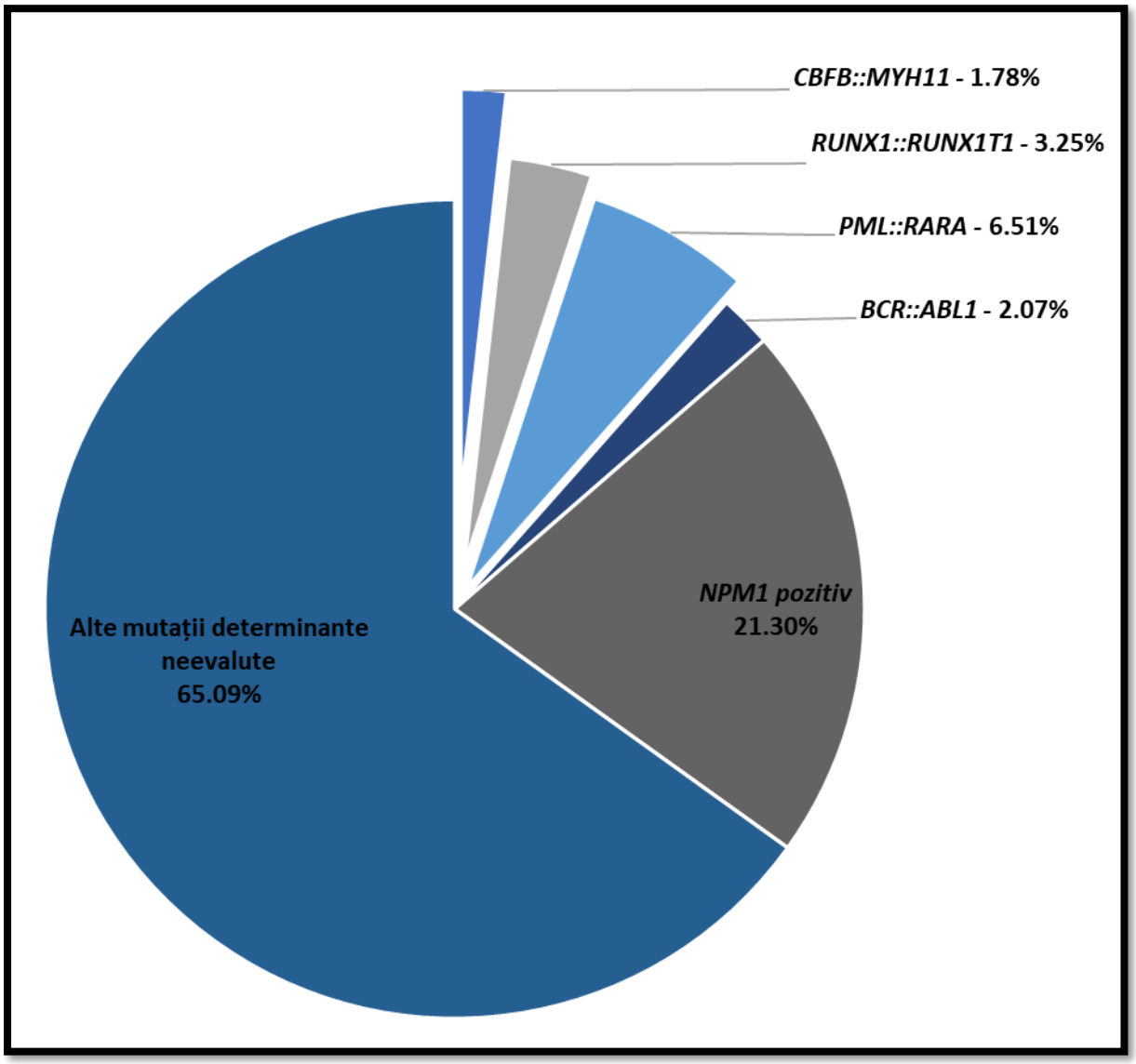


Figure 5.1 Pie chart showing the frequencies of driver mutations for AML diagnosed at the UEHB. Between February 2015 and February 2023, a total of 336 patients had validated results for the evaluation of the presence of recurrent mutations in AML.

AML – acute myeloid leukemia, UEHB – University Emergency Hospital Bucharest

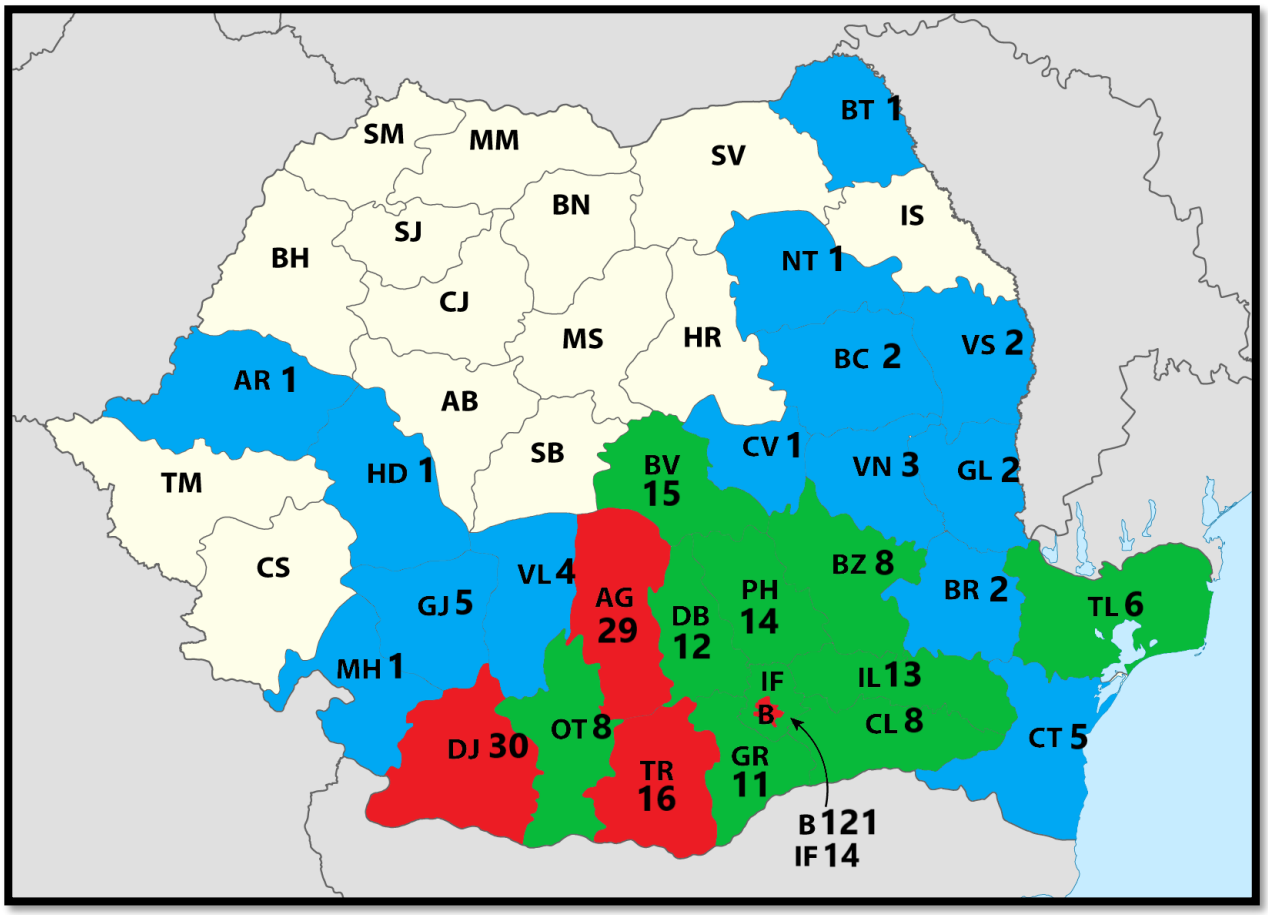


Figure 5.2 Distribution by counties of AML cases that benefited from complete molecular evaluation at the UEHB. Between February 2015 and February 2023, a total of 336 patients had validated results for the evaluation of the presence of recurrent mutations in AML. The numbers next to the counties represent the total number of AML cases diagnosed during that six-year period.

AML – acute myeloid leukemia, UEHB – University Emergency Hospital Bucharest

5.4 Conclusions

The implementation of testing for the six recurrent mutations in AML allowed for the biomolecular diagnosis of almost half of the evaluated cases, 43.15% (145/336 cases).

The implementation of FLT3-ITD and NPM1 mutation testing using multiplex PCR coupled with capillary electrophoresis enabled the simultaneous evaluation of two of the most frequent recurrent mutations in AML, and allowed us to determine the allelic ratio FLT3-ITD/FLT3-WT.

The implementation of molecular diagnosis allowed for a more accurate classification of AML cases into genetic risk categories according to ELN 2017 [15] and ELN 2022 [25].

Chapter 6 – FLT3-ITD DNA allelic burden, but not mRNA levels, influences the biological characteristics of AML patients

6.1 Introduction

This article was published in the Romanian Journal of Laboratory Medicine [50]

FLT3-ITD mutations confer an unfavorable prognosis [51,52], and the 2017 European LeukemiaNet (ELN) guideline [15] recommends that each patient be tested for the presence of the *FLT3*-ITD mutation with the determination of the allelic ratio (AR) *FLT3*-ITD / *FLT3*-WT (*FLT3*-WT = *FLT3* wild type – the normal allele of the *FLT3* gene) to establish the genetic risk category.

In this study, we evaluated whether the current method for evaluating *FLT3*-ITD mutations can be adapted for the qualitative and semi-quantitative analysis of *FLT3*-ITD mRNA from primary AML samples in adults. We also characterized and compared different parameters of *FLT3*-ITD mutations (length, ITD/WT ratio) for both DNA and mRNA, with different demographic and biological characteristics of our patient population..

6.2 Patients, Materials, and Methods

6.2.1 Patients

From a total of 42 patients with AML diagnosed with FLT3-ITD between March 2016 and June 2019, 32 cases had good quality total RNA samples available for analysis.

6.2.2 *FLT3*-ITD Evaluation and Statistical Methods

The methods used in this study, including semi-quantitative determinations of the *FLT3*-ITD mutation (AR, RR), and the statistical tests used are described in **Chapter 4 – General Methodology of the Research**.

6.3 Results

There was a very good correlation between the length of the *FLT3*-ITD mutation determined from gDNA and mRNA for the primary mutant populations (Kendall's tau coefficient $\tau b = 0.937$, $P < 0.001$) and for the secondary mutant populations (Kendall's tau

coefficient τb = 1.0, P < 0.001). There was a strong correlation between *FLT3*-ITD/WT AR and RR (Kendall's tau coefficient τb = 0.488, P < 0.001).

6.3.1 Biological Characteristics of Patients According to *FLT3*-ITD Mutation Parameters

To analyze the relationship between ITD insertion length from DNA and mRNA samples and clinical/biological parameters, only patients with a single detectable *FLT3*-ITD population were considered. For the analysis of the *FLT3*-ITD/WT AR from DNA, patients were divided into two groups based on the AR value for genetic risk stratification according to ELN 2017 (=0.5) [15], a group with AR < 0.5 and a group with AR ≥ 0.5. For the analysis of the *FLT3*-ITD/WT RR from mRNA, patients were divided into two groups based on the median RR value (=0.72) (**Table 6.1**): a group with RR < 0.7 and a group with RR ≥ 0.7.

According to the AR value, patients with AR ≥ 0.5 had a higher white blood cell count (Mann-Whitney U test, P = 0.01), higher LDH levels (Mann-Whitney U test, P = 0.037), and higher percentages of blasts in peripheral blood (t-test, P = 0.023) than the AR < 0.5 group (**Figure 6.1.A**). We further compared the white blood cell count, LDH levels, and percentages of blasts in peripheral blood between the FLT3-ITD negative control group and the two AR groups. We observed no significant differences between the AR < 0.5 group and the FLT3-ITD negative group (**Figure 6.1.A**). There were no statistically significant differences between the two RR groups.

We also performed a bivariate correlation analysis between AR levels and white blood cell count, LDH values, and percentages of blasts in peripheral blood (**Figure 6.1.B**). We observed moderate but significant correlations between AR levels and the three parameters: (i) AR and white blood cell count (Kendall's tau coefficient τb = 0.384, P = 0.003); (ii) AR and LDH values (Kendall's tau coefficient τb = 0.348, P = 0.011); and (iii) percentages of blasts in peripheral blood (Kendall's tau coefficient τb = 0.300, P = 0.036).

6.4 Conclusions

Our study presents new data on the utility of *FLT3*-ITD analysis based on mRNA in the context of diagnosing patients – mRNA-based testing being an alternative due to the higher chance of identifying mutations. This aspect is supported by the ability to identify multiple ITD insertions, as well as by the fact that RR is generally higher than AR, being very useful for confirming mutations that show an ASV < 5% on DNA testing for the presence of *FLT3*-ITD.

Regarding the correlation between *FLT3*-ITD mutational burden and *FLT3*-ITD mRNA expression with the biological characteristics of patients – from our results, a

possible independent dose effect of *FLT3*-ITD mutations can be proposed. We observed statistically significant differences only between biological parameters and *FLT3*-ITD/WT AR, suggesting that the studied parameters are influenced only by the proportion of *FLT3*-ITD positive cells and not by the level of *FLT3*-ITD mRNA expression.

Table 6.1 Clinical and Biological Characteristics of *FLT3*-ITD Positive Patients According to *FLT3*-ITD/WT Allelic Ratio (AR) and *FLT3*-ITD/WT mRNA Ratio (RR)

Characteristic	*FLT3*-ITD/-WT AR <0.5 (n= 14)	*FLT3*-ITD/-WT AR ≥0.5 (n= 18)	P	*FLT3*-ITD/-WT RR <0.7 (n= 15)	*FLT3*-ITD/-WT RR ≥0.7 (n= 16)	P
Age, years	Median 68 Range 27 – 80	Median 62 Range 24 – 78	P= NS	Median 63 Range 24 – 80	Median 65 Range 29 – 78	NS
Sex M/F, n	4 / 10	9 / 9	P= NS	6 / 9	7 / 9	NS
Rural/Urban, n	4 / 10	10 / 8	P= NS	9 / 6	5 / 11	NS
Leucocytes, $x10^9$/L	Median 29070 Range 2610 – 150000	Median 98750 Range 1860 – 287000	**P= 0.01**†	Median 73 250 Range 2 610 – 150 000	Median 50 855 Range 1 860 – 287 000	NS
Hgb, g/dL	Median 7.5 Range 6 – 10.7	Median 8.5 Range 5.6 – 13.5	P= NS	Median 7.8 Range 5.6 – 13.5	Median 8.25 Range 5.6 – 11.6	NS
Thrombocytes, $x10^9$/L	Median 60 000 Range 6 000 – 148 000	Median 32 500 Range 13 000 – 103 000	P= NS	Median 23 000 Range 8 000 – 148 000	Median 51 000 Range 6 000 – 120 000	NS
LDH, UI/dL	Median 526 Range 288 – 1617	Median 700 Range 367 – 1613	**P= 0.037**†	Median 535 Range 333 – 1 617	Median 628 Range 288 – 1 613	NS
Peripheral blood blasts, %	Median 27 Range 0 – 92	Median 73.5 Range 38 – 97	**P= 0.023**†	Median 45 Range 0 – 92	Median 76 Range 20 – 97	NS
Marrow blasts, %	Median 60 Range 32 – 95	Median 75 Range 48 – 96	P= NS	Median 69 Range 35 – 95	Median 67 Range 32 – 96	NS
NPM1-MUT / total, n	9 / 14	8 / 10	P= NS	9 / 15	8 / 16	NS

†test t, ‡Mann-Whitney U-test, Range= min – max values

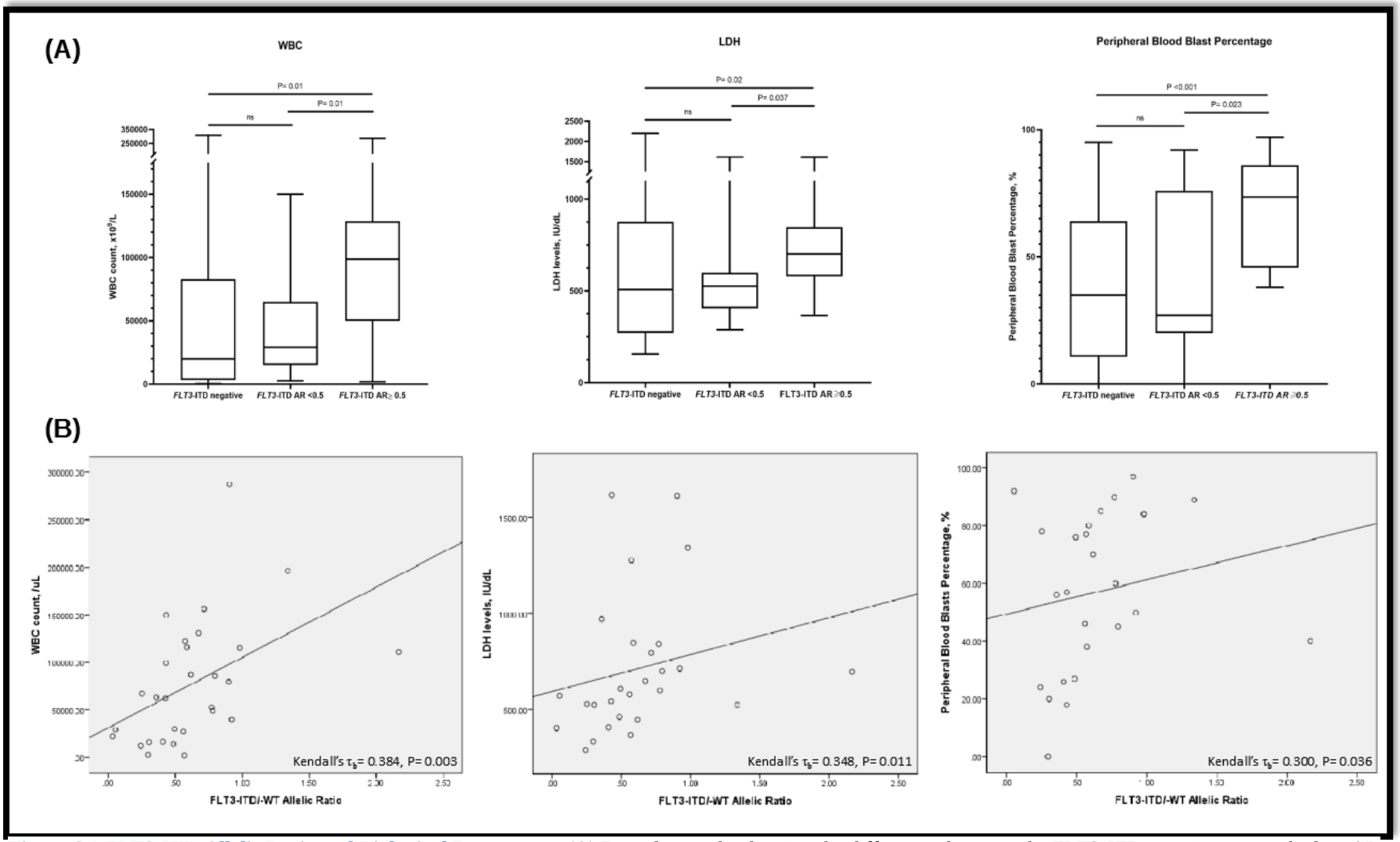


Figure 6.1 FLT3-ITD Allelic Ratio and Biological Parameters. (A) Box-plot graphs showing the differences between the FLT3-ITD negative group, the low AR group < 0.5, and the high AR group ≥ 0.5 for white blood cell count, LDH values, and the percentage of blasts in peripheral blood. (B) Correlation between FLT3-ITD AR and white blood cell count, LDH values, and the percentage of blasts in peripheral blood. AR = allelic ratio.

Chapter 7 – FLT3-ITD DNA and mRNA levels in AML do not correlate with CD7, CD33 and CD123 expression

7.1 Introduction

The study presented in this chapter was published in the Journal of Cellular and Molecular Medicine [53].

At diagnosis, patients with *FLT3*-ITD AML present with a high white blood cell count, a high percentage of blasts in peripheral blood and bone marrow. Additionally, *FLT3*-ITD leukemic cells have high expression of CD33 and CD123 [34,54,55], which has been shown to be directly proportional to the *FLT3*-ITD/FLT3-WT AR (allelic ratio) [34].

In this study, we investigated the quantitative expression of cell surface markers in *FLT3*-ITD AML within our local population of diagnosed patients and evaluated the impact of DNA and mRNA ratios between *FLT3*-ITD/*FLT3*-WT on surface antigen expression levels.

7.2 Patients, Materials, and Methods

7.2.1 Patients

Forty-two patients with AML were diagnosed with *FLT3*-ITD following DNA sample testing. Of these, 32 patients had good quality mRNA samples available. Sample UPN-8334 had the *FLT3*-ITD mutation identified only from the DNA sample, while only the *FLT3*-WT amplicon was detected in the mRNA sample. In the end, 31 mRNA samples presented *FLT3*-ITD mutations and were analyzed.

A control group of 104 *FLT3*-ITD negative patients was selected based on sex, age, FAB subtype, cytogenetic diagnosis, and *NPM1* mutation status, compared to *FLT3*-ITD positive patients.

All patients provided written informed consent, and the study was conducted in accordance with the Declaration of Helsinki and good clinical practice guidelines.

7.2.2 Immunophenotyping

All primary samples were analyzed according to EuroFlow protocols: (i) sample preparation, instrument setup, and calibration [56]; and (ii) the antibody panel, staining procedure, and data acquisition according to [57]. The samples were analyzed on a FACSCanto II cytometer (BD Biosciences). A total of 20,000 events per tube were recorded.

Measurements and data analysis were performed using FlowJo X software (Tree Star, Ashland, OR, USA). The gating strategy was based on the CD45/SSC gate (SSC – side scatter). Leukemic blast populations were defined as CD45-mid and SSC-low. To verify the gating strategy, the selected populations were cross-checked according to the expression of CD117, CD34, and HLA-DR.

The following antigens were analyzed: CD4, CD7, CD9, CD13, CD14, CD33, CD34, CD56, CD64, CD71, CD117, and CD123.

Quantitative surface antigen expression was determined by calculating the geometric means of fluorescence intensities (MFI) for leukemic blast populations of the selected antigens and normalizing them to the expression determined on control lymphocyte populations (CD45-bright & SSC-low), negative for the respective markers, as previously described [34].

7.2.3 *FLT3*-ITD Testing and Statistical Methods

The remaining methods used in this study, including the semi-quantitative determinations of the *FLT3*-ITD mutation (AR – **A**llelic **R**atio, RR – m**R**NA **R**atio), and statistical evaluation are described in **Chapter 4 – General Methodology of the Research**.

7.3 Results

7.3.1 Antigen Expression Profile Specific to *FLT3*-ITD Mutations

FLT3-ITD positive cases showed significantly higher expressions of CD7, CD33, and CD123 antigens compared to the *FLT3*-ITD negative control group (Mann-Whitney U test) – **Figure 7.1**.

7.3.2 *FLT3*-ITD Mutation Parameters & Patient Grouping Based on Them

To analyze the impact of AR on MFI values, patients were divided into two groups according to ELN 2017 genetic risk stratification [15]: a group with AR < 0.5 (n = 16) and a group with AR ≥ 0.5 (n = 26). For the analysis of the impact of RR on antigen expression, patients were divided into two groups based on the median RR (= 0.72): a group with RR < 0.7 (n = 16) and a group with RR ≥ 0.7 (n = 15). To analyze the relative abundance of FLT3-ITD mRNA to DNA (RR/AR), patients were divided into two groups based on the median RR/AR (= 1.27): a group with RR < 1.3 (n = 16) and a group with RR ≥ 1.3 (n = 15).

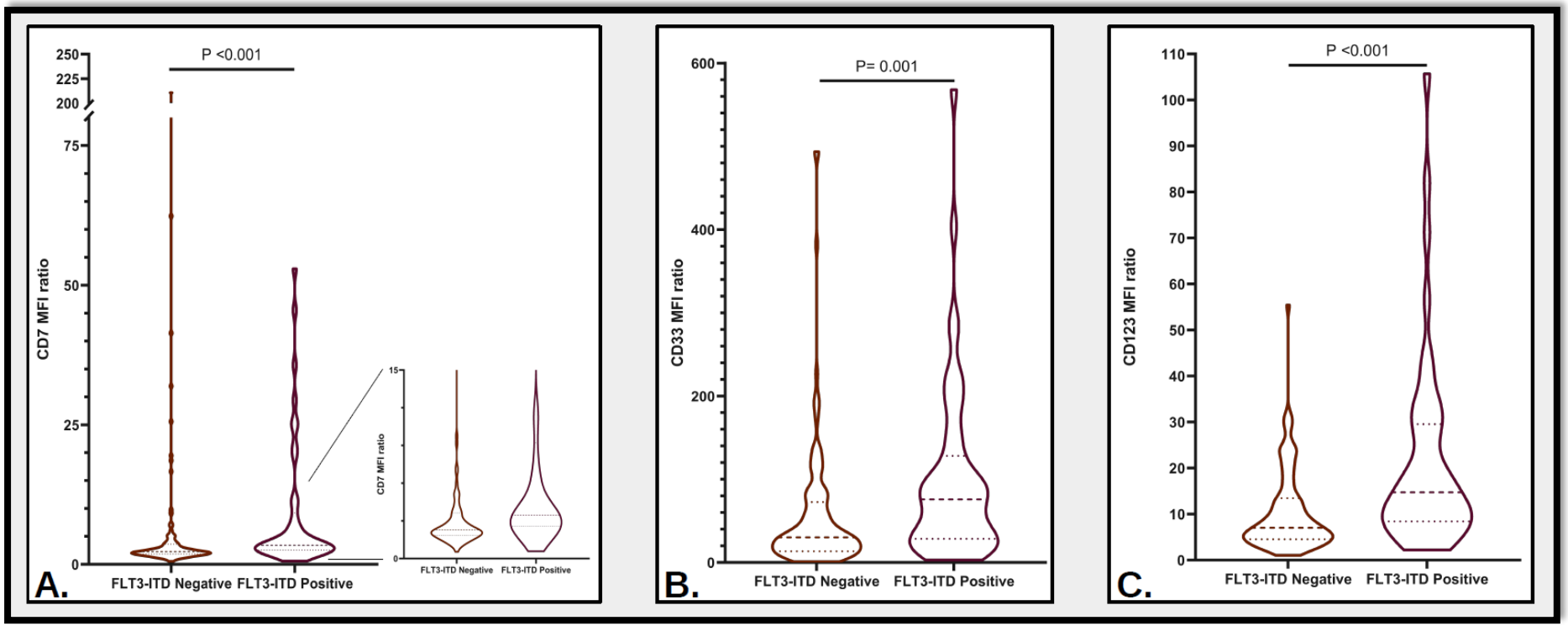


Figure 7.1 MFI values for: **A.** CD7 (insert: detail of the chart); **B.** CD33; and **C.** CD123 in *FLT3*-ITD positive AML patients vs. negative patients.
AML – acute myeloid leukemia; ITD – internal tandem duplication; MFI – mean fluorescence intensity.

7.3.3 Quantitative Parameters of *FLT3*-ITD Mutations in Relation to Surface Antigen Expression of CD7, CD33, and CD123

To evaluate the association of quantitative FLT3-ITD parameters with the expression of CD7, CD33, and CD123, FLT3-ITD positive patients were grouped for each parameter into two cohorts using the following threshold values.

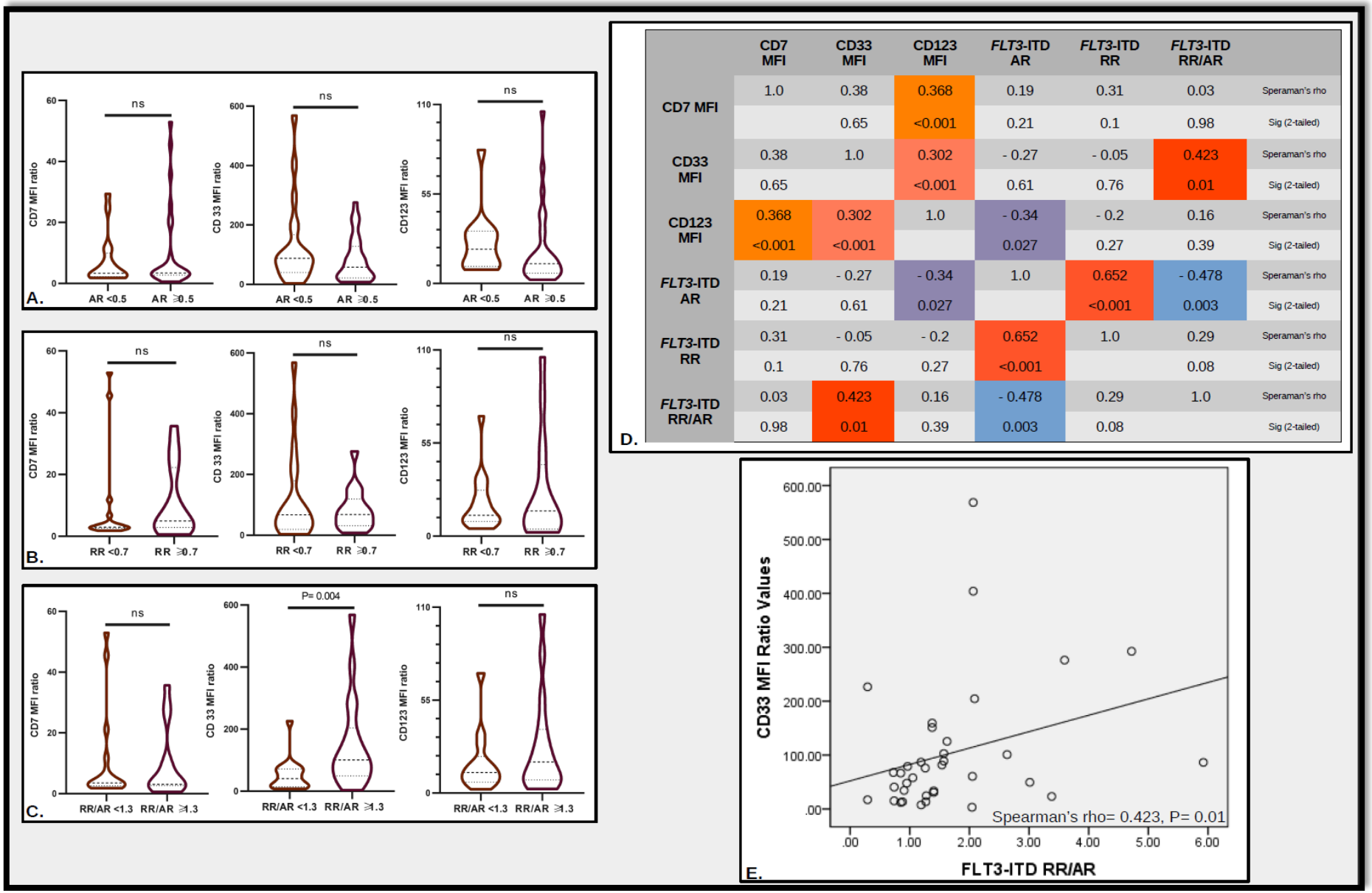
The only observed difference was between the groups with the RR/AR ratio, with the high RR/AR group (≥1.3) showing higher CD33 values than the low RR/AR group (**Figure 7.2 C**). There were no other statistical differences between the levels of CD7, CD33, and CD123 in the other FLT3-ITD groups compared to the control group (Mann-Whitney U test), **Figure 7.2**.

We also tested the bivariate correlation between the expression of CD7, CD33, CD123, and the three quantitative FLT3-ITD parameters. There was a moderate correlation between CD33 MFI values and the RR/AR ratio values (Spearman's rho = 0.423, P = 0.01) (**Figure 7.2 D, E**).

7.5 Conclusions

In our patient population, *FLT3*-ITD mutations were associated with a specific antigen expression profile consisting of high MFI values for CD7, CD33, and CD123. However, the expression levels of specific antigens are not evidently influenced by the quantitative parameters of *FLT3*-ITD mutations, determined from both DNA and mRNA samples. Thus, the relatively increased expression of CD7, CD33, and CD123 antigens is more likely secondary to the mere presence of the *FLT3*-ITD mutation.

Unlike previously published data, which described a statistically significant direct relationship between the *FLT3*-ITD/*FLT3*-WT allelic ratio and the antigen expression of CD33 and CD123, this association was not observed in our patient cohort. This may be secondary to differences between the two patient cohorts. Further studies are needed to elucidate the impact of the *FLT3*-ITD mutation on antigen expression.



	CD7 MFI	CD33 MFI	CD123 MFI	*FLT3*-ITD AR	*FLT3*-ITD RR	*FLT3*-ITD RR/AR	
CD7 MFI	1.0	0.38	0.368	0.19	0.31	0.03	Speraman's rho
		0.65	<0.001	0.21	0.1	0.98	Sig (2-tailed)
CD33 MFI	0.38	1.0	0.302	- 0.27	- 0.05	0.423	Speraman's rho
	0.65		<0.001	0.61	0.76	0.01	Sig (2-tailed)
CD123 MFI	0.368	0.302	1.0	- 0.34	- 0.2	0.16	Speraman's rho
	<0.001	<0.001		0.027	0.27	0.39	Sig (2-tailed)
FLT3-ITD AR	0.19	- 0.27	- 0.34	1.0	0.652	- 0.478	Speraman's rho
	0.21	0.61	0.027		<0.001	0.003	Sig (2-tailed)
FLT3-ITD RR	0.31	- 0.05	- 0.2	0.652	1.0	0.29	Speraman's rho
	0.1	0.76	0.27	<0.001		0.08	Sig (2-tailed)
FLT3-ITD RR/AR	0.03	0.423	0.16	- 0.478	0.29	1.0	Speraman's rho
	0.98	0.01	0.39	0.003	0.08		Sig (2-tailed)

Figure 7.2 Detailed Analysis of CD7, CD33, and CD123 MFI Values According to Quantitative *FLT3*-ITD Parameters. Violin plot graphs of antigen expression levels according to the FLT3-ITD/-WT ratio (**A**) AR, (**B**) RR, (**C**) RR/AR. **D.** Results of the correlation analysis between CD7, CD33, CD123 MFI values and AR, RR, and RR/AR ratios.E. Correlation between CD33 MFI values and the RR/AR ratio.

AR – allelic ratio; RR – mRNA ratio; RR/AR – relative abundance of FLT3-ITD mRNA to DNA; ITD – internal tandem duplication; MFI – mean fluorescence intensity.

Chapter 8 –Conclusions and personal contributions

8.1 Discussions

Based on the data presented in **Chapter 7**, obtained from the patient cohort in Romania, a smaller than expected impact of the semi-quantitative *FLT3*-ITD parameters (allelic ratio and mRNA expression level of *FLT3*-ITD) is observed, particularly regarding the evaluation of surface antigen expression of leukemic blasts—where no statistically significant correlation was observed between the expression level of specific antigens (CD7, CD33, and CD123) and the semi-quantitative parameters of the *FLT3*-ITD mutation.

Form previously publications we confirm the increased expression of CD33 and CD123 [34,54,55], however we do not confirm a direct correlation between the FLT3-ITD/FLT3-WT allelic ratio and the expression level of CD33 and CD123 antigens [34]. This difference may initially be explained by differences between the study populations (Romanian patient cohort compared to the patient cohort from Germany).

However, when comparing the epidemiological data obtained from the local patient cohort with the data obtained from a patient cohort in Germany and Austria (AMLSG BiO) [14], it can be observed that the two patient populations are relatively similar—having the same median age of 65 years, and similar percentages of *NPM1* and *FLT3*-ITD mutations.

Another observation regarding the impact of the semi-quantitative parameters of the FLT3-ITD mutation is that, based on the data presented in **Chapter 6**, a statistically significant correlation is observed between biological parameters (total white blood cell count, LDH values, and percentage of peripheral blasts) only with the FLT3-ITD/FLT3-WT AR and not with the mRNA expression level of FLT3-ITD. From this, it can be suggested that the studied parameters are influenced only by the proportion of FLT3-ITD positive cells and not by the level of mRNA expression of FLT3-ITD.

Regarding prognostic evaluation, there is no consensus, especially concerning the impact of the *FLT3*-ITD/*FLT3*-WT AR. The genetic prognostic evaluation model proposed in the ELN 2017 guidelines [15] is based on a series of retrospective articles [26–29] and states that patients with FLT3-ITD/FLT3-WT AR < 0.5 have a better outcome than patients with AR ≥ 0.5. The ELN 2017 genetic prognostic model was later confirmed in a 2020 article published by K. Döhner *et al* [31].

Studies evaluating the *ex-vivo* impact of the *FLT3*-ITD/*FLT3*-WT ratio showed that in a pediatric AML FLT3-ITD positive population [58] , higher levels of FLT3-ITD/FLT3-

WT AR (≥ 0.5) had a better ex-vivo response to the inhibitor gilteritinib than samples with AR < 0.5 or FLT3-WT control samples. These data were later confirmed by Kivioja *et al* [59].

However, a series of articles that evaluated the ELN 2017 genetic prognostic evaluation model concluded that AR does not allow clear prognostic stratification [60–63]. Additionally, another study investigating the importance of the FLT3-ITD/FLT3-WT ratio determined on mRNA as an alternative biomarker for determining the prognosis of AML patients [64] , showed that *FLT3*-ITD mRNA levels have a high prognostic impact on overall survival and relapse-free survival only in AML patients with coexisting *NPM1* mutations and did not present any prognostic value in *NPM1*-WT AML cases.

With the publication of the new ELN 2022 guidelines [25], prognostic evaluation of *FLT3*-ITD cases based on AR was discontinued, citing the lack of standardization of the AR evaluation method, as well as referencing the previously mentioned article by K. Döhner *et al* [31], which additionally showed that the addition of the TKI midostaurin significantly improves prognosis, especially in patients with FLT3-ITD/FLT3-WT AR ≥ 0.5.

Combining the results presented in **Chapters 6 and 7** with the cited data, we can conclude that the impact of determining FLT3-ITD AR or FLT3-ITD mRNA levels presents relatively contradictory results. Further studies are needed to standardize the methodology for determining FLT3-ITD/FLT3-WT AR as well as to further evaluate the clinical impact of the FLT3-ITD/FLT3-WT ratio on both gDNA and mRNA samples. Discontinuing AR determination in centers that previously performed it may not be appropriate, as this semi-quantitative determination can still provide valuable information for clinicians, especially if the evaluation is performed serially, allowing assessment of the patient's response dynamics to specific treatment.

8.2 General conclusions

Below is a list of personal contributions to the field:

- I demonstrated the feasibility and sustainability of molecular biology diagnosis of acute myeloid leukemia at the University Emergency Hospital Bucharest.
- I described the positive association between the biological parameters of patients (total white blood cell count, percentage of leukemic blasts in peripheral blood, and LDH value) and the value of the *FLT3*-ITD/*FLT3*-WT

allelic ratio, but not the association with the expression level of *FLT3*-ITD (reported by the *FLT3*-ITD/*FLT3*-WT mRNA ratio).

- I described and confirmed the surface antigen expression associated with the *FLT3*-ITD mutation: significantly increased expression of CD7, CD33, and CD123 antigens, but without a direct association between the expression values of these antigens and the *FLT3*-ITD/*FLT3*-WT allelic ratio or the *FLT3*-ITD/*FLT3*-WT mRNA expression level.
- The impact of determining *FLT3*-ITD AR or *FLT3*-ITD mRNA levels presents relatively contradictory results, suggesting the need for further studies.

8.3 Personal contributions

Based on the working hypotheses and the associated objectives, the personal contributions consist of:

1. **Improving AML Diagnosis:** this **was achieved** by implementing fusion gene testing through RT-PCR and combined NPM1 & FLT3-ITD testing. The details and results of the implementation process of molecular testing in AML are presented in **Chapter 5 – Implementation of Molecular Testing for AML**.

2. **Formation of Study Cohorts:** **study cohorts were defined** using information generated from molecular testing, clinical, and paraclinical data. The cohorts were formed to investigate specific parameters of interest for each chapter/subchapter, taking into account the available cases at the time of designing the experimental studies.
   - For **Chapter 5,** based on the implementation of testing for 6 recurrent mutations in AML—4 fusion genes and *NPM1* & *FLT3*-ITD mutations—we were able to analyze and describe a total of 336 patients. Of this population, 43.15% of cases (145/336 cases) were positive for at least one of the 6 evaluated mutations.
   - For **Chapter 6**: a cohort of 117 patients diagnosed with AML was formed, for whom complete paraclinical data were available, including 32 patients diagnosed with the FLT3-ITD mutation and a control group of 85 patients.

- For **Chapter 7**: a cohort of 146 patients diagnosed with AML was formed, for whom all raw immunophenotyping data were available, including 42 patients diagnosed with the FLT3-ITD mutation and a control group of 104 patients.

3. **Characterization of *FLT3*-ITD Mutations:** **This was described** in **Chapter 6 & 7.**
   i. **Chapter 6 – FLT3-ITD DNA allelic burden, but not mRNA levels, influences the biological characteristics of AML patients**. The main observation is that statistically significant correlations were observed between biological parameters (total white blood cell count, LDH values, and percentage of leukemic blasts in peripheral blood) and only the *FLT3*-ITD/WT AR (AR – allelic ratio), suggesting that the studied parameters are influenced only by the proportion of *FLT3*-ITD positive cells and not by the level of *FLT3*-ITD mRNA expression.
   ii. **Chapter 7 – FLT3-ITD DNA and mRNA levels in AML do not correlate with CD7, CD33 and CD123 expression**. The main observation is that although *FLT3*-ITD mutations were associated with a specific antigen expression profile represented by increased expressions of CD7, CD33, and CD123 antigens, the expression levels of these antigens are not evidently influenced by the semi-quantitative parameters of *FLT3*-ITD mutations, determined from both DNA and mRNA samples.

4. **Improving the Management of *FLT3*-ITD Cases:**

   Regarding clinical management, through the implementation of combined NPM1 & FLT3-ITD testing using capillary electrophoresis (**Chapter 5 – Implementation of Molecular Testing for AML**) patients were correctly evaluated according to the ELN 2017 [15] clinical guidelines and later ELN 2022 [25]. Based on the released results, the indication for administering TKI with specific anti-*FLT3*-ITD activity was established, and/or the indication for allogeneic hematopoietic stem cell transplantation was determined depending on the *NPM1* and *FLT3*-ITD status.

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