MEDICAL UNIVERSITY

“PROF. D-R PARASKEV STOYANOV“ – VARNA

DEPARTMENT OF MEDICAL GENETICS

d-r Dinnar Ali Yahya

Analysis of molecular-genetic markers in patients with acute myelogenous leukemia

EXTENDED SUMMARY

OF DISSERTATION WORK

TO ACQUIRE A SCIENTIFIC AND EDUCATIONAL DEGREE “DOCTOR OF PHILOSOPHY”

Doctoral program: „Genetics”

Supervisors:

Prof. Dr. Ilina Dimitrova Micheva, MD, PhD

Assoc. Prof. Dr. Trifon Georgiev Chervenkov, MD, PhD

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Reviewers:

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Prof. Dr. Lyudmila Boncheva Angelova, MD, PhD

Varna, 2024

The dissertation is presented in 130 pages, contains 15 figures and 12 tables. The bibliography covers 201 literary sources, of which 5 are in Cyrillic and 196 are in English.

The dissertation work was presented at a meeting of the expanded Departmental Council of the Department of Medical Genetics, Medical University - Varna, on 03.06.2024.

All research included in the dissertation work was carried out in:

- Department of Medical Genetics at the Medical University "Prof. Dr. Paraskev Stoyanov" - Varna
- Laboratory of Medical Genetics at the at "Sveta Marina" University Multidisciplinary Hospital for Active Treatment JSC Varna (will be referred as “Sveta Marina University Hospital in this work)

The current research is partially subsidized under project No. 21008 "Molecular-genetic analysis in newly diagnosed patients with Acute Myelogenous Leukemia", financed by the "Science" Fund (competition session 2021) at Varna Medical University.

The official defense of the dissertation work will take place on 20.06.2024 at the Department of Medical Genetics, Medical University of Varna, 1 Hristo Smirnenski Blvd., Varna.

Defense materials are available at the Department of Medical Genetics, Medical University - Varna.

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Frequently used abbreviations

ALL - Acute lymphoblastic leukemia

AML - Acute myelogenous leukemia

APL - Acute promyelocytic leukemia

CCA - Conventional cytogenetic analysis

CML - Chronic myelogenous leukemia

DNA - Deoxyribonucleic acid

ELN - European Leukemia Net

FISH - Fluorescent In Situ Hybridization

MDS – Myelodysplastic syndrome

MLPA - Multiplex Ligase-dependent Probe Amplification

NGS - Next-generation Sequencing

NK- Normal karyotype

OGM - Optical Genome Mapping

PCR - Polymerase Chain Reaction

WHO - World Health Organization

1. INTRODUCTION

Acute myelogenous leukemia (AML) is a heterogeneous group of diseases due to abnormal proliferation of progenitor cells of the myeloid lineage. It is particularly common in individuals over the age of 45 and accounts for about 80% of leukemia cases in adults. However, AML is a rare disease, with an overall incidence of 4.2 per 100,000 people, with a slight predilection for males over females *(Vakiti & Mewawalla, 2021)*.

Based on the established mechanisms of etiology and pathogenesis, it is known that at the cellular level it is a primarily genetic disease associated with a wide range of genetic mutations and epigenetic modifications. Genetic mutations are most often acquired and lead to disorders in hematopoiesis in terms of proliferation, differentiation, maturation, cell cycle control and apoptosis. AML is characterized by extreme genetic heterogeneity, rapid clinical course and variable prognosis. These features, on the one hand, complicate the diagnostic process and require precise and efficient collaboration between specialists from the fields of clinical hematology, medical genetics, clinical immunology, clinical pathology, etc. On the other hand, the characterization of the specific genetic events leading to a given case of the disease is decisive for making an accurate diagnosis, predicting its course and predicting the therapeutic response *(Vakiti & Mewawalla, 2021).*

In terms of diagnosis, the conventional cytogenetic analysis (CCA) takes an essential place, which is crucial for the classification of cases and their risk stratification. The first steps in oncocytogenetics are associated with the discovery of the small Philadelphia chromosome in cells of patients with Chronic Myelogenous Leukemia (CML) in 1960 in the American state of the same name *(Ferguson-Smith, 2015)*, but the method is still used today, which is also discussed by the latest European Leukemia Net (ELN) revision of 2022 *(Döhner et al, 2022).* Given its well-known drawbacks and risks such as low resolution, labor-intensive manual work, long processing time, and the possibility of missing metaphase plates, however, it is complemented by more detailed and modern molecular studies.

The current dissertation draws attention to an actual health problem - the need to routinize an informative molecular-genetic method for parallel conduct with KCA.

The lack of sufficient studies on the applicability of molecular genetic methods for the diagnosis of patients with AML in our country against the background of the growing role of genetic markers in international work recommendations and assessment systems for these patients was a prerequisite for the development of the current dissertation work.

2. GOAL AND TASKS OF THE STUDY

2.1 Goal of the study

To evaluate the applicability of the MLPA (Multiplex Ligase-dependent Probe Amplification) method for reporting AML-specific molecular genetic markers in the routine clinical-diagnostic activity of evaluating patients with newly diagnosed AML.

2.2 Tasks of the study

In order to realize the goal set in the dissertation, we have identified the following five tasks:

1. To introduce a molecular genetic method for the identification of significant molecular genetic markers associated with AML.

2. To select patients with newly diagnosed AML meeting the criteria for inclusion in the prospective study.

3. To conduct a molecular genetic analysis of DNA isolated from leukocytes from venous blood from patients with newly diagnosed AML before treatment and from a control group of healthy individuals.

4. To compare the data with those from a parallel KCA, and to summarize and analyze the results of the conducted molecular genetic research.

5. To summarize the role of the used molecular genetic method in the initial genetic screening and to derive guidelines for improving the genetic evaluation of the contingent of newly diagnosed patients with AML.

On this basis, we defined the following working hypothesis: MLPA is a suitable method for the study of molecular genetic markers and it would be suitable to be implemented in the routine evaluation of the genetic basis in patients with newly diagnosed AML, in parallel with the cytogenetic analysis.

3. PATIENTS AND METHODS

3.1 Location of the study

Our study is prospective in nature and covers the period February 2022 - May 2023. It was conducted on the territory of the Laboratory of Medical Genetics at "Sveta Marina" University Hospital, Varna.

3.1.1 Patients

Patients meeting the following criteria are eligible for inclusion in the study:

- Newly diagnosed and untreated patients with AML, meeting the clinical-morphological criteria (according to the current climate classification of the WHO (World Health Organization));

- Patients in whom APL (Acute Promyelocytic Leukemia) is not suspected;

- Age ≥18 years;

- After giving written informed consent to participate in the study;

Accordingly, the exclusion criteria are as follows, one of which would be sufficient to exclude participants from the study:

- Patients with AML, previously diagnosed, with a specified therapeutic plan or who underwent transplantation of hematopoietic stem cells, or those who do not meet the clinical-morphological criteria;

- Patients suspected of APL;

- Age under 18 years;

- Refusal to sign an informed consent to participate in the study;

The recruitment of suitable participants in this group was carried out with the help of the attending physician, as they were selected from the contingent of inpatients at the Clinic of Clinical Hematology of the "Sveta Marina" University Hospital.

MLPA is a method that requires the presence of DNA (deoxyribonucleic acid) from a source of analogous biological material from clinically healthy individuals in each run of the assay. Using the latter is important to ensure maximum sensitivity and specificity (*https://support.mrcholland.com*). Volunteers who meet the following criteria are also included in the study:

- Absence of clinical and morphological signs of AML or of acute or chronic diseases.

- Age over 18 years.

- With similar gender and age distribution of the patient group.

- Given written informed consent to participate in the study.

Accordingly, the exclusion criteria are as follows, one of which would be sufficient to exclude the participant from the study:

- Presence of clinical-morphological signs of AML, and/or acute or chronic diseases.

- Age under 18 years.

- Lack of similar distribution by gender and age in relation to the patient group.

- Refusal to sign an informed consent to participate in the study.

As healthy control individuals, we invited women and men (randomly) who met the described criteria, mostly employees of the Medical University - Varna and "Sveta Marina" University Hospital, who signed an informed consent, to participate in the study.

The study was conducted under project No. 21008: "Molecular-genetic analysis of newly diagnosed patients with acute myelogenous leukemia", financed by the "Science" Fund (competition session 2021) at Varna Medical University.

The study was approved by the Committee on Ethics of Scientific Research at Varna Medical University with protocol No. 111 of the Meeting on 20.01.2022.

3.1.2 Biological material

For the purposes of molecular genetic analysis, DNA was isolated from leukocytes from venous blood. A closed system was used for each study participant following standard sterility procedures. The material was collected by a single sampling of peripheral venous blood in the amount of 6-10 ml, and for the group of newly diagnosed patients it was parallel to the routine sampling during the hospital stay for the patients. Isolation of DNA was carried out within 24 hours after taking the sample, and the obtained DNA was in TE buffer and was stored in a dissolved state at a temperature of -20 ºC until the samples were analyzed.

3.2 Methods

3.2.1 Clinical methods

All patients were selected by their attending physician at the Clinical Hematology Clinic, “Sveta Marina” University Hospital, and referred for consultation with the principal investigator from the Medical Genetics Laboratory, “Sveta Marina” University Hospital. During the consultation, the nature, objectives, expected benefits and risks of the study are explained in detail, and an informed consent to participate in the study is signed. The available medical documentation from laboratory, imaging and other tests from the current and previous hospitalizations was also reviewed.

3.2.2 Genetic laboratory methods

3.2.2.1 Isolation of DNA from venous blood (preanalytical procedure)

Genomic DNA was isolated by the salting out method. The concentration of isolated DNA was measured using a NanoDrop™ 2000c absorption spectrophotometer. The resulting DNA was stored dissolved in TE buffer at -20°C until the samples were analyzed.

3.2.2.2 MLPA

We performed MLPA on the isolated genomic DNA with the SALSA MLPA probemix X060-X2 MDS-AML kit (MRC Holland, Netherlands), targeting AML/MDS (Myelodysplastic syndrome)-specific chromosomal regions and their corresponding genes, as well as 11 reference probes for regions, characteristically stable in MDS and AML (Table 1) – a total of 59 probes for analysis and quality control, according to the protocol described by the manufacturer.

Table 1. *Probes included in the SALSA MLPA probemix X060-X2 MDS-AML kit.*

Single-gene somatic variants	**Copy number aberrations with respective genes and exons**	**Referrent probes with respective genes**
DNMT3A (R882H)	4(q24) - *TET2-4, -11*	1(q23.3) - *PPOX*
SF3B1 (K700E)	5(q31.2) - *CTNNA1-3, -6* 5(q35.1) - *NPM1-2, -5*	1(q41) - *USH2A*
IDH1 (R132H & R132C)	6(p22.3) - *JARID2-8, -19*	2(p21) - *SLC3A1*
NPM1 (865insTCTG)	7(p12.2) - *IKZF1-4, -6*	3(p12.3) - *GBE1*
FLT3-TKD (D835Y)	7(q22.1) - *CUX1-6, -23* 7(q22.2) - *KMT2E(MLL5)-4, -11* *7(q36.1) - EZH2-4, -14*	3(q25.31) - *KCNAB1*
IDH2 (R140Q)	11(q22.3) - *ATM-13, -22, -63* 11(q23.3) - *KMT2A*(*MLL*)-3, -4, -5, -36	6(q12) - *EYS*
*ASXL1 (G646fs*12)*	12(p13.2) - *ETV6-1, -8* 12(p12.3) - *AEBP2-3, -9b*	9(q21.13) - *PCSK5*
	17(p13.1) - *TP53-8, -7*	13(q14.3) - *RNASEH2B*
	17q(11.2) - *NF1-34, -53, SUZ12-10, -15*	14q11.2 - *RPGRIP1*
	20(q11.21) - *ASXL1-1, -8*	15(q15.3) - *SPG11*
	21(q22.12) - *RUNX1-6, -2* 21(q22.3) - *U2AF1-1, -7*	16(p13.2) - *ABAT*

Capillary electrophoresis was performed with a GeXP Beckman Coulter Genetic Analyzer (Sciex, USA) with a 600 nucleotide size standard. Data were exported and analyzed with specialized software Coffalyser version 220513.1739 (MRC Holland, Netherlands). For quality control, negative controls (in the absence of DNA) as well as samples from healthy control subjects were included. As a positive control and to increase the quality of the analysis of the data from the fragment analysis of the eight monogenic somatic variants, the so-called SD041 binning DNA - synthetic DNA with the above-described eight variants simultaneously present - is included in the test set.

The variants detected by MLPA in the *FLT3-TDK (D835Y)* gene were promptly compared with the data from a routine parallel molecular genetic analysis from the Laboratory of Clinical Immunology, "Sveta Marina" University Hospita, where the polymerase chain reaction method - polymorphism of restriction fragment length polymorphism (PCR-RFLP, Polymerase Chain Reaction - restriction fragment length polymorphism), using restriction enzymes that recognize and cut specific sites in the wild-type gene, but not in the mutant alleles *(Rasmussen, 2012).*

3.2.2.3 CCA

We compared the MLPA data with a concurrent CCA (in cases where one was performed) as a routine part of the diagnostic process. Cytogenetic analysis was performed as standard on bone marrow aspirate material after a short-term 24- or 48-hour culture according to an established protocol in accordance with the Medical Standard "Medical Genetics". Staining was applied with GTG banding technique on a minimum of 15, preferably 20 metaphase plates with varying resolution depending on the source of the plates. The results were interpreted and described according to the current international nomenclature (ISCN 2020, International System for Human Cytogenomic Nomenclature 2020).

3.2.3 Statistical methods

For statistical analysis we used the following methods:

- Graphical analysis
- Processing of quantitative indicators
- Non-parametric analyzes - *Mann-Whitney test*, *Kruskal-Wallis test*, as well as *chi-square test, Fisher's exact test.*
- Evaluation of survival by *Kaplan-Meier test*

Software for work - GraphPad Prism v. 9.5.1 (GraphPad Software, USA) and Microsoft Excel 2016 (Microsoft, USA). We accepted a two-tailed p value <0.05 as a statistically significant difference. All numbers except p values are rounded to one decimal place.

4. RESULTS

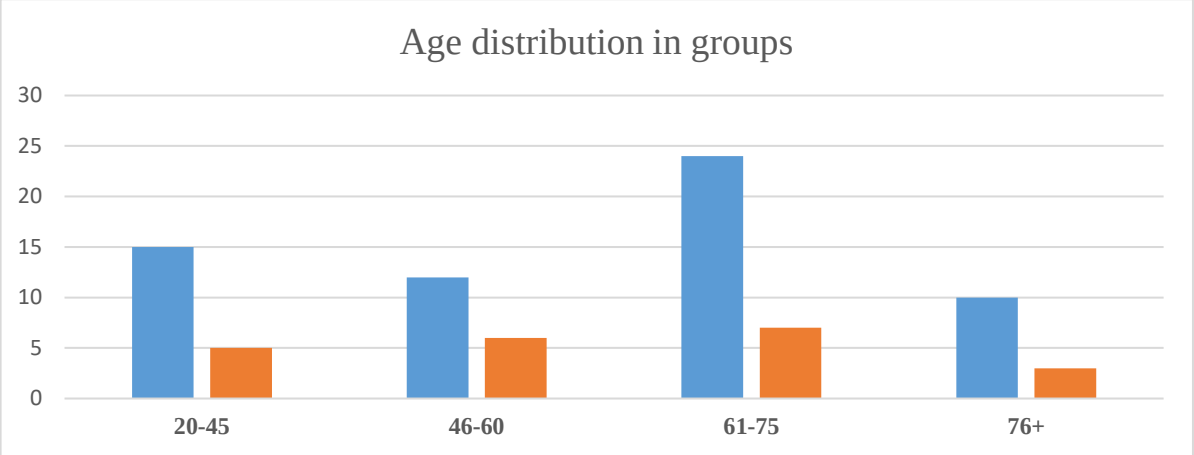
4.1 Descriptive – epidemiological characteristics of patients and control individuals included in the study

4.1.1 Age and sex characteristics

We included a total of 61 patients – 29 (47.5%) women and 32 (52.5%) men (M : F = 1.1 1). The age of the patients ranged from 20 to 89 years (median 62 years) with no significant differences between the two sexes – women were aged 20 to 89 years with a median of 61 and men – 29 to 83 with a median of 65 years *(p= 0.3867, Mann-Whitney test).* Patients over 60 years old prevailed - 59% (n=36) *(p=0.0466, Chi-square test),* and the most represented age group was that of 61-75 years old - 39.3% (n=24) (Figure 1).

We included a total of 21 healthy control subjects - 10 women aged 20-79, median 64.5 years, and 11 men aged 37-73, median 62 years, with no significant difference with the distribution and median age of the patients *(p=0, 8557, Kruskal-Wallis test)* (Figure 1).

Figure 1. *Age distribution of patients and healthy control individuals (blue – patients, orange – control individuals)*



4.1.2 Distribution according to AML type

According to the initial diagnosis, the studied contingent of patients was conditionally divided into 4 main groups:

- **Group I** – newly diagnosed patients without other previous hematological (including malignant) diseases (*de novo* AML) – 48 (78.7%), **dominant one.**
- **Group II** – patients with previously diagnosed MDS – 7 (11.5%)

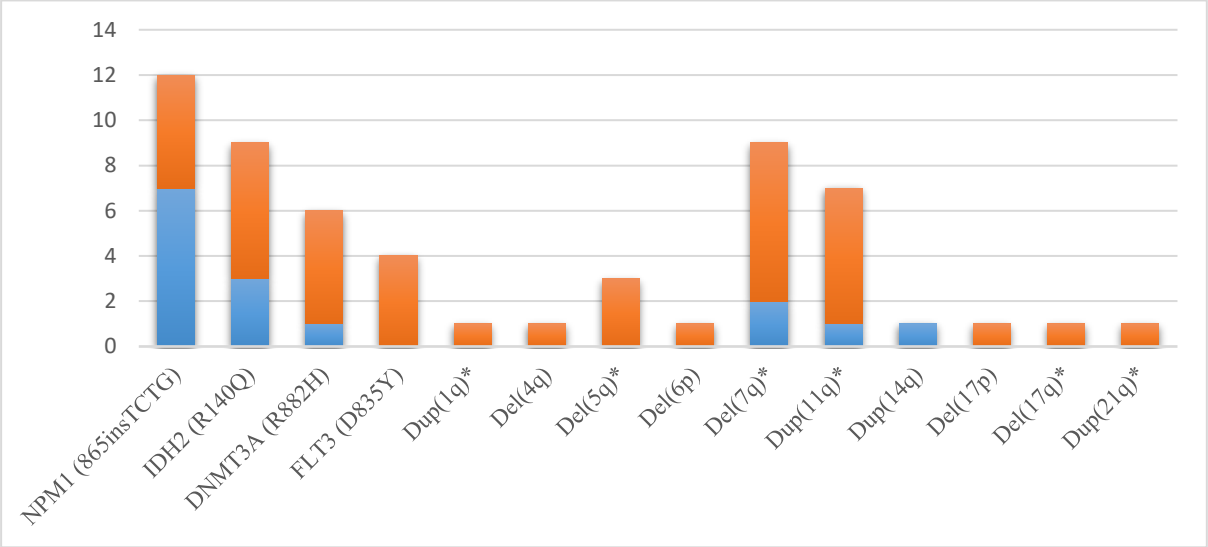
- **Group III** – patients with previously established BCR::ABL1 (-) Myeloproliferative neoplasia – 4 (6.6%)
- **Group IV** – patients with previously diagnosed CML – 2 (3.3%)

4.2 Presentation and analysis of the results of conducted genetic studies

4.2.1 Results from MLPA

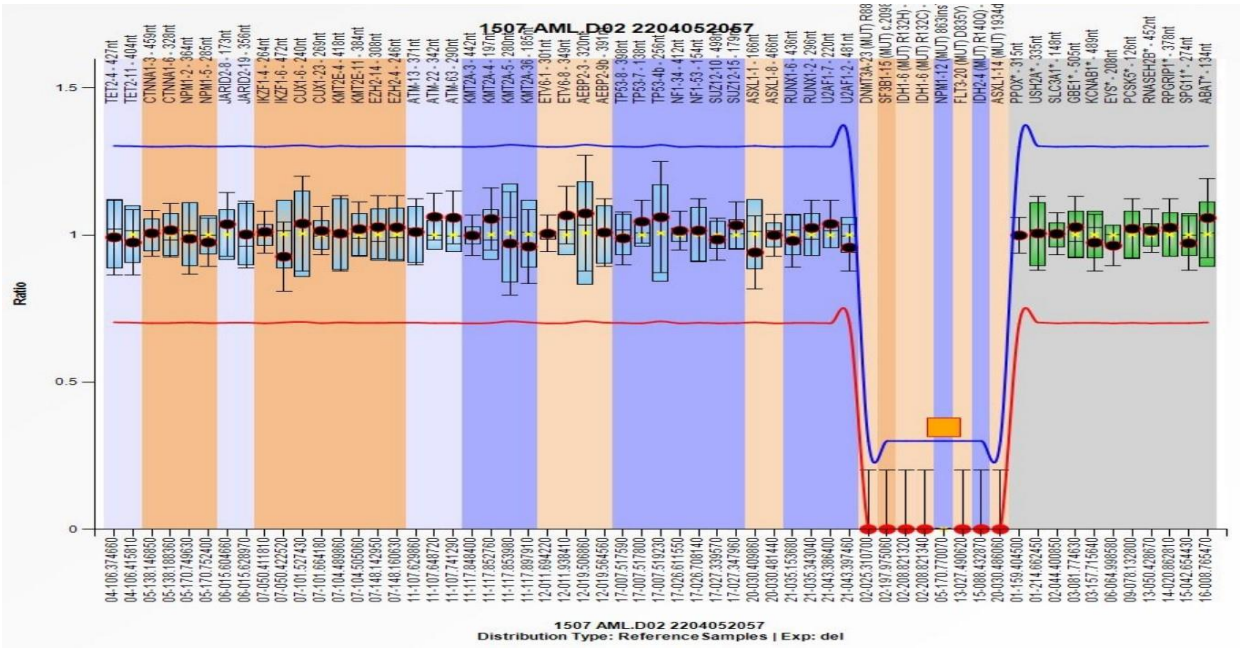
Of the examined 61 patients, genetic changes were detected in 34 (55.7%) and no such changes were detected in the remaining 27 (44.3%). Of the 34, a total of 31 monogenic somatic variants were found in 22 (64.7%) – in isolated form in 11 and in combination with another genetic finding in the remaining 11 (Figure 2). Leading in frequency was the ***NPM1 (865insTCTG)*** variant (Figure 3) – 35.3% of all patients with pathology and 19.7% of all examined patients (n=12).

Figure 2. *Genetic changes detected through MLPA (blue – isolated, orange – combined)*



** For simplicity of view, the total of all probes in the chromosome arm is marked, and the frequency is given per number of patients. The combinations are discussed in detail in Table 3.*

Figure 3. *A patient with an NPM1 (865insTCTG) variant (orange rectangle), deteted through MLPA (Coffalyser™ tool, MRC Holland, Netherlands)*



As can be seen in Figure 2, the *NPM1* variant occurs more often in an isolated state – in 7 patients, while in the remaining 5 it is in combination, but only with other monogenic variants from the performed molecular genetic analysis (Table 2).

Table 2*. Patients with an NPM1 varianr in combination with other single-gene vatiants*

Patient	**First variant***	**Second variant***	**Third variant***
1.	*NPM1* (865insTCTG)	*IDH2* (R140Q)	*FLT3-TDK (D835Y)*
2.	*NPM1* (865insTCTG)	*IDH2* (R140Q)	*DNMT3A (R882H)*
3.	*NPM1* (865insTCTG)	*IDH2* (R140Q)	-
4.	*NPM1* (865insTCTG)	*FLT3-TDK (D835Y)*	*DNMT3A (R882H)*
5.	*NPM1* (865insTCTG)	*DNMT3A (R882H)*	-

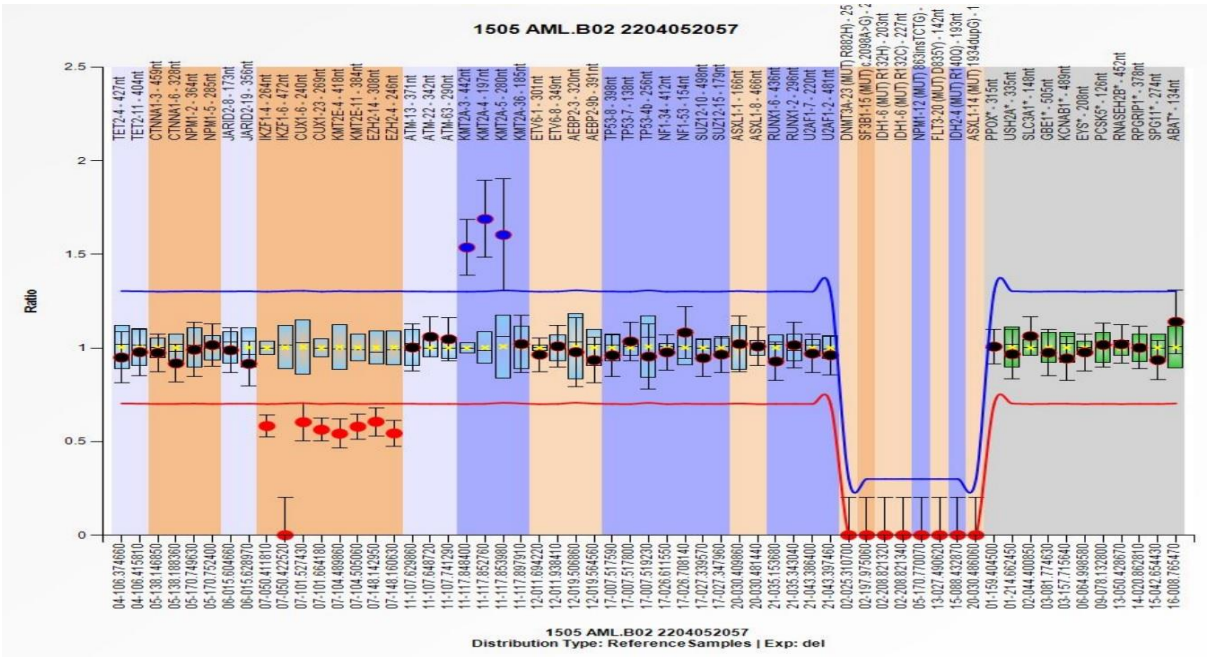
**the sequence of the variants is conditional and does not reflect the evolutionary antiquity of the various clonally occurring monogenic events*

The monogenic variant in *IDH2* was in isolated form in three patients. In three others, as seen above (Table 2), it was in combination with other variants in the *NPM1*, *FLT3* and *DNMT3A* genes. In the remaining three, the combination was with structural chromosomal variants – two patients with 5q deletion and one with 11q duplication. *DNMT3A* was in an isolated form only in 1 patient, while in the rest it was in combination with another monogenic (n=3), with a monogenic and structural chromosomal variant – *FLT3* and duplication 11q (n=1), or only with a structural chromosomal variant (n =1) – 7q deletion. The *FLT3-TKD* variant was

found only in a combined form - 2 patients with one or more other monogenic variants in *NPM1*, *DNMT3A*, *IDH2* (Table 2), and another 2 with monogenic mutations and structural chromosomal variants - *DNMT3A* and duplication 11q (n=1) and 11q duplication (n=1). The detected *FLT3-TKD* variants were confirmed by identical results from routine RFLP-PCR in 3 of the 4 patients (the 4th was not tested by this method).

Regarding chromosomal changes, 18 (52.9%) of the same 34 patients (29.5% of all in the study) had repeat number changes in chromosomes 1, 4, 5, 6, 7, 11, 14, 17 and 21 (Figure 2). As a frequency distribution, the most frequent were the MDS-associated **deletion in the 7th chromosome** (Figure 4) – in 50% of patients with detected structural chromosomal pathology (n=9), as well as a **duplication in the long arm of the 11th chromosome** – 38.9% (n=7) (Figure 2, Table 3).

Figure 4. *Patient with deletion in chromosome 7 - long and short arm (in red) and duplication in long arm of chromosome 11 (in purple) (Coffalyser™ tool, MRC Holland, Netherlands)*



Of all the structural chromosomal findings described, 4 were in an isolated state, and 14 were in combination with other genetic changes (Table 3).

Table 3. *Structural chromosomal aberratioms found through MLPA with respective genes and exons; single gene variants found in combination also included.*

Patient	**MLPA result**
1.	del(4)(q24) – *TET2-4, -11*; del(5)(q31.2) - *CTNNA1-3, -6* del(6)(p22.3) – *JARID2-8, -19*; del(17)(p13.1) – *TP53-8, -4b;* del(17)(q11.2) – *NF1-34, SUZ12-10*; dup(21)(q22.12) – *RUNX1-2*; dup(21)(q22.3) – *U2AF1-7*
2.	del(5)(q31.2) – *CTNNA1-3, -6*; del(5)(q35.1) – *NPM1-2, -5*
3.	del(7)(p12.2) – *IKZF1-4, -6;* del(7)(q22.1) – *CUX1-6* del(7)(p22.2) – *KMT2E-4, -11;* del(7)(q36.1) – *EZH2-4, -14*
4.	del(7)(p12.2) – *IKZF1-4, -6;* del(7)(q22.1) – *CUX1-6* del(7)(q22.2) – *KMT2E-4, -11;* del(7)(q36.1) – *EZH2-4, -14* dup(11)(q23.3) – *KMT2A-3, -4, -5*
5.	del(7)(p12.2) – *IKZF1-4, -6;* del(7)(q36.1) – *EZH2-4, -14*
6.	***DNMT3A (R882H);*** del(7)(q22.1) – *CUX1-23* del(7)(q22.2) – *KMT2E-4, -11; d*el(7)(q36.1) – *EZH2-4, -14*
7.	del(7)(q22.1) – *CUX1-6;* del(7)(q22.2) – *KMT2E-4, -11* del(7)(q36.1) – *EZH2-4, -14;* dup(11)(q22.3) – *ATM-13, -22, -63* dup(11)(q23.3) – *KMT2A-3, -4, -5, -36*
8.	del(7)(q22.1) - *KMT2E; CUX1-6;* del(7)(q36.1) – *EZH2-4, -14*
9.	dup(1)(q23.3) – PPOX; dup(1)(q24) – USH2A; del(7)(q22.1) – *CUX1-6, -23;* del(7)(q22.2) – *KMT2E-4, -11* del(7)(q36.1) – *EZH2-4, -14*
10.	***DNMT3A (R882H); FLT3-TDK (D835Y);*** dup(11)(q22.3) – *ATM-13, ATM-22, ATM-63;* dup(11)(q23.3) – *KMT2A-3, KMT2A-4, KMT2A-5, KMT2A-36*
11. и 12.	***IDH2 (R140Q);*** dup(11)(q23.3) – *KMT2A-3, -4, -5*
13.	***FLT3-TKD (D835Y);*** *dup(11)(q23.3) – KMT2A-3, -4, -5*
14.	***IDH2 (R140Q);*** del(5)(q31.2) - *CTNNA1-3, -6*
15.	dup(14)(q11.2) - *RPGRIP1*
16.	del(7)(p12.2*) – IKZF1-4, IKZF1-6*
17.	del(7)(q36.1) – *EZH2-4, -14*
18.	dup(11)(q23.3) – *KMT2A-3, -4, -5*

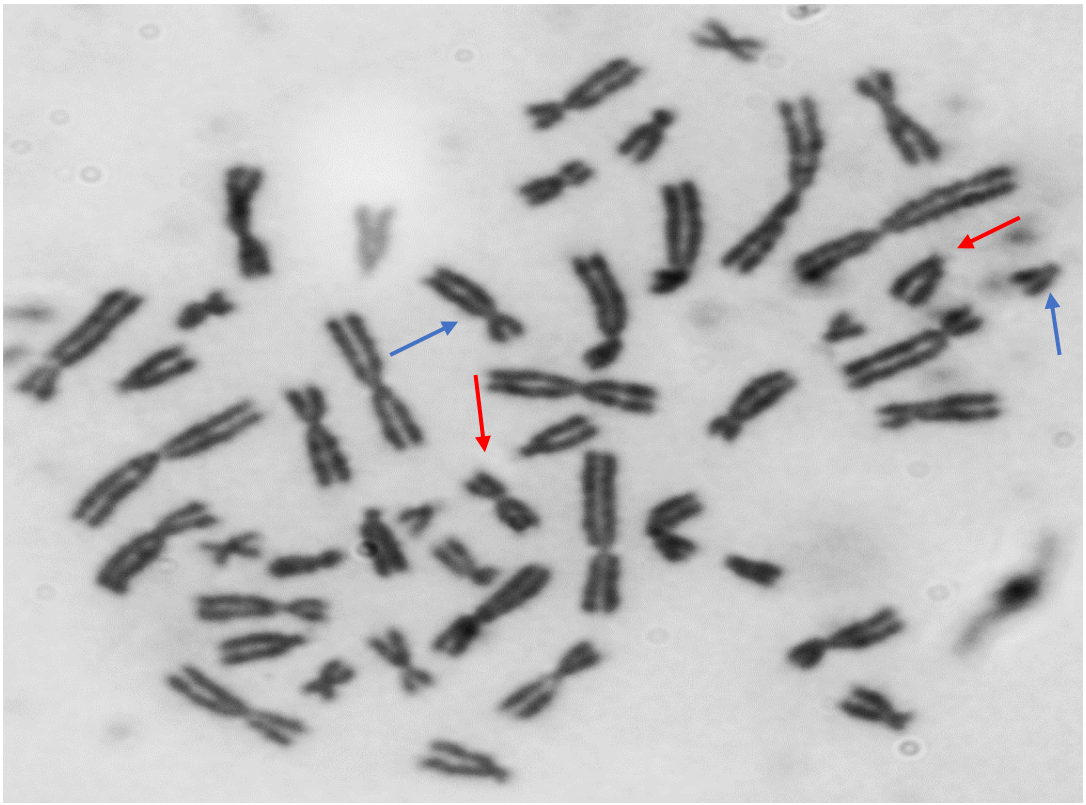
4.2.2 CCA results and comparison with MLPA fidings

The analysis was performed for 53 (86.9%) of all patients - successfully in 38 (71.7%), and without detectable metaphase plates in the remaining 15 (28.3%). In 21 (55.3%) of those successfully performed (or 39.6% of all examined), CCA found a pathological result (Table 4). The leading finding in frequency was **t(8:21)(q22;q22)** (Figure 5) - in 6 (28.6%) of these patients, followed by the complex karyotype - in another 4 (19%).

Table 4*. CCA pathological results*

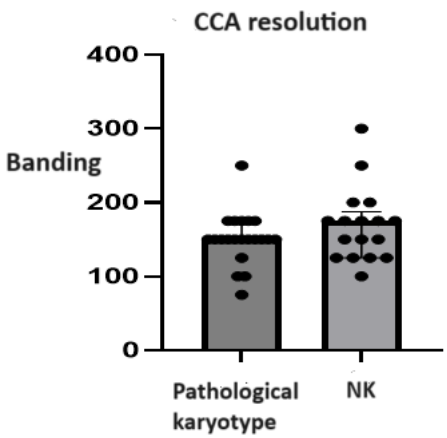
№	Result	Resolution (bands)
1	45,XY,-?D[7]/46,XY[8]	<100
2	46,XY,add(19)(q13.3?)[20]	150
3	43~46,XY,del(?5)(q?13q?33),+16,-17,-17,+mar{cp9}/46,XY[1]	100
4	46~48,XX,-7?[3],del(11)(q22),+11,del(12)(p?12),+16,+mar{cp14}	150-200
5	45,XX,-20[3]/46,XX[17]	150
6	45,XY,-7[20]	150
7	45,XY,-8(?)[3]/46,XY[17]	150
8	46,XX,add (14)(q32) [20]	150-200
9	46,XX,add(3)(q?29),del(4)(q?25), del(5)(q12(13);q33),del(11)(q23),del(?13)(q?34),-17,+21[20]	150
10	46,XX,del(16)(q21(22))[2]/46,XX[18]	150
11	46,XX,del(5)(q12(13)q?33)[10] / (46,XX,-C,+mar[2])	100
12	46,XX,inv(16)(p13q22)[6]/46,XX[6]	150
13	46,XX,t(2;21)(p11(12);q22)[15]	150
14	46,XY,t(7;15)(p?15;q?15)[20]	150-200
15	47,XY,+8[16]/46,XY[4]	150
16	46,XX,t(8;21)(q21;q22)[20]	150
17	46,XY,t(8;21)(q21(22);q21(22))	100-150
18	46,XY,t(8;21)(q21;q22)[20]	150-200
19	45,X,t(8;21)(q21(q22);q22)[20]	150-200
20	46,XX,t(8;21)(q21;q22)[20]	150
21	47,XY,t(8;21)(q22;q22),+8[20]	200-300

Figure 5. *A photograph of karyotype with t(8;21)(q22;q22) (red arrows – derivative chromosomes, blue arrows – normal chromosomes), GTG banding, 150-200 bands.*



It is noteworthy that the leading resolution and at the same time the median in patients with detected chromosomal aberration is 150 bands - 11 (52.4%). We compared it with that of patients with NK (normal karyotype) and found no statistically significant difference despite a slightly higher median in the second group *(p=0.2906, Mann-Whitney test)* (Figure 6).

Figure 6. *A graphical representation of the resolution distribution with the medians of the two sets of results plotted (GraphPad Prism)*



If we focus on the combinations of the results of the two methods as a whole, the following analysis can be made:

- Patients with a *NPM1* variant from MLPA

Cytogenetic analysis in these 12 patients demonstrated NK in 7 of them, as well as 3 pathologic findings with trisomy 8, monosomy 20, and deletion of an undefined group D chromosome, respectively. The other two patients were test-failed and no-test, respectively.

- Patients with an *IDH2* variant from MLPA

In these patients, there was no combination of pathology detected by the two methods – 4 had NK, 3 had a unsuccessfully performed CCA, and 2 had no such performed.

- Patients with a *DNMT3A* variant from MLPA

Here, 4 were with NK, 1 with monosomy on chromosome 20, and 1 with failed CCA.

- Patients with a *FLT3* variant MLPA

The distribution in these patients was – 2 with NK, 1 with monosomy 20 (the same patient was reported with three monogenic variants - in *NPM, DNMT3A*, and *FLT3*, and monosomy 20), and one with failed CCA.

- Patients with structural chromosomal abnormalities from MLPA

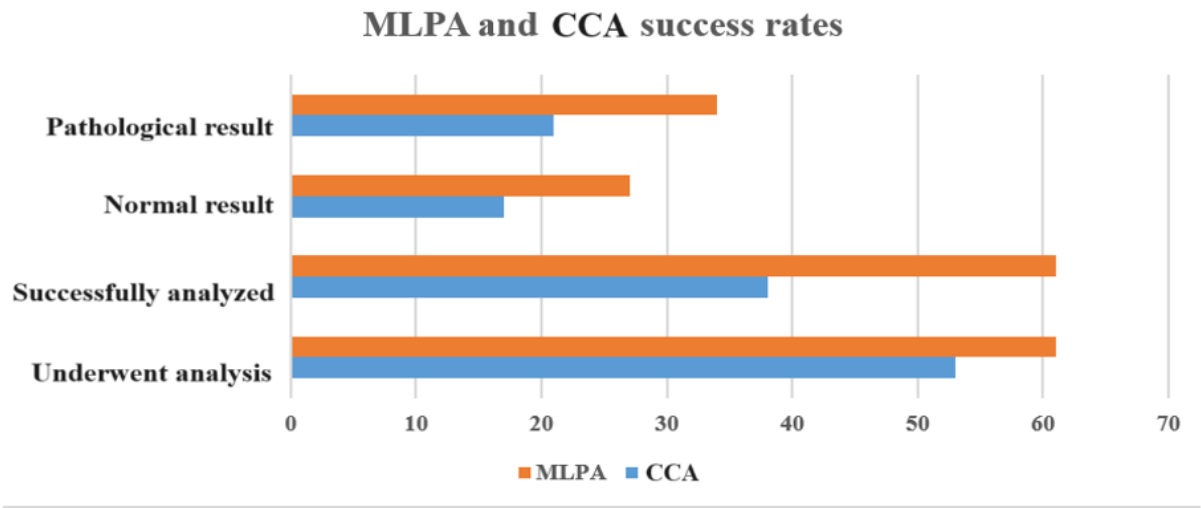
From a total of 18 patients with detected structural chromosomal aberration by MLPA, 6 (30%) had failed and 4 (22.2%) had no CCA. Another 4 (22.2%) had NK and in another 4 (22.2% or only 7.5% of all those with a CCA) a pathological result was found, and here are the cases of concordance between the two methods. This overlap included structural and/or numerical chromosomal aberrations on chromosomes 4, 5, 6, 11, 14, 17, and 21, which overlapped between the two methods (Table 5). Monosomies and trisomies detected by CCA were recorded by MLPA as duplications and deletions affecting several probes on one chromosome, respectively.

Table 5. *MLPA and CCA findings concordance*

	CCA finding	**MLPA finding**
1	Monosomy 7, trisomy 11	del(7q), dup(11q)
2	del(4q), del(5), monosomy 17, trisomy 21	del(4q), del(5q), del(17p), del(17q), dup(21q)
3	Add(14q)	dup(14q) – referrent probe
4	Monosomy 7	del(7q), dup(11q)

It is noteworthy that in the last patient the duplication of the 11th chromosome, detected by MLPA, was not reported by the CCA - in this case, it is not a numerical, but a structural change. The comparison of the two methods in terms of their overall success rate and informativeness is graphically presented in Figure 7.

Figure 7. *MLPA and CCA success rate comparison*



Several features stand out from the comparison:

1) A statistically significant difference was noted between those successfully examined - 61 vs. 38 for MLPA and CCA, respectively *(p<0.00001, Chi-square test).* This difference of 23 patients - 37.7% of all patients in the study - is the sum of the patients without (n=8) and the patients with failed cytogenetic analysis (n=15)

2) Still, the apparent difference of about 16% for detected pathology – 55.7% for MLPA and 39.6% for CCA, did not show statistical significance *(p=0.08544, Chi-square test).* When comparing the chromosomal pathology detected, the success rate is higher for CCA, as MLPA detects this type of pathology in only 29.5% of all examined.

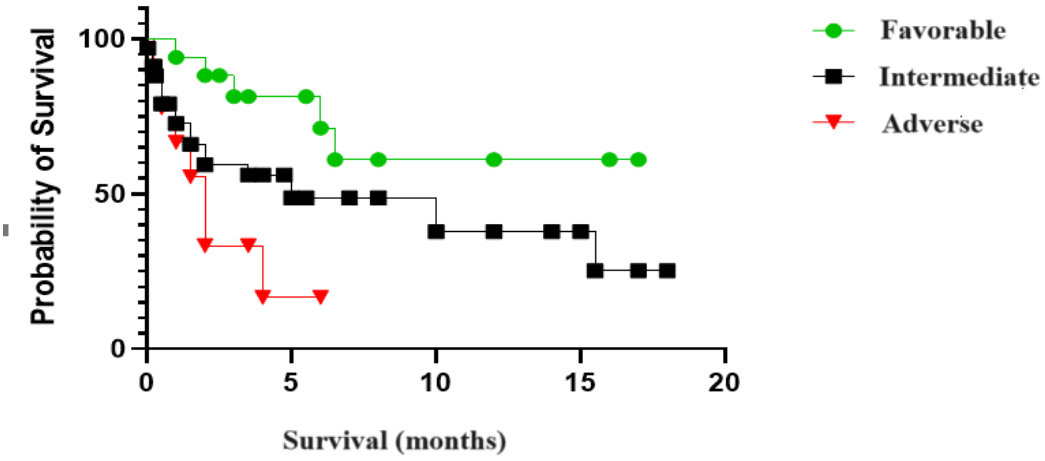
In total from both methods, the success rate of their combination provides information for 48 (78.7%) patients. Of the remaining 13 (21.3%), 5 (8.2%) were without detected pathology by both methods, and another 8 (13.1%) – were without findings from MLPA and with unsuccessful or not performed CCA. Excluding monogenic changes, as they are not covered by CCA, chromosomal pathology was detected in 35 (57.4%) by the combination of CCA and MLPA - 17.8% more than by chromosome analysis alone. In 18% (n=11) of all examined, MLPA provided information on findings missed by CCA.

ELN (European Leukemia Net) 2022 risk stratification

According to the risk stratification derived from these results, 18 (29.5%) belonged to the favorable, 34 (55.7%) to the intermediate, and 9 (14.8%) to the unfavorable risk group. The individual role of the MLPA method was such that it contributed to the stratification of 26 (42.6%) of the patients in the study. For 14 (22.9%) patients, classification was based entirely on method information (Figure 2) due to missing or failed CCA. For the remaining 12 (19.7%), the information from the CCA (mainly NK) was upgraded and led to a change in the initial stratification.

We performed an analysis of median survival using the *Kaplan-Meyer test* with the null hypothesis that the survival of the three groups was identical. Median survival for the favorable risk group was undefined and adjusted by *Machin's* method *(Machin et al, 2006)* - 11.8 months of survival for 61.1% of the risk group. For the intermediate and unfavorable groups, it was respectively 5 and 2 months with a statistically significant difference between the three *(p=0.0190, Log-rank (Mantel-Cox test), p=0.0054, Log-rank test for trend).* This result rejected the null hypothesis (Figure 8):

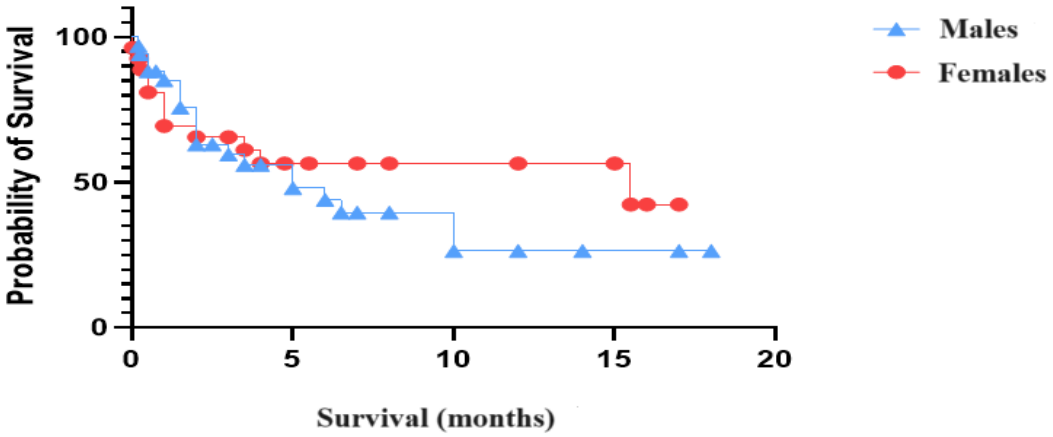
Figure 8*. Median survival of patients in respect of ELN 2022 risk stratification (Kaplan-Meyer test, GraphPad Prism)*



Analysis of median survival by gender and age

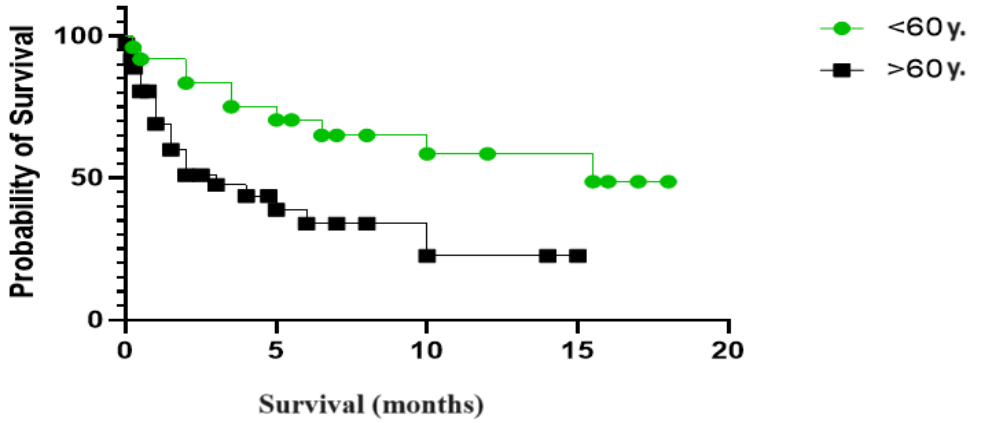
We also performed a *Kaplan-Meyer* analysis of survival according to available demographic data for the included patients. Gender - a median survival of 15.5 months was demonstrated for women and three times lower for men - 5 months, although the *p-value* did not reach a statistically significant value *(p=0.4046, Log-rank (Mantel-Cox) test, p= 0.8603, Gehan-Breslow-Wilcoxon test)* (Figure 9):

***Figure 9**. Median survival for both sexes (Kaplan-Meyer test, GraphPad Prism)*



We also compared survival in patients under and over 60 years of age. The result showed a statistically significant difference in median survival -15.5 months for patients under 60 and 3 for those over 60 *(p=0.0074, Log-rank (Mantel-Cox) test, p=0.082, Gehan- Breslow-Wilcoxon test)* (Figure 10).

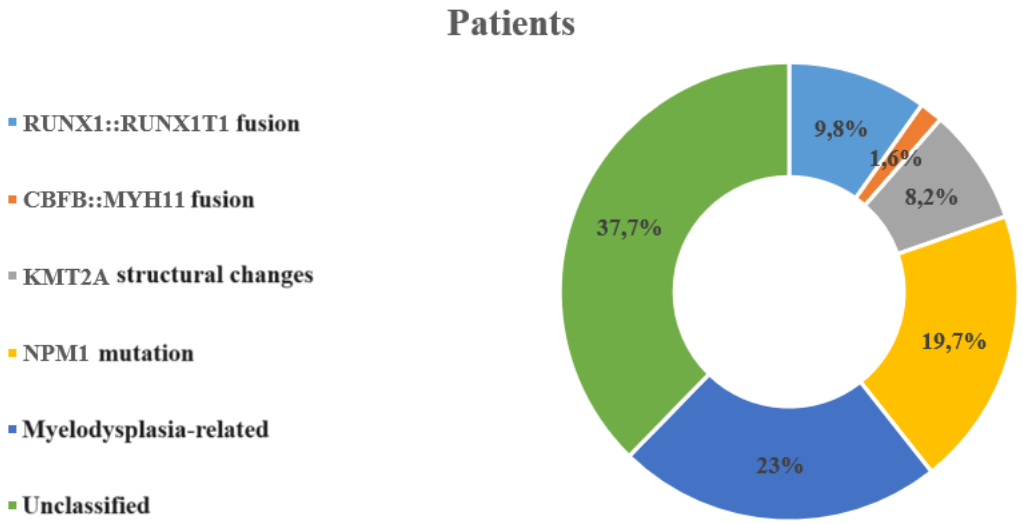
***Figure 10.** Age-dependent median survival (Kaplan-Meyer test, GraphPad Prism)*



AML subtype stratification according to WHO 2022

Based on the information obtained from the study, we divided the patients into the following groups (Figure 11):

Figure 11. *AML subtype stratification according to WHO 2022*



As can be seen from the table, among the classified patients, the group of **MDS-related changes** was the leading group - 23% (n=14). In practice, there are the most unclassified patients - 37.7% (n=23) due to the absence of a detected genetic change in 21.3% (n=13) of both methods or due to the impossibility of classifying the available genetic change - in the remaining 16.4% (n=10).

5. DISCUSSION

5.1 Discussion of results of descriptive-epidemiological characterization of study participants

Age-sex characteristics and median survival relative to these factors

Our results showed great similarity with those reported in the literature regarding the average age of diagnosis – 63-65 years *(Vakiti & Mewawalla, 2021, Gercheva et al., 2010).* Also, AML was found more often in older patients - median age 62 years, with 59% (n=36) over 60 and 75.4% (n=46) over 45 years of age. Similar are the data of Gercheva and co-authors with 55% of patients over 60 years of age *(Gercheva et al., 2010).* The advanced age of newly diagnosed AML patients is a well-known prerequisite for a lower rate of achieving remission, higher early mortality and shorter overall survival *(Appelbaum et al, 2006),* also established by local studies *(Gercheva et al., 2010, Shemelekova et al., 2010).* The survival of our patients under and over 60 years of age also demonstrated a worsening of the prognosis for older patients with a fivefold lower survival in the latter (Figure 10). Of course, this mortality is also influenced by other factors such as type of therapy, comorbidities (our study period coincides with the newly subsided COVID-19 pandemic in addition to the expected cardiovascular, endocrine, and other chronic diseases), ELN risk group, etc.

Much younger was the patient cohort in a long-term study from India by Srivastava et al *(Srivastava et al, 2023)* – a median of 42 years, and only 13% were over 60 years of age. It may be a question of different age of onset of the disease in different ethnic groups or of differences caused by the larger volume (1860 patients) of the cited study.

We did not notice a big difference in the involvement of both sexes - M:F=1.1:1. According to literature data, a larger one is expected - 1.67:1 *(Vakiti & Mewawalla, 2021),* 1.34:1 *(Stabellini et al, 2023),* 1.63:1 *(Shemelekova et al., 2010).* It is possible that our ratio is due to the small sample of patients and less likely to the age restriction imposed in our study. Our analysis of median survival (Figure 9) reported a threefold difference between the two sexes (M:F = 5:15.5 months). In comparison, Stabellini and co-authors reported similar survival for both - 10.8 and 10.1 months for men and women, respectively, but a two-fold difference in favor of women - 36.7 versus 70.9 months, when taking into account the history of previous hospitalization *(Stabellini et al, 2023).* Probably, as with the age factor, gender cannot be considered without the other factors determining the course of the disease.

Distribution by types of indications

A major risk factor for the development of AML is known to be MDS, as well as myelofibrosis and aplastic anemia *(Vakiti & Mewawalla, 2021).* In our study, 11 (18%) patients fit these categories. A total of 11.5% had AML after MDS, which coincides with the rate of secondary AML (11%) in patients over 60 years of age in the study by Shemelekova et al. *(Shemelekova et al., 2010).* In these, as well as in the few patients with previous CML (n=2), knowledge of these conditions and the systematic conduct of control examinations and clinical laboratory studies suggest a timely diagnosis of AML. However, prior haematological disease predisposes to a worse prognosis compared to de novo AML *(Hochman et al, 2023).*

However, for the majority of those included in the study, this was a first hematological disease. These 48 (78.7%) people were diagnosed on the basis of complaints manifested over time, their severity, as well as sought medical help. Some of them have extremely short survival (less than 30 days) and correspondingly early mortality, including emergency admissions and deaths of acute circulatory failure before or during the initial diagnostic evaluation and therapy. Beyond the scope of the hematologist, as well as the entire team involved in the initial evaluation of a patient with AML, would be the initiative and speed of seeking medical care and referral from general practitioners or smaller hospital settings. However, these are factors determining the time of diagnosis and hence the outcome of the disease of these patients. Although this issue is not a specific subject of the present dissertation and would be difficult to control, it addresses the need for mass awareness of society for effective prevention of this disease, as well as the need to improve the mass health culture in general.

5.2 Discussion of results from laboratory genetic analyses

5.2.1 MLPA

Regarding the success rate of the method, we reported the presence of a genetic change in 55.7% of the examined (Figures 2 and 7). Assuming a target leukemogenic success rate of 100%, MLPA fails to detect genetic alterations in the remaining 44.3%. This is due to the targeted nature of the method, and many WHO 2022 forms are omitted here. According to literature data, the overall frequency of all these forms combined is of the order of 25-45% in adults, which may explain the lack of a higher success rate of the molecular genetic method used by us. A factor is also the known limitation in the sensitivity of the method with respect to monogenic variants, deletions and duplications – 5-10%, 20% and 40% respectively *(Hömig-*

Hölzel & Savola, 2022). It is possible that the use of the generally accepted first choice for biological material in myeloid neoplasia - bone marrow *(Rack et al, 2019)* will increase the informativeness of the method and this is one of the future directions of work of this study.

We found only a few studies using MLPA as a method of an initial assessment of patients with AML - Bănescu et al and Tripon et al *(Bănescu et al, 2019, Tripon et al, 2019)* used a total of 5 panels to study newly diagnosed patients over 18 years of age. The team also included somatic variants in the *NPM1, FLT3,* and *DNMT3A* genes. Overall, their results were similar to those of our study in terms of chromosomal (31.8%) and monogenic findings - *NPM1* variant in 17%, *FLT3-TKD* – 5.3%, *DNMT3A* – 12.4%. Our approach was likely more practical as we used only one MLPA panel with lower consumption of reagent and biological material and lower cost of molecular genetic evaluation.

In another study, Marcinkowska-Swojak and co-authors *(Marcinkowska-Swojak, 2016)* used a proprietary panel and focused on detecting three of the most common in AML *NPM1* variants or rearrangements involving this gene, with a total of 12 specific probes. Their success rate of 20.9% (14 of 67) for detecting *NPM1* type A is comparable to ours.

The team of Donahue and co-authors *(Donahue et al, 2011)* conducted a study of a total of 110 samples - 56 of them were from patients with MDS/AML. They used a P145-MDS2 panel comprising 31 chromosomal locations without monogenic somatic variants and FISH with the Vysis MDS/myeloid panel (Abbot Molecular, USA) with probes for 11 chromosomal regions. Their success rates were lower at 8.9% for MLPA and 10.7% for FISH with high concordance between the two methods. This difference with our data is probably due on the one hand to the selection of patients and the lack of somatic variants in their selected panel. However, the reported high concordance between the two methods and between types of biological material is encouraging for our future work.

If we compare the detected pathology with the known frequency from literature data, we see that the expected frequency for *NPM1* variants is 30% *(Heath et al, 2022).* It is possible that our lower rate was due to missed patients with other types of variants in the gene, to the use of venous blood, or to the small size of our study. However, the success rate in our study is comparable to that of other teams - Balatzenko and co-authors *(Balatzenko et al, 2014),* Bănescu and co-authors *(Bănescu et al, 2019)* and Marcinkowska-Swojak and co-authors *(Marcinkowska-Swojak, 2016).*

Regarding the *IDH2* variant *(R140Q)* we found in 14.6% of patients, our reported frequency coincides with that of literature data – 10-15% *(Stein, 2023).* Abbas et al *(Abbas et al, 2010)* reported a frequency of 11% (n=97) of variants in this gene in a larger study of newly diagnosed AML patients by reverse transcriptase-PCR followed by direct sequencing. The most common variant they reported was *R140Q* – 8.3% (n=74) of all tested. Apart from improved diagnostics purposes, recognition of these patients could enable targeted therapy *(Amaya et al, 2018).*

Regarding the patients in our study with a detected *DNMT3A* variant - 9.8% - we compared the work of several teams. Bănescu et al *(Bănescu et al, 2019)* found one in 12.4% of their patients by simultaneously checking for a total of 6 point variants in the gene), while the kit we used had a probe for one of them – *R882H (c. 2645G>A).* Since the frequency of each of these 6 variants has not been commented on separately, we can speculate that the frequency of the variant studied by us will be to some extent lower than the one indicated in general.

In a study from China by Yuan et al *(Yuan et al, 2019),* including 870 newly diagnosed patients over 14 years of age, two point variants in the *DNMT3A* gene, *R882H* and *R882C*, were investigated. They used pyrosequencing and found *R882H* in 6.2% percent of all tested. Notably, both our study and the data from these two studies reported a lower than literature-expected detectability of 20% regardless of volume or method used *(Park et al, 2020).* This may be because we are examining only one, albeit the most common, variant in this gene, with others of similar prognostic significance already present.

Also interesting is the presence of a concomitant *NPM1* and/or *FLT3* variant in a total of 66.7% of our patients with an established *DNMT3A* variant, 50%, and 33.4%, respectively, with one patient having all three variants simultaneously. A similar trend was found in the study by Yuan et al – nearly 27% of patients with the *DNMT3A* variant also had one in *FLT3*, given that they checked for the presence of *FLT3-ITD*, which is more common in the literature. Unfortunately, they did not report a specific frequency of *NPM1* variants *(Yuan et al, 2019).* The significance of these combinations is not fully understood, but probably the initially good prognosis in the presence of an NPM1 variant is adversely affected, as is known for the *FLT3-ITD* co-mutation *(Döhner et al, 2022).*

Concomitant presence of *DNMT3A* variant and structural and/or number chromosomal variants was also observed in 50% of our patients (combined by both methods) and in 33.3%

specifically by MLPA. Bănescu et al. *(Bănescu et al, 2019)* reported similar data, and Yuan et al.'s team at 20.6% found by CCA *(Yuan et al, 2019).*

The *FLT3-TKD* variant is the rarest in our study and is still outside the known classifications and risk stratifications. Our reported incidence of 6.6% is consistent with that reported in the literature *(Kennedy & Smith, 2020).* Similar is the data from the large-scale study from Germany by Bacher and co-authors of 3082 patients over the age of 17.5 years - 4.8% *(Bacher et al, 2008)* using the so-called melting curve analysis – a variant of PCR with consideration of the difference in melting points in the presence of differences in the sequences of the PCR products. In a retrospective study by Shankaralingappa et al *(Shankaralingappa et al, 2022*), *FLT3-ITD* and *FLT3-TKD* were investigated in 424 newly diagnosed adults and children. The *FLT3-TKD* variant was detected in 5.9% of those examined using a PCR-based method. The frequency of the other variant in the gene is much higher - 16.5%, which once again confirms the need for its examination in every newly diagnosed patient.

As for the detection of structural chromosomal pathology, the leading in frequency in our country was deletion in the 7th chromosome - y (14.6%) and duplication in the long arm of the 11th chromosome at (11.5%). In the study by Donahue et al *(Donahue et al, 2011)*, their overall leading finding was a duplication in chromosome 8 (4%) - a probable trisomy 8 characteristic of AML. The panel we selected did not contain a probe targeting chromosome 8, so changes on it would have gone completely unrecognized. The team also reported a combination of 5q and 17p deletions in 1 patient, as reported in us, but in combination with several other disorders (Table 3). It can be seen that the comparison is made difficult by the different success rates and the different probes used in the two studies. A better comparison would be with the work of Bănescu and co-authors, but they do not present more detailed data on the structural chromosomal pathology they found.

In a study by Konialis et al *(Konialis et al, 2014)* they examined mostly bone marrow samples from adults and children, 56% of which were from newly diagnosed patients and 14% from patients with probable AML. The team created a P377 panel comprising a total of 54 probes for structural chromosomal findings and worked with DNA isolated after short-term culture, and used 10 samples from healthy control individuals for quality control. They also perform CCA and FISH - available panels (Vysis, Abbott Molecular, USA) or selected and synthesized according to chromosomal region (Empire Genomics LLC, USA, Bluegnome Ltd., UK). Successful interpretation of MLPA results was reported in 98.4% of cases with the most

frequent finding - deletion in 9p21.3 (*CDKN2A,-B*) in 2.2% of all examined, but patients were suspected of acute lymphoblastic leukemia (ALL). The X060 panel does not have such a probe. In another 1.9% - loss or additional genetic material in 22q22.1 - *RUNX1*, mainly in pediatric patients with probable acute leukemia. We reported a duplication in a single patient (1.6%). In 1.3% of patients in the same age and clinical group, they reported a deletion in *ETV6*, which was not reported in our study. Further comparison is not possible due to a lack of description of all findings in the study - the authors are limited to clinically significant in the background of NK or unrelated findings so determined by them. The comparison is greatly hampered by the differences in the design of the two studies – patient cohort such as age and initial diagnosis, time since diagnosis. Here, only half of the patients are newly diagnosed. The selected panel is also a factor – P377 is broader and could find application in various oncohematological conditions, but at the same time the findings for a specific nosological unit are severely limited. The difficulty in the interpretation of 1.6% described by the authors was not observed in us. A possible explanation for this could be the larger number of healthy controls in our study at a ratio of 1:3 to the patients, against a background of 1:31.3 for the Konialis team and co-authors, or the larger study volume of (n= 313).

Due to the limited number of publications describing the application of MLPA, we decided to compare its success rate for structural chromosomal rearrangements with that of alternative variants. In a recent study from the US, Levy et al *(Levy et al, 2023)* studied 100 adult AML patients – 98 newly diagnosed and 2 post-relapse, with a median age of 58 years. As a source of DNA, the team worked with venous blood or bone marrow in the presence of at least 20% blasts. The method of choice is Optical Genome Mapping (OGM) (Bionano Genomics, USA) - a molecular genetic whole-genome method visualizing a large range of structural genetic changes (resolution over 500 bases), including balanced ones, with high sensitivity (5%). All of them are subject to CCA, 19 - to FISH, 3 - to microchip analysis. In total, a finding of OGM is found in 45%, which exceeds the success rate of the molecular genetic method used by us in relation to the specific type of pathology. According to the results they presented, 11 of the 16 types of findings were translocations and inversions that were not within the analytical scope of MLPA. Among them for the above-described t(8;21), inv(16p), t(6;9) and others included in the current WHO classification and ELN risk stratification. Regarding results comparable to our study, 11% of subjects were found to have a monosomy or deletion in chromosome 5, a higher rate than reported in our results. A monosomy or deletion in chromosome 7 was reported in 11% of those studied by Levy et al, similar to our MLPA frequency. Monosomy or deletion in chromosome 17 is found in 8%, compared to 1.6% in us. Another 6% of the cited study had

trisomy 8, for which there was no probe in the kit we used, and 3% had a deletion in chromosome 20 not reported by MLPA. It is worth noting, however, that considering all the methods used in Levy et al.'s study, a total of 46% had pathology with high concordance between the methods they used. In parallel, a total of 57.4% of us have a structural or numerical chromosomal pathology from the combination of CCA and MLPA. This difference may be due to the different size of the studies or to unknown differences in study design and patient selection. Our concordance is far lower, most likely because of the targeted nature of the molecular genetic method, but the overall success rate is higher. In 13% of cases, OGM (Optical Genome Mapping) provided information on findings missed by CCA, compared to 18% in our experience with MLPA. Also, in 27% of cases, the method used in the cited study specified the points of these chromosomal changes, refining the information available and the genes involved, which also led to changes in the initial risk stratification. This clarification mostly concerns the translocations discovered by the team. OGM demonstrated high concordance with CCA and FISH, accounting for miss potential only in low-grade clones below 5-10%. This makes the method unsuitable for examining patients with the aim of reporting minimal residual disease. In our case, however, the concordance was much smaller, both because of the targeted nature of MLPA and because of the lower sensitivity to OGM and CCA. From these data, it can be seen that the comparison of OGM with MLPA clearly points to the former as a superior method in the detection of chromosomal alterations with different resolution. Easier would be the comparison with the combination of CCA and MLPA, suggesting that OGM could successfully replace it.

The results of this study show that OGM is a potential alternative for the detection of numerical and structural chromosomal disorders, including balanced and complex, with high sensitivity and specificity. It outperforms both MLPA in sensitivity and CCA in specifying some cryptic (to submicroscopic) but key alterations such as translocations, small deletions, inversions, and cases of chromoplexy. Also important is the speed of the analysis – a few days for lab run and data analysis, as well as the lack of need for cell culture, which is often a challenge with this type of sample. Considering these advantages, as well as the automation of the process, it is very likely that the method will replace CCA in the future. The main challenge for the target is the still high cost of the assay – about US$500 per sample *(Levy et al, 2023).* Including an additional method for detection of monogenic changes, the cost per patient would probably double. OGM is performed with specialized equipment requiring a significant initial investment. This technique is not compatible with any other method, unlike practical thermocyclers and sequencers in PCR or MLPA. Last but not least, the whole genomic nature

of the method implies a large volume of data obtained, requiring complex bioinformatic processing and interpretation by specialized personnel. Such a person does not always appear as a standard part of the personnel of hospital facilities. However, this is more of a temporary problem – modern genetic research tends to be more comprehensive and working with large amounts of information is expected to become routine.

Returning to our data, it appears from Table 3 that in most cases there is a change with respect to several probes marking different genes and/or different exons thereof on the same chromosome arm. On the one hand, this correlates with the amount of aberration that occurs, although absolute information cannot be given due to the targeted nature of the analysis. On the other hand, affecting several adjacent probes increases the reliability of the result, as the possibility of accidental ligation errors in the reaction is excluded. However, although partial, this information could be supplemented by that available from routinely administered CCA, as will be discussed below.

5.2.2 CCA

With this method, there is an initial reduction of patients whose results are subject to discussion. On the one hand, in 8 (13.1%) of them, CCA was not conducted for technical reasons. A further reduction was brought about by the failure of cell culture, in 24.6% of all patients in the study - a high rate compared to the study by Cirakoglu et *al (Cirakoglu et al, 2022)* - 12.1%. Yuan et al *(Yuan et al, 2019)* successfully performed CCA in 90.7% of patients in the study—significantly lower than our failure rate. In the study by Gercheva et al. *(Gercheva et al., 2010)*, most of the patients (60%) were not amenable to CCA, and the failed analysis in them was observed in 14.3%. The cited differences in successful cell cultivation are an indication of the need to review and attempt to improve the working protocols we use. It should be noted that our success rate in practice corresponds to the permissible threshold of 60% established by the "Medical Genetics" standard of the Ministry of Health (*https://www.mh.government.bg/media/filer_public/2015/11/18/medicinska-genetika.pdf*).

Success in terms of detected pathology is another aspect that could vary. The proportion of NK reported by us (Figure 7) - 44.7% is similar to that of Cirakoglu and co-authors - 42.8% *(Cirakoglu et al, 2022).* Yuan et al *(Yuan et al, 2019)* reported 60.7%. It is possible that this difference is due to subjective factors such as criticality of the assayer, sensitivity of the karyotyping software if used, or others not discussed in this team's paper. A comparison with

the Bulgarian studies cited above would be interesting, but Shemelekova et al. *(Shemelekova et al., 2010)* studied a small cohort and the data were not included, while in Gercheva et al. *(Gercheva et al., 2010),* the findings were not specified, but only related to a risk group.

Regarding the type and frequency of findings, Cirakoglu et al. *(Cirakoglu et al, 2022)* found monosomal (27.8%) and complex karyotype (25.3%). This differs from our results - the favorable t(8;21) was the leading one in 28.6% of patients with an available finding, followed by a complex karyotype in 19%. In the cited study, the same translocation occurred in only 8.7% of patients with pathology, significantly lower than our reported frequency. This difference may decrease as our study size increases, as the difference in the samples compared here is fourfold.

Also interesting is the parallel with our own experience for a period of 11 years - 2010-2020, not including the present study *(Yahya et al, 2021).* The results of 424 AML patients, mostly adults (97.4%) – a total of 723 CCA samples – were reviewed retrospectively. Since it is not only about newly diagnosed patients but also about already treated patients, some of them were examined more than once during the period. Success was reported in 83.8% of cases, which is closer to the related studies cited above. However, the success rate in terms of reported pathology was lower, NK reported 60.9%, which is closer to that commented by Yuan and co-authors *(Yuan et al, 2019).* A relevant difference between these two studies is the selection of patients – our retrospective study included previously treated patients, which is a well-known and expected prerequisite for NK *(Marcucci et al, 2004, Chen et al, 2011).* With this in mind, the most common finding during this period was the complex karyotype, occurring in 35% of the cases with detected pathology, while t(8;21) is characteristic of only 4.2%.

In a study by Velizarova et al. *(Velizarova et al., 2009),* 49 newly diagnosed adult patients were examined - 28 with AML and the rest with ALL. They performed CCA on bone marrow material as well as FISH with five Vysis fusion gene probes (Abbot Molecular, USA). Findings were not broken down by patient diagnosis. One of the patients did not have a CCA, and in another 8 (16.7% of the examined) it was not unsuccessful. 37.5% of the rest have NC - lower than the studies described above. The design of this study included in addition cases of ALL and patients with APL, usually excluded due to the expected finding there (*https://atlasgeneticsoncology.org/haematological/1035/t(15;17)(q24;q21)-pml-rara, Liquori et al, 2020).* A translocation (15;17) or its equivalent gene fusion was found in 7.1% of AML patients by the combination of KCA and FISH. Out of 25 patients with detected pathology, in

2 with AML, a Philadelphia chromosome was found, absent in us. In another 2, the authors report t(8;21). Although the diagnosis of the latter is not specified, it is probably about those with AML, i.e. the frequency would also be 7.1%, lower than ours, which for all examined would be 11.3%. The same can be assumed for 1 (3.6%) patient with an inversion in chromosome 16, also the only one in us, as well as 2 patients with trisomy 8 for both studies. A complex karyotype was also reported in 3 patients, but it was initially characteristic of various oncohematological diseases and further interpretation was not possible.

In the study by Srivastava et al *(Srivastava et al, 2023)*, 1860 adults with AML were examined through CCA on bone marrow material with additional application of FISH. They initially excluded treated patients and those with absent or insufficient metaphase plates. These criteria were met by 96.3% (n=1791) – a higher success rate than ours and that of the other cited studies. The share of discovered chromosomal aberrations is also leading - pathology was reported in as many as 63.9%, but patients with OPL were not excluded. Not by chance, the most common finding is t(15;17) - 26.1% of the pathological results, the complex karyotype - 17.5% and the monosomal karyotype - 13.8% are also common. These rates are similar to our study, although far less. Also as in the study by Velizarova et al. *(Velizarova et al., 2009)*, t(8;21) was less common than in our patients. Chromosome 16 inversion was reported in 2.6% of patients as a chromosomal aberration, which is less than our frequency, but this is probably related to the size of the two samples (a single patient in our study). The team also described an interesting association of advanced age with NK and complex karyotypes. Despite the aforementioned difference in age between the two studies, the difference in findings across their target age groups is striking. Due to the small number of patients, we did not perform such an analysis, but it would be informative to compare in a larger cohort in the future.

Levy and co-authors *(Levy et al, 2023)* demonstrated a lower success rate than reported in our results concerning CCA – 42% with pathological results and 58% with NK. Monosomy/deletion in chromosomes 5, 7, and 17 were leading findings with 23.8% of each of the samples with a chromosomal aberration present, followed by inv(16) in 19%, far exceeding our reported frequencies for this method. It may be a resolution limitation that prevents our team from reporting some structural changes, in this case, deletions and inversions. Again, the representation of t(8;21) in the data from this study is comparatively weaker - 11.9%. Some of these findings combined, with 11.9% having a complex or monosomal karyotype, twice as common in our patients.

An important aspect of the reliability of CCA is also the achieved resolution. Although not cited in the studies we reviewed, it is a known limit both for the method itself and for working with bone marrow samples. In each standard CCA result *(https://www.mh.government.bg/media/filer_public/2015/11/18/medicinska-genetika.pdf, Hastings et al, 2012, Hastings et al, 2013)* state the resolution and the limitations it imposes. In reporting the resolution we achieved (Table 4) without reporting a significant difference in NC and pathology (Figure 6), we are aware of the inevitably missed findings. Invariably, this is a prerequisite for failure to register more detailed changes, which are by definition within the resolution of the method. Such, for example, are some deletions and duplications, insertions, inversions, etc. That is, NK can quite adequately be called a "conditional" NK. Also, some of the results lack specificity regarding an affected chromosome or its region – only a chromosome group or a marker chromosome is noted. Some results have a question mark due to uncertainty in the particular subband. This is also due to the low-resolution characteristic of bone marrow samples and carries an additional limitation in the informativeness of the method.

In this sense, MLPA overcomes or is independent of several common problems in performing cytogenetic analysis - the need to culture cells, the lack of metaphase plates for analysis, and the technical problems associated with the source of biological material for it, including the refusal to perform bone - brain biopsy or temporary inability to carry out this procedure. Working with venous blood in the presence of sufficient circulating blasts is accepted as a reliable source of biological material for genetic analysis *(Döhner et al, 2022, Rack et al, 2019).*

Excluding the expected discordance with monogenic somatic variants, such is observed in the results of both methods in patients (where CCA was successfully performed). On the one hand, in 4 patients a structural chromosomal aberration was detected by MLPA – 2 with dup(11q), 1 with del(5q) and 1 with del(7q), without a corresponding finding by CCA with NK result. At the same time, in another patient, the discordance is partial - the chromosome analysis does not report the presence of a structural, but only a numerical change (Table 5, patient 4). A likely explanation is the resolution, in this case 100-200 bands for these 4 results, and its limitations discussed above. In another 17 patients, pathology was detected by the cytogenetic analysis without an analogous result by the molecular genetic method:

- Translocations – 8;21 (n=6), 2;21 (n=1), 7;15 (n=1): except for loss of genetic material detectable with a probe targeting the region, or one for a specific fusion gene, MLPA is unable to detect this type of change.

- Monosomy 20 in 15% of analyzed metaphases (n=1): here the size of the clone probably does not allow detection by MLPA, which in the particular patient detected 3 monogenic variants, but no chromosomal aberration. There may also be a difference in the size of this branch in blasts in bone marrow and venous blood.

- Trisomy/monosomy on chromosome 8 (n=2): the absence of a probe for this chromosome causes the omission of aberrations on it.

- Inversion in chromosome 16 (n=1) – similar to translocations.

- Deletion in the long arm of chromosome 16 in 10% of analyzed metaphases (n=1) – here the lack of concordance is due to both the size of the branch and the lack of a probe for the deleted region.

- Group D chromosome monosomy in 46.7% of analyzed metaphases (n=1) – expected missing probe for this chromosome. However, given the information on available reference probes for chromosomes from this group, it can be assumed that the clone was not sufficiently well represented in the venous blood sample for MLPA, or that the resolution (>100 bands) and/or possible aberrations here prevented correct determination of chromosome group.

- Composite karyotype with various aberrations (n=1) – by definition, the composite karyotype is extremely variable between individual metaphases, which accounts for poor representation of individual branches beyond the sensitivity of MLPA.

- Additional material on chromosome 19 (n=1) – missing probes for this chromosome.

- Deletion in the short arm of chromosome 5 (n=1) – lack of targeting probe.

In summary of our data from genome-wide genetic studies, CCA maintains its leading role as a method for the detection of numerous and gross structural chromosomal alterations. Sensitivity to low-grade branches and balanced rearrangements further stabilizes its role in the initial evaluation of patients with AML. However, the information from it, especially at low resolution, should be taken critically, since limited informativeness can "mask" the presence or change the perception of a given aberration. For example, extra material on one chromosome and missing on another could be due to an unrecognized translocation, but the two methods performed would not provide enough information. As with marker chromosomes, the origin could be specified by methods such as Multicolor FISH, microarray analysis of unbalanced changes, or OGM.

The results of our study indicate that the combination of CCA and MLPA significantly increases (Figure 7) the amount of detected genetic markers. In 44.3% of patients, MLPA findings were established - monogenic or structural chromosomal, on the background of NK,

absent or failed CCA. In another 6.6%, those complementing or clarifying the result of the chromosome analysis were found. The level of detection of pathological findings by the two methods is also in contrast - 36.9% and 55.7% for CCA and MLPA, respectively. Despite the absence of a statistically significant difference between these two values, the effect of the additional molecular genetic method increases the amount of information about the genetic basis of the disease in these patients.

21.3% of patients remain with an unexplained genetic characteristic - 8.2% have a normal result from both methods and 13.1% - with a normal result from MLPA and missing or failed CCA. This suggests the need for a more comprehensive approach to the diagnosis of patients with the inclusion of more genetic markers - monogenic and (numerical and structural) chromosomal. This goal is mostly directed at the molecular genetic method or methods, but the very high rate of failed CCAs compared to the studies cited above should also be considered. The success rate could be increased by choosing another method such as PCR, NGS (Next-generation Sequencing), Single nucleotide polymorphism-array, FISH, OGM, or by adding probes or a panel to the current MLPA approach. These are practices described in other studies and international recommendations as a solution to the problem *(Döhner et al, 2022, Shimony et al, 2023, Bănescu et al, 2019, Tripon et al, 2019).*

Of course, the method we used could be completely replaced by one based on next-generation sequencing. However, NGS is not a particularly cost-effective method in a decentralized setting with smaller sample numbers. This is the case both here and in other countries *(Bănescu et al, 2020).* Also, like OGM and other types of analysis producing large amounts of information, the interpretation of the latter requires complex bioinformatic processing.

Discussion of the ELN 2022 risk stratification

Risk stratification data demonstrated a significant preponderance of patients in the intermediate, with the least number of patients in the adverse risk group. In the study by Bănescu and co-authors *(Bănescu et al, 2019)* the distribution was with similar ratios in the three groups. In other of the cited studies *(Gercheva et al, 2010, Levy et al, 2023, Cirakoglu et al, 2022)* the distribution has swapped positions for a favorable and unfavorable group, while the intermediate group retains the leading position. The presence of the least number of patients in the unfavorable group in our study is probably due to underdiagnosis of these patients. From the methods we used and their capabilities, it can be seen that many elements of the risk group

are outside the scope of this combination. The capabilities of CCA are also limited by the low resolution, as commented above, and this could affect the detection of some more detailed structural changes. No less important is, of course, the large share of patients with missing or failed CCA - a total of 37.7% of all in the study.

A recent study from the USA *(Lachowiez et al, 2023)* included 513 newly diagnosed adult patients with AML, excluding cases of relapse and APL. Information on available genetic markers in this cohort is based on performed CCA, FISH, RNA sequencing, as well as whole exome and targeted next-generation sequencing. According to ELN 2022, their distribution was 48% for unfavorable, 34% for intermediate, and 29% for the favorable risk group, which differs from ours and the studies cited above. Notably, they reported the presence of somatic variants in *SRSF2, SF3B1, U2AF1, ZRSR2, BCOR, EZH2,* and *STAG2* genes in 33% of all included patients, which explains the increased frequency of patients in the high-risk group. These genes did not appear in any of the 3 studies listed above, and only a variant in *SF3B1* was included in ours. The results of this study and the differences with our stratification demonstrate once again the advantage of combining multiple methods with different scopes and resolutions of maximum detection of genetic changes and correct prediction for patients. Quite literally, of course, the cost of such combinations remains a problem in routinizing such practices in developing economies.

Another large study from Germany *(Rausch et al, 2023)* evaluated 1138 adult patients with AML. Their distribution is dominated by unfavorable risk groups, followed by favorable and intermediate. As a source of information, the team used CCA and targeted next-generation sequencing, selecting 68 frequent somatic variants typical in myeloid neoplasias. Here, too, the preponderance of the group with the richest range of included somatic monogenic variants speaks for better informativeness and more correct stratification of patients.

However, the majority of patients in the intermediate risk group in our and some of the cited studies is not surprising. It is due both to the above-mentioned underdiagnosis of some markers in the other groups and to the inclusion of NK and all chromosomal changes not assigned to another group, which invariably exceed the distinct favorable and unfavorable in number *(Döhner et al, 2022).* This trend is subject to change as more and more detailed genetic methods are introduced, elucidating more of the genetic characterization of more AML cases. The enrichment of the risk stratifications used is also a matter of time, which is observed with each subsequent correction of the ELN *(Döhner et al, 2022, Döhner et al, 2017).*

Regarding the contribution of MLPA specifically, our data demonstrate the benefit of detecting monogenic variants and structural chromosomal aberrations that led to the classification of 42.7% of patients (Figure 2, Table 10). This clarification of the genetic basis of the leukemogenic process makes it possible to personalize the approach to patients and select the most adequate measures to mediate better survival for the specific case.

Regarding our reported survival of these patient groups (Figure 8), a statistically significant difference in survival was expected. It would be more informative to follow up with the patients for a longer period, a minimum of 5 years, to obtain more definitive data on overall survival and to compare with other Bulgarian and foreign studies.

Discussion of AML subtype stratification according to WHO 2022

The results of our study allowed a new classification of patients based on cytogenetic and molecular genetic markers from the two methods used (Figure 11). Among those classified, leading in frequency (23%) are those with MDS-associated changes, which can be attributed to the lower limit of frequency according to literature data – 25-48% *(Koenig et al, 2020, Arber et al, 2020)*. Of these, 64.2% were classified in the group only by MLPA, 14.3% by CCA, and in the remaining 21.4% there was overlap (Figure 2, Tables 4 and 5). The proportion of this group may increase with the inclusion of a larger number of patients or with an improvement in the genetic coverage of the methods used. Our results show a gap in the reporting of molecular genetic markers of this subtype – only *ASXL1* and *SF3B1* appear in the kit we used, while *BCOR, EZH2, SRSF2, STAG2, U2AF1* and *ZRSR2* remain unclear. At the same time, we have reason to conclude that both methods - MLPA and CCA, are limited in reporting available changes in the group of structural chromosomal changes. While cytogenetic analysis is limited by its success rate and resolution, MLPA has disadvantages such as targeted nature and lower sensitivity. It is highly likely that the majority of patients remaining unclassified (37.7%) belonged at least partially to this initially large group.

It is noteworthy that, according to the WHO 2022 classification, the group of AML with MDS-associated changes includes patients with de novo MDS and with secondary AML after MDS. From our data, it is clear that the information obtained from the performed analyzes doubles the number of patients in the group, judging by those initially referred with a known previous MDS (n=7). MDS and AML are known to be etiologically and pathogenetically

related, and their distinction is currently becoming more tentative and uncertain *(Jagurinoski et al, 2022).*

The next largest group (19.7%) was that of AML with an *NPM1* somatic variant detected by MLPA. The comparability of this group as a frequency with other data was discussed above. The prognosis of these patients, initially favorable, depends on the status of the *FLT3* gene *(Heath et al, 2022, Kunchala et al, 2018).* In two, a *FLT3-TKD* variant was also identified, which likely has a negative impact on disease outcome, although it is currently not listed in the latest version of ELN *(Döhner et al, 2022, Kennedy et al, 2020, Kiyoi et al, 2020).* In another, information on *FLT3-ITD* present outside the study was available and he was classified in the intermediate group.

The favorable *RUNX1::RUNX1T1* fusion accounts for the third largest number (9.8%) of this group of patients. Its establishment is entirely thanks to CCA and the most common finding of the method. We allow for the possibility of missing other patients in this group in the quoted 37.7% with a missing karyotype.

KMT2A structural alterations characteristic of 8.2% of our patients were reported by the specific probes of the MLPA X060 panel we used. It is noteworthy that none of them have data on chromosomal changes from CCA - two have NK, one with failed CCA and 2 without a chromosomal analysis.

The *CBFB::MYH11* fusion group was represented by a single patient (1.6%), suggesting significant underdiagnosis according to frequencies reported in the literature and cited studies *(Lv et al, 2020, Levy et al, 2023).* Improved detection of the inversion and translocation leading to the characteristic gene fusion by CCA or another method would increase the number of patients both in classifying them and adequately risk stratifying them according to ELN 2022.

As commented above, there was limited to missing representation of some groups according to WHO 2022. This is due, on the one hand, to the small number of patients studied, as well as to the age restriction inherent in the study design. However, the omission on the part of the methods we used suggests the need for an even more comprehensive evaluation of these patients and is one of the directions for future work.

6. CONCLUSION AND FUTURE WORK PERSPECTIVES

Molecular genetic analyzes have an invariable position in the initial evaluation of patients with newly diagnosed AML. At the moment, the recommendations include mandatory application of CCA and one or more molecular genetic methods to fully characterize each case *(Döhner et al, 2022, Rack et al, 2019).* These data are greatly needed for diagnostic, prognostic and predictive refinement and refinement of the approach to patients with AML. The choice of the most suitable method or a combination of them depends on many factors - analytical scope, complexity of implementation and technical requirements, number of examined patients per unit of time, staffing, available laboratory equipment, etc.

Based on the comparison of the results of MLPA and those of cytogenetic analysis performed, and on the comparison with other similar studies, we can conclude that MLPA can be a useful and informative method for initial genetic screening in parallel with cytogenetic analysis. Through molecular genetic analysis data, genetic changes were revealed in more than half (55.7%) of the patients. Also, slightly less than half (42.6%) of them were classified and stratified by MLPA alone. The combination of the two methods - cytogenetic and molecular genetic - provided information for the majority (78.7%) of our patients and provided an opportunity to individualize their treatment. Used as a screening method, MLPA provides additional information on more detailed genetic alterations, overcoming the well-known limitations of CCA. Compared to the application of several other MLPA kits or methods simultaneously in other studies *(Bănescu et al, 2019, Marcinkowska-Swojak et al, 2016, Donahue et al, 2011, Balatzenko et al, 2014, Abbas et al, 2010),* the selected from us X060 kit presented no less findings of monogenic and chromosomal origin, making it a valuable part of the evaluation of this contingent of patients. In our opinion, the benefit of MLPA would be particularly felt in countries with limited coverage of molecular genetic analysis by national health insurance. Since Bulgaria is a good example of such limited funding, we consider the method promising at least until the volume of clinical pathways and procedures available for the care of these patients is changed and improved.

Based on our study and its results, and with the aim of improving the genetic evaluation of the contingent of newly diagnosed AML patients, the following guidelines can be drawn:

• Introduction of a routine molecular genetic method (or a combination thereof) for initial genetic evaluation of the commented contingent of patients in addition to CCA.

• Selection of molecular genetic markers of interest - both for adults and pediatric patients, taking into account the current international recommendations and opportunities for targeted therapy, as well as their regular updating in view of the rapidly progressing scientific data in the field.

• Whenever possible, use of bone marrow material as a DNA source to ensure the most accurate assessment of neoplastic genotype.

• Good knowledge of the characteristics, limitations and options for improving the informativeness of each of the methods used.

However, the need for a larger study volume in the future is evident in order to draw lasting conclusions about its role in routine use. The significant percentage of results without a defined genetic finding by MLPA – 44.3%, is an indication of the need to evaluate more genetic markers in accordance with current recommendations. The need for patients with NK, without or with unsuccessful cytogenetic analysis, is particularly significant, since the presence of such is a permanent trend in longer-term domestic and foreign studies.

Regarding our prospects for future work, one of them is related to the time factor. As our study was relatively short, it would be appropriate to continue the evaluation of MLPA with a longer follow-up of the present cohort and inclusion of more patients. Extending the follow-up period would allow the calculation of 5-year survival. Also, our team will be able to form a more definitive conclusion about the benefits of a method giving more detailed information with a shorter time to result compared to CCA. A parallel with another molecular genetic method such as NGS would provide useful information on the effectiveness of our proposed alternative, although this would depend mainly on additional financial resources. Also, a parallel study of different sources of neoplastically altered genetic information—venous blood and bone marrow—would provide an opportunity to compare quality and findings.

As for the contingent of examined patients, we consider it promising to include children, taking into account the described differences and peculiarities of this group. The inclusion of patients with MDS would also be appropriate given the recent changes in the WHO, ICC and ELN classifications and the changing understanding of the association with AML *(Döhner et al, 2022, Khoury et al, 2022, Arber et al, 2022).*

Last but not least, collective activity is undoubtedly the most promising in modern science. Data from consortia engaged in the study of a given group of diseases or a specific one are particularly informative and valuable. We consider it extremely useful to combine the data for Bulgaria and future inclusion in a consortium to study and clarify the genetic etiology in

patients with AML. So far, these consortia under-represent patients from Eastern Europe, and the interest in working with and involving these countries is well known.

7. INFERENCES

1. Among the selected patients with newly diagnosed AML, there was a clear preponderance (more than three quarters - 78.7%) of those without other previous hematological (including malignant) diseases.

2. MLPA allowed detection of monogenic and chromosomal alterations in more than half (55.7%) of patients, which contributed to their successful classification and stratification of most of them according to risk according to current international classifications.

3. Through the method used, new for us, monogenic markers new to our practice were diagnosed (in the NPM1, IDH2, DNMT3A genes), which were reported in one third (34.4%) of all examined patients.

4. The MLPA method allowed additional detection of chromosomal changes compared to CCA, with MLPA providing information on findings missed by CCA in one fifth (18%) of all examined.

5. In nearly one third (27.9%) of the patients, conventional cytogenetic analysis revealed a pathology without an analogous result from the molecular genetic method.

6. The combination of the two methods - cytogenetic and molecular genetic - provided information for the majority (78.7%) of our patients.

8. CONTRIBUTIONS OF THE DISSERTATION

Original contributions

1. For the first time in our country, systematized information, and analysis of the results of the application of the MLPA method with an assessment of its contribution to the care of newly diagnosed AML patients over the age of 18 is performed and presented. The obtained results are a basis for comparative studies at the national and international level.

2. The present work is one of the few prospective studies in our country examining and comparing the diagnostic success rate of genetic laboratory methods - in this case, CCA and MLPA, in relation to this contingent of patients, drawing conclusions for routine clinical-diagnostic practice.

Confirmatory contributions

1. The lasting role of the cytogenetic method as a practical and tested one in specifying significant numerical and large structural chromosomal markers included in modern classifications and algorithms for therapeutic behavior has been confirmed.

2. The need to upgrade the application of conventional cytogenetics with modern high-resolution molecular genetic methods to prevent frequent problems with the latter and specify more detailed, including monogenic, somatic changes in these patients has been confirmed.

3. The leading role of the combination of cytogenetic and molecular genetic methods in laboratory diagnostics for revealing the genetic basis of the disease in patients with AML has been confirmed.

Practical contributions

1. A method for molecular genetic analysis - MLPA, was introduced for genetic screening of patients with newly diagnosed AML, in parallel with the routinely conducted cytogenetic analysis within the scope of the Laboratory of Medical Genetics, Varna.

2. Guidelines for the selection of genetic laboratory methods, biological material and genetic markers have been derived to improve the genetic evaluation of the contingent of newly diagnosed patients with AML.

9. SCIENTIFIC PUBLICATIONS RELATED TO THE THEME OF THE DISSERTATION

1. Full-text publications:

- **Dinnar Yahya**, Mari Hachmeriyan, Ilina Micheva, Trifon Chervenkov. Acute myelogenous leukemia - current recommendations and approaches in molecular-genetic assessment. Romanian Journal of Internal Medicine. 2022;60(2):103-114

- **Dinnar Yahya**, Milena Stoyanova, Mari Hachmeriyan, Lyudmila Angelova, Ilina Micheva, Trifon Chervenkov. Molecular- geneticmarkers in acute myeloid leukemia – the good, the bad and the intermediate. Varna Medical Forum. 2022;11(2):7-19

- **Yahya, Dinnar**, Hachmeriyan, Mari, Ruseva, Tsanka, Chervenkov, Trifon and Micheva, Ilina. "MLPA in the initial genetic screening of patients with acute myeloid leukemia"; Romanian Journal of Internal Medicine, vol.0, no.0, 2023, pp.-. https://doi.org/10.2478/rjim-2023-0027

2. Scientific proceedings with published abstracts:

- **D. Yahya**, T. Ruseva, M. Tsvetkova, M. Stoyanova, M. Levkova, V. Miteva ,M. Hachmeriyan, L. Angelova1, I. Micheva, T. Chervenkov. Tendencies of cytogenetic analysis of patients with acute myeloid leukemia an 11-year single-centre experience report. 13th EUROPEAN CYTOGENOMICS CONFERENCE. ECA newsletter (2021).

- **D. Yahya**, T. Ruseva, M. Hachmeriyan, L. Angelova, I. Micheva, T. Chervenkov. Cytogenentic stratification according to risk in acute myeloid leukemia – retrospective study over a 10-year period. National Conference of Hematology (2021)

- **D. Yahya**, T. Ruseva, M. Hachmeriyan, L. Angelova, T. Chervenkov, I. Micheva. Cytogenetic profile of newly diagnosed patients with acute myeloid leukemia – a single centre retrospective study. European Journal of Human Genetics (2022), vol 3. Sup. 1

- **Yahya D.**, Hachmeriyan M., Ruseva T., Chervenkov T., Micheva I. MLPA as a genetic screening for patients with acute myeloid leukemia. XII National Congress of Hematology (2023)

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