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ACUTE LEUKEMIAS IN CHILDREN AND ADOLESCENTS: MOLECULAR ANALYSIS AND ITS IMPACT ON MANAGEMENT

SUMMARY OF Ph.D. THESIS

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List of abbreviations

AIEOP - Associazione Italiana di Ematologia e Oncologia Pediatrica

ALL- Acute lymphoblastic leukemia

AML - Acute myeloid leukemia

APL- Acute promyelocytic leukemia

ATO- Arsenic trioxide

ATRA- All trans retinoic acid

BFM- Berlin-Frankfurt-Munster

CBF- Core binding factor

CCG -Childhood Cancer Group

CI- Confidence interval

CLL1- C-type lectin-like molecule 1

CNS- Central Nervous System

COG- Children's Oncology Group

CSF-Cerebrospinal fluid

CTCAE- Common Terminology Criteria for Adverse Events

DIC- Disseminated intravascular coagulation

DS- Down Syndrome

EFS- Event-Free Survival

EORTC -European Organization of Research and Treatment of Cancer

FAB- Franco-American-British

FISH- Fluorescent in situ hybridization

FLT3- FMS-like tyrosine kinase 3

Hb-Hemoglobin

HDAC- Histone deacetylase

iAMP21- Intrachromosomal amplification of chromosome 21

IgH- immunoglobulin heavy chains

ITD- Internal tandem duplication

IV- intravenous

MRC - Medical Research Council

MRD- Minimal residual disease

NGS- Next-Generation Sequencing

OS-Overall survival

Ph- Philadelphia

RT-PCR- Polymerase Chain Reaction

TKD- Tyrosine kinase domain

WHO- World Health Organization

Introduction

Acute leukemia is the most common malignancy in children and adolescents and at the same time an important cause of cancer mortality in these age groups.

Acute lymphoblastic leukemia (ALL) is the most common type of cancer in children, accounting for approximately 25% of the diagnosed neoplasms in children, with an annual incidence of approximately 36 cases per million (Inaba, 2021; Horibe, 2013).

The probability of survival in pediatric ALL has improved significantly over the past 5 decades due to intensive chemotherapy, stratified and individualized therapy according to the risk category of the disease, and appropriate supportive therapy. The 5-year survival rate for children >1 years of age with ALL in the US and Europe has increased from less than 5% in the early 1960s to 85-90% today. Genetically, ALL is a heterogeneous disease. Genomic analysis has revealed over 30 molecular subtypes of ALL that are associated with either chromosomal aneuploidy, rearrangements involving oncogenes or transcription factors, or point mutations (Inaba, 2021). Genetic characterization in ALL is of uttermost importance, as it identifies molecular abnormalities with unfavorable risk, influences therapeutic decisions by placing patients in the risk categories established by the current therapeutic protocols or by justifying the indication for allogeneic hematopoietic stem cell transplantation, and equally contributes to the transition to molecularly targeted therapy that reduces the risk of relapse. The main new therapies in ALL, molecularly targeted or at the level of surface markers present in leukemic blasts are: immunotherapy (bispecific antibodies such as Blinatumomab, CAR-T cell therapy and conjugated antibodies such as Inotuzumab), tyrosine kinase inhibitors, BCL2 inhibitors, proteasome inhibitors.

Acute myeloid leukemia (AML) is a relatively rare disease in children, with an incidence of about 7 cases per million representing 15-20% of all pediatric acute leukemia cases. The incidence is higher in children under 2 years of age and also in teenagers aged over 15 years (Reinhardt, 2022; De Kouchkovsky, 2016). Significant progress has been made in recent years in improving overall survival in pediatric AML, mainly due to intensified treatment protocols according to which patients are included in various risk categories and sustained and appropriate supportive therapy. However, overall survival and 3-year EFS rates are much lower than in pediatric ALL, reaching values of 65% and 45%, respectively (Aplenc, 2020; Conneely, 2021), whereas relapse rates range between 25-35% (Zwaan, 2015).

As in the case of pediatric ALL, the molecular genetics of AML plays a very important part both in understanding the mechanisms of leukemogenesis, as well as in determining the prognosis and choice of the therapeutic strategy. The latest WHO classification of AML includes subtypes of AML with recurrent genetic abnormalities (Arber, 2016).

In the general part of this thesis (**Chapter 1** and **Chapter 2**), I have provided a brief presentation of theoretical data on the current state of knowledge in pediatric AML, respectively pediatric ALL, emphasizing the particularities of molecular abnormalities and their implications in establishing new classifications and risk stratifications, in justifying therapeutic decisions, and in integrating new, molecularly targeted therapies.

In the past ten years, in the tertiary Hematology-Oncology Unit of "Sf. Maria" Children's Hospital in Iaşi, molecular biology analyses have been implemented and included in the standard diagnostic and follow-up panel of each newly diagnosed case (both ALL and AML) in collaboration with the Molecular Biology Laboratory of the Regional Institute of Oncology Iaşi. **Study no. 1** presented in **Chapter 3** in the section dedicated to personal contributions is entitled **Molecular analysis and its impact on management in pediatric AML** and includes a number of 54 patients with AML diagnosed in the Hematology-Oncology Unit of "Sf. Maria" Children's Hospital in Iaşi, between 2010- 2019. **Study no. 2** (presented in **Chapter 4** in the section dedicated to personal contributions) focuses on molecular analysis and its impact on management and survival in pediatric ALL, and represents a retrospective observational study including a number of 165 patients with ALL diagnosed in the Hematology-Oncology Unit of "Sf. Maria" Children's Hospital Iasi, in the interval 2010-2019. The two studies presented in the chapters dedicated to personal contributions (Chapter 3 and Chapter 4) analyzed a large number (219) of pediatric acute leukemia patients. Both studies presented data on post-chemotherapy complications, detailed according to the treatment phase. Study no. 2 presents also the analysis of different types of L-asparaginase toxicity in first-line ALL treatment and the correlations of different types of adverse effects with the biological, molecular and survival profile of the patients. In the research literature there are few studies, made on such a large group of pediatric ALL patients, which analyze in detail the full spectrum of adverse effects of asparaginase in the chemotherapy protocol. The category of patients with Down syndrome and acute leukemia (DS-ALL and DS-AML) was addressed in detail in the discussion subchapters, where we presented the results of the retrospective observational study conducted on a group of 21 patients with acute leukemia and Down syndrome from three tertiary hemato-oncology centers of the country.

Personal contributions

Chapter 3

Study no. 1: Molecular analysis and its impact on management in pediatric AML

3.1. Objectives

This study aims to analyze the results of molecular evaluation of pediatric AML patients diagnosed in the past 10 years and to assess the role of molecular analysis in determining the appropriate therapeutic approach. Furthermore, another objective of the study was to identify the differences in the overall survival rate of patients and to identify prognostic factors that had a significant impact on the survival rate. The research also aimed at providing a separate analysis of the group of patients with AML and FLT3 mutations compared to the group of patients with AML without FLT3 mutations, as well as at analyzing the group of patients diagnosed with APL. Another group of patients that was analyzed in detail was that of patients with Down syndrome and AML.

3.2 Patients and methods

Eligibility criteria:

1. Newly diagnosed AML according to WHO and FAB criteria in “Sf. Maria” Children's Hospital Iasi in the period 2010-2019.
2. Patients aged between 0-18 years.
3 Consent signed by one of the patient's parents with regard to medical investigations and the use of data from the observation chart in scientific research.

Exclusion criteria:

1. Lack of data on molecular or immunophenotypic analysis at onset.
2. Lack of data needed to monitor patient’s progress.
3. Parents’ refusal to sign the consent for medical investigations and use of data from the observation record in scientific research.

A total of 56 patients diagnosed with AML were evaluated for eligibility of which 54 patients diagnosed with AML between January 2010 and December 2019, in the Hematology-Oncology Unit of “Sf. Maria” Children's Hospital Iasi, were enrolled in this retrospective observational study. Two patients were excluded as there was no data available on molecular analysis at onset. The retrospective study of the patients' observation chart was approved by the Ethics Committee of “Sf. Maria” Children's Hospital Iasi (6877/26.02.2020).

The diagnosis was performed according to WHO and FAB criteria, all patients underwent bone marrow aspiration with flow cytometry and molecular analysis (NPM1, FLT3-ITD, FLT3 D835, PML-RARα, CBFβ-MYH11, AML1-ETO). Molecular analysis was performed at the Regional Institute of Oncology Iasi, Laboratory of Molecular Biology.

Complete remission was defined according to International Working Group criteria (Cheson, 2003) as <5% blasts in bone marrow, no blasts in peripheral blood, platelet count >50,000/mm^3 without thrombocyte support, neutrophils >1000/mm^3 after Induction phase. Partial remission was defined as having a percentage of blasts in the bone marrow between 5-25% after the first induction treatment. Central nervous system (CNS) leukemic infiltration was defined as the presence of more than 5 blasts/mm^3 in the cerebrospinal fluid. Relapse was defined as recurrence of leukemia regardless of location. Post-therapy toxicity was defined according to the Common Terminology Criteria for Adverse Events (CTCAE) version 5.0.

Patients diagnosed with AML 3 were treated according to the **GIMEMA-AIDA 2000** protocol and those who had a leukocyte count at diagnosis > 10,000/mm^3 were classified as high risk. Patients with AML who did not have AML3 were treated according to the **AIEOP-LAM 2002** treatment protocol. Patients with AML and Down syndrome performed the AIOEP-LAM 2002 protocol, the version adapted for patients with Down syndrome.

Statistical processing of data

Statistical analysis was performed using IBM SPSS statistical analysis software, version 25.0 (Armonk, NY). The descriptive analysis was performed using frequency and percentage analysis for categorical variables and median with minimum and maximum values for quantitative variables. Early death was defined as death occurring within the first 28 days after diagnosis.

Overall survival was calculated as the time from the date of diagnosis to the date of death or the date of last assessment. Event-free survival (EFS) was calculated as the time from the date of diagnosis to the date of the first event such as death, relapse, treatment resistance,

second malignancy, or date of last follow-up if none of the above events occurred. Overall survival and event-free survival (EFS) were estimated using the Kaplan-Meier curve. The subgroups were compared using the log-rank test (Mantel-Cox). The p-value was considered statistically significant at values <0.05. The Cox regression model was used for multivariate analysis of overall survival and event-free survival (EFS). Bivariate analysis was used for the analysis of the correlations between observed variables. We used multiple logistic regression for correlation analysis between two or more independent variables and a single dependent variable. The date of reference for the statistical analysis is 1st of June 2021.

3.3. Results

3.3.1. General characteristics of the group of patients with AML

This study included 54 patients diagnosed with AML between January 2010 and December 2019 in the Hematology-Oncology Unit of "Sf. Maria" Children's Hospital Iasi. The clinical, hematological and molecular characteristics of the group of patients with AML are summarized in Table 3.1.

In terms of molecular biology, the CBF beta MYH11 gene rearrangement was identified in two (3.7%) patients and the PML-RARA fusion gene was detected in ten patients (18.5%). Six patients (11.1%) had FLT3 mutations and three patients had NPM1 gene mutations (5.6%). Two patients presented FLT3 ITD mutations associated with NPM1 mutations and two patients presented PML RARA associated with FLT3 ITD mutations. 70.7% of patients had no molecular abnormalities identified at diagnosis (Figure 3.1.). Cytogenetic examination was available for 14 patients, four of them had trisomy 21, one patient had trisomy 8, one patient had translocation t(3:5)(q21:q31), and 8 patients had normal karyotype.

After the first Induction treatment, on day +28 after the initiation of Induction, 39 (72.2%) patients showed remission, out of which 29 (53.7%) showed complete remission and 10 patients (18.5%) presented partial remission. Thirteen patients (24.1%) died within the first 28 days after diagnosis. Causes of death were: severe hemorrhages (6), severe infections (4) and disease progression (3). It is also worth noting that all four patients who, at diagnosis, had leukocyte counts > 200,000/mm^3, died within the first week of onset. Three of them had severe hemorrhages due to leukostasis and coagulation disorders, and one of them died of severe systemic infection.

Table 3.1. Clinical, hematological and molecular characteristics of the whole group of patients with AML

	Number =54	**Percent %**
Sex		
Female	29	53.7%
Male	25	46.3%
Down Syndrome	4	7.4%
Median age at diagnosis (range)	**10.9 years (0.1-17.9)**	
Age		
<3 years	10	18.5%
3-10 years	16	29.6%
>10 years	28	51.9%
Median Leukocyte at diagnosis	**19.580/mm^3 (900-593.260)**	
Leukocyte count		
< 10.000/mm^3	19	35.2%
10,000-99,000/ mm^3	22	40.7%
≥100,000//mm^3	13	24.1%
Median Thrombocytes at diagnosis	**26,500/mm^3 (2000-261.000)**	
Platelet count		
<10,000/mm^3	11	20.3%
10,000-30,000/mm^3	19	35.2%
>30,000/mm^3	24	44.4%
CNS infiltration at onset	4	7.4%
Molecular abnormalities		
AML-ETO	0	0%
CBF beta-MYH11	2	3.7%
PML-RARA	10	18.5%
NPM1	3	5.6%
FLT3	6	11.1%
Subtype FAB		
M0	0	0%
M1	3	5.6%
M2	13	24.1%
M3	10	18.5%
M4	6	11.1%
M5	16	29.6%
M6	0	0%
M7	6	11.1%
DIC at onset	21	38.9%

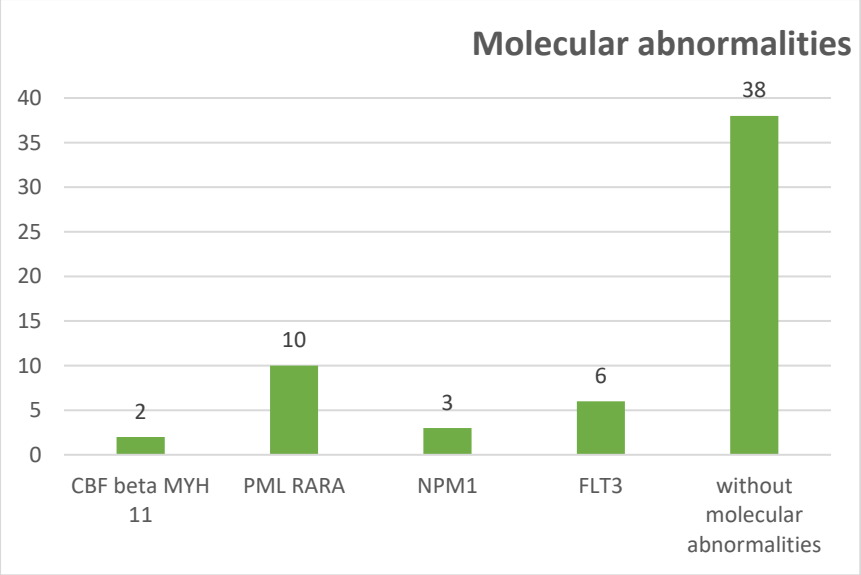

Figure 3.1. Distribution of molecular abnormalities identified in the group of patients diagnosed with AML

Table 3.2. Outcome and response to treatment for the whole group of patients with AML

	Number of patients (%)
Complete remission	29 (53.7%)
Partial remission	10 (18.5%)
Early death in Induction	13 (24.1%)
Therapy-related mortality	10 (18.5%)
Relapse	8(14.8%)
Overall survival	46.3%
EFS	44.4%
Transplant	8(14.8%)

The estimated overall survival of the whole group of patients with AML, using the Kaplan Meier curve, was 46.3% (as illustrated in Figure 3.2.), with an estimated mean survival period of 5.28 years ± 0.72 years, 95% CI: 3.86-6.70 years. The probability of event-free survival related to disease relapse, death or second malignancy was 44.4% ± 6.8%, while the estimated mean event-free survival period was 5 years ± 0.72 years with 95% CI: 3.66-6.49

years. Overall survival was found to vary according to the FAB classification of AML, with overall survival being 66.7% in patients with AML7, 53.8% in patients with AML2, 50% in patients with AML3, 43.8% in patients with AML5, 33.8% in patients with AML1 and only 16.7% in patients with AML4.

12 patients left during or after the chemotherapy protocol to centers in the country or abroad where 8 of them underwent hematopoietic stem cell transplantation (4 haplo transplants, 3 allotransplant from related donor and 1 allotransplant patient from unrelated donor). Six patients underwent transplantation at the time of first complete remission and two underwent transplantation at the time of first relapse. Of those patients who underwent transplantation, 4 died (either because post-transplant complications or disease progression after post-transplant relapse) and 4 survived. The overall survival rate of patients who underwent transplantation was 50% compared to 45.7% of those who did not undergo transplantation (p = 0.463), thus implying no statistically significant difference between the two groups of patients. However, this data should be interpreted with caution due to the small number of patients who underwent the allotransplant procedure.

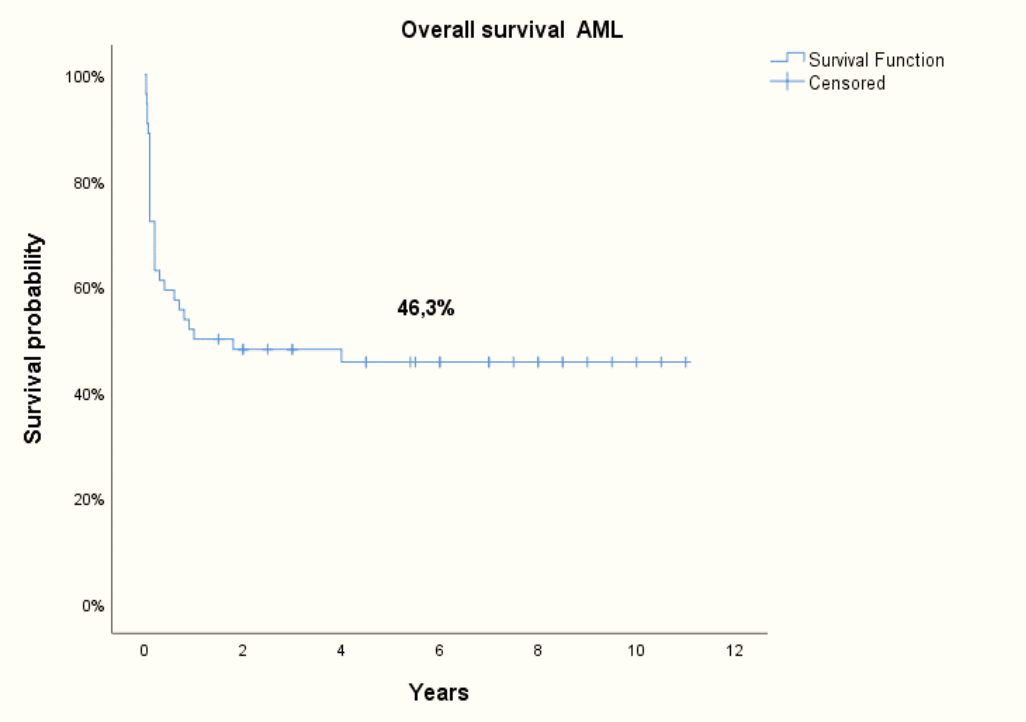

Figure 3.2. Kaplan-Meier curve of overall survival rate for the whole group of patients with AML

29 (53.7%) of the patients diagnosed with AML died, the main causes of death being fulminant hemorrhage (8), infection (10), disease progression (8), post-transplant complications (3), liver failure (1).

Multivariate analysis by Cox regression model revealed that male sex patients (p=0.004, HR: 3.589, 95%CI:1.516-8.499), the presence of significant hepatosplenomegaly at diagnosis (p=0.008, HR:3.887, 95%CI:1.415-10.679) and the age at diagnosis > 10 years (p=0.03, HR:2.921, 95% CI:1.106-7.710) are statistically significant independent predictors of shorter survival (Table 3.3.).

Also, as shown in Table 3.3, for the whole group of patients with AML, the leukocyte count at diagnosis >100,000/mm^3 (p=0.650, HR:1.284), the identification of FLT3-ITD mutations (p=0.392, HR:1.815), as well as the presence of DIC syndrome at diagnosis (p=0.591, HR:1.372), have a negative impact upon the overall survival, but this impact is not statistically significant. NPM1 mutations, the presence of PML-RAR alpha and CBF beta-MYH11 increase survival duration, but this increase is not statistically significant.

Table 3.3. Multivariate Cox regression analysis for independent prognostic factors of overall survival in the group of patients with AML

	p	HR	95% CI for HR	
Sex	**.004**	3.589	1.516	8.499
Age >10 years	**.030**	2.921	1.106	7.710
Leukocyte count >100,000/mm^3 at onset	.650	1.284	.437	3.774
Hepato-splenomegaly	**.008**	3.887	1.415	10.679
Gingival hypertrophy at onset	.592	.746	.256	2.174
Peripheral blood blasts > 50% at onset	.819	.892	.335	2.378
DIC at onset	.591	1.372	.433	4.346
NPM1 mutations	.249	.242	.022	2.703
FLT3-ITD mutations	.392	1.815	.464	7.091
PML-RAR alpha	.744	.781	.177	3.448
CBF beta-MYH11	.735	.663	.061	7.159

Multivariate Cox regression analysis revealed that male patients (p=0.005, HR:3.352, 95%CI:1.444-7.779), significant hepatosplenomegaly at diagnosis (p=0.006, HR: 3.931, 95%

CI: 1.484-10.412) and age at diagnosis over 10 years (p=0.029, HR:2.846, 95%CI:1.111-7.291) are statistically significant independent predictors for a lower EFS rate.

It can also be noted that, for the whole group of patients with AML, the leukocyte count at diagnosis >100,000/mm^3 (p=0.454, HR:1.476), the identification of FLT3- ITD mutations (p=0.383, HR: 1.818) at diagnosis and the presence of DIC at diagnosis (p=0.795, HR: 1.160), are factors that negatively impacts on the EFS rate, but this impact is not statistically significant. In the group of patients we assessed, although the NPM1 mutations (p=0.254, HR: 0.244), the presence of PML-RAR alpha (p=0.893, HR: 0.904), and the presence of CBF beta-MYH11(p=0.540, HR: 0.481) increase the EFS rate, the increase remains not statistically significant.

3.3.2. Comparative analysis of patients with PML-RARA positive AML3 and patients with AML excluding AML3

In the period 2010-2019, 10 cases of **AML3** were confirmed out of a total of 54 AMLs diagnosed cases, which represent 18.5% of the total AML cases. Two patients had associated FLT3-ITD mutations and one patient had the NPM1 mutation associated with the PML-RARA transcript. The PML-RARA fusion gene was detected in all ten patients with AML3, in two patients the isoform being also described (one of these patients had the bcr1 isoform and the other displayed the bcr2 isoform).

According to the Sanz classification (Sanz et al, 2019) which takes into account the leukocyte count and platelet count at the time of diagnosis, 4 cases were included in the high-risk group, 4 cases in the intermediate risk group and 2 cases in the low-risk group.

What stands out is the number of three early deaths of children with AML3 in the first few days after diagnosis, all three deaths being caused by severe bleeding in the context of DIC syndrome with fibrinolysis at onset. The median of the number of days from diagnosis to the time of death was 5 days. Two of the cases of cerebral hemorrhage occurring in the first days of induction were among patients from the high-risk group with a leukocyte count at diagnosis > 10,000/mm^3 (143,000/mm^3, respectively 13,590/mm^3).

Only one patient relapsed, the death of this patient being due to hemorrhagic manifestations secondary to disease progression. One patient died from disease progression

following the refusal of the treatment protocol. Five patients are alive, in complete remission, all having completed the chemotherapy protocol.

None of the patients diagnosed with AML 3 underwent stem cell transplant. The overall survival of AML3 patients was 50%, with an estimated average survival of 5 ± 1 years (1.9 years-8 years), whereas the probability of event-free survival (EFS rate) was 50%. The overall survival rate was noted to be higher in female patients (75%) as compared to male patients (33.3%), yet the difference was not statistically significant (p = 0. 234).

The overall survival for **the group of patients with AML, patients diagnosed with AML3 excluded,** was 45.5%, with an estimated average of survival of 5.2 ± 0.7 years (3.6 - 7.6 years). The probability of event-free survival (EFS) was 43.2%, with an estimated average duration of event-free survival of 4.9 ± 0.7 years (3.3 years - 6.5 years). The survival of patients who achieved complete remission at the 28th day of follow-up, after the first Induction treatment, was 73.9%, significantly higher than the overall survival of patients who achieved only partial remission after the first Induction treatment, which was 20% (p = 0.003, HR: 3.729; 95% CI: 1.894-7.341).

The overall survival rate of patients who experienced disease relapse was low (14.3%) compared to the overall survival of 51.4% for patients who did not experience disease relapse (p = 0.292). The estimated average survival period for relapse patients was 1.6 ± 0.63 years (0.4 years - 2.8 years), and for patients without relapse, the estimated average survival period was 5.7 ± 0.88 years (4 years - 7, 4 years).

In terms of overall survival for patients who underwent hematopoietic stem cell transplantation, the overall survival was 50% compared to 44.4% overall survival for patients who did not undergo transplantation, without no identified statistically significant difference between the two groups of patients (p = 0.418).

Considering the variables that might influence the likelihood of early death in induction, multivariate regression model analysis revealed that teenagers aged over 14 years (p = 0.031, Odds Ratio: 20.219, 95% CI: 1.326-308.349) and patients with an LDH value > 1,000 U/l at disease onset (p = 0.037, Odds Ratio: 13.116, 95% CI: 1.164-147.797) have a statistically significantly higher risk of death within the first 28 days.

Table 3.4 Comparative analysis of the group of patients diagnosed with AML excluding AML3 and the group of patients diagnosed with AML3

	AML n=44	**AML3 PML-RARA positive n=10**	**p**
Sex			0.345
Male	19 (43.2%)	6 (60%)	
Female	25 (56,8%	4 (40%)	
Down Syndrome	4 (9.1%)	0 (0%)	0.331
Median of age at diagnosis	11.3 years (0,1-17,9)	9.6 years (1,4-15,5)	0.404
Median leukocyte at diagnosis	25.190/mm^3 (14.805-93.260)	7.125/mm^3 (900-143.000)	**0.049**
Median Thrombocytes at diagnosis	27,500/mm^3 (2.000-261.000)	15.000/mm^3 (4.000-231.000	0.084
Median LDH at onset	1.221 U/l (316-11.550)	665 U/l (313-3.164)	**0.031**
FAB Subtype			
M1	3(6.81%)		
M2	13 (29.54%)		
M4	6 (13.63%)		
M5	16 (36.36%)		
M7	6 (13.63%)		
DIC at onset	11 (25%)	10	< **0.0001**
Molecular abnormalities			
AML-ETO	0 (0%)		
CBF beta-MYH11	2 (4.5%)		
NPM1	2 (4.5%)	1 (10%)	0.506
FLT3	4 (9.1%)	2 (20%)	0.331
PML-RARA		10 (100%)	
Complete remission after the first Induction treatment	23(52.3%)	6 (60%)	0.124
Early deaths	10 (22.7%)	3 (30%)	0.635
Relapse	7 (15.9%)	1(10%)	0.643
Treatment-related mortality	10 (22.7%)	0 (0%)	0.098
Overall survival	20 (45.5%)	5(50%)	0.799
EFS	19 (43.2%)	5 (50%)	0.702
Transplant	8 (18.2%)	0 (0%)	

3.3.3. Comparative analysis of the group of patients with AML FLT3 and the group of patients with AML without FLT3 mutations

Table 3.5. Comparative analysis of the group of patients with FLT3-ITD AML and the group of patients without FLT3-ITD mutations (biological and molecular aspects)

	AML FLT3-ITD positive	**AML without FLT3-ITD mutations**	***p***
Number	6	48	
Median age at diagnosis	13,65 (8-16,9)	8,6 (0,1-17,9)	0.104
Age over 10 years	5 (83.3%)	23 (47.9%)	0.105
Sex			0.850
M	3 (50%)	22 (45.8%)	
F	3 (50%)	26 (54.2%)	
Median Leukocyte count at diagnosis	32.655/mm^3 (3.380-593.260/mm^3)	18.790/mm^3 (900-487.000)	0.315
Median Hb	6.5g/dl (5-10.8)	7,2 (2,3-15)	0.794
Median platelet count at diagnosis	19,500/mm^3	27,000/mm^3	0.378
Blasts in the peripheral blood >50%	4 (66.7%)	16 (33%)	0.149
Hepatosplenomegaly at onset	2 (33.3%)	22 (45.8%)	0.570
DIC at onset	5 (83.3%)	16 (33.3%)	**0.017**
Infiltration of CNS	0 (0%)	4 (8.3%)	0.472
FAB Subtype			
M1	0 (0%)	3 (6.3%)	
M2	3 (50%)	10 (20.8%)	
M3	2 (33.3%)	8 (16.7%)	0.331
M4	1 (16,7%)	5 (10.4%)	
M5	0 (0%)	16 (33.3%)	0.095
M7	0 (0%)	6 (12.5%)	
Molecular abnormalities			
PML-RARA CBF beta-MYH11	2 0	8 2 (4.2%)	0.326 0.618
NPM1	2 (33,3%)	1 (2.1%)	**0.001**

Table 3.5 reveals that the median age at diagnosis is higher for patients with FLT3 - ITD mutations (median age at diagnosis of 13.6 years compared to median age at diagnosis of 8.6 years for AML patients without FLT3-ITD mutations, p = 0.104). Furthermore, patients with FLT3 ITD mutations have higher median leukocyte counts at diagnosis than patients without FLT3 mutations (32,655/mm^3 compared to 18,790/mm^3), however, the difference is not statistically significant (p = 0.315).

It is, noteworthy that 83.3% of patients with FLT3 mutations had DIC syndrome at the time of diagnosis, compared to only 33.3% of patients without FLT3 mutations, showing a statistically significant difference between the two groups of patients (p = 0.017).

Table 3.6. Outcome and response to treatment for the group of patients with FLT3-ITD mutations versus the group of patients without FLT3-ITD mutations

	AML FLT3 positive n = 6	**AML FLT3 negative n = 48**	**p**
Complete remission	3 (50%)	26 (54,2%)	0.780
Partial remission	1 (16,7%)	9 (18,8%)	
Early death	1(16,7%)	12 (25%)	0.660
Relapse	1(16,7%)	7 (14,6%)	0.859
Survival	2 (33,3%)	23 (47,9%)	0.509
EFS	2 (33,3%)	22 (45,8%)	0.588
Transplant	1(16,7%)	7 (14,6%)	0.859

The overall survival for the group of AML patients with FLT3 ITD mutations was 33.3% ± 10.2% compared to 47.9% ± 7.2% overall survival in the group of AML patients without FLT3 ITD mutations (p = 0.509). The estimated average survival period for patients with FLT3 mutations was 2.8 ± 1.2 years (0.4 - 5.2), while the estimated average survival period for patients without FLT3 mutations was 5.4 ± 0.7 years (3.9 - 6.9).

The probability of event-free survival (EFS) was 33.3% for the group of AML patients with FLT3 mutations versus 45.8% for patients without FLT3 mutations (p = 0.588) (Figure 3.3.), with no statistically significant difference. The average period of event-free survival was 2.6 ± 1.2 years (0.14 years-5.13 years) for patients with FLT3 mutations and 5.2 ± 0.76 years (3.7 years-6.7 years) for patients without FLT3 mutations.

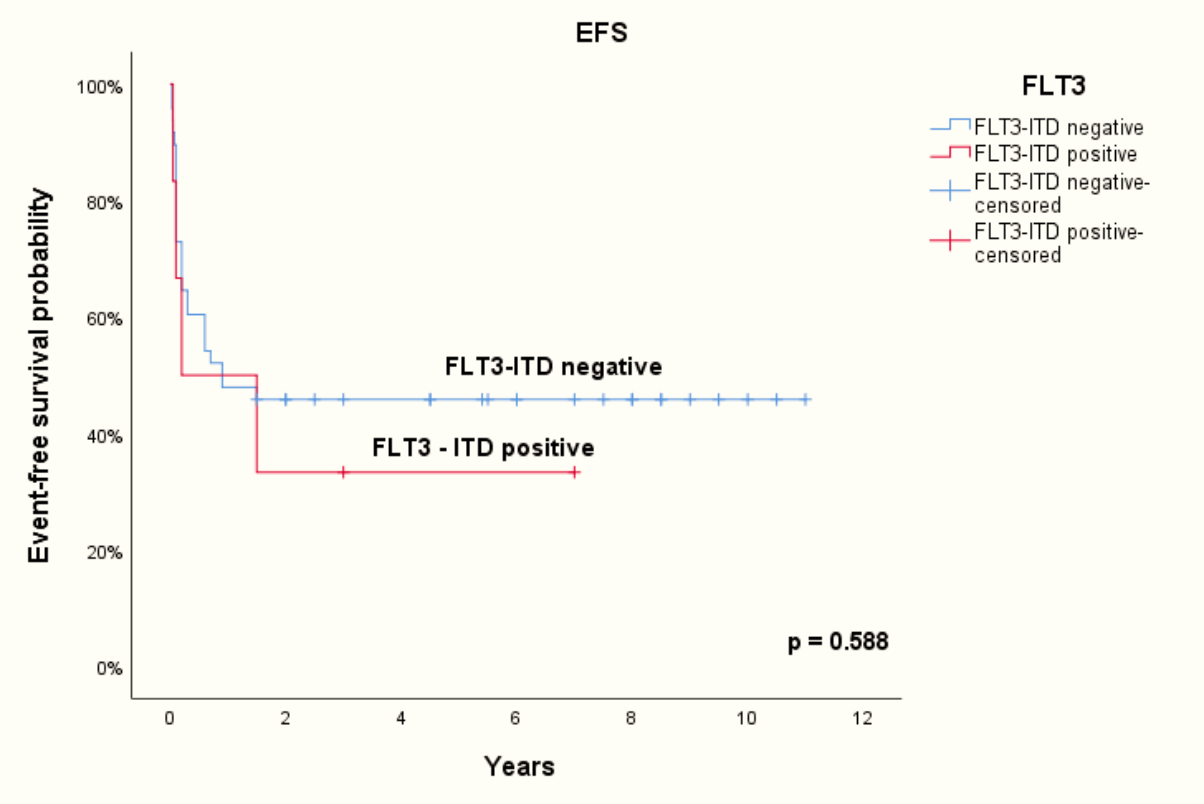

Figure 3.3. Kaplan-Meier curves for event-free survival (EFS) for the group of patients without FLT3 mutations (blue line) versus the group of patients with FLT3 mutations (red line)

3.3.4. AML and Trisomy 21 (Down Syndrome)

From the group of patients included in this study, only four children presented the association of Down syndrome and AML, representing 7.4% of all patients diagnosed with AML. Since the number of patients with Down Syndrome and acute leukemia is small, we tried to assessed a larger group of children with Down Syndrome and AML by adding to the four patients, another patients diagnosed in two other centers of the country, one pediatric center (Fundeni Clinical Institute, Pediatrics Department) and one center for adults (Fundeni Clinical Institute, Hematology Department). Thus, in the retrospective observational study published in 2021, a group of 21 patients with Down syndrome and acute leukemia diagnosed in the three hematology-oncology centers was assessed, out of which seven patients were diagnosed with AML and Down syndrome. This group was analyzed in comparison with the group of AML patients without Down syndrome. (**Schmidt** et al, 2021 a).

The study indicated that the age at diagnosis was lower for patients with Down syndrome and AML (2.5 years) than for those with AML alone (11.2 years) ($p = 0.151$). Median leukocyte counts at diagnosis were higher for children with Down syndrome than for those with AML alone (93,040 /mm^3, versus 21,500/mm^3, but without a statistically significant difference, $p = 0.834$). There was a significant correlation ($p = 0.002$) between the morphological subtype AML 7 according to the FAB classification and the association of Down syndrome and AML. The cytogenetic examination revealed that one of the patients associated trisomy 21, trisomy 11, inversion of chromosome 11 and duplication of the long arm of chromosome 1.

Post-chemotherapy complications in children with AML and Down syndrome were less severe than those in children with ALL and Down syndrome and consisted mainly of severe mucositis (42.8% of patients), sepsis (14.2%), skin infections (28.5%), and hemorrhagic manifestations (28.5%). Mortality associated with therapy toxicity was 14.2%, similar to that seen in AML without Down syndrome (16%).

Overall survival was better for patients with Down syndrome and AML than for those with AML alone (57.1% versus 45.1%, $p = 0.479$), but with no statistically significant difference. The data related to overall survival was consistent with other studies in the field that have indicated a better survival rate for children with Down syndrome and AML (Qiao, 2018).

One should note the particularity of extramedullary recurrence in the right lower eyelid in a patient with AML7 and Down syndrome. The treatment cycles of high-dose Cytarabine were highly effective and confirmed previous results published in the literature on the topic in ex-vivo studies regarding the increased sensitivity of myeloblasts in AML associated with Down syndrome (Taub, 1999). Also, the AAML 0431 trial demonstrated that early use in the first-line protocol of high-dose Cytarabine treatments improves survival and response rates in children with Down syndrome and AML (Taub, 2017).

Chapter 4

Study no. 2. Molecular analysis and its impact on management and survival in children and adolescents with ALL

4.1. Objectives

This study aims at analyzing the results of molecular evaluation of pediatric ALL patients diagnosed in the last 10 years, identifying correlations between the presence of molecular abnormalities, outcome, treatment complications, survival and EFS rates, as well as analyzing the role played by the molecular analysis in determining therapeutic behavior. Moreover, another objective of the study was to identify the differences in overall patient survival and determine the factors that had a significant impact on survival. The study also aimed at providing a separate analysis for the group of patients with ETV6-RUNX1 rearrangement compared to patients without ETV6-RUNX1 rearrangement. Another group of patients that was analyzed in detail was that of patients with Down syndrome and ALL.

Patients and methods

Eligibility criteria:

1. Patient with newly diagnosed ALL according to WHO and FAB criteria at "Sf. Maria" Children's Hospital Iasi between January 2010 and December 2019.
2. Patient aged 0-18 years.
3 Consent signed by one of the patient’s parents agreeing to medical investigations and to the use of data from the observation chart in scientific research.

Exclusion criteria:

1. Lack of data on molecular or immunophenotypic analysis at onset.
2. Lack of data needed to monitor patient progress.
3. Parents’ refusal to sign their consent to medical investigations and use of data from the observation chart in scientific research.

A total of 173 patients with the diagnosis of ALL were evaluated for eligibility and 165 patients diagnosed with ALL between January 2010 and December 2019 in the Hemato-Oncology Department of the Children's Hospital "Sf. Maria" Iasi were enrolled in this retrospective observational study. Eight patients were excluded because the necessary follow-up data was not available. Patients were monitored until the time of death or until the reference date of 1st of July 2021. Median follow-up duration was 5 years (1month -11.5 years). The retrospective study of patient observation charts was approved by the Ethics Committee of "Sf. Maria" Children's Hospital Iasi (6877/26.02.2020).

Diagnosis of patients was performed according to WHO criteria (Arber, 2016), all patients had a bone marrow aspirate with flow cytometry according to the European Leukemia Immunological Characterization Group criteria (Bene, 1995) and molecular analysis for ETV6-RUNX1(TEL-AML1), TCF3-PBX1(E2A-PBX1), BCR-ABL1 p190, MLL-AF4, SIL-TAL fusion genes performed by RT-PCR. Molecular analysis was performed at the Regional Institute of Oncology Iasi, Molecular Biology Laboratory. Since 2017, treatment response was also assessed by minimal residual disease performed by multiparametric flow cytometry (Theunissen, 2017). Complete remission was defined according to International Working Group criteria (Cheson, 2003). Response on day 8 of treatment is the response assessed after 7 days of corticosteroid therapy, and poor response to prednisone was defined as a number of blasts >1000/mm^3 in peripheral blood on day 8 of treatment. Minimal residual disease is considered positive at values > 0.01%. Relapse was defined as recurrence of leukemia regardless of location, post-therapy toxicity was defined according to the Adverse Common Terminology Criteria for Adverse Events (CTCAE) version 5.0. Early death was defined as death occurring within the first 33 days after diagnosis. Infiltration of the central nervous system (CNS) is the presence in the cerebrospinal fluid of more than 5 blasts/mm^3.

Patients aged 1-18 years were treated with the ALL IC BFM 2002 chemotherapy protocol (Stary, 2014). Patients with BCR-ABL1-positive ALL had Imatinib added to the 2002 ALL IC BFM chemotherapy protocol. Patients < 1 year old performed the INTERFANT 1999 protocol (Pieters, 2007).

The statistical analysis was conducted using IBM SPSS statistical analysis software, version 25.0 (Armonk, NY). The descriptive analysis was performed using frequency and percentage analysis for categorical variables and the median with minimum and maximum values for quantitative variables. Death in Induction was defined as death occurring within the first 33 days of treatment. Overall survival was calculated as the time from the date of diagnosis to the date of death or the date of last assessment. Event-free survival (EFS) was calculated as the

time from the date of diagnosis to the date of the first event such as death, relapse, treatment resistance, second malignancy or date of last assessment if none of the above events occurred. Overall survival and event-free survival (EFS) were assessed using the Kaplan-Meier curve. Subgroups were compared using the log-rank test (Mantel-Cox). The p-value was considered statistically significant at values <0.05. The Cox regression model was used for multivariate analysis of overall survival and event-free survival (EFS). Bivariate analysis was used to analyze correlations between observed variables. Multiple logistic regression was used for correlation analysis between two or more independent variables and a single dependent variable.

4.3. Results

4.3.1 Characteristics of the group of patients with ALL

Table 4.1. summarizes the biological and molecular characteristics of the analyzed group of patients with ALL.

In terms of molecular biology, 37 patients (22.4%) presented changes detected by RT-PCR, BCR-ABL1 rearrangement was identified in three (1.8%) patients, in 7(4.2%) patients TCF3-PBX1 (E2A-PBX1) fusion gene was detected, 25 patients (15.2%) had the ETV6-RUNX1(TEL-AML1) fusion gene, 3 patients (1.8%) had the MLL-AF4 rearrangement present at diagnosis and 3 patients (1.8%) had the BCR-ABL P190 rearrangement. (Figure 4.1.).

The karyotype examination was available for 20 patients, of which three patients presented trisomy 21, in one patient associated with the Robertsonian translocation t(14q;21q), three patients presented the Philadelphia chromosome, one patient displayed the association t(21;21) with der(1)p22, del1p13, der(1:6), one patient had del cr2, del cr5, del cr6, addcr1, addcr11, addcrX, and the remaining 12 patients had normal karyotype.

For the entire group of 165 patients with ALL and a median follow-up duration of 5 years (1 month-11.5 years), the 5-year overall survival was 74.5% ± 3.6%. The estimated average of overall survival was 8.71 ± 0.3 years (7.9-9.4) years. The 5-year EFS rate was 71.3% ± 3.6%, and the estimated average for the event-free survival duration was 8.35 ± 0.3 years (7.6-9.1) years. The overall survival was higher in patients diagnosed in the period 2015-2019 compared to those diagnosed in the period 2010-2014, 82.2% versus 68.5%, with no statistically significant difference (p = 0.082).

Table 4.1. Biological and molecular characteristics of the group of patients with ALL

Number of ALL cases	**n=165**
Median age at diagnosis Adolescents ≥14 years old Age 10-13.9 years old Age 1-10 years old Age < 1 year od	5.4 years (0.1-17.1 years) 25 (15,2%) 24 (14,5%) 112 (67,9%) 4 (2,4%)
Sex Male Female	 108 (65,5%) 57 (34,5%)
Down Syndrome	3(1,8%)
Leukocyte count at diagnosis (median, range)	12,250/mm3 (420-1,000,000)
Hb at diagnosis (median, range)	6.8g/dl (2-13.5)
Platelet count at diagnosis (median, range)	37,000/mm^3(2,000-573,000/mm^3)
Percentage of blasts in peripheral blood (median, range)	46% (0-90%)
Median LDH value at diagnosis	951 U/l (151-18.297)
T-ALL/ precursor cell B ALL	26/139 (15,8%/84,2%)
Infiltration of central nervous system (CNS)	12 (7,3%)
Molecular abnormalities TCF3-PBX1 (E2A-PBX1) ETV6-RUNX1 (TEL-AML1) MLL-AF4 SIL-TAL BCR-ABL P190	37(22,4%) 7(4,2%) 25(15,2%) 3(1,8%) 0(0%) 3(1,8%)

The relapse rate was 14.5%, with 24 of the patients presenting disease relapse, 15 (9%) had bone-marrow relapse, 6 (3.63%) patients had CNS relapse, and three (1.8%) patients had combined relapse (CNS + bone marrow). 19(11.51%) presented early relapses and 5(3%) patients had late relapses. Three patients had a second disease relapse

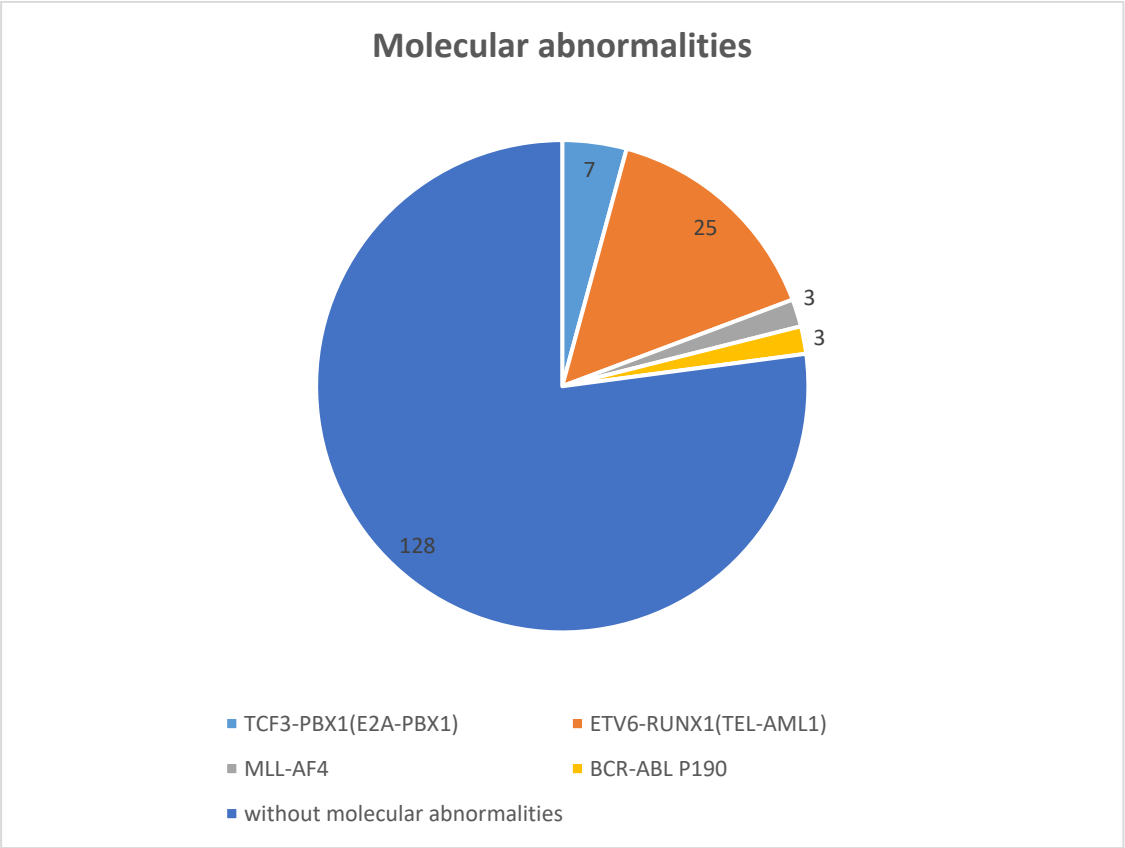

Figure 4.1. Analysis of molecular abnormalities in ALL patients

Three patients underwent hematopoietic stem cell transplantation, two allotransplants from an unrelated donor and one haplotransplant where the donor was the mother of the patient. In the case of two of the patients, the allotransplant took place after first relapse (early CNS relapse in one case and late combined relapse in another), and in the case of the third patient the haplo transplant took place during the first complete remission. One patient had a second relapse of the disease about one year after allotransplantation. Of the three patients who underwent transplantation, two survived, while the third died due to post haplo transplant complications.

42 of the patients died, the causes of death being disease progression (19), infections (15), post-chemotherapy toxicity (5), hemorrhagic manifestations (2), post haplo transplant complications (1) (Figure 4.2.).

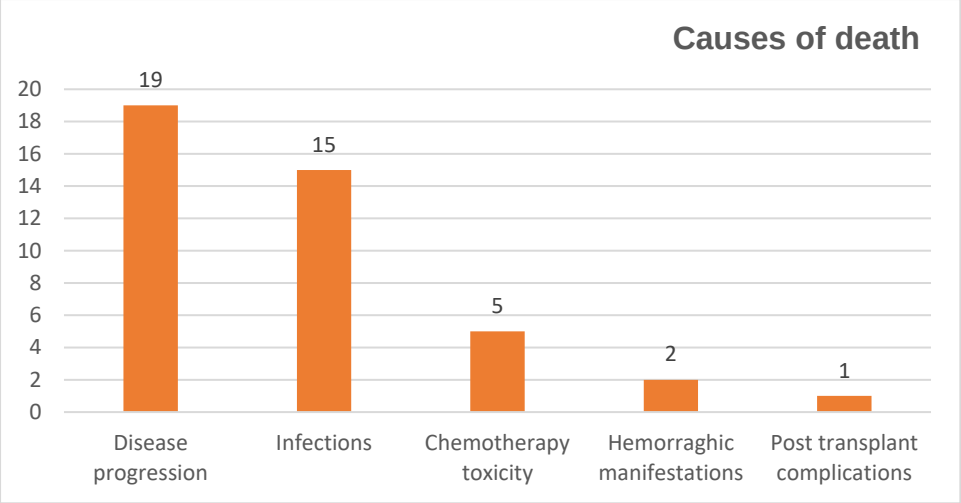

Figure 4.2. Causes of death in patients with ALL

Table 4.2. Evolution and response to treatment of patients with ALL

	Number (percentage)
Response on day 8 of treatment Poor prednisone response day 8 Good prednisone response day 8	 23 (13,9%) 141 (85,5%)
Complete remission day 33	155 (93,9%)
Minimal residual disease Negative Positive	n=34 26 (76,47%) 8 (23,5%)
Early deaths	9(5,4%)
Deaths in complete remission after day 33	6 (3,63%)
High risk group classification	33 (20%)
Relapse Bone marrow CSN Combined Early Late	24 (14,5%) 15 (9%) 6 (3,63%) 3 (1,8%) 19 (11,51%) 5 (3%)
Transplant	3 (1,8%)
Overall survival	123 (74,5%)
Event Free Survival (EFS)	117 (70,9%)
Median follow-up duration	5 years (1 month - 11.5 years)

Minimal residual disease (MRD) performed by multiparametric flow cytometry for day 33 assessment was available for 34 patients. In all patients who had fusion genes revealed by RT-PCR at diagnosis, minimal residual disease was also performed by RT-PCR examination. 8 of the patients had positive MRD (> 0.01%) and 26 patients had negative MRD. It was found that the overall survival of patients with negative MRD was significantly higher compared to the survival of patients with positive minimal residual disease (92.3% vs. 50%, p = 0.002) (Figure 4.3.).

Overall survival was significantly higher in the group of patients who had a good response to Prednisone on day 8 compared to the group of patients who were found to be corticosteroid resistant on day 8 (80.1% vs. 39.1%, p < 0.0001). The relapse rate was higher among patients with poor prednisone response on day 8 of treatment compared to patients with good prednisone response on day 8 (26.1% versus 12.8%, p = 0.098).

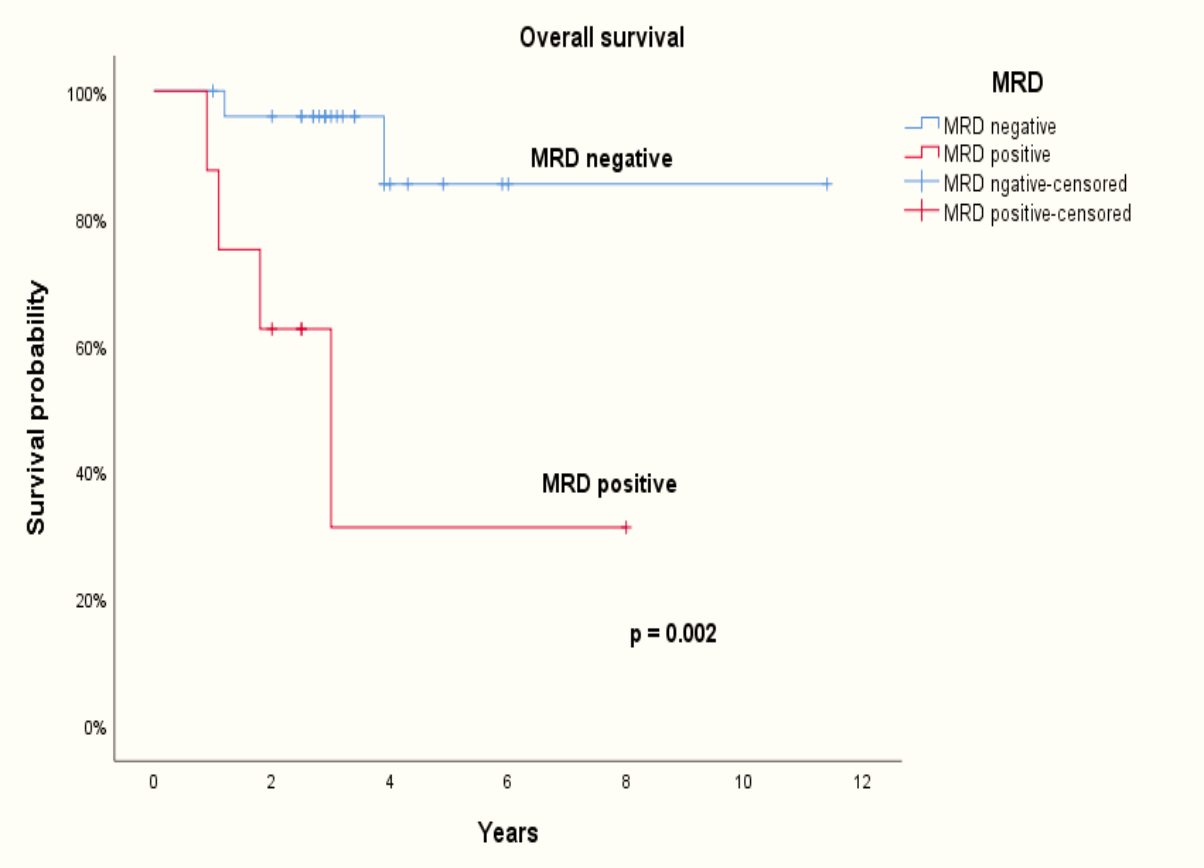

Figure 4.3 Kaplan-Meier curve of overall survival for MRD-negative patients (blue line) compared to overall survival for MRD-positive patients on day 33 (red line).

4.3.2. Analysis of prognostic factors for survival in pediatric ALL

Univariate analysis of prognostic factors influencing overall survival of patients with ALL identified age < 1 year (p =0.021, HR: 9.389; 95% CI:0.949-92.381), leukocyte value at onset > 50,000/mm^3 (p =0.021,HR: 2.412; 95% CI:1.126-5.169), infiltration of CNS at onset (p =0.007, HR:4.720; 95% CI:1.410-15.798), presence of BCR-ABL1 transcript (p =0.03, HR:1.077; 95% CI:0.990-1.171), poor response to Prednison at day 8 (p <0.0001, HR: 6.278; 95% CI:2.467-15.975), positive residual minimal disease at day 33 as negative prognostic factors (p =0.006, HR:12.00; 95% CI:1.623-88.702). Also, male patients (p =0.015, HR:0.414; 95% CI:0.202-0.849) and age at diagnosis in the range 1-10 years old (p =0.027, HR:0.444; 95% CI:0.215-0.918) were identified as favorable prognostic factors (Table 4.3.).

Table 4.3. Univariate analysis of factors that impact survival

Variable	**p**	**HR**	**95% CI**
Sex M/F	**0.015**	0.414	0.202-0.849
Age < 1 year old	**0.021**	9.385	0.949-92.831
Age 1- 10 years old	**0.027**	0.444	0.215-0.918
Adolescents >14 years old	0.070	2.250	0.922-5.491
L > 50.000/mm^3	**0.021**	2.412	1.126-5.169
Infiltration of CNS at onset	**0.007**	4.720	1.410-15.798
ALL-T	0.243	1.701	0.693-4.172
TEL-AML1	0.239	0.511	0.165-1.586
E2A-PBX1	0.488	0.476	0.056-4.070
MLL-AF4	0.098	6.100	0.539-69.073
BCR-ABL1	**0.03**	1.077	0.990-1.171
Poor prednisone response day 8	< **0.0001**	6.278	2.467-15.975
Minimum residual disease day 33	**0.006**	12.00	1.623-88.702
High risk arm	0.108	1.943	0.858-4.401

In order to further analyze prognostic factors in ALL and to identify the existence of independent predictors of survival, we used the multivariate analysis using the Cox regression model, adding the variables that had a significant impact in the univariate analysis and the variable TEL-AML1, the most common molecular abnormality in pediatric ALL (Table 4.4.).

Multivariate analysis for overall survival identified in the ALL group that minimal residual disease on day 33 of induction was the only independent predictor of survival (p=0.042; HR:24.432; 95% CI:1.123-531.486). Likewise, minimal residual disease on day 33 was also the only independent predictor of event-free survival such as relapse, death or second malignancy (p=0.007; HR:40.312; 95% CI:2.798-580.691).

Table 4.4. Multivariate survival analysis using the Cox regression method

Survival analysis	B	SE	p	Hazard Ratio	95.0% CI for HR	
Poor prednisone response day 8 of treatment	2.226	2.397	.353	9.264	.084	1017.364
Minimal residual disease	3.196	1.571	**.042**	**24.432**	1.123	531.486
Sex	-.414	1.531	.787	.661	.033	13.284
Age 1-10 years	-1.987	1.276	.119	.137	.011	1.671
Infiltration of CNS at onset	2.417	2.643	.360	11.214	.063	1991.986
Leukocytes > 50,000 at onset	1.211	2.284	.596	3.357	.038	295.280
TEL-AML1	.131	1.743	.940	1.140	.037	34.724

EFS	B	SE	p	HR	95.0% CI for HR	
Poor prednisone response day 8 of treatment	1.583	2.534	.532	4.870	.034	699.247
Minimal residual disease	3.697	1.361	**.007**	40.312	2.798	580.691
Sex	.607	1.163	.602	1.835	.188	17.947
Age 1-10 years	-1.907	1.059	.072	.148	.019	1.183
Infiltration of CNS at onset	2.894	2.692	.282	18.061	.092	3531.847
Leukocytes >50,000 at onset	.226	2.444	.926	1.254	.010	150.960
TEL-AML1	-.073	1.359	.957	.929	.065	13.337

4.3.3 Survival analysis for patients with molecular abnormalities at onset

Overall survival for the group of patients with ***ALL and ETV6-RUNX1(TEL-AML1) rearrangement***, the most common molecular abnormality identified, was 84% versus 72.9% for the group of patients without ETV6-RUNX1(TEL-AML1) rearrangement (p = 0.260), with an estimated average survival period of 9.6 ± 0.8 years (8-11.2 years) in patients with ETV6-RUNX1(TEL-AML1) rearrangement compared to 8.5 ± 0.4 years (7.7-9.3 years) in patients without ETV6-RUNX1(TEL-AML1) rearrangement. The EFS rate in patients with ETV6-RUNX1(TEL-AML1) LAL was 80% compared with 69.3% in the group without this rearrangement (p = 0.294), with an estimated average of the event-free survival period of 9.2 ± 0.8 years (7.5-10.9 years) in patients with ETV6-RUNX1(TEL-AML1) rearrangement compared with 8.1 ± 0.4 years (7.3-9 years).

The estimated average survival period for patients with ***E2A-PBX1 ALL*** was 7.3 ± 0.9 years (5.3-9.3 years) versus 8.6 ± 0.3 years (7.9-9.4 years) for patients with ALL without E2A-PBX1 rearrangement. We should also note that patients with E2A-PBX1 ALL had very high median leukocyte values at onset (139,000/mm^3 versus 11,760/mm^3, p = 0.015) and also that a high percentage of patients with this type of rearrangement had a poor prednisone response on day 8, statistically significantly higher compared to patients without E2A-PBX1 who presented corticosteroid resistance on day 8 (42.9% versus 12.7%, p = 0.025) (Table 4.5.).

It can be noted that patients with ***BCR-ABL1 rearrangement*** had both the estimated 5-year survival (p = 0.001) and event-free survival (p = 0.001), significantly lower than patients without this type of rearrangement. Also, the presence of ***MLL-AF4*** was associated with both 5-year survival rate (33.3%) and 5-year EFS rate (33.3%) which are lower than in patients without MLL-AF4 (73.9% and 72.1%, respectively; p = 0.07 and p = 0.095, respectively).

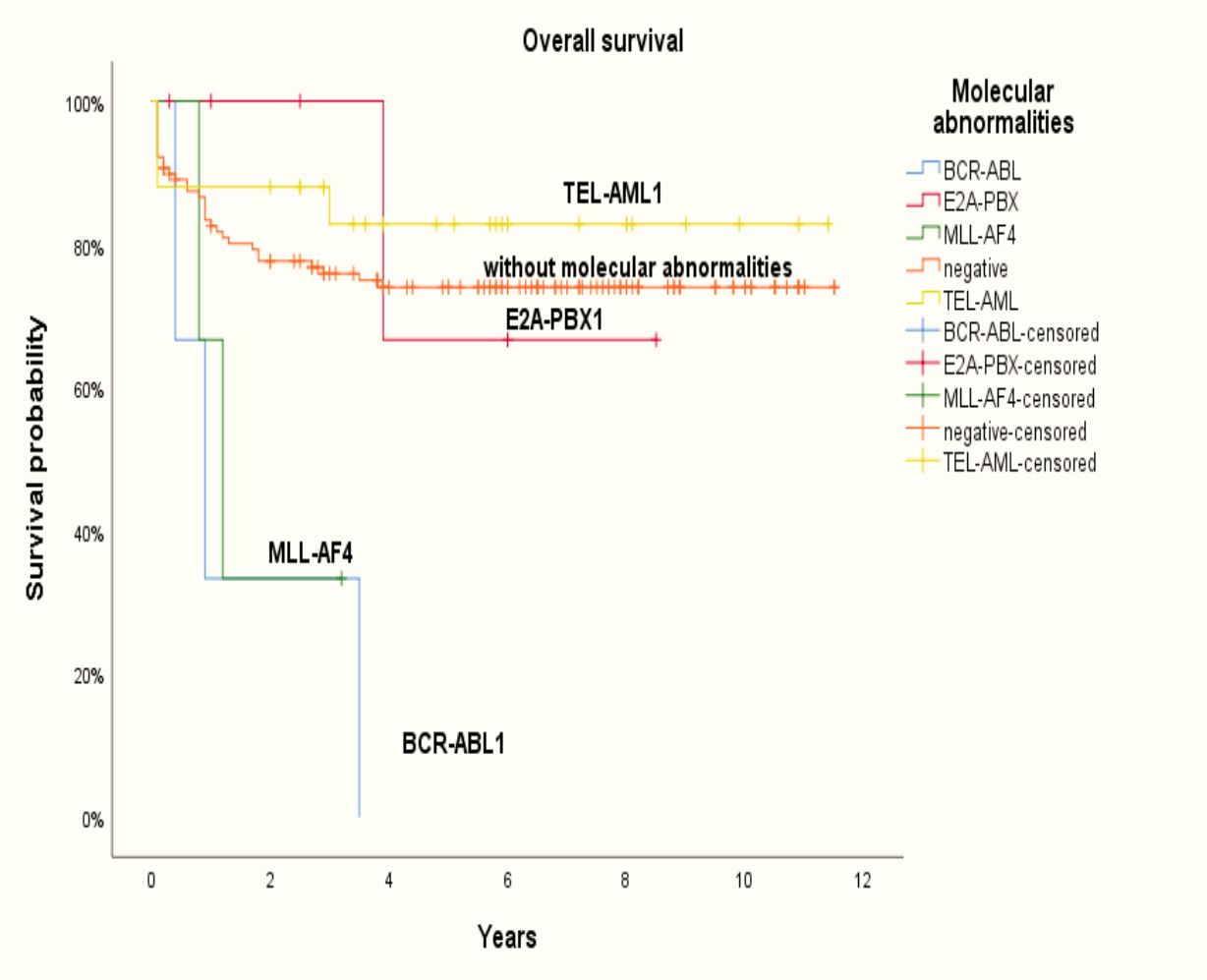

Figure 4.4 Kaplan-Meier survival analysis of patients diagnosed with ALL according to molecular abnormalities detected at onset: TEL-AML1 (yellow line), no molecular abnormalities detected (orange line), E2A-PBX1 (red line), BCR-ABL1 (blue line), MLL-AF4 (green line)

Figure 4.4 indicates that the estimated 5-year survival period is the highest for patients with TEL-AML rearrangement (82.8%±7.9%), followed by that of patients without molecular abnormalities detected by RT-PCR at onset (74%±4%) and the survival of patients with E2A-PBX rearrangement (66.7%±7%).

Patients with MLL-AF4 rearrangement and those with BCR-ABL1 rearrangement have very low survival rates (33.3% and 0%, respectively); however, the number of patients with these molecular abnormalities is quite small (three patients in each of the two categories), thus the data should be interpreted with caution.

Table 4.5. Comparative analysis of patients with molecular abnormalities at onset

	ALL TEL-AML1 (ETV6-RUNX1)	**ALL E2A-PBX1**	**ALL BCR-ABL P190**	**ALL MLL- AF4**
Number of patients	25 (15,15%)	7(4,2%)	3(1,8%)	3(1,8%)
Median age at diagnosis	3.9 years (2,1-13,9)	4.8 years (2,9-16)	9.2 years (5,9-15,9)	2 months (21 days-14.5 years)
Sex F/ Sex M	8/17 (32%/68%)	4/3 (57,1%/42,9%)	1/2 (33%/66%)	1/2 (33%/66%)
Median Leukocyte value at diagnosis	16,620/mm^3 (2.800-303.000)	139,000/mm^3 (4.800-248.000)	20,570/mm3 (4.870-45.740)	79.230/mm3 (24.600-169.000)
Patients with Leukocytes >100,000 at diagnosis	6(24%)	4(57,1%)	0(0%)	1(33,3%)
Infiltration of CNS at diagnosis	0(0%)	1(14,3%)	0(0%)	0(0%)
Poor prednisone response on day 8	1(4%)	3(42,9%)	0(0%)	1(33%)
Relapse	2(8%)	1(14,3%)	2(66%)	2(66%)
Overall survival	84%	85,7%	0%	33,33%

The TEL-AML1 (ETV6-RUNX1) rearrangement was the most common molecular change, identified in 25 (15.15%) of all patients.

A comparative analysis between the group of patients with TEL-AML1 (ETV6-RUNX1) and the group of patients without this rearrangement revealed that patients with TEL-AML1(ETV6-RUNX1) had significantly lower median age at diagnosis (3.9 years versus 6.3 years, p=0.034) (Table 4.6.) Hematological values (median leukocyte count, median Hb, median platelet count, peripheral blood blasts) at disease onset did not differ significantly between the two groups of patients.

Table 4.6. Analysis of the group of patients with ETV6-RUNX1 rearrangement versus the group of patients without ETV6-RUNX1

	ALL with ETV6-RUNX1 n=25	**ALL without ETV6-RUNX1 n=140**	**p**
Median of age at diagnosis	3.9 years (2.1-13.9)	6.3 years (0.1-17.1)	**0.034**
Sex Male Female	 17 (68%) 8 (32%)	 91 (65%) 49 (35%)	0.807
Down Syndrome	0 (0%)	3(2,1%)	0.463
Leukocyte at diagnosis (median)	16.620/mm ^{3} (2.800-303.000)	12.020/mm^3 (420-1.000.000)	0.448
Hb at diagnosis (median)	6.1g/dl (2.9-12.4)	6.9 g/dl (2-13.5)	0.522
Thrombocytes at diagnosis (median)	25,000/mm^3 (7.000-224.000)	38,000/mm^3 (2.000-573.000)	0.943
Peripheral blood blasts (percentage, median)	60% (0-90%)	40% (0-90%)	0.614
Infiltration of Central nervous system (CNS)	0 (0%)	12 (8,6%)	0.130
Early death	3 (12%)	6 (4,3%)	0.451
Poor prednisone response day 8	1 (4%)	22 (15,7%)	0.118
Complete remission day 33	23 (92%)	125 (89,3%)	0.339
Positive MRD day 33	2/8=25%	6/26=23%	0.151
Relapse	2 (8%)	22 (15,7%)	0.311
Overall survival	21 (84%)	102 (72,9%)	0.290
EFS	20 (80%)	97 (69,3%)	0.294

4.3.4. ALL and the Down syndrome

A special category of patients, with distinct biological and outcomes peculiarities, is represented by patients with Down syndrome and ALL. Existing clinical trials tend to exclude patients with ALL and Down syndrome, so there are only a few clinical trials examining differences in outcome, response and complications to treatment of this subgroup of patients compared to patients with ALL without Down syndrome, diagnosed and treated in the same time period and under similar conditions in terms of economic resources. As the proportion of children with Down syndrome from the total number of children diagnosed with ALL was small (1.8%), in the study published in 2021 (**Schmidt, 2021a**) we added patients from two other hematology-oncology centers of the country (Fundeni Clinical Institute, Pediatric Department and Fundeni Clinical Institute-Hematology Department) and established a group of 14 patients with ALL and Down syndrome. The aim of the study was to analyze the differences in survival and outcome between patients with ALL and Down syndrome and those who had only ALL.

Outcome of patients with Down syndrome and ALL was unfavorable. Overall survival was only 35.7%, statistically significantly lower than that of patients without Down syndrome which was 75% ($p = 0.001$, odds ratio, 2.12; 95% CI: 1.047-4.316). The main causes of death in patients with Down syndrome were severe infections and toxicity to chemotherapy. A statistically significantly greater difference in therapy-related mortality was observed in patients with Down syndrome ($p < 0.0001$, odds ratio, 1.7; 95% CI, 1.005-2.88) compared with patients without Down syndrome, this result being in agreement with that of the Ponte di Legno working group (Buitenkamp, 2014).

Chapter 5. Conclusions and personal contributions

Conclusions of Study no. 1

1. Unlike pediatric ALL, AML in children and adolescents has an unfavorable outcome, the overall survival rate for the entire group of patients with AML, analyzed in Study 1, being 46.3% and EFS rate of 44.4%.

2. Early death rate, in the first 28 days after diagnosis, was high (22.4%). The main cause of death in the first 14 days was cerebral hemorrhage, while deaths between 15-28 days after diagnosis were mainly due to severe infections. Multivariate regression model analysis revealed that adolescents aged over 14 (p=0.031, Odds Ratio: 20.219, 95% CI: 1.326-308.349) and patients with an LDH value > 1,000 U/l at disease onset (p=0.037, Odds Ratio: 13.116, 95% CI:1.164 - 147.797) had a statistically significant higher risk of death in the first 28 days after diagnosis.
3. A total of 29.3% of the patients with AML had molecular abnormalities at diagnosis, 18.5% (10 patients) had the PML-RARA fusion gene, 3.7% (2 patients) had the CBF beta MYH11 rearrangement, 11.1% (6 patients) had FLT3-ITD mutations and 5.6% (3 patients) had NPM1 gene mutations.
4. The incidence of AML with PML-RARA (LAM3) was higher (18.5% relative to the total number of AMLs) than the one described in most European studies (6-10%) (Creutzig, 2012; Testi, 2018). The overall survival rate in PML-RARA AML was 50%, being negatively influenced by early deaths caused by cerebral hemorrhage.
5. The overall survival of patients with FLT3-ITD mutations was lower than that of patients without FLT3-ITD mutations, but with no statistically relevant difference (33% versus 47.9%, p =0.588). For patients with AML, excluding AML3, a statistically significant correlation was identified between the presence of FLT3-ITD mutations and DIC syndrome at onset (p=0.017)
6. Overall survival was better for patients with Down syndrome and AML than for those with AML alone (57.1% compared to 45.1%). A statistically significant correlation was observed between M7 subtype and AML associated with Down syndrome.
7. Multivariate analysis by Cox regression model revealed that male sex (p=0.004, HR: 3.589, 95% CI:1.516-8.499), the presence of significant hepatosplenomegaly at diagnosis (p=0.008, HR:3.887, 95%CI:1.415-10.679) and age at diagnosis > 10 years (p=0.03, HR: 2.921, 95% CI: 1.106-7.710) are statistically significant independent predictors of shorter survival. Also, for the whole group of patients with AML, leukocyte count at diagnosis>100,000/mm^3 (p=0.650, HR:1.284), identification of FLT3-ITD mutations (p=0.392, HR:1.815) and presence of DIC syndrome at diagnosis (p=0.591, HR:1.372), influence in a negative way the overall survival, but not statistically significant. NPM1 mutations, presence of PML-RAR alpha and CBF beta-MYH11 increase survival period, but not significant from a statistical point of view.

8. The number of patients with AML, excluding AML3, who underwent allogeneic hematopoietic stem cell transplantation was small (8 patients, 18.2%), 75% of whom underwent allogeneic hematopoietic stem cell transplantation during the first complete remission. Overall survival rate for patients who underwent transplantation was 50%.
9. A patient with FLT3-ITD AML underwent FLT3 inhibitor therapy as a bridge therapy to allogeneic hematopoietic stem cell transplantation. As the intensification of chemotherapy protocols that already result in a high toxicity rate is not feasible, it is necessary in the future to move from the complete identification of molecular particularities at disease onset or relapse to molecularly targeted therapy, for as many patients as possible.

Conclusions of Study no. 2

1. ALL in children and adolescents has a favorable outcome, with a 5-year overall survival probability rate of 75.8% for the patients aged > 1 year old included in this study, but overall survival remains below that of highly developed countries which have reported overall survival rates of over 85% in recent years.
2. Children aged between 1 and 10 years at diagnosis had the highest overall survival rate (80.4%), while adolescents aged over 14 had an overall survival rate of 60% and patients aged < 1 year old at diagnosis had an overall survival rate of only 25%.
3. Univariate analysis demonstrated that age <1 year, leukocyte value at onset >50,000/mm^3, presence of BCR-ABL1 transcript, poor prednisone response on day 8 of treatment, and positive MRD on day 33 were identified as factors with a negative impact on survival, while the male gender and age at diagnosis between 1-10 years were identified as factors with a favorable impact on survival. Multivariate analysis identified MRD disease at day 33 as the only independent predictor of overall survival and EFS rate.
4. The survival rate of patients who relapsed was significantly lower (22.7%) compared to the survival rate of patients who did not relapse (84.1%, $p < 0.0001$) and was negatively influenced by the fact that only two patients (8.33%) out of 24 patients who relapsed underwent allogeneic hematopoietic stem cell transplantation.
5. The main causes of death during Induction treatment were severe infections and L-asparaginase toxicity. Mortality associated with L-asparaginase toxicity was 3%.

Approximately 50% of patients experienced at least one adverse effect related to L-asparaginase treatment, the most important adverse effects of L-asparaginase being hypersensitivity reactions (24.1%), hepatotoxicity (19.4%), thrombosis (2.4%), hypoproteinemia (9.1%), hyperglycemia (4.2%), hypertriglyceridemia (6.7%), pancreatitis (3%). A statistically significant correlation (p=0.001) was found between the occurrence of thrombosis and the presence of ETV6-RUNX1 (TEL-AML1) transcript.

6. 22.4% of patients had molecular abnormalities at diagnosis. The ETV6-RUNX1(TEL-AML1) rearrangement was the most common molecular abnormality identified, with a statistically significant correlation between the existence of this type of rearrangement and the age range between 1-10 years old at diagnosis (p = 0.021). The outcome of patients with ALL and ETV6-RUNX1 (TEL-AML1) was favorable, with an overall survival rate of 84% for this subtype of patients. Patients with ALL BCR-ABL1 as well as those aged < 1 year old and MLL-AF4 rearrangement had a particularly unfavorable evolution.
7. While the overall survival rate for patients with ALL without Down syndrome has continuously improved in recent years, the evolution of the disease remains unfavorable for patients with Down syndrome and ALL who have both a higher relapse rate (28.5% versus 13.3%, p=0.13) and significantly higher treatment-related mortality (p<0.0001). Overall survival rate for patients with ALL and Down syndrome was only 35.7% compared to 75% for patients with ALL without Down syndrome (p = 0.001). The main causes of death for patients with Down syndrome were severe infections and post-chemotherapy toxicity, making it difficult to balance the chemotherapy doses needed to achieve a long-lasting remission and avoid therapy toxicity against the particular fragile background of these patients.
8. Another particular category of patients was adolescents >14 years old who had a lower overall survival rate (60%), a higher percentage of T-ALL (24%), a higher likelihood of being placed in the high-risk arm of therapy, a higher toxicity associated with chemotherapy leading to a mortality rate in the first 33 days of Induction of 4% and a mortality rate in complete remission after day 33 of 12%. None of the teenagers presented the ETV6-RUNX1(TEL-AML1) favorable prognostic rearrangement.
9. The difference in the survival rate between the T-ALL group and the B-cell precursor ALL group was approximately 10% (65.4% versus 76.3%), with a particularly

unfavorable outcome for patients with T-ALL and poor prednisone response on day 8 who had a survival rate of only 12.5%.

10. In recent years, international efforts have been made to introduce molecularly targeted individualized therapy in the treatment of ALL, which can improve survival rate and provide a bridge to allogeneic hematopoietic stem cell transplantation and has a lower risk of toxicity. Four of the patients included in this study underwent molecularly targeted therapies (tyrosine kinase inhibitors, anti-CD38 monoclonal antibody, anti-CD22 monoclonal antibody) both in the first line of therapy as well as after relapse in an attempt to achieve a second complete remission prior to allo hematopoietic stem cell transplantation.
11. Further development of molecular biology testing and expansion of the panel of standard molecular tests performed at diagnosis are absolutely necessary because they will result in better classification of the patient into the genetic risk group, rapid orientation towards individualized molecular targeted therapy and the possibility to use the latest therapeutic protocols that include both immunotherapy and molecular risk stratification criteria. ALL treatment in children and adolescents should have a long term high goal, taking into account as far as possible both cure with minimal toxicity associated with therapy and subsequent social integration of patients.

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