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"**NEW PROGNOSTIC MARKERS IN ACUTE MYELOID LEUKEMIA:**

FOCUS ON BCL2 EXPRESSION"

Scientific disciplinary sector: MED/15 - Blood Diseases

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1. ABSTRACT

Acute myeloid leukemia (AML) comprises a genetically and clinically heterogeneous group of aggressive haematological neoplasms, characterized by clonal proliferation, reduced maturation of malignant myeloid precursors and resistance to apoptosis.

The BCL2 protooncogene encodes an inner mitochondrial membrane protein that blocks programmed cell death, and it can be over overexpressed in AML, but is uncertain if its overexpression could have a prognostic impact.

We perform immunophenotypic analysis to identify blast cells population from bone marrow samples of all newly diagnosed AML patients in our haematology unit.

At data cut off, we enrol 68 newly diagnosed AML pts from December 2019 to January 2023. 36 male 32 female, the median age 68.5ys (range 20-92ys), 42/68 were treated with intensive chemotherapy, 26/68 were treated with hypomethylating agents or low dose cytarabine. We studied the prognostic biologic futures of the AML samples, especially NPM1, FLT3 mutations and karyotype. The expression of BCL2 is classified by using as a parameter the MFI, mean fluorescence intensity, obtained by calculating the ratio between the median intensity of BCL2 in the sample and the corresponding isotipic control. After several statistical analysis we couldn't prove a prognostic impact of the over expression of BCL2 about OS, as some papers had shown before. We tried to find also a statistical correlation between over expression of BCL2 and other biological futures with prognostic role, but neither mutations of NPM1, FLT3 were related to the expression of BCL2. Finally we study OS of our cohort of patients treated with intensive chemotherapy and low dose therapies and we found a very good prognosis for responder patients treated with intensive chemotherapy with a median OS not reached after 2 years of follow up. Unfortunately the prognosis of older patients, event with the introduction of new target therapies remain poor, with a median OS less then one year.

2. INTRODUCTION

Acute myeloid leukemia (AML) is a neoplastic disease caused by genetic alterations affecting haematopoietic stem cells already differentiated by myelopoiesis. These alterations give the leukemic clone a selective advantage in terms of: uncontrolled proliferation, independent of normal mitogenic stimuli, resistance to apoptosis and differentiating block.

In the last decades the progress in understanding the pathophysiology of the AML had improved the prognosis of this severe diseases, especially finding new molecular abnormalities that can be use as a target for new drugs.[1-5] Unfortunately the prognosis of these disease remain disappointing especially in older patients, who frequently are not eligible for intensive chemotherapy or clinical studies for new drugs, with more or less 1 year of survival form diagnosis.

DIAGNOSIS AND RISK STRATIFICATION IN AML

AML can be diagnosed by morphological examination and further analysis of the bone marrow aspirates or immunohistochemistry on a core biopsy may be made if an aspirate is unable to be obtained. The diagnosis can be confirmed by the identification of cell surface and intracellular markers using immunophenotyping with multiparameter flow cytometry, this technique is very useful even in order to identify a specific leukemic pattern used to monitor MRD (minimal residual disease) after treatment.

Cytogenetic analysis is considered mandatory, with fluorescence in situ hybridization (FISH) and analysis of the karyotype searching some specific chromosomes abnormalities with prognostic impact.

Molecular testing, should be used to screen for genetic abnormalities, such NPM1 and FLT3 alterations, that can define disease's risk categories, or identify a specific target of new drugs other then find another way to monitor MRD.

The newest AML classification and risk stratification was recently updated and published in 2022[6-7], (Table 1), it allows to arrange AMLs in different prognostic subgroups adapting chemotherapy's strategies and indication to allogeneic bone marrow transplant.

Table 1 ELN 2022 risk stratification

Risk category†	Genetic abnormality
Favorable	• t(8;21)(q22;q22.1)/*RUNX1*::*RUNX1T1*†,‡ • inv(16)(p13.1q22) or t(16;16)(p13.1;q22)/*CBFB*::*MYH11*†,‡ • Mutated *NPM1*†,§ without *FLT3*-ITD • bZIP in-frame mutated *CEBPA*‖
Intermediate	• Mutated *NPM1*†,§ with *FLT3*-ITD • Wild-type *NPM1* with *FLT3*-ITD (without adverse-risk genetic lesions) • t(9;11)(p21.3;q23.3)/*MLLT3*::*KMT2A*†,¶ • Cytogenetic and/or molecular abnormalities not classified as favorable or adverse
Adverse	• t(6;9)(p23.3;q34.1)/*DEK*::*NUP214* • t(v;11q23.3)/*KMT2A*-rearranged# • t(9;22)(q34.1;q11.2)/*BCR*::*ABL1* • t(8;16)(p11.2;p13.3)/*KAT6A*::*CREBBP* • inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2)/*GATA2*, *MECOM(EVI1)* • t(3q26.2;v)/*MECOM(EVI1)*-rearranged • −5 or del(5q); −7; −17/abn(17p) • Complex karyotype,** monosomal karyotype†† • Mutated *ASXL1*, *BCOR*, *EZH2*, *RUNX1*, *SF3B1*, *SRSF2*, *STAG2*, *U2AF1*, and/or *ZRSR2*‡‡ • Mutated *TP53*[a]

STATE OF THE ART OF TREATMENT

The treatment's choice in AML is based on clinical evaluation, performance status of the patients, comorbidities, age, and biological characteristics of the disease.

The induction therapy, for younger and fit patients, is based, since 1973, on the 3+7 regimen (3 days of daunorubicin + 7 days of cytarabine), a second induction could be done if the response to treatment is considered poor. After this initial chemotherapy cycles, patients are allocated to consolidation or allogenic stem cell transplant based on the risk stratification at diagnosis and response to treatment. The percentage of response after this kind of strategy is 50-80%, but relapses remain high[15].

For older and unfit patients prognosis is very poor, presence of frequent genetic abnormalities and the reduce intensity regimens caused a frequent relapses either then refractoriness and short survival. Current therapies with hypomethylating agents (HMAs) azacitidine or decitabine show about 10-50% overall response rate (including haematological improvement) with a median overall survival 1 year[8-9]. But in recent times the use of HMAs plus BCL2 inhibitor, venetoclax, can improve this outcomes[10-11], in fact randomized studies [14] had shown an overall survival longer and the incidence of remission higher among patients who received azacitidine plus venetoclax than among those who received azacitidine alone. These reports have led to changes in clinical practice.

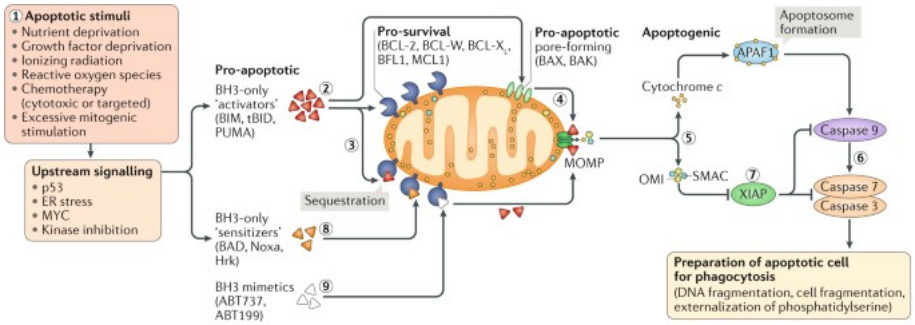
Molecular abnormalities like mutation of FMS-like tyrosine kinase 3 (FLT3), one of the most common aberrations found in AML, are recently used as a target for new drugs even in younger or older patients [16].

BCL2

The mitochondrial pathway of apoptosis is governed by the B-cell lymphoma 2 (BCL-2) family of pro- and anti apoptotic proteins play an important role in the survival and persistence of AML blasts.[10-12] When BCL-2 is antagonized, BAX is released, resulting in mitochondrial outer membrane permeabilization and cell death. BCL-2 maintains myeloblast survival by sequestering pro-apoptotic BAX, resulting in mitochondrial dependence on BCL-2. (figure 1) [12].

Figure 1.

Role of BCL2



Due to the important role in apoptosis, BCL2 was investigated since its discovery in many studies even for its possible implication in the treatment choice, and prognostic impact.

In literature there are several articles which tried to suggest that high expression of BCL2 could be associated with an inferior response to chemotherapy and poor survival among patients with AML[17-18-20], as if its over expression, alone or combined with other proteins, could modify cells survival.

Moreover in other papers over expression of BCL2 was associated with particular AML subtypes[21-22] both in adults and children, but they didn't found any correlations with survival.

Another important issue is the heterogeneity in the used technique to find out the expression of BCL2. Some authors used PCR to find a qualitative data and the specific amount of BCL2 in the samples[19], others prefer to use the flow cytometry to identify the protein inside the mithocondria[18], but there isn't a specific guideline about the preference use of PCR o flow cytometry and this method's differences make difficult to compare data from different studies.

Turning to therapy, venetoclax, a potent, selective, oral inhibitor of BCL-2, has demonstrated single-agent clinical activity and a tolerable safety profile in patients with relapsed or refractory AML[9]. Recently venetoclax in combination with azacitidine or decitabine [12-13] shows a good impact on overall survival with a median OS 14.5 months in previously untreated AML[11-14]and due to this results the drug was approved for therapy in NDAML for older o frail patients who couldn't face an intensive chemotherapy.

3. AIM OF THE STUDY

Due to the poor prognosis of AML patients, several studies tried to ameliorate prognostic stratification and find new molecular targets in order to improve therapeutic regimens, in young o older patients.

There are some well know prognostic factors like NPM1, FLT3 mutations and abnormalities of the karyotype and other new alteration that can modify prognosis identifying by new techniques like Next Generation Sequencing (NGS) and recently included in the ELN AML classification.

The best identification of relapse risk and correct management of patients is crucial to improve prognosis and choose the right treatment, for example considering or not stem cell transplant in younger patients.

After a review of the literature about the role of BCL2 even in the normal cells ad in leukemic cell, while its function in apoptosis is established, remain uncertain how it can remodel survival in abnormal haematologic cells.

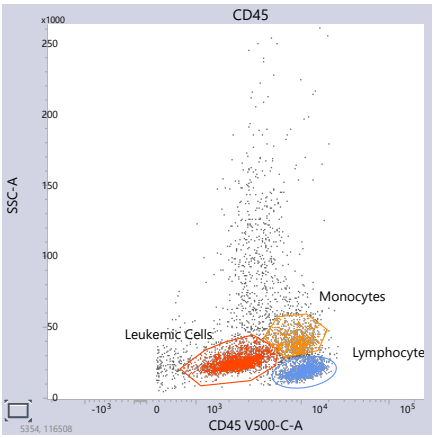
In this pilot prospective study we try to show if the expression of BCL2 can modify prognosis in AML and if there is any correlation between this marker and other well known prognostic factors. Furthermore we decided to use the flow cytometry to measure the expression of BCL2 apply the MFI (mean fluorescence intensity) to compare data in order to find a level of expression of BCL2 and not only a qualitative information.

4. MATERIALS AND METHODS

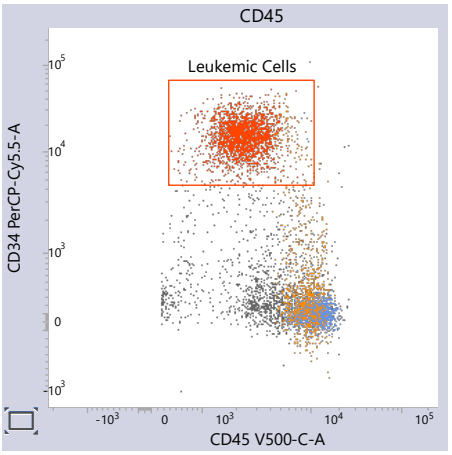
We decided to enrol all newly diagnosed AML from December 2019 to January 2023, regardless of age and type of treatment, we decided to exclude only patients with APL (acute promyelocytic leukaemia). According to the international guidelines (ELN, European Leukemia Net) we perform bone marrow aspirates at the time of diagnosis to evaluate by flow cytometry immunophenotipic assessment, identifying when it was possible, a specific leukaemic associated pattern, mutations of NPM1 and FLT3 and other abnormalities of karyotype. In the next lines we explain how we perform the immunophenotic assay.
Figure 2 A-B shows a representative gating strategy for AML blast population.

Figure 2 A. CD45 versus SSC, Leukemic Cells in red colour, Monocytes in orange colour and Lymphocytes in light blue colour. Figure 2B. CD45+ versus CD34+, Leukemic Cells CD34+ in red colour.

2A



2B



Bone Marrow (BM) samples at diagnosis are collected at room temperature, and processed on the same day of collection. BCL-2 expression is assessed using the fixation and permeabilization protocol of BD IntraSureTM Kit (BD Biosciences, San Jose, CA) according to manufacturer's recommendation. Briefly, 100 µL of cells suspension were initially incubated with with monoclonal antibodies directed against patient specific antigen for leukemic cells for 15 min at room temperature. Subsequently, cells were fixed adding 100 µL of BD IntraSure™ Reagent A to each tube and incubating for 5 minutes at room temperature (20°C–25°C). Red blood cells were then lysed by adding 3 mL of BD FACS Lysing solution (BD

Biosciences, San Jose, CA) to each tube, and incubated at room temperature for 10 min after gently shaking. After centrifugation step and removal of supernatant, cells were permeabilized by adding 50 µL of BD IntraSure™ Reagent B. At this point, cells are incubated for 15 minutes at room temperature (20°C–25°C), protected from light, with an IgG1-FITC isotype controls (BD) and the anti-human Bcl-2 (FITC-conjugated). After incubation, cells were washed with BD FACSTM Lyse Wash Assistant (LWA, BD Biosciences, San Jose, CA).

Analysis of BM samples was performed on a BDFACSCantoTM II and a BD FACSLyricTM instrument, using the FACSDivaTM and the FACSSuiteTM softwares (BD Biosciences, San Jose, CA). Instruments setup were monitored daily and, to ensure reproducible results over time, we followed standardized protocols that implied adjustments of FACS internal parameters, using the FACSTM CS&T IVD Beads to keep constant the instrument perfomance by correcting wear of lasers and fluidic instability.

Analysis of results were performed by gating both lymphoid cells and leukemic clone, and comparing BCL-2 expression as Mean Fluorescence Intensity (MFI) calculated as ratio on positive leukemic cell to that lymphoid cells that normally express BCL2. In our cohort the median lymphocytes MFI is 12, so we divided patients as over express or under express BCL2 if the resulting MFI is over under the value of 12.

In Figure 3 (A-B) is represented the population con leukemic blast cells by the expression of BCL2.

Figure 3 A. BCL-2 expression in Leukemic Cells (red colour) and IgG isotype control (blue colour).

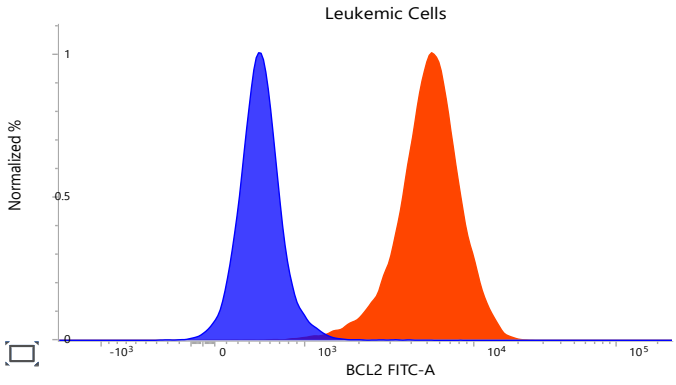
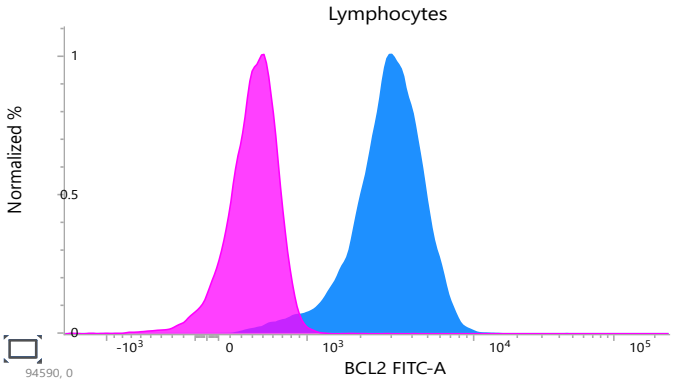


Figure 3B. BCL-2 expression on lymphocyte population (light blue colour) and IgG isotype control (pink colour).



STATISTICAL ANALYSIS

For the statistical analysis we used the software “R” version 4.0. The correlation between expression of BCL2 and the mutations of NPM1 e FLT3 was tested with the Fisher's Exact Test. We made the overall survival curves by Kaplan Meier curves, and the differences between groups were tested with the log rank test. Finally for the multivariate analysis we used a Cox proportional hazards model.

5. RESULTS

We enrol in our study 68 newly diagnosed AML, 36 male and 32 female, the median age was 68.5 yrs (range 20-90). 42 pts were treated with different intensive chemotherapy regimens, while 26 pts not eligible for intensive treatment due to age or comorbidities, were treated with hypomethylating agents or low dose cytarabine. 20 patients, were treated also with BCL2 inhibitor most of them in association with low dose chemotherapy. The molecular analysis were performed for NPM1 and FLT3 mutations according to the ELN guidelines.

The characteristics of our cohort are represented in table 2.

Table 2. Patients characteristics

AGE	68.5 (range 20-90)	
SEX	M F	36 (53%) 32 (47%)
NPM1	MUT WT	20 (29%) 48 (71%)
FLT3	MUT • ITD • TKD WT	17 (25%) • 10 (15%) • 7 (10%) 51 (75%)
KARIOTYPE	ABN WT NE	19 (22%) 43 (68%) 6 (10%)
BCL2	OVER EXPRESS UNDER EXPRESS	39 (57%) 29 (43%)
TREATMENT	INTENSIVE CHT NON INTENSIVE CHT	42 (62%) 26 (38%)

Among the 68 patients, 51 were negative for any FLT3 alteration while 10 were positive per ITD mutations and 7 were positive for TKD mutation (figure 4).

About NPM1 mutation 48 samples were negative and 20 patients were positive for one of the most common mutations (figure 5).

43 patients were also negative for any alteration of the karyotype, for 10 patients we were able to find a complex karyotype, t(8;21) e inv16 were found in 2 patients each, 3 patients with probably secondary AML had a chromosome 7 deletion and 1 patients each had a rare mutations such add (11p15) and t(9;11) +8. Unfortunately 6 samples were not evaluable for the analysis (figure 6).

Figure 4. FLT3 mutations in our cohort

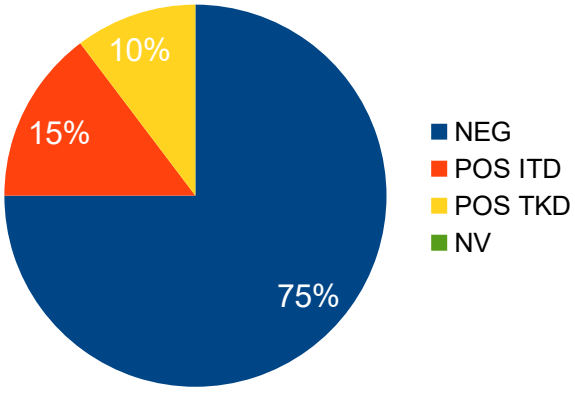


Figure 5. NPM1 mutations in our cohort

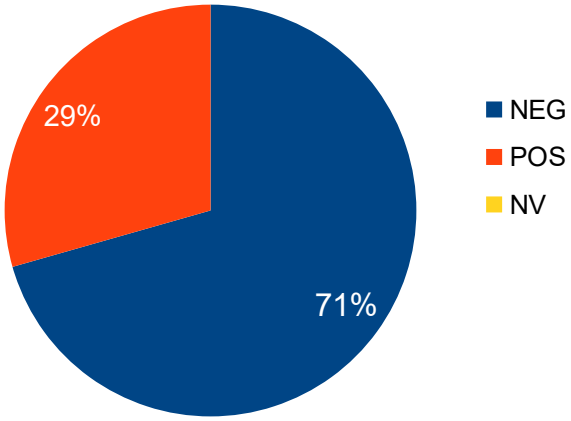
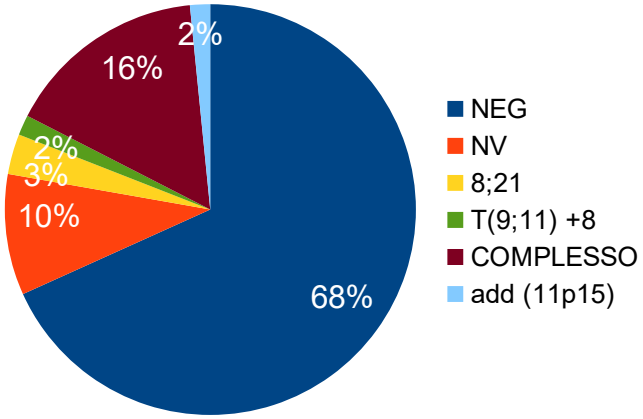


Figure 6. Abnormalities of karyotype in our cohort



As said before, for the analysis of BCL2 we classify the expression of BCL2 by using as a parameter the MFI, obtained by calculating the ratio between the median intensity of BCL2 in the sample and the corresponding isotipic control.

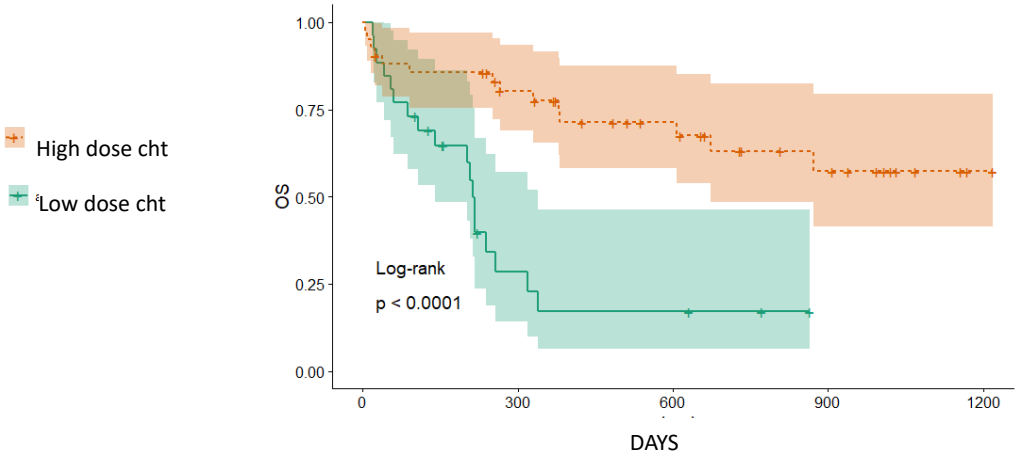
Samples will be classified for BCL2 as over express or under express if the resulting MFI is over or under the value of 12, that is the median value of lymphocyte's MFI that normally express BCL2, in our cohort, 39 out of 68 pts's samples over express BCL2 while 29 out of 68 samples BCL2 was under express.

After a median follow up of 233 days for patients treated with low dose chemotherapy and 541 days for patients treated with intensive chemotherapy, 36 patients are alive, 32 are death.

According to the survival data, median OS is not reached for patients treated with intensive chemotherapy, while is under one year for older patients.

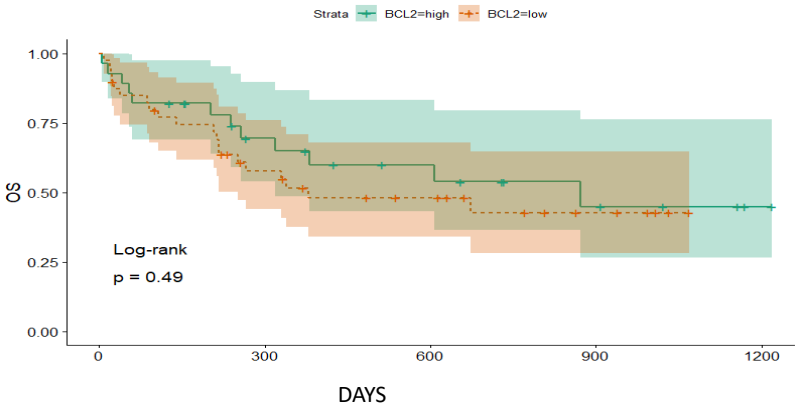
There is a significantly OS benefit for patients treated with intensive chemotherapy, as expected ($p < 0.0001$) compared to patients treated with hypomethilating agents. In the figure 7 is represented OS of all patients included in the study.

Figure 7 OS of our cohort treated with intensive chemotherapy or not



After this initial analysis of survival we decided to stratify patients by the expression of BCL2, regardless the treatment choice, in the total cohort we can't find a prognostic implication of the protein's levels (p 0.49).

Figure 8. OS by BCL2 expression



Our analysis therefore continued with the stratification of our cohort by therapeutic regimens and BCL2 expression, obtained 4 subgroups. In the two subgroups of patients treated with high or low intensive chemotherapy regimens, mean BCL2 MFI was slightly different, even it wasn't statistically significant, in fact in the cohort of patients treated with intensive treatment mean BLC2 MFI was 14.36, in the other cohort was 11.89.

Considering 42 patients treated with intensive chemotherapy 18/42 over express BCL2, 24/42 not. For patients treated with low dose chemotherapy 10/26 over express BCL2, 16/26 not (Table 3). Studying the survival of patients by the expression of BCL2, we find that patient with high expression of BCL2 and treated with low dose therapy have the worst prognosis, while patients treated with intensive chemotherapy with high expression of BCL2 have the better prognosis. As before we can find a survavl benefit for patients treated with intensive chemotherapy, but we can't find a correlation between the expression of BCL2 and survival even when patients were treated almost in the same way.

The median OS for younger patients treated with intensive chemotherapy isn't reached after mean follow up time of 540 days, either in patients with high or low expression of BCL2. Interestingly there is a trend, even if it isn't statistically significant, for a better outcome for patients with high expression of BCL2. Moreover for patients treated with low dose chemotherapy the prognosis remain poor, in fact after mean follow up time of 223 days the median OS for patients treated with non intensive regimens is only 212 days if the expression of BCL2 is high, while is 239 days if the expression is low (figure 9).

Figure 9

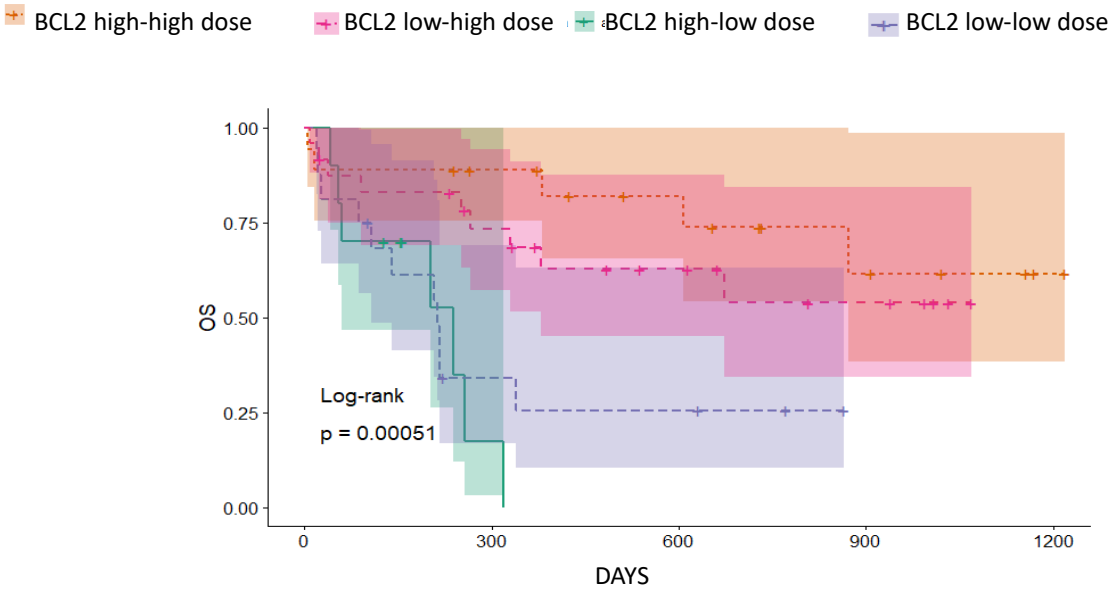


Table 3

		HIGH DOSE NO	HIGH DOSE YES	p
BCL2 (%)	HIGH LOW	10 (38%) 16 (62%)	18 (42%) 24 (58%)	0.917
FLT3 (%)	WT MUT	21 (81%) 5 (19%)	30 (72%) 12 (28%)	0.564
NPM1 (%)	WT MUT	19 (76%) 6 (24%)	18 (67%) 14 (33%)	0.595
DAYS FU (MEAN)		223	540	
BCL2 MFI (MEAN)		11.89	14.36	0.248

After the analysis about survival we also try to find a correlation between the high or low expression of BCL2 and other prognostic markers, such FLT3 and NPM1 mutations, also because some authors reported a statistical correlation between BCL2 and other markers. In our cohort 20 patients have a mutation of NPM1, 6 of them have also a high expression of BCL2, 14 patients under express BCL2, for 48 patients who don't have any mutation 23 patients over express BCL2, while 25 patients have low level of the protein. The p value, 0,28, isn't statistically significant.

Analysing data form patients with mutations of FLT3, 17 samples are positive for one of the to most frequent alteration, in this group 6 patients over express BCL2 and 11 patients under express the protein. 51 samples are negative for any alteration, about this group 22 patients over express BCL2 e 29 have a low expression of the protein. Even in this case the difference is not statistically significant with a p value of 0.78. The results are shown in the table 4-5.

Table 4.

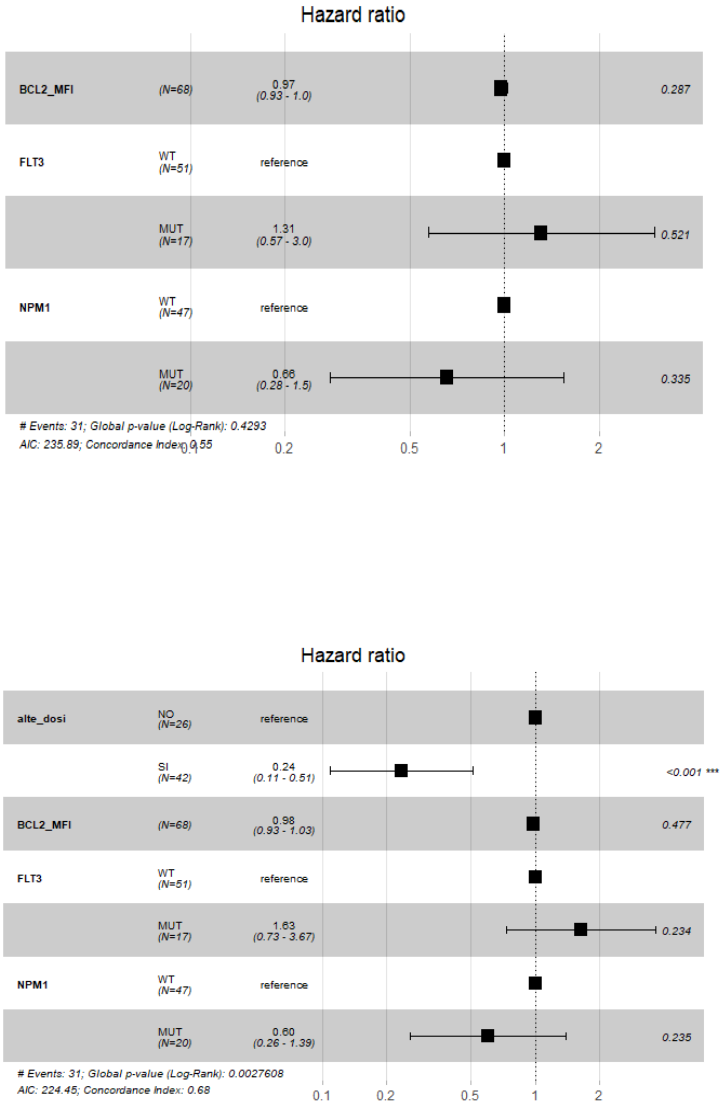
	NPM1 MUT	**NPM1 WT**	**p**
BCL2 HIGH	6	23	0.28
BCL2 LOW	14	25	

Table 5.

	FLT3 MUT	**FLT3 WT**	**p**
BCL2 HIGH	6	22	0.78
BCL2 LOW	11	29	

Finally we perform a multivariate analysis, shown in figure 10, to expose the possible correlation between the expression of BCL2 and choice of therapy or other prognostic alterations. Even in this analysis the only item with a statistical implication is the treatment choice, while the other analysed variable don't show a statistical correlation to the outcome.

Figure 10 Multivariate analysis



6. DISCUSSION

We observe a cohort of 68 consecutive newly diagnosed AML, classified as current ELN guidelines and treated appropriately to age and comorbidities.

We decided to study the expression of BCL2 in our AML samples due to its role in apoptosis, and to attempt this issue we used the flow cytometry to identify different levels of BCL2 expression in our samples and compared results with the MFI.

As reported in some articles[17-18-19-20-23] the expression of BCL2 can be link to response to treatment and survival, but there is a heterogeneity in term of used technique to determined the BCL2 expression, and it's very difficult to compare results from different studies.

Furthermore several authors linked the expression of BCL2 to other molecular markers such FLT3[16], MDR1[23], WT1[19] in order to identify new prognostic subgroups, but the results were not confirmed in the work of other subsequent colleagues[21].

Our data, even in a small cohort of newly diagnosed AML, are consistent with some papers[21-22] from the literature which can't support, at this moment, the prognostic role of BCL2 either in younger or older patients, in fact after a considerable follow up time the survival for patients treated uniformly with high dose chemotherapy or hypomethilating agents is almost the same regardless the BCL2 expression.

Looking at results we focus our attention on the younger patients subgroup treated with intensive chemotherapy, interesting the median OS for patients with high expression of BCL2 is a little bit better then median OS of patients who under express BCL2, even this difference isn't statistically significant already, is interesting to follow these patients with a longer follow up and analyse deeper specific AML futures, to find other prognostic implication.

About older patients who receive low dose chemotherapy, some of them were treated (as standard of care since 2021-2022) with hypomethylating agents (azacitidine or decitabine) and BCL2 inhibitor, venetoclax. We can't see in our study a more favourable prognosis based on the expression of BCL2 for these patients the mean OS remains very short about 1 year, these data are aligned to previous studies.[14]

Our results suggest that the underlying mechanisms of drug resistance and apoptosis is more complex for AML's cells and their survival isn't related only to one mechanism.

The expression of BCL2 is probably one of the most interesting issue talking about new prognostic markers especially since the introduction of venetoclax as standard of care for

older patients and the use of flow cytometry to analyse its the expression level is probably the best way to compare data, because the MFI not only provide a qualitative data but also different level of expression.

7. CONCLUSIONS AND FUTURE PROSPECTIVE

To sum up, our data couldn't verify a prognostic role for the expression of BCL2 in AML patients, even stratifying patients by treatment choice, probably due to the small cohort or a short follow up.

In the next future we will enrol new patients in our study, even young and eligible to high dose chemotherapy, and older e frail patients. We decided also to study the expression of BCL2 in bone marrow samples not only at the time of diagnosis but also after first cycle of treatment, it could be more interesting especially for patients with high expression of BCL2 to see if there is any kind of correlation to its reduction to the response to treatment.

For patients treated with hypomethylating agents and venetoclax is also interesting to see if a reduction of the expression of BCL2 is related with a better response to treatment or not, even because in the large approval studies of the drugs combination it was not investigated.

Finally, since recently new prognostic markers identified by NGS have been introduced in the clinical practice and prognostic stratification, could be interesting, especially for patients who will undergo to intensive chemotherapy, to find a correlation with the BCL2 expression.

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