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INVESTIGATING NOVEL PROGNOSTIC FACTORS IN PATIENTS WITH ACUTE MYELOID LEUKEMIA: ABSOLUTE LYMPHOCYTE COUNT AND IMMUNE TRANSCRIPTOMIC BONE MARROW PROFILE

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Abstract

A better and more-in-depth understanding of the interactions between acute myeloid leukemia (AML) and immune system is likely to enable the development of new therapeutic and prognostic scenarios.

Concurrently to the extensive characterization of AML blast cell, which has led to crucial changes and implications in AML prognostic evaluation and to the addition of new targeted therapies to the armamentarium, in recent years the study and interest of microenvironment (ME) in bone marrow (BM) in which AML cells form and grow up and the immunological network established has been rapidly raising.

Absolute lymphocyte count (ALC) is known to be an independent prognostic factor for overall survival (OS) in patients with lymphomas as long as many solid cancers. In AML patients, enhanced ALC recovery after intensive chemotherapy (IC) has been associated with superior OS. However, there are still poor data correlating ALC recovery with novel therapy regimens, prognostic molecular European Leukemia Net (ELN) classification, allogeneic stem cell transplantation (HSCT) and measurable residual disease (MRD).

This research project is closely focused, as a central theme, on the complex relationship between the immune system network and AML. In particular, our study explores the immune transcriptomic profile variation in AML patients treated with chemotherapy and, concurrently, the impact of a clinical prognostic parameter as ALC.

To explore the gene expression profile variation in AML treated with chemotherapy, firstly, we performed a Pancancer Human NanoString panel, using BM samples collected from 48 AML patients, both at onset and post therapy, to describe distribution expression before and after therapy. Of note, Nanostring analysis showed significant differences in transcriptomic immune environment between onset and post therapy samples, especially in gene belonging to immune cells response.

Secondly, we investigated ALC recovery in the total newly diagnosed AML patient population, which includes 188 patients, from Bologna Seràgnoli Hematology Institute, who were responsive to IC. Based on the experimental hypothesis, we stratified patients according to different factors such as the type of chemotherapy regimen used, the molecular status at diagnosis, the impact of HSCT and the rate of

MRD. We defined 4 ALC time-points (TPs) from IC start and an ALC cut-off of 500/mm^3. While ALC recovery did not significantly impact on survival in the whole population, among patients treated with "3+7-based" regimen (51,6%), a relevant correlation with OS of patients with ≥500/mm^3 ALC (31,9%) vs those under the cut-off (19,6%) was observed at ALC-15. This finding was further statistically significant in patients with ALC≥500/mm^3 in all 4 TPs (25,5%) compared to those who had <500/mm^{3}ALC in at least one TP (26%)(p=0.02).

ALC recovery was also correlated with MRD value. Among "3+7-based" patients who obtained MRD negativity (n = 52), those who had ≥500/mm^{3}ALC in all ALC TPs (n=29) and in ALC-15 (n=35) resulted in a globally better OS as compared to patients with <500/mm^3 ALC at the two different evaluations (n=23 and n=17, respectively).

Pursuing new biological and clinical prognostic tools in management of AML, it has been demonstrated that ALC recovery after IC can be a promising independent survival predictor, in AML patients who achieve CR. Its impact is different according to the used IC regimen type (3+7 versus fludarabine-based). Importantly, ALC recovery seems to emphasize an OS difference among MRD negative patients and may represent a predictive biomarker to identify a group of responding patients who may benefit from HSCT. A difference can be spotted when comparing BM onset samples analyzed with NanoString panel, according to ALC and MRD, but further and more extensive subanalysis are required.

In order to explore ALC potentiality as a prognostic predictor, we should further subdividing AML patients in more groups, considering the use of a third drug added to the backbone (such as FLT3 inhibitors, gemtuzumab-ozogamycin, venetoclax) or the differentiation in categories according to ELN risk, including a more precise molecular characterization. Along with these subdivisions, studies with larger cohorts of patients are needed and highly warranted, in order to consolidate the results.

Introduction and aim of the research

The investigation of the immunologic network within solid neoplasms and hematologic malignancies, specifically in the setting of acute myeloid leukemias (AMLs), is one of the most interesting recent research topics in hematology. In the last years major strides have been made in the identification of new prognostic disease biomarkers and innovative drugs, including monoclonal antibodies, that harness the immune system against neoplastic cells with the potential to change the clinical management of AML patients.

The field which still is aim of research is the immunological microenvironment (ME) of AML bone marrow (BM), with the focus on the complex interaction mechanisms existing between AML blast and all other partners belonging to the hematopoietic niche. The advances in the knowledge of these areas of interest are the key to find out the AML blast behavior in its ME and discover new potential targets to exploit, towards a therapeutic implementation.

In this scenario, the absolute lymphocyte count (ALC) as survival predictor has already been investigated in cancer. ALC is known to be an independent prognostic factor for overall survival (OS) in patients with lymphomas as long as many solid cancers. In AML patients, enhanced ALC recovery after intensive chemotherapy (IC) has been associated with superior OS. However, there are still poor data correlating ALC recovery with novel therapy regimens, prognostic molecular ELN classification, allogeneic stem cell transplantation (HSCT) and measurable residual disease (MRD).

This research project is closely focused, as a central theme, on the complex relationship between the immune system network and AML. In particular, our study explores the immune transcriptomic profile variations in AML patients treated with chemotherapy and, concurrently, the impact of a clinical prognostic parameter as ALC.

Firstly, we performed a Pancancer Human NanoString panel, using BM samples collected from 48 AML patients, both at onset and post therapy, to describe immune transcriptomic distribution in AML treated, through the identification of gene expression modifications before and after therapy.

Secondly, we investigated ALC recovery in the total newly diagnosed AML patient population, which includes 188 patients, from Bologna Seràgnoli Hematology

Institute, who were responsive to IC. Based on the experimental hypothesis, we stratified patients according to different factors such as the type of chemotherapy regimen used, the molecular status at diagnosis, the impact of HSCT and the rate of MRD.

Other potential issues about ALC and hematological recovery have been reported, including the interaction with gut microbiota in AML.

In the last part of the project, we matched the biological data obtained from NanoString panels and the exploited clinical prognostic factors such as ALC itself or MRD status.

Within the context of immunological profiling in AML, this project represents an initial step pursuing the immune system enhancement in AML.

1. Prognostic evolution in AML

AML is an entity that encompasses an extremely heterogeneous group of hematologic malignancies, characterized by hematopoietic maturational blockage and rapid accumulation of immature forms in the BM and, consequently, in the peripheral blood (PB). The median age of onset is 65 years, with a peak at around 80 years (Estey, 2018; Papayannidis et al., 2019). Currently, about 40% of patients who are under 60 years can be curable with chemotherapy regimens and subsequent consolidation with HSCT; conversely, elderly patients, who are not candidates for such treatments, have a median survival rate that can be calculated in months (Döhner et al., 2015).

Over the past 15 years, in addition to the known cytogenetic features, the knowledge about the enormous molecular heterogeneity of the disease has greatly increased, through the genomic diversity and spectrum of possible somatic mutations acquired in AML, using new diagnostic techniques such as Fluorescence in situ hybridization (FISH), Sanger Sequencing, single nucleotide polymorphism profiling (SNP), whole genome and exome sequencing (WGS, WES), RNA sequencing (RNA-seq), laying the new foundation for the complex pathogenetic background of AML (Döhner et al., 2015; Papaemmanuil et al., 2016). This advance has gradually redefined the old morphological classification systems toward a classification system integrated with new genomic insights (Papaemmanuil et al., 2016).

In addition, such efforts have led to the identification, precisely within the most frequently studied mutations, of potential new therapeutic targets that can be exploited with appropriate therapies (Perl, 2017; Short et al., 2020). Consequently, in the need of improving outcomes in the adult patient, despite the benefit derived from transplantation, and in order to ensure a therapeutic chance for the elderly population not eligible for intensive therapies, there has been, mainly since 2017, a radical change in induction schemes and a marked lengthening of the list of salvage therapies, due to the approval by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) of about a dozen of new drugs, produced to target a specific molecule and turn off one of several mechanisms of leukemic development (Papayannidis et al., 2019; Perl, 2017; Short et al., 2020).

Therefore, after several decades poor in novelties, currently the armamentarium has been rapidly enriched, moving from an era when a single standard of intensive

chemotherapy had to be adapted to all patients (Perl, 2017) toward a model according to which patient characteristics (fitness and comorbidities), together with the cytogenetic-molecular profile of the disease, at onset and relapse, can guide the therapeutic choice, with addition of target drugs to standard regimens and increasing personalization of treatment (DiNardo and Wei, 2020; Papayannidis et al., 2019; Short et al., 2020). Thus, research has improved outcomes and has begun to improve a therapeutic need, especially in the elderly population with AML, which was previously considered untreatable (DiNardo and Wei, 2020; Palmieri et al., 2020).

Prognostic factors have been profoundly affected by the expansion of knowledge in the last decade. Prognostic factors can be divided into those related to the patient (age, performance status, comorbidities) and those attributable to the disease, including hyperleukocytosis, leukemia secondary to a pre-existing myeloproliferative syndrome or therapy-related ones, karyotype analysis (Döhner et al., 2015). In addition to the prognostic risk historically defined by cytogenetic data, in 2010 an international panel of experts from the European Leukemia Net (ELN) proposed a standardized prognostic system, reporting recurrent cytogenetic alterations and selected molecular alterations that in studies were correlated with outcome to conventional treatment significantly, so that cytogenetically normal AML could also be distinguished (Mrózek et al., 2012). There were three new molecular alterations introduced: mutation of Nucleophosmin-1 (*NPM1*), FMS-like tyrosine kinase 3 - Internal Tandem Duplications (*FLT3-ITD*), and biallelic CCAAT Enhancer Binding Protein Alpha (*CEBPA*). The innovative feature of ELN classification was the division into a model of 4 prognostic categories, combining cytogenetics with these 3 molecular alterations, summarized in Tab 1.1: favorable, intermediate-1, intermediate-2 and unfavorable risk (Döhner et al., 2015; Mrózek et al., 2012).

Genetic Group	Subsets
Favorable	t(8;21)(q22;q22); *RUNX1-RUNX1T1*
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
	Mutated *NPM1* without *FLT3*-ITD (normal karyotype)
	Mutated *CEBPA* (normal karyotype)
Intermediate-I	Mutated *NPM1* and *FLT3*-ITD (normal karyotype)
	Wild-type *NPM1* and *FLT3*-ITD (normal karyotype)
	Wild-type *NPM1* without *FLT3*-ITD (normal karyotype)
Intermediate-II	t(9;11)(p22;q23); *MLLT3-MLL*
	Cytogenetic abnormalities not classified as favorable or adverse
Adverse	inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
	t(6;9)(p23;q34); *DEK-NUP214*
	t(v;11)(v;q23); *MLL* rearranged
	–5 or del(5q)
	–7
	abnl(17p)
	Complex karyotype*

Abbreviations: AML, acute myeloid leukemia; ITD, internal tandem duplication.
*Complex karyotype is defined as three or more chromosome abnormalities in the absence of one of the WHO designated recurring translocations or inversions: t(8;21), inv(16) or t(16;16), t(15;17), t(9;11), t(v;11)(v;q23), t(6;9), inv(3) or t(3;3).

Tab 1.1 ELN 2010 classification. From (Mrózek et al., 2012)

This prognostic index had also been applied to prospective clinical trials and had strongly predicted the different possible outcomes in both young adults and the elderly, in an independent fashion regardless of the chemotherapy regimens used in induction and consolidation (Mrózek et al., 2012).

Nevertheless, when considering patients who were addressed toward HSCT, the validity of the 2010 ELN classification got reduced, as favorable risk patients had significantly higher OS than other risk categories (Grimm et al., 2020)**.** Therefore, in 2017, a new prognostic classification had been proposed, being able to take advantage of insights in genomic architecture and the most frequent mutations at the molecular level in AML, supplementing the previous one from 2010 (Döhner et al., 2017). In addition to the already established mutational screening including *NPM1*, *FLT3* and *CEBPA* genes, mutations in *RUNX1* and *ASXL1*, common in older patients, identify high-risk patients associated with poorer survival. *TP53* mutation, commonly associated with complex, monosomal karyotype or aneuploidy, indicates a very poor prognosis.

The other characteristic of the 2017 ELN recommendations concerns the different prognostic impact of mutations depending on the remaining molecular context. For example, mutation of *NPM1*, even when associated with *FLT3* positivity (low allelic

ratio) confers favorable prognosis, but this advantage is lost in the presence of mutated *RUNX1* or *ASXL1*. In core-binding factor (CBF) AML, particularly in AML with t(8;21), the presence of *KIT* mutation is associated with poorer prognosis; however, MRD negativity after therapy abrogates the negative effect of mutated *KIT* status. In addition, there are additional genetic markers whose prognostic value has yet to be discussed, but they equally represent therapeutic targets (*IDH1*, *IDH2*, *KMT2A*) (Döhner et al., 2017).

In conclusion, the intent of the new prognostic classification was the standardization of the genetic markers of the disease, in order to correlate them with clinical features and outcome; infact, the division between the intermediate-1 and intermediate-2 category in the ELN 2010, which considered mere genetic features, was repealed in the ELN 2017, unifying the two groups, as they are prognostically indistinguishable, especially in the elderly population, which represents the majority.

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 or with *FLT3*-ITD^{low}†
	Biallelic mutated *CEBPA*
Intermediate	Mutated *NPM1* and *FLT3*-ITD^{high}†
	Wild-type *NPM1* without *FLT3*-ITD or with *FLT3*-ITD^{low}† (without adverse-risk genetic lesions)
	t(9;11)(p21.3;q23.3); *MLLT3-KMT2A*‡
	Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1); *DEK-NUP214*
	t(v;11q23.3); *KMT2A* rearranged
	t(9;22)(q34.1;q11.2); *BCR-ABL1*
	inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2,MECOM(EVI1)*
	−5 or del(5q); −7; −17/abn(17p)
	Complex karyotype,§ monosomal karyotype‖
	Wild-type *NPM1* and *FLT3*-ITD^{high}†
	Mutated *RUNX1*¶
	Mutated *ASXL1*¶
	Mutated *TP53*#

Tab 1.2 ELN 2017 classification. From (Döhner et al., 2017)

The 2017 ELN prognostic classification (shown in Tab 1.2) thus reconfirms the favorable prognostic role of *NPM1* and biallelic *CEBPA*; the high recurrence rate in the presence of the *FLT3-ITD* mutation is strictly dependent on the allelic load ratio,

considered as low if below the ratio cut-off of 0.5, high if above; infact, the latter, if above 0.5, reduces the positive effect on prognosis of a concomitant *NPM1* mutation (Döhner et al., 2017). Infact, the 4 possible *NPM1/FLT3-ITD* genotypes, depending on the allelic ratio, differ significantly in terms of 5-year OS probability (Döhner et al., 2020). In contrast, mutations in other genes including *DNMT3A*, *IDH1*, *IDH2* or others involved in chromatin and spliceosome had not enough evidence to constitute ELN risk (Döhner et al., 2017).

Finally, among the included recommendations of the ELN 2017 document, the role of MRD as a prognostic criterion after therapy has been introduced and also the criteria for disease progression (Döhner et al., 2017).

In the context of transplantation, the assignment to the 3 2017 ELN groups retained its prognostic significance, with patients at adverse risk having a worse prognosis; also analyzing the impact of MRD, transplanted patients with MRD positivity had worse prognosis with a higher incidence of cumulative recurrence (Grimm et al., 2020; Hansen et al., 2021).

More recently, a new AML ELN classification edition have been published (Tab 1.3). ELN 2022 includes some significant amendments to the previous one, such as the reclassificaton of all *FLT3-ITD* patients as intermediate risk; the abrogation of *FLT3-ITD* allelic ratio; the addition of the so-called myelodysplastic syndrome (MDS)-related mutations as *ASXL1*, *BCOR*, *EZH2*, *SF3B1*, *SRSF2*, *STAG2*, *U2AF1*, *ZRSR2*, *RUNX1* in the definition of adverse risk; a more defined characterization of *NPM1* mutated AML according to comutations and cytogenetic alterations (Döhner et al., 2022).

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]

Tab 1.3 ELN 2022 classification. From (Döhner et al., 2022)

Lastly, as ELN 2022 can be only applied to AML patients treated with intensive regimens, a post retrospective analysis Beat-AML risk classification has recently redefined the prognostic impact of ELN risk in patients with newly diagnosed AML aged ≥60 years. The new classification sorting of group, with the addition of other independent prognostic mutations such as *IDH2* among favorable ones and *KRAS* among adverse ones, improved discriminations of survival and treatment choices (Hoff et al., 2024).

2. Immunological microenvironment in AML

Concurrently to the extensive characterization of AML blast cell, which has led to crucial changes and implications in AML prognostic evaluation, as shown in previous paragraph, and to exploration of new targeted therapies, in recent years the study and interest of AML cells concomitant ME in BM has been rapidly raising up.

In this paragraph, a brief overview of immunological ME in AML is reported. Previously, leukemia-cell-intrinsic cytogenetic and molecular aberrations had always been considered the only determining factors in AML disease onset and progression. This concept has been challenged, thus establishing that AML development also depends on the ME. Indeed, the interplay between leukemic cells and a variety of immune cells resulting in the dysregulation of both innate and adaptive immune responses has been shown to influence the disease outcome (Davidson-Moncada et al., 2018; Isidori et al., 2021), being a crucial determinant of AML pathophysiology.

2.1 Microenvironment characteristics

BM is a primary lymphoid organ with a distinctive ME that provides support and regulates self-renewal or differentiation of hematopoietic stem cells (HSCs) and contains most developing and mature immune cell types. Cells belonging to the tumor ME activate gene expression and protein patterns that promote tumor growth and are intrinsically immune suppressive. The ability of AML cells to shape the BM niche to their advantage and to determine tumor progression is emerging as a hallmark of this cancer. Recent evidence has shed light on the mechanisms deployed by AML blasts to confer a survival advantage over normal HSCs. Infact AML blasts interact with different cell type, both immunological and non-immunological, as highlighted in Fig. 2.1, forming a network of molecular interactions. To understand the role of the niche in leukemia development or propagation, the various cellular components of the BM ME must be considered. This array of cell types includes immune cells, adipocytes, bone-forming osteoblasts, mesenchymal stromal cells, and vascular endothelial cells. Under healthy conditions, the ME has been proposed to instruct HSC fate via multiple mechanisms, including proximal interactions with the stroma, such as VE-cadherin^{+} vascular endothelial cells and leptin receptor–expressing (Lepr^{+}) perivascular stroma cells that secrete stem cell factor (SCF) and CXCL12 to regulate HSC quiescence and survival. Osteolineage cells also contribute

to regulation of HSC differentiation, as well as immune cell types, such as macrophages and megakaryocytes, which have been shown to regulate HSC retention and quiescence via proximal interactions (Witkowski et al., 2020).

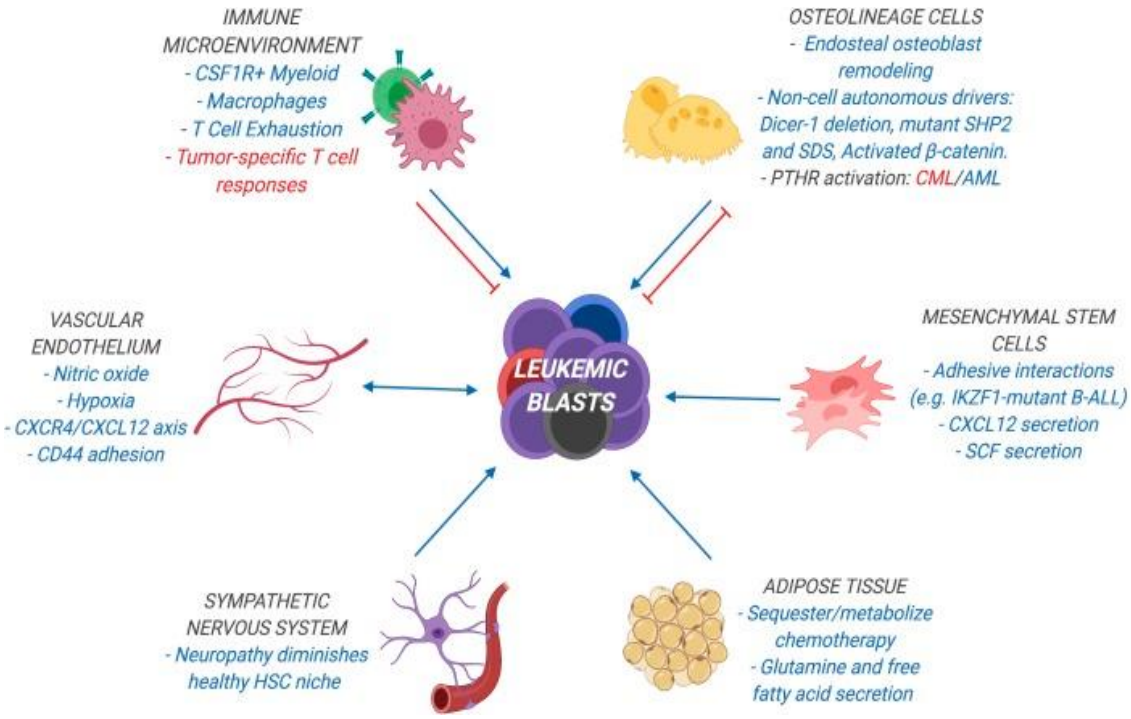


Fig 2.1 Schematic representation of different cell types interacting with AML blast. From (Witkowski et al., 2020)

2.2 Immunological network and AML blast evasion

Similarly to most hematological malignancies, AML cells are poorly immunogenic and highly immunosuppressive and are known to reshape immune ME toward the induction of immune tolerance. However, while potently acting by reducing the response to therapies, these immunosuppressive mechanisms may be reversible. Indeed, some chemotherapeutic agents, such as anthracyclines, may remodel the AML immune ME through the activation of an antitumor immune response in a process known as chemotherapy-induced immunogenic cell death (ICD) (Isidori et al., 2021; Vago and Gojo, 2020). Given the relevance of these immunosuppressive mechanisms and pathways for the full exploitation of immunotherapies, a brief description is provided in this paragraph.

Tumor escape strategies help adaptation of the AML cells to hide from immune system and further modifications of the immune cell compartment that include

effector T-cells, natural killer (NK)-cells, and dendritic cells (DCs) (Tettamanti et al., 2022).

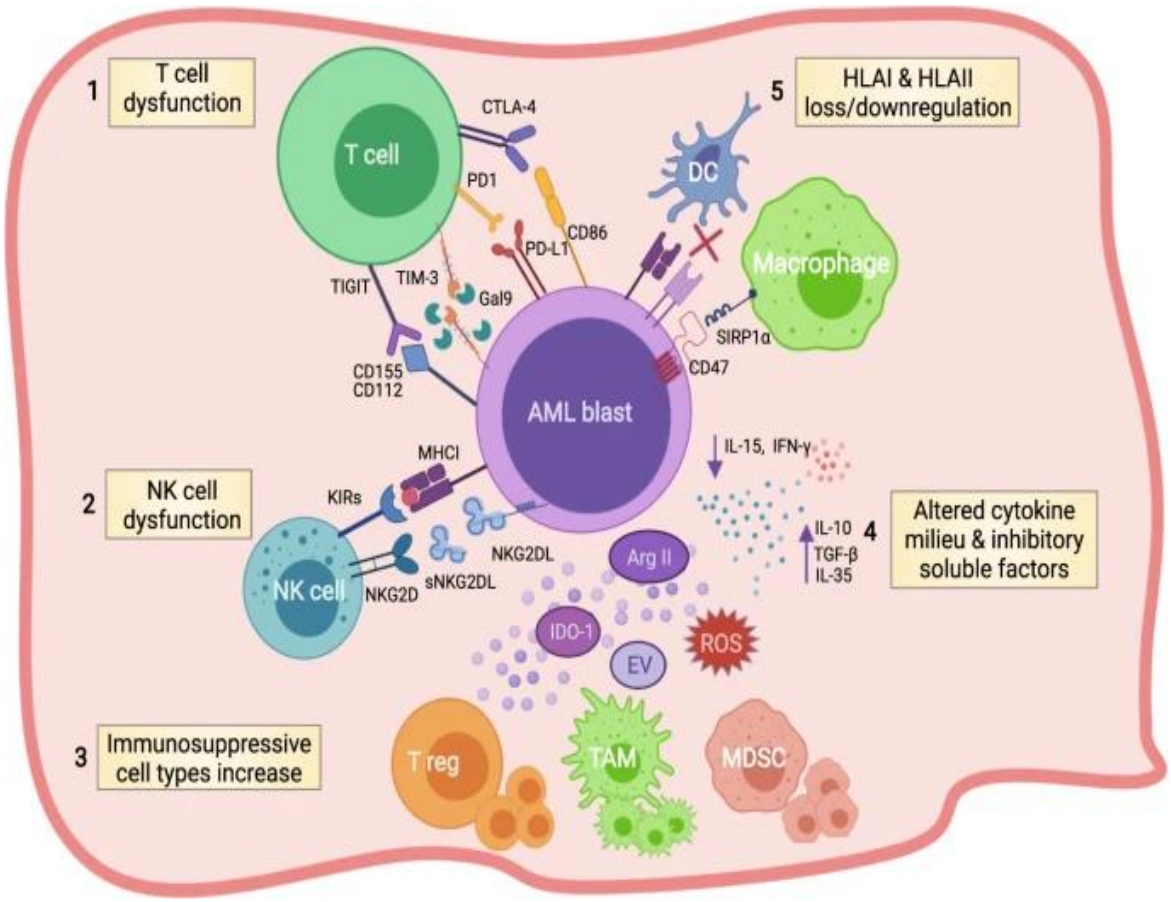


Fig 2.2 Mechanisms of immune escape in high-risk AML. The most relevant mechanisms underlying the capacity of high-risk AML cells to evade immune response are depicted: altered HLA molecules machinery presentation and HLA loss mechanism; PD-L1 overexpression on AML cells in TP53 mutated patients, through the p53-dependent MYC transactivation axis, thus leading to reduction of OX40+ CD8 T cells and increase of ICOS+ Tregs, the most suppressive Tregs population; and IDO1 is a critical mediator of Tregs induction, and it is both overexpressed on MSCs through IFN-γ, which is also constitutively released by AML, and on AML blast itself, contributing to Tregs immune tolerance. From (Tettamanti et al., 2022)

A resume of mechanisms of immune network in AML and immune escape is shown in Fig 2.2. AML blasts have developed immunoediting processes, such as genetic deletion of HLAs, especially in the context of the haploidentical HSCT, and epigenetic downregulation of HLA class II molecules in different donor transplant settings, which preclude conventional recognition of AML blasts by CD8 and CD4 T-cells (Tettamanti et al., 2022; Vago and Gojo, 2020).

To evade immune surveillance, AML blasts aberrantly express the ligands for immune checkpoints. Among them, as examples, Programmed-cell-death ligand-1 (PD-L1), once recognized by the PD-1 receptor on T-cells, provides a co-inhibitory signal that causes T-cell exhaustion. Moreover, the PD-1/PD-L1 axis can also promote the expansion of regulatory T-cells (Tregs). T-cell immunoglobulin and mucin domain 3 (TIM-3) binds to its ligand galectin-9, which is highly expressed on AML blasts, and has been found to promote self-renewal via stimulatory β-catenin and NFκB-signaling, and to reduce the release of pro-inflammatory cytokines, ultimately resulting in NK- and T-cell dysfunction (Tettamanti et al., 2022; Williams et al., 2019). Inhibitory receptors such as Cytotoxic T-lymphocyte associated protein 4 (CTLA4) and lymphocyte activating-3 (LAG-3) in AML blasts resulted similarly overexpressed. Of note, AML blasts alter the formation of T-cell immune synapses with an aberrant T-cell activation signature in AML patients, thus impairing T-cell cytotoxic potential and granules (Tettamanti et al., 2022).

Mechanisms of NK-cell evasion have been documented as well and include an altered expression, through epigenetic changes, of NK-cell ligands of activating receptor NKG2D (NKG2DL). Moreover, AML blasts were shown to release a soluble form of NKG2DL, which causes the downregulation of NKG2D receptor on NK-cells. AML blasts may also escape NK-cells by induction of co-inhibitory receptors in NKs that include T Cell Immunoreceptor With Ig And ITIM Domains (TIGIT), which inhibits IFN-γ release. High TIGIT expression at engraftment has been associated with a reduced number of NKs in the BM, reduced incidence of acute graft-versus-host disease and poor survival.

Moreover, AML blasts lead to an altered cytokine and soluble factors milieu. The higher amount of Tregs in AML BM has been associated with capacity of AML blasts to secret immunoinhibitory factors, such as IL-10, IL-35, transforming growth factor-beta (TGF-β), and indoleamine 2,3-dioxygenase 1 (IDO1), promoting immune tolerance and disease progression. IDO1 is an enzyme that catalyzes the rate-limiting step of tryptophan degradation along the kynurenine pathway. Similarly to many solid tumors, IDO1 overexpression is associated with worse outcomes in AML, contributing to the immunosuppressive ME. Proinflammatory signals such as IFN-γ are known to induce IDO1 expression on macrophages and dendritic cells (DCs). In

addition to IDO1 induction *via* inflammatory signals, recent reports have demonstrated that certain subsets of human myeloid DCs might constitutively express IDO1 to suppress allogeneic T-cell immune responses. These data could help identify an IDO1-mediated tolerogenic signature in DCs (Curti et al., 2009; Isidori et al., 2021; Tettamanti et al., 2022). This mechanism belongs to those implemented by myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages, leading to further T-cell tolerance.

Importantly, IDO1 is negatively controlled by the BIN1 tumor suppressor, which in turn is regulated by the RBM25 splicing factor, generating a dominant-negative BIN1 isoform that is unable to repress MYC activity. In previous studies, IDO1 messenger RNA (mRNA) expression in the BM, evaluated by gene expression profiling or quantitative realtime polymerase chain reaction (RT-PCR), was a predictor of shorter survival. In this study, targeted transcriptomic profiles of 24 diagnostic BM samples were analyzed using the NanoString nCounter platform. BIN1 and IDO1 were inversely correlated and individually predicted OS. PLXNC1, a semaphorin receptor involved in inflammation and immune response, was the IDO1-interacting gene retaining the strongest prognostic value. The incorporation of PLXNC1 into the 2-gene IDO1-BIN1 score gave rise to a powerful immune gene signature predicting survival, especially in patients receiving chemotherapy. Taken together, these data indicate that IDO1 is pivotal for the construction of an immune gene signature predictive of survival in AML patients (Ragaini et al., 2022).

Lastly, crucial actors in this interplay are known to be mesenchymal stromal cell (MSCs). Notably, MSCs are not constitutively immune suppressive but rather acquire this capacity, including the ability to induce Tregs, in response to proinflammatory stimuli. Inflammation has recently emerged as a hallmark of cancer, especially in terms of influence over host immune response inhibiting tumor development. One such signal is IFN-γ, a cytokine produced predominantly by T-cells and NK-cells. IFN-γ suppresses hematopoiesis and is a master regulator of innate and adaptive immunity. In the tumor ME, IFN-γ orchestrates an array of antiproliferative, proapoptotic, antitumor, immune-activating features. However, recent emerging paradoxical findings are indicating that IFN-γ can also be involved in pathways belonging to tumorigenesis and immune evasion. In this paper, AML BM aspirates were subdivided according to IFNG expression. Gene expression

profiles in *INF-γ*^{high} and *IFN-γ*^{low} samples were compared by microarray and NanoString analysis. High IFNG expression was associated with poor OS. Of note, IFN-γ release by AML cells positively correlated with a higher BM suppressive Treg frequency. In coculture experiments, IFNghigh AML cells modified MSC transcriptome by upregulating IFN-γ-dependent genes related to Treg induction, including IDO1. In vivo, the genetic ablation of IFN-γ production by AML cells reduced IDO1expression on MSC and Treg infiltration (Corradi et al., 2022).

2.3 Immunogenic cell death

In the view of studying AML patients which have been treated with chemotherapy, beside the assessment of immunological ME of AML at onset, it is duty to report the modification of AML ME after chemotherapic interactions and to introduce the concept of ICD.

The explanation of ICD has been introduced only in recent years, after the identification of many different factors after cell death which can induce tolerance or immunity, overcoming the old concept which previously had assigned this role only to the dichotomic distinction of two principal forms of cell death (apoptosis or necrosis) (Ocadlikova et al., 2019).

The cell death process called ICD induces a type of death which is apoptotic in morphology, but caspase-dependent and highly efficient in immune response induction without any adjuvant. Recent data support the role of chemotherapy in activating the immune response, both in solid tumors and hematologic malignancies, with important therapeutic implications. Several agents capable of triggering ICD have already been identified, which include chemotherapeutic drugs such as cyclophosphamide, oxaliplatin, mitoxantrone, and anthracyclines and in addition ionizing radiations (Fucikova et al., 2011; Ocadlikova et al., 2019).

In particular, as shown in Fig 2.3, as a result of the cytotoxic effect of chemotherapy , the tumor cell undergoes different intracellular and extra/pericellular modifications. Tumor cell death induced by these treatments is able to result in an efficient release of a high amount of tumor-associated antigens (TAAs) from dying

tumor cells, with some factors, known as damage-associated molecular pattern molecules (DAMPs), generated in cell-stress conditions, hypoxia or nutrient depletion. Subsequently, innate immune response begins with presentation to antigen presenting cells (APCs), particularly DCs, which in turn can initiate the anti-tumor TAAs-specific T-cell immune response (Hou et al., 2013; Ocadlikova et al., 2019).

Specifically, during apoptosis, cells undergo a wide variety of biochemical and molecular events resulting in alteration of the plasma membrane, secretion of proteins into the surrounding ME, and activation of intracellular catabolic pathways. Apoptotic bodies, which result altered in composition and surface, induce changes in local infiltration by immune system cells with systemic effects on the immune response (Hou et al., 2013).

The DAMPs produced during ICD include the translocation of the endoplasmic reticulum (ER) chaperones such as calreticulin (CRT) and heat shock proteins 70 and 90 (HSP70 and 90) on cell surface, the adenosine triphosphate (ATP) active secretion, the non-histone chromatin-binding protein high mobility group box 1 (HMGB1) release from nucleus in extracellular milieu and finally, the release of immunostimulatory cytokines, such as type IFN I (Ocadlikova et al., 2019).

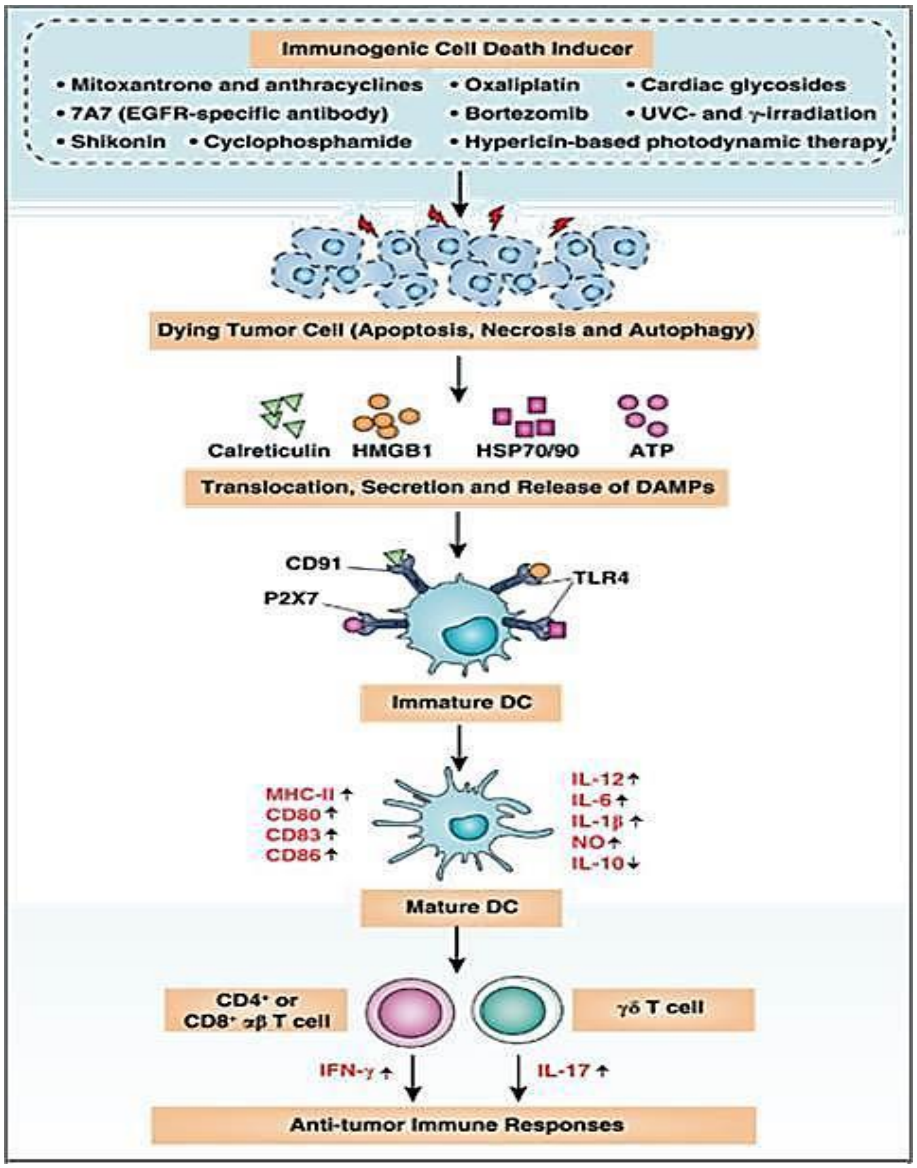


Fig 2.3 Schematic representation of the key events of immunogenic cell death induced by chemotherapy and ionizing radiation. In dying tumor cells, calreticulin and HSPs are translocated to the cell surface; pro-inflammatory factor HMGB1 is released into the extracellular medium and ATP is secreted. These molecules interact with their receptors (CD91, TLR4, P2X7) on DCs determining their maturation and inducing the secretion of pro-inflammatory cytokines that activate the anti-tumor immune response. From (Hou et al., 2013)

Among the processes induced by ICD, one of the most known is the ATP release after the cell death and autofagy. In the subsequent phases of the process, autophagy-dependent secretion of ATP, which binds purinergic receptors present on APCs, promotes recruitment, survival, and differentiation of DCs.

This mechanism of ATP secretion begins in the latest phase of the ICD entire process and it is highly dependent on the apoptotic phase and the type of stress or stimulus that induced it (Fig 2.4). Autophagy is a multistep process that involves

cytoplasmic material sequestration within double-membraned organelles, autophagosomes, and their fusion with lysosomes (Ocadlikova et al., 2019). Autophagy is essential in promoting the formation of LAMP1 (lysosomal-associated membrane protein 1) autolysosomes in which ATP is accumulated and then released outside the cell as a result of vesicle translocation to the cytoplasmic membrane. Exocytosis depends on the caspase-mediated opening of annessin 1 channels. Pharmacological inhibition or gene silencing of autophagosome components, such as Atg5, Atg7, or Beclin-1, results in a significant reduction in ATP release, limiting the immunogenicity of dying tumor cells (Zitvogel et al., 2012).

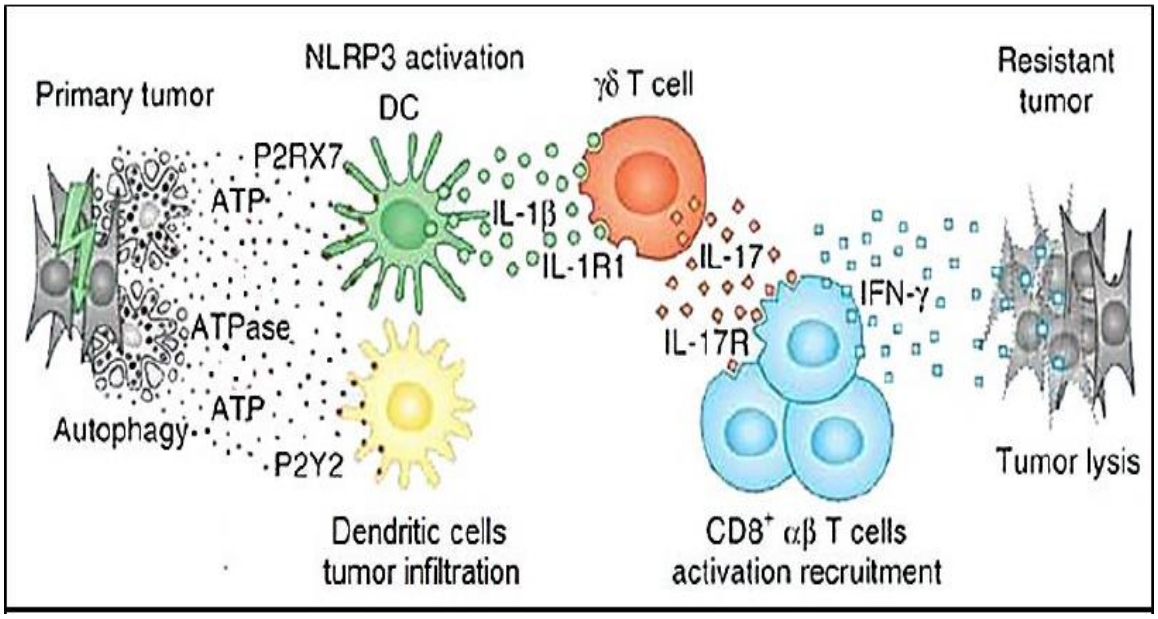


Fig 2.4 Anti-tumorigenic effects of ATP released during immunogenic cell death. From (Zitvogel et al., 2012)

Notably, besides its role as a DAMP molecule, extracellular ATP represents a strong “find me” signal which facilitates APCs, such as DCs, recruitment in sites of massive apoptosis. DCs recruitment in tumor sites is mediated by P2Y2 receptors, whereas activation of P2Y11 receptors on monocytes and DCs induces their maturation (Ocadlikova et al., 2019). Recruitment of DCs to tumor sites occurs through P2Y2 receptor-mediated signaling, binding to P2Y11 subtype receptors, present on monocytes and DCs, however, also induces their maturation (Li et al., 2022; Zitvogel et al., 2012).

Once recruited, naive immune cells need activation signals to increase their anti-tumor activity. Recently, it has been shown that P2X7-type purinergic receptors are

essential for chemotherapy-induced immune response (Ocadlikova et al., 2019). ATP released from dying cells binds P2RX7 receptors present on DCs, resulting in the assembly and activation of NLRP3/ASC/caspase-1 (NOD-like Receptor protein 3) inflammasome, following the generation of Ca2+ and K+ ions flux, and driving the secretion of IL-1β. IL-1β is critical for adequate recruitment of IL-17-secreting T lymphocytes and generation of tumor-specific CD8+IFN-γ+ cytotoxic T lymphocytes (Hou et al., 2013; Zitvogel et al., 2012).

3. Methods NanoString

As for the translational biological section, within the total study population, we analyzed a cohort of AML patients (n=35) whose BM samples were collected.

BM at onset had been collected for all 35 patients of this cohort; among them, 13 samples were also matched with the respective post therapy BM samples.

RNA samples used for these studies had been extracted from the AML BM samples, previously collected and stored, after obtaining informed consents for storage and genetic analysis, in accordance with GCP rules and as approved by the local ethics committee. BM samples were stored at a temperature between -78ºC and -80ºC with continuous temperature monitoring.

3.1 NanoString platform nCounter-Gene expression analysis

As for translational biological section, within the total study population, we analyzed a cohort of marrow samples, which were analyzed after extraction of total RNA from mononuclear cells using technology "*KIT maxwell CSC blood RNA kit (Promega)*". Gene expression analysis was performed using the *nCounter* platform (*NanoString Technologies Inc., Seattle, WA)* (Kulkarni, 2011).

The NanoString technology used in the nCounter analysis system is a digital detection method capable of direct profiling of single molecules in a single reaction without the need for enzymatic reactions and with a very high degree of multiplexing. It therefore enables the study of gene expression by direct, digital detection of nucleic acids using a fully automated system in both the preparation and readout phases and without the need for gene amplification. The principle is based on marking each individual target nucleic acid molecule with a unique color coded-barcode. Infact, mRNA molecules are identified through the use of target-specific, color-coded probe pairs. Specifically, each target gene is detected using a probe pair, a "*reporter*" probe and a "*capture*" probe consisting of specific sequences ranging in length from 35 to 50 bases that bind the target RNA strand making it visible through a fluorescent signal (Kulkarni, 2011).

The gene expression panel used was the PanCancerIO360, a gene expression panel built for the purpose of evaluating genes involved in the immune response in different types of cancer diseases. With this panel, it is possible to analyze the gene expression of 770 key genes that allow the identification of 24 different categories of

infiltrating immune cells, assess immunologic changes in response to immunotherapy, identify tumor-specific antigens and "*housekeeping*" genes, i.e., constitutively expressed, in order to facilitate normalization of samples (Cesano, 2015). Each nCounter reaction involves a total of 150 ng of unfractionated RNA. Probe hybridization is carried out at 65°C for a period of 20 hours. Following hybridization, samples are purified by *NanoString Prep Station* technology and fixed on cartridges. Raw data are acquired through the *nCounter FLEX* analysis system, with a scanning resolution of 555 fields of view. In order to check the correct correlation between RNA-marker and biological pathway of belonging, the "*CodeSet probe annotation file NS_IO360_V1*" was used. Advanced *nSolver* software *(version 2.0.115)* was used for quality control, data normalization and differential expression analysis, according to the manufacturer's instructions.

4. Results NanoString analysis

RNA extracted from mononuclear cells of patients' BM samples was analyzed by NanoString technology using the *PanCancer IO-360* preconstituted gene panel (as explained in the previous paragraph). To assess potential differences in immunological gene expression and different pathways, the strategy of this analysis has been to compare BM samples at diagnosis and samples collected after induction therapy.

Considering the whole 48 BM samples used, 35 were collected at onset and 13 after therapy, as mentioned above. All these 35 patients have been treated with IC and belong to the *"3+7" based* backbone regimen (see further details in paragraph 7). Hence, only 13 pre therapy samples had the corresponding matched post therapy data. It is important to remark that, as for the whole population included in this PhD research, the 13 BM post induction samples represent patients who obtained a complete response (CR) to chemotherapy. Hence, we decided to consider the differences spotted between all 35 onset samples and 13 post induction ones, not only among the matched patients.

The following figures describe the application of the the *PanCancer IO-360* gene expression with 770 key genes involved in the immune response.

4.1 Volcano plot representation

The figure below (Fig 4.1) shows a Volcano plot graph which indicates the gene expression distribution among the whole 48 BM samples from AML patients, subdivided in 35 onset samples and 13 post therapy ones. Volcano plot shows genes of major interest expressed. The onset samples are represented by negative Log2 fold change on the left side of the graph, while post induction samples are represented by positive Log2 fold change on the right side of the graph. Genes who are statistically significant (FDR <0.05, FDR 5%) are marked in red and are shown in the upper part, while at the bottom or the graph non significant genes are marked in blue.

The Volcano plot highlights remarkable gene expression differences between the onset samples and the post induction ones.

Different specific genes expressed in the two subgroups are listed below and properly described according to their function.

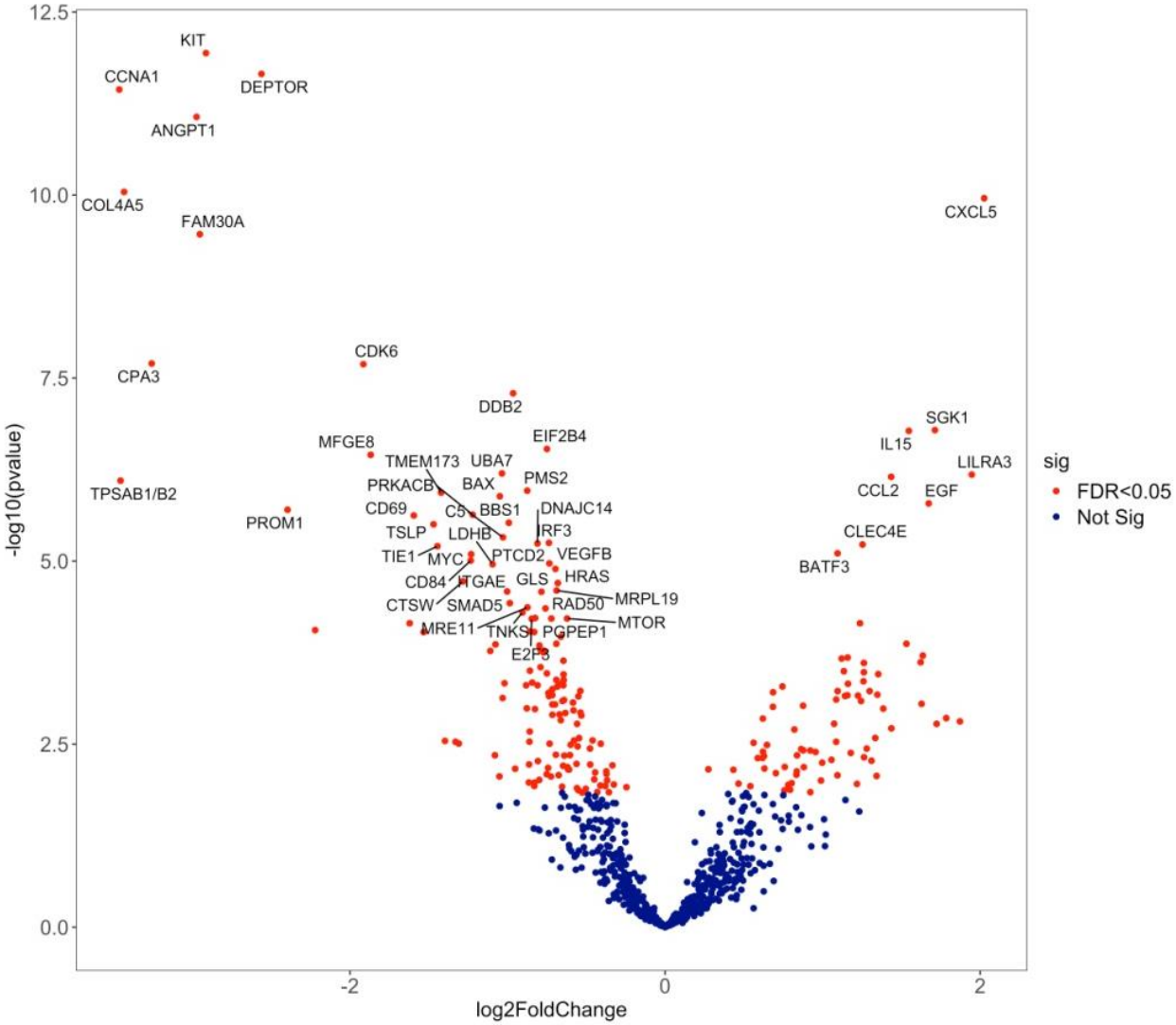


Fig 4.1 Volcano plot shows different gene expression in comparison between onset samples, represented by negative Log2 fold change, and post induction samples, represented by positive Log2 fold change. Genes who are statistically significant (FDR <0.05) are marked in red.

Among onset samples, the most significant gene highlighted are 12: KIT, CCNA1, DEPTOR, ANGPT1, COL4A5, FAM30A, CPA3, CDK6, DDB2, TPSAB1/B2, PROM1 and MFGE8. Secondarily, other genes to be reported significant are EIF2B4, UBA7, TMEM173, PMS2, PRKACB, BAX, CD69, C5, BBS1, DNAJC14, TSLP, LDHB, IRF3, TIE1, MYC, PTCD2, VEGFB, CD84, HRAS, ITGAE, GLS, CTSW, SMAD5, RAD50, MRPL19, MRE11, TNKS, MTOR, PGPEP1 and E2F3. One gene (KIT), commonly known as a proto-oncogene and for

its receptor tyrosine kinase activity, is involved in the process of killing of cancer cells. Three genes are found to be involved in processes of interaction between tumor cells and cells of the immune system (TPSAB1/B2, CD69, FAM30A). Among these only TPSAB1/B2 is physiologically expressed by mast cells; also, CPA3 is expressed by mast cells; both TPSAB1/B2 and CPA3 are further involved in immune cell localization to tumors; PROM1, MFGE8 and CD84 are three other genes involved in this last function and specifically CD84 is expressed normally by macrophages. CCNA1, DDB2, TNKS, PMS2, MRE11 and RAD50 are involved in release of cancer cell antigens. Three genes (CD69, CCNA1, PROM1) are involved in tumor cell recognition by T-cells, and also in T-cell and NK-cell activation and priming. In addition, one gene is involved in antigen presentation processes (UBA7). COL4A5, PGPEP1, E2F3 and BBS1 are included among stromal factors and in common signaling pathways. Five other genes are related to cancer cell "*killing*" processes (TPSAB1/B2, CD69, BAX, IRF3, PROM1). IRF3 participates directly in pathways of response to cytokine signals and is also connected to regulation of interferon-mediated cellular activation. As long as previous listed different functions, PROM1 and MFGE8 belong also to tumor-intrinsic factor and to those with myeloid-cell activity. UBA7 is also are involved in DNA damage repair pathway and involved in interferon-mediated immune responses. Three genes are involved in pathways related to functional regulation of myeloid-derived cells (CD69, TIE1, ANGPT1). In addition, TIE1 is expressed by endothelial cells but also by HSCs, appearing to be involved in processes regulating angiogenesis and cell-matrix adhesion. Three genes, DEPTOR, PTCD2 and GLS, are related to immunometabolic pathways, in particular GLS is expressed in glutamate metabolism and stress response processes. MRPL19 is expressed in proteic metabolism. Among genes described, four are directly involved in pathways that control survival, proliferation, and cell cycle regulation (CCNA1, CDK6, BAX, MTOR). MYC encodes a nuclear phosphoprotein that plays a role in cell cycle progression, apoptosis and cellular transformation; however, it can be found in immunometabolism gene group and common signaling pathways; HRAS shows a similar profile. MTOR belongs to a family of phosphatidylinositol kinase-related kinases, which mediate cellular responses to stresses such as DNA damage and nutrient deprivation; it is also involved in autofagy. VEGFB belongs to family members and regulates the

formation of blood vessels and are involved in endothelial cell physiology. TMEM173, LDHB, ITGAE and TSLP are gene members of different immunological functions already listed, including T-cell and NK-cell activation and priming, immune cell localization to tumors, cancer antigen presentation, immunometabolism but also in tumor-intrinsic factor and myeloid-cell activity. CTSW is normally expressed in cytotoxic cells and can be found in cancer antigen presentation and in immune cell localization to tumors. EIF2B4, SMAD5 and PRKACB are to be included in common signaling pathways genes. DNAJC14 and MRPL19 are listed in internal reference genes. BAX is strictly related to apoptosis.

Among the post induction samples, the most significant genes highlighted are 8: CXCL5, SGK1, IL15, LILRA3, CCL2, EGF, CLEC4E and BATF3. CXCL5 is a member of the CXC subfamily of chemokines; chemokines, which recruit and activate leukocytes, are classified by function (inflammatory or homeostatic) or by structure. CXCL5 is thus involved in immune cell localization to tumors. SGK1 is a gene connected to immunometabolism; encodes a serine/threonine protein kinase that plays an important role in cellular stress response. IL15 is a cytokine that regulates T- and NK-cells activation and proliferation. This cytokine and interleukine 2 share many biological activities. They are found to bind common hematopoietin receptor subunits, and may compete for the same receptor, and thus negatively regulate each other's activity. This cytokine induces the activation of JAK kinases, as well as the phosphorylation and activation of transcription activators STAT3, STAT5, and STAT6. IL15 is involved in T-cell priming and activation; costimulatory signaling; in immune cell localization to tumors; recognition of cancer cells by T-cells; killing of cancer cells, but also myeloid cell activity. LILRA3 belongs to cancer antigen presentation genes. CCL2 is a chemokine member of the CC subfamily which is characterized by two adjacent cysteine residues. This cytokine displays chemotactic activity for monocytes and basophils but not for neutrophils or eosinophils. It has been implicated in the pathogenesis of diseases characterized by monocytic infiltrate; among its functions there are T-cell priming and activation; in immune cell localization to tumors; recognition of cancer cells by T-cell; killing of cancer cells, but also myeloid cell activity. EGF acts a potent mitogenic factor that plays an important role in the growth, proliferation and differentiation of numerous cell types; it is present in common signaling pathways. CLEC4E gene functions are in immune

cell localization to tumors, immune cell adhesion and migration, and myeloid cell activity. BATF3 encodes a member of the basic leucine zipper protein family. The encoded protein functions as a transcriptional repressor when heterodimerizing with JUN. The protein may play a role in repression of interleukin-2 and matrix metalloproteinase-1 transcription; among its functions T-cell priming and activation and cancer antigen presentation, as well as immunometabolism and common signaling pathways. The most involved pathways with potential correlation in oncology are JAK-STAT, MAP-kinase cascade, NfKB pathway and PI3K-AKT pathway.

4.2 Gene pathways heatmap representation

In the following representation of the different gene expression fashion between onset and post induction samples, we have chosen the heatmap representation graph (Fig 4.2) showing the most statistically significant gene pathways (n = 100) involved in the samples in study.

The columns represent every one of the 48 BM samples of the study, both onset and post therapy, including the 13 ones which are matched. Onset samples are represented in blue which are mainly located in the left side of the graph and post induction ones in red, in the right side of it. The horizontal lines identify the most significant gene pathway selected from the panel which has been applied to our samples. Each sample is matched with every single lines of pathway defined. The level of expression of every single pathway is represented by a different grade of colour, from the “purple” which indicates down-regulated pathway to the “pink” which stands for up-regulated pathway (GVSA score).

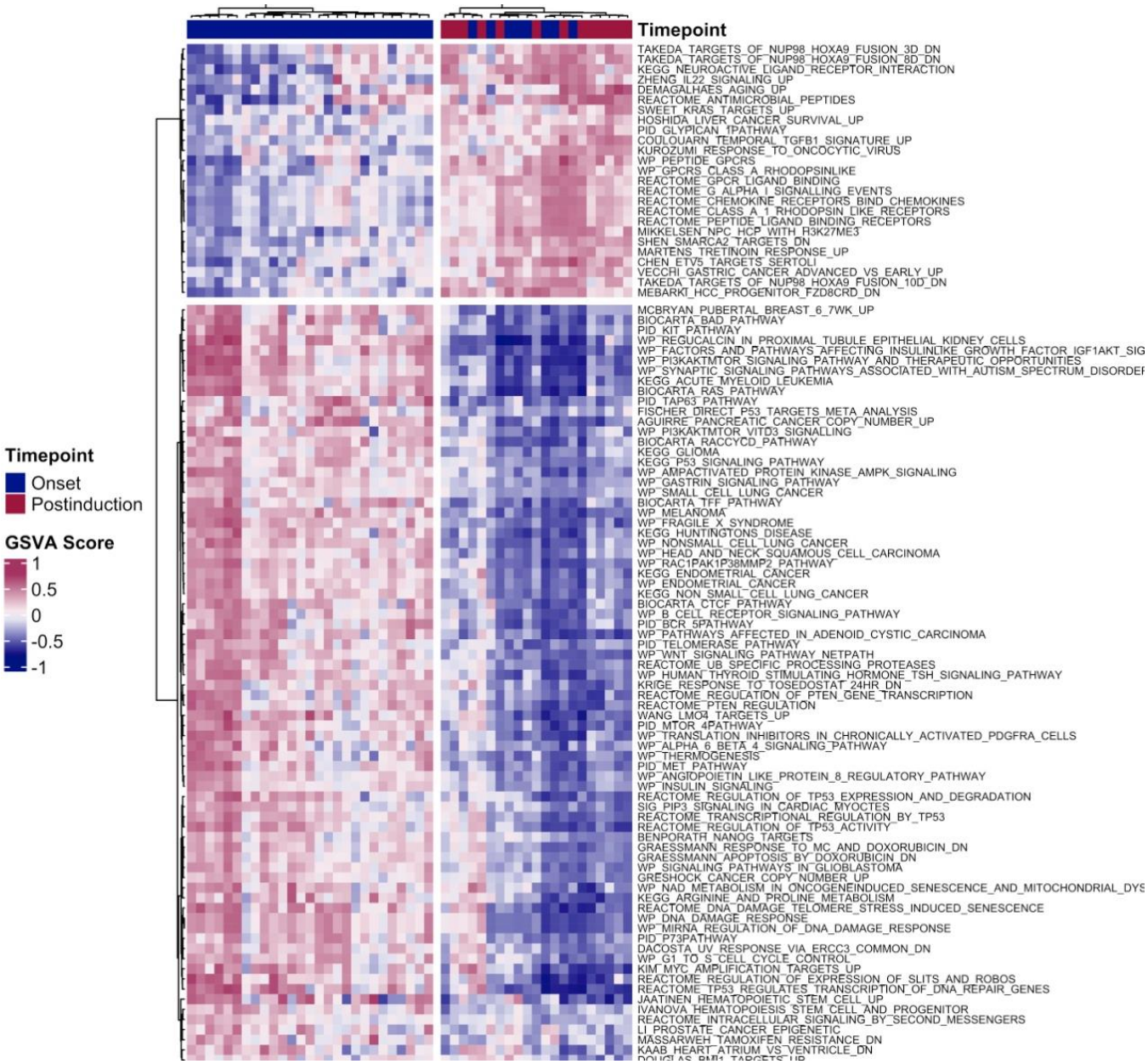


Fig 4.2 Heatmap showing the most statistically significant gene pathways involved in the samples in study. Each sample is matched with every single line of pathway defined. The level of expression of the pathways is represented by a different grade of colour, from the "purple" which indicates down-regulated pathway to the "pink" which stand for up-regulated pathway. The comparison between the two-column group, representing the two timepoints group studied, shows a quite clear and specular distinction in gene pathways expression.

Considering the different pathways shown in the heatmap, in a similar way described by the previous Volcano plot, there is quite a straight differentiation in terms of gene pathways expression between onset and post therapy samples. In fact, as indicated in the upper part of the graph, all the pathways which are up-regulated (towards pink) in the post induction (collected from patients who obtained CR) result down-regulated (towards purple) in the onset samples; instead, in a specular fashion,

all the pathways which are up-regulated at onset disease correspond to a down-regulated expression of the same pathway in the post induction phase.

Of note, the heatmap reveals that the difference of gene expression is not perfectly specular; in fact some of the onset samples have been included among the post induction group, as these samples shared the level of gene expression (regarding most of the pathways) together with the other subgroup. We can conclude that further analysis are needed in order to explain the reason of these exceptions and this behavior of expression which results similar to remission phase.

Among the 100 gene pathways considered and statistically significant in the heatmap, the most remarkable in the immunological network described in AML are listed below, with a brief resume of their functions.

- COULOUARN_TEMPORAL_TGFB1_SIGNATURE_UP: this gene encodes a secreted ligand of the TGF-beta (transforming growth factor-beta) superfamily of proteins. It regulates cell proliferation, differentiation and growth, and can modulate expression and activation of other growth factors including interferon gamma and tumor necrosis factor alpha.
- ZHENG_IL22_SIGNALING-UP: IL22 takes effect on non-hematopoietic cells, mainly stromal and epithelial cells, stimulating survival, proliferation and sintesis of antimicrobial peptides and chemokines; IL22 partecipates in would healing, tissue remodelling and in protection against microbes.
- REACTOME_CHEMOKINE_RECEPTORS_BIND_CHEMOKINES: chemokine receptors are cytokine receptors found on the surface of certain cells, which interact with a type of cytokine called chemokines. Following the interaction, these receptors trigger a flux of intracellular calcium which leads to chemotaxis. Chemokine receptors are divided into different families, CXC chemokine receptors, CC chemokine receptors, CX3C chemokine receptors and XC chemokine receptors that correspond to the 4 distinct subfamilies of chemokines they bind to.
- REACTOME_PEPTIDE_LIGAND_BINDING_RECEPTORS: these receptors, a subset of the Class A/1 (Rhodopsin-like) family, all bind peptide ligands which include the chemokines, opioids and somatostatins.
- BIOCARTA_BAD_PATHWAY: BAD is a pro-apoptotic molecule; its phosphorylation in three sites results in loss of the ability of BAD to

heterodimerize with the survival proteins BCL-XL or BCL-2. Phosphorylated BAD is sequestered in the cytoplasm. Its phosphorylation is concordant with the activation of Akt and of the Ras-MAPK pathway.

- REACTOME_ANTIMICROBIAL_PEPTIDES: Antimicrobial peptides (AMPs) are small molecular weight proteins with broad spectrum of antimicrobial activity against bacteria, viruses, and fungi. The majority of known AMPs are cationic peptides with common structural characteristics where domains of hydrophobic and cationic amino acids are spatially arranged into an amphipathic design, which facilitates their interaction with bacterial membranes. Besides the direct neutralizing effects on bacteria, AMPs may modulate cells of the adaptive immunity (neutrophils, T-cells, macrophages) to control inflammation and/or to increase bacterial clearance.
- SWEET_KRAS_TARGETS_UP: Kirsten ras oncogene, encoder for a member of the small GTPase superfamily. Mutated protein is involved in many type of malignancies.
- WP_PI3KAKTMTOR_SIGNALING_PATHWAY_AND_THERAPEUTIC_OPPORTUNITIES: this signaling pathway is highly conserved signal trasduction network in eukaryotic cells and promotes cell survival, cell growth and cell cycle progression. It is strongly regulated by multiple cross-interaction with several more pathways and their dysregulation can lead to cancer development, treatment resistance and disease progression.
- KEGG_ACUTE_MYELOID_LEUKEMIA: a pathway which resumes both activating mutations of signal transduction intermediates and alterations in myeloid transcription factors governing hematopoietic differentiation, genetic events which are crucial for the molecular pathogenesis of AML. Both aberrant and constitutive activation of signal transduction molecules are found in about 50% of primary AML BM samples, such as FLT3, NRAS, KRAS, KIT.
- BIOCARTA_RAS_PATHWAY: the effectors mediate Ras stimulation to a diverse set of cellular signals. Many of these signals are interpreted differently depending on the cell type or ME receiving the stimulus. Not all of these effectors are activated in any given cell type.
- PID_TAP63_PATHWAY: TAP63 is a p53 omologous which plays an important role in various biological processes, including cell proliferation,

differentiation, and apoptosis. However, how TAp63 is regulated remains largely unclear. It has been demonstrated that NF-κB induces TAp63 gene expression.

- KEGG_P53_SIGNALING_PATHWAY: p53 activation is induced by a number of stress signals, including DNA damage, oxidative stress and activated oncogenes. The p53 protein is employed as a transcriptional activator of p53-regulated genes. This results in three major outputs: cell cycle arrest, cellular senescence or apoptosis. Other p53-regulated gene functions communicate with adjacent cells, repair the damaged DNA or set up positive and negative feedback loops that enhance or attenuate the functions of the p53 protein and integrate these stress responses with other signal transduction pathways.
- WP_B_CELL_RECEPTOR_SIGNALING_PATHWAY: the B cell antigen receptor (BCR) is composed of membrane immunoglobulin (mIg) molecules and associated Igα/Igβ (CD79a/CD79b) heterodimers (α/β). The mIg subunits bind antigen, resulting in receptor aggregation, while the α/β subunits transduce signals to the cell interior. BCR aggregation rapidly activates the Src family kinases Lyn, Blk, and Fyn as well as the Syk and Btk tyrosine kinases. This initiates the formation of a 'signalosome' composed of the BCR, the mentioned tyrosine kinases, adaptor proteins such as CD19 and BLNK, and signaling enzymes such as PLCγ2, PI3K, and Vav. Signals emanating from the signalosome activate multiple signaling cascades that involve kinases, GTPases, and transcription factors. This results in changes in cell metabolism, gene expression, and cytoskeletal organization. The complexity of BCR signaling permits many distinct outcomes, including survival, tolerance (anergy) or apoptosis, proliferation, and differentiation into antibody-producing cells or memory B cells. The outcome of the response is determined by the maturation state of the cell, the nature of the antigen, the magnitude and duration of BCR signaling, and signals from other receptors such as CD40, the IL-21 receptor, and BAFF-R. Many other transmembrane proteins, some of which are receptors, modulate specific elements of BCR signaling, including CD45, CD19, CD22, PIR-B, and FcγRIIB1 (CD32). The magnitude and duration of BCR signaling are limited by negative feedback loops including

those involving the Lyn/CD22/SHP-1 pathway, the Cbp/Csk pathway, SHIP, Cbl, Dok-1, Dok-3, FcγRIIB1, PIR-B, and internalization of the BCR. In vivo, B cells are often activated by antigen-presenting cells that capture antigens and display them on their cell surface. Activation of B cells by such membrane-associated antigens requires BCR-induced cytoskeletal reorganization. BIOCARTA_CTCF_PATHWAY: CTCF (a First Multivalent Nuclear Factor) is central to signaling pathways in immature B cells elicited by cross-linking the Ig BCR and stimulation with TGF. Both stimuli result in induction of cell cycle arrest and apoptosis. BCR ligation stimulates a transient induction of MYC that leads to high level CTCF expression and feedback suppression of MYC transcription. BCR ligation also activates PTEN, opposing PI3K activation of MYC. Pharmacologic inactivation of PI3K or mTOR/FRAP results in suppression of S6K resulting in activation of CTCF and suppression of MYC. CTCF activation induces transcriptional activation of p19ARF, with its downstream consequences, and of p27. Growth arrest is occasioned by co-expression of p21 and p27 and inhibition of MYC. CTCF, is bona fide multivalent DNA-sequence binder whose specificity is mediated by different sets of zinc fingers (ZFs).

- WP_WNT_SIGNALING_PATHWAY_NETPATH: Wnt family of proteins are a large family of cysteine-rich secreted glycoproteins that regulate cell-cell interactions. They bind to members of the Frizzled family of 7 transmembrane receptors. Binding of Wnt to its receptors leads to activation of at least 3 distinct pathways: the canonical beta catenin pathway, the planar cell polarity pathway and the calcium pathway.
- REACTOME_REGULATION_OF_PTEN_GENE_TRANSCRIPTION: transcription of the PTEN gene is regulated at multiple levels. It identifies a tumor suppressor gene that is mutated at high frequencies in a large numebr of cancers. It negatively regulates the AKT/PKB signaling pathway.
- WP_ALPHA_6_BETA_4_SIGNALING_PATHWAY: the alpha6beta4 integrin is a component of Hemidesmosomes (HDs). Increased expression of alphaEbeta4 and changes in its distribution is found to be correlated with increased aggressiveness of tumors and poor prognosis. The cancer cell invasion is regulated through a IRS/PI3K dependent process while the effect

on carcinoma cell survival in a PI3K/Akt and dependent manner. Activation of the transcription factors NFkB and NF-IL6 in a p38Mapk dependent pathway and subsequent activation of IL6 gene expression was shown to be mechanism of alpha6beta4 induced survival of thymocytes and proliferation of thymic epithelial cells. Integrin alpha6beta4 is also known to activate the Ras/Raf/MEK/ERK cascade which is found to be involved in the regulation of cell cycle.

- REACTOME_REGULATION_OF_TP53_EXPRESSION_AND_DEGRADATION: TP53 tumor suppressor protein is a transcription factor that functions as a homotetramer. The protein levels of TP53 are low in unstressed cells due to MDM2-mediated ubiquitination that triggers proteasome-mediated degradation of TP53. The E3 ubiquitin ligase MDM2 functions as a homodimer/homo-oligomer or a heterodimer/hetero-oligomer with MDM4 (MDMX). Activating phosphorylation of TP53 at serine residues S15 and S20 in response to genotoxic stress disrupts TP53 interaction with MDM2. Binding of MDM2 to TP53 is also inhibited by the tumor suppressor p14-ARF, transcribed from the CDKN2A gene in response to oncogenic signaling or oxidative stress.
- KIM_MYC_AMPLIFICATION_TARGETS_UP: a proto-oncogene that encoder for a phosphoprotein that plays a role in cell cycle progression, apotosis and cellular transformation. It forms a heterodimer with the related transcription factor MAX, regulated the transcription of targeted genes. Its amplification is frequently observed in many human cancers.
- JAATINEN_HEMATOPOIETIC_STEM_CELL_UP: characteristics of primitive hematopoietic cells, in particular note with genes associated with metabolism, cellular physiological processes, cell communication, and development; express primitive markers and possess clonogenic progenitor capacity.
- IVANOVA_HEMATOPOIESIS_STEM_CELL_AND_PROGENITOR: genes up-regulated in HSCs and progenitors from adult BM and fetal liver; considering global gene expression profiles for mouse and human HSCs and other stages of the hematopoietic hierarchy, murine and human HSCs share a number of expressed gene products, which define key conserved regulatory

pathways in this developmental system, producing a molecular signature of stem cells.

4.3 Transcriptional factors heatmap representation

Another heatmap representation is shown below. In a similar way to the previous one, the differences in gene expression between the two timepoints included (onset and post induction) are highlighted in the following heatmap graph, matching the transcriptional factors (Fig 4.3) which have resulted in the most statistically significant (n = 63) involved in the samples in study.

In this heatmap, onset samples are represented in blue which are mainly located in the middle part of the graph; post induction samples are in red and most are shown in the left side of it. The horizontal lines identify the most significant transcriptional factors selected from the panel which has been applied to our samples. Each sample is matched with every single line of pathway defined. The level of expression of every single pathway is represented by the same different grades of color (purple or pink) as the previous graph. Of note, the right-sided group of columns represents both onset and also some post therapy samples and this third part of the population has been separated according to a different and less evident behavior.

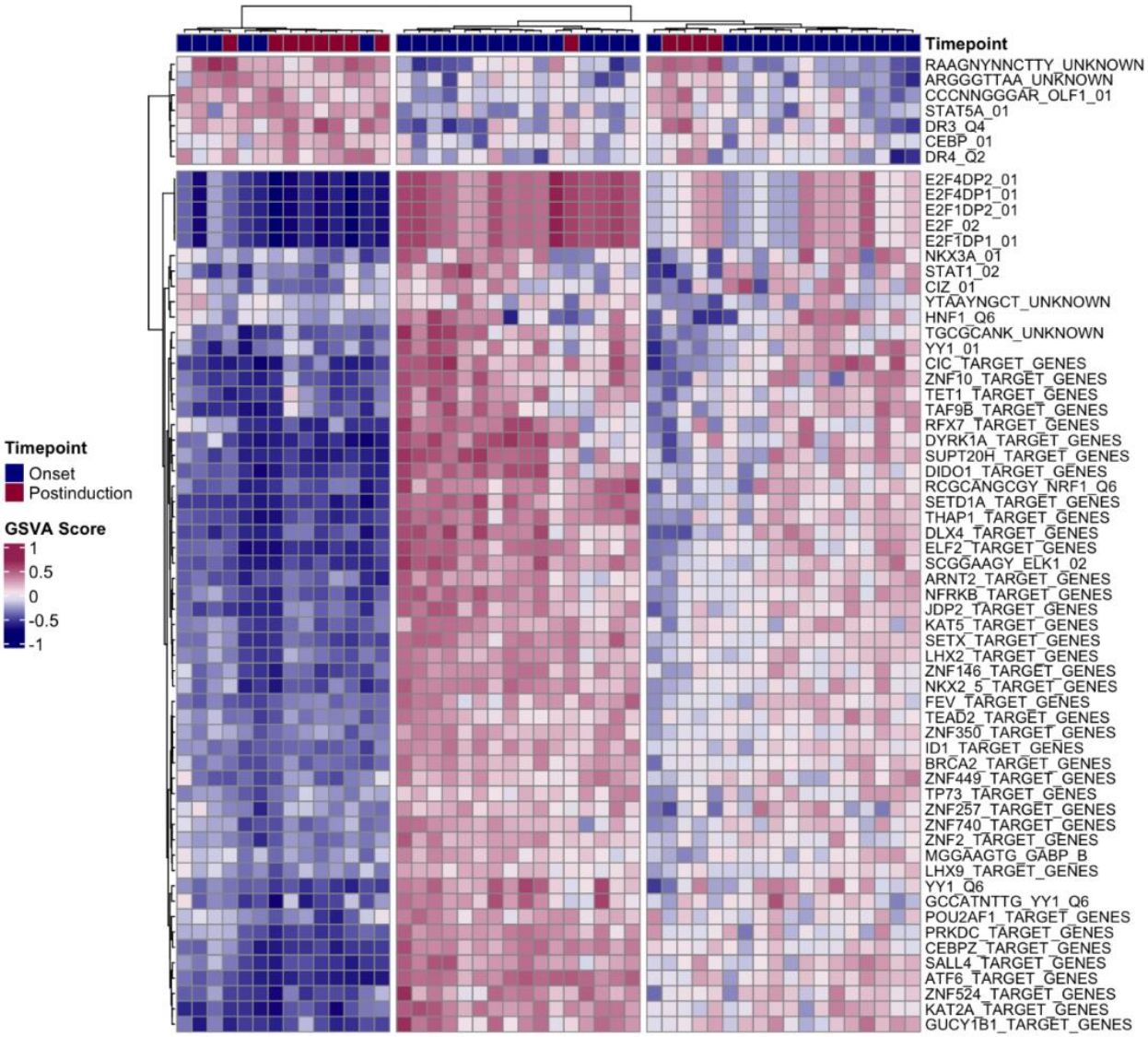


Fig 4.3 Heatmap showing the most statistically significant transcriptional factors involved in the samples in study. Each sample is matched with every single lines of pathway defined. The level of expression of the pathways is represented by a different grade of color, from the "purple" which indicates down-regulated pathway to the "pink" which stand for up-regulated pathway. The comparison between the left column group (mainly post induction samples) and the middle one (mainly onset samples) confirm a substantial difference in gene involved. The third column on the right, which both timepoints have been included in, shows a more heterogeneous fashion, which needs deeper analysis.

Considering the different transcriptional factors shown in the heatmap, the comparison between the onset samples, which mainly belong to the middle column group, and the left side, which mainly includes post induction ones, highlights a clear and specular distinction in transcriptional factors gene expression. Infact, as shown in the upper part of the graph, transcriptional factors which are up-regulated (towards

pink) in the post induction responsive samples correspond to down-regulated result (towards purple) in the onset samples in the middle column; instead, specularly, in the lower part of the heatmap, all genes up-regulated at onset disease in the middle part of the graph are found with a down-regulated expression in the post induction timepoint.

Of note, the third column on the right side represents an exception, which both timepoints samples have been included in. Different genes are more heterogeneously expressed among both timepoints fashion. This needs more extensive analysis focusing on the explanation of these similarities in these patients.

Among the 63 transcriptional factors considered and statistically significant in the heatmap, the most remarkable in the immunological network described in AML are listed below, with a brief resume of their functions and their specific behavior evaluated in our cohort.

- CCCNNGGGAR_OLF1_01 (annotation for EBF2): hematopoiesis requires the interaction of HSCs with various stromal MEs. Early B cell factor 2 (EBF2), a transcription factor expressed in a subset of immature osteoblastic cells, when knocked out induces decreased frequencies of HSCs and lineage-committed progenitors. This defect is cell nonautonomous, as shown by the fact that transplantation of EBF2-deficient BM into wild-type hosts results in normal hematopoiesis. EBF2 acts as a transcriptional determinant of an osteoblastic niche that regulates the maintainance of hematopoietic progenitors, in part by modulating Wnt signaling. In the entire heatmap, it is evident how it is upregulated in post therapy (remission) samples.
- STAT5A: this gene is a member of the STAT family of transcription factors. In response to cytokines and growth factors, STAT family members are phosphorylated by the receptor associated kinases, and then form homo- or heterodimers that translocate to the cell nucleus where they act as transcription activators. This protein is activated by and mediates the responses of many cell ligands, such as IL2, IL3, IL7, GM-CSF, erythropoietin, thrombopoietin and different growth hormones. Activation of this protein in myeloma and lymphoma associated with a TEL/JAK2 gene fusion is independent of cell stimulus and has been shown to be essential for tumorigenesis. The mouse counterpart of this gene is found to induce the

expression of BCL2L1/BCL-X(L), which suggests the antiapoptotic function of this gene in cells.

- E2F4: Members of the E2F family contain many important genes that regulate the cell cycle, DNA damage repair and development. E2F4 is a transcription factor that contributes to controlling the cell cycle. E2F1-3-deficient hematopoietic cells have defects in myeloid cell differentiation, with an accumulation of granulocyte/macrophage progenitor (GMP) cells and a decrease in CD11b+ myeloid cells in the BM. Therefore, E2F1-3 are essential for cell survival and proliferation during the differentiation of BM cells. Regarding E2F4 and its role in the progression of AML, human clinical data revealed that increased E2F4 expression was associated with poor prognosis in AML patients. Moreover, experimental results showed that E2F4 was aberrantly overexpressed in human AML patients and cell lines. Depletion of E2F4 inhibited the proliferation, induced the differentiation and suppressed the growth of AML cells in a nude mouse model. By contrast, overexpression of E2F4 promoted the proliferation and inhibited the differentiation of AML cells in vitro. Additionally, E2F4 expression not only is positively correlated with EZH2 but also can bind to EZH2. RNA microarray results also showed that E2F4 can regulate MAPK signaling pathway. EZH2 can reverse the inhibitory effect of E2F4 silencing on MAPK signaling pathway. In perspective, E2F4 may be a potential therapeutic target for AML therapy (Feng et al., 2020). In the presented heatmap, its expression among onset and post induction is perfectly specular, being strongly up-regulated in new onset patients.
- CEBP_01 (annotation for CEBPa): CEBPa encodes a transcription factor that contains a basic leucine zipper (bZIP) domain and recognizes the CCAAT motif in the promoters of target genes. The encoded protein functions in homodimers and also heterodimers with CCAAT/enhancer-binding proteins beta and gamma. Activity of this protein can modulate the expression of genes involved in cell cycle regulation as well as in body weight homeostasis. Mutation of this gene is associated with AML onset.
- STAT1: in response to cytokines and growth factors, STAT family members are phosphorylated by the receptor associated kinases, and then form homo-

or heterodimers that translocate to the cell nucleus where they act as transcription activators. The protein encoded by this gene can be activated by various ligands including interferon-alpha, interferon-gamma, EGF, PDGF and IL6. This protein mediates the expression of a variety of genes, which is thought to be important for cell viability in response to different cell stimuli and pathogens. Our graph enlights its slight down-regulation during post therapy.

- CIZ_01 or ZNF384 (Zinc Finger protein 384): this gene encodes a C2H2-type zinc finger protein, which may function as a transcription factor. The protein appears to bind and regulate the promoters of the extracellular matrix genes MMP1, MMP3, MMP7 and COL1A1. Studies in mouse suggest that nuclear matrix transcription factors (NP/NMP4) may be part of a general mechanical pathway that couples cell construction and function during extracellular matrix remodeling. Recurrent rearrangements of this gene with the Ewing's sarcoma gene, EWSR1 on chromosome 22, or with the TAF15 gene on chromosome 17, or with the TCF3 (E2A) gene on chromosome 19, have been observed in acute leukemias. Diseases associated with ZNF384 include Mixed Phenotype Acute Leukemias. Similarly to the previous gene, it results down-regulated post therapy.
- YY1_01: YY1 is a transcription factor that acts as an activator, repressor, or initiator of transcription. These functions are realized due to its ability to interact with DNA. However, the role of YY1 goes beyond the classic functions of the transcription factor, since it can perform some of its functions without interacting with DNA, but rather with other proteins and RNA. However, even when considering YY1's function only as a transcription factor, its interaction with other elements, particularly with proteins, seems to be as important as its interaction with DNA. Many possible interactions that modify the components of the transcriptional machinery and the disordered flexible structure make YY1 a likely candidate for a hub protein, thus influencing transcription on many levels. According to this, in our cohort it is shown to be importantly up-regulated at AML disease onset.
- ZNF10: the protein encoded by this gene contains a C2H2 zinc finger, and has been shown to function as a transcriptional repressor. The Kruppel-

associated box (KRAB) domain of this protein is found to be responsible for its transcriptional repression activity. RING finger containing protein TIF1 was reported to interact with the KRAB domain, and may serve as a mediator for the repression activity of this protein. It results upregulated at onset.

- TET1: DNA methylation is an epigenetic mechanism that is important for controlling gene expression. The protein encoded by this gene is a demethylase that belongs to the TET (Ten-Eleven Translocation) family. Members of the TET protein family play a role in the DNA methylation process and gene activation. Strongly overexpressed in onset BM.
- DIDO1: in mice, the Death Inducer-Obliterator 1 is upregulated by apoptotic signals and encodes a cytoplasmic protein that translocates to the nucleus upon apoptotic signal activation. When overexpressed, the mouse protein induced apoptosis in cell lines growing in vitro. This gene is similar to the mouse gene and therefore is thought to be involved in apoptosis. Diseases associated with DIDO1 include myeloproliferative neoplasm.
- ELF2: enables DNA-binding transcription factor activity, RNA polymerase II-specific and sequence-specific double-stranded DNA binding activity. Involved in negative regulation of transcription, positive regulation of transcription and regulation of transcription by RNA polymerase II. Among its related pathways are gene transcription and immune response IL23 signaling pathway.
- ELK1_02: This gene is a member of the Ets family of transcription factors and of the ternary complex factor (TCF) subfamily. Proteins of the TCF subfamily form a ternary complex by binding to the the serum response factor and the serum response element in the promoter of the c-fos proto-oncogene. The protein encoded by this gene is a nuclear target for the ras-raf-MAPK signaling cascade. Among its related pathways are Toll Like Receptor 7/8 (TLR7/8) cascade and prolactin signaling.
- NFRKB: enables protease binding activity. Predicted to be involved in DNA recombination and DNA repair. Diseases associated with NFRKB include Fanconi Anemia and High-Grade B-Cell Lymphoma Double-Hit/Triple-Hit.

- JDP2: Jun Dimerization Protein 2 enables DNA-binding transcription repressor activity, RNA polymerase II-specific and sequence-specific double-stranded DNA binding activity. Involved in negative regulation of transcription by RNA polymerase II. Diseases associated with JDP2 include Granulomatous Amebic Encephalitis and Primary Amebic Meningoencephalitis. Among its related pathways are IL-1 Family Signaling Pathways and Tacrolimus/Cyclosporine Pathways.
- TP73: this gene encodes a member of the p53 family of transcription factors involved in cellular responses to stress and development. It is frequently deleted in neuroblastoma and other tumors, and thought to contain multiple tumor suppressor genes. Participates in the apoptotic response to DNA damage. Isoforms containing the transactivation domain are pro-apoptotic, isoforms lacking the domain are anti-apoptotic and block the function of p53 and transactivating p73 isoforms. May be a tumor suppressor protein. Its expression in our population results actually pretty heterogeneous.
- PRKDC: Protein Kinase, DNA-Activated, Catalytic Subunit encodes the catalytic subunit of the DNA-dependent protein kinase (DNA-PK). The protein encoded is a member of the PI3/PI4-kinase family. Diseases associated with PRKDC include Immunodeficiency 26 With Or Without Neurologic Abnormalities and Severe Combined Immunodeficiency. It is strongly associated with AML onset in our samples.
- KAT2A: Lysine Acetyltransferase 2A is a histone acetyltransferase (HAT) that functions primarily as a transcriptional activator. It also functions as a repressor of NFkB by promoting ubiquitination of one subunit in a HAT-independent manner.

5. ALC in hematological and solid neoplasms

The role of ALC has been a fairly unexplored topic in the field of malignancies to date. The research group of Porrata LF and colleagues at the Mayo Clinic College of Medicine is the one that had evaluated it most.

Some studies had shown the correlation between low recipient ALC during thymoglobulin (ATG) administration and shorter relapse-free survival (RFS) after HSCT (Modi et al., 2020). Early observations had arisen from the setting of autologous transplantation in non-Hodgkin B lymphomas (NHL). A retrospective study had considered ALC at day 15 after autologous transplantation in patients with aggressive NHL and also multiple myeloma (MM); considering a cut-off of ALC 500/mm^3, this parameter in multivariate analysis had been shown to be an independent predictor of OS and progression-free survival (PFS), with higher medians in case of ALC ≥500/mm^3 (L. F. Porrata et al., 2001). More recent studies had similarly confirmed the validity of ALC at recovery after transplantation in patients with diffuse large B-cell lymphoma (DLBCL) (Kim et al., 2017) and MM, despite the adoption of different cut-offs for the analyses (Jimenez-Zepeda et al., 2015; Suriu et al., 2016).

The value of ALC at post autologous transplantation recovery in Hodgkin's lymphomas (HL) was found to be similarly predictive of better survival (L. F. Porrata et al., 2002) and a more recent prospective study confirmed the clinical significance of early immunological recovery in these patients (Valtola et al., 2016). Some authors have also studied the same parameter at disease onset in patients with lymphoma and in particular follicular lymphoma, with significant results in terms of predictive of response to therapy, PFS, and OS in retrospective analyses, both considering ALC individually and as a relationship with absolute monocyte count (AMC), which conversely had an inverse correlation with outcome (Mohsen et al., 2020).

Pursuing the same goal, attention has also recently been paid to the lymphocyte count assessed on the units of autologous cells infused in the setting of DLBCLs. Compared with any other historically known prognostic parameter for survival in lymphomas, a count ≥500/mm^3/Kg reconfirmed the prognostic value of ALC as an independent predictor of outcome and suggesting, accordingly, that the infused

lymphocyte proportion may be equally important to that of autologous stem cells for the transplant success (Porrata et al., 2021).

It is worth reporting how similar observations are also derived from immunologic recovery (ALC) post autologous transplantation in the experience of solid neoplasms, including breast cancer (L. Porrata et al., 2001) and melanoma, regardless of the addition of immune checkpoint inhibitors to treatment (Postow et al., 2020).

In the setting of acute lymphoblastic leukemia (ALL) in children, this study revealed, like previous retrospective analyses (De Angulo et al., 2008), considering the value of ALC $\geq 750/mm^3$ at day 29 from induction therapy, using a multiple linear regression model, an inverse relationship with the number of post therapy BM blasts, proposing ALC as an important prognostic factor (Hirase et al., 2015).

6. ALC in AML

Similarly to the significant data from other malignancies, ALC as a prognostic factor has also been evaluated in the setting of AML.

The literature on the impact of ALC regarding the outcome post HSCT in AML reports conflicting data (Thoma et al., 2012). In these analysis of 191 patients transplanted for AML, an ALC $\geq 500/mm^3$ at day 28 post HSCT was associated with a reduced rate of post-transplant relapse on multivariate analysis; however, no impact on OS had been demonstrated (Michelis et al., 2014).

For the post autologous transplantation recovery phase in AML, the evaluation of ALC at day 15 after transplantation, with $500/mm^3$ as the cut-off, had again been proposed by the research group. Compared with patients who did not reach this threshold, AML patients with a count $\geq 500/mm^3$ had higher median OS and disease-free survival (LFS) (L. Porrata et al., 2002). In addition, elevated ALC even at disease onset showed an impact on reduced RFS after both one and two cycles of chemotherapy (Le Jeune et al., 2014).

These studies had shown a role of ALC as a predictive index in AML mainly in the post-transplant setting, but not after chemotherapy.

Therefore, in order to estimate the impact of ALC in the recovery phase after induction chemotherapy (IC), a study evaluated 103 newly diagnosed AML patients treated with chemotherapy from 1998 to 2002 at the Mayo Clinic (Behl et al., 2006). These were mainly patients treated with the standard “3+7” chemotherapy scheme and some with acute promyelocytic leukemia (APL) treated with all-trans retinoic acid (ATRA) and idarubicin. The primary end point was to evaluate the impact of early ALC recovery on OS and LFS. ALC had been collected at 4 separate time points, on days +15, +21, +28 from the start of therapy and also before consolidation therapy (CC). The main data of ALC had been compared with the main known prognostic factors of AML: age, sex, FAB classification, cytogenetics, secondarily, LDH, hyperleukocytosis. Among the 60 patients who died from disease relapse or progression, only 28% had reached the $500/mm^3$ ALC cut-off in all time-points, while 72% had not. Multivariate analysis, to estimate the significance of ALC as an independent predictor of CR, had considered only the 85 out of 103 patients who had

not benefited from HSCT and, in a logistic regression model, both proved to be significant compared with the other prognostic indices (Behl et al., 2006).

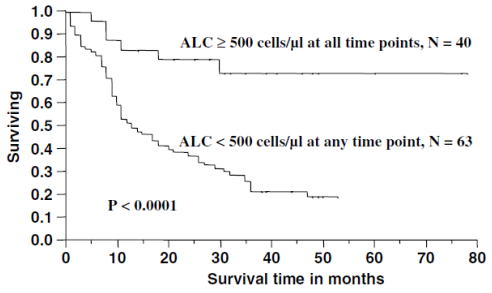


Figure 1 Kaplan–Meier estimates of overall survival (OS) of patients achieving an ALC recovery ⩾500 cells/μl at all time points vs patients achieving ALC recovery <500 cells/μl at any time point. The median OS was not reached in the group with an ALC recovery ⩾500 cells/μl at all time points and 13 months in the group with an ALC recovery <500 cells/μl at any time point. The OS rates at 4 years were 73 vs 19%, respectively, $P<0.0001$.

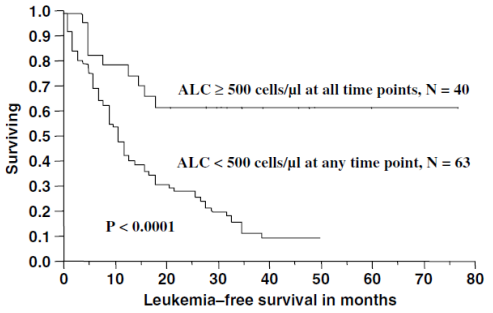


Figure 2 Kaplan–Meier estimates of leukemia-free survival (LFS) of patients achieving an ALC recovery ⩾500 cells/μl at all time points vs patients achieving ALC recovery <500 cells/μl at any time point. The median LFS was not reached in the group with an ALC recovery ⩾500 cells/μl at all time points and 11 months in the group with an ALC recovery <500 cells/μl at any time point. The OS rates at 4 years were 62 vs 9%, respectively, $P<0.0001$.

Fig 6.1 ALC impact correlated with OS and LFS. From (Behl et al., 2006)

The key who had been used from the study working group to generated the results described in the study, as is shown by the Kaplan-Meier curves in Fig 6.1, was the choice to separate the patients in the study population into two groups in order to simplify and to better correlate with the other risk factors: patients who had reached 500/mm^3 lymphocytes at all 4 time-points (TPs) considered versus all others, who had missed the 500/mm^3 ALC mark in at least one of the four TPs. Based on this subdivision, they were also distinguished in descriptive analysis for all other prognostic risk characteristics.

Considering the value of 500/mm^3 as the cut-off, higher OS and LFS were observed in the 40 patients who had reached this cut-off at all TPs than in the 63 patients who had not reached it in at least one of the TPs (Behl et al., 2006).

To highlight the role of ALC and exclude that complete hematologic recovery could affect its variablity after chemotherapy, data on platelets (PLT) and neutrophils (PMN) counts at the same TPs were also collected. The distribution of PLT and PMN recovery data at days 15, 21, 28 and CC from chemotherapy had been found to be similar in both groups. Infact, no association had been identified between platelets or neutrophils counts in relation to the respective lymphocyte TP.

In conclusion, these analysis had definitely given a first demonstration of a crucial role of immunological recovery in OS after IC in AML, despite the high number of

secondary and cytogenetically unfavorable AML in the group with <500/mm^3 lymphocytes, which could have compromised the results. Moreover, considering the multivariate logistic regression model, lymphocyte count above the cut-off at all 4 TPs remained a significant independent predictor for both complete response (CR with $p<0.007$) and outcome parameters (OS with $p<0.0008$ and LFS with $p<0.0011$), when compared with the other known risk factors including age at diagnosis, cytogenetics, FAB morphotype, and secondarily. It had therefore been hypothesized, despite the lack of association with platelets and neutrophils recovery in the study population (probably due to the small size of the study sample), that early lymphocyte recovery, considering the known data about platelets recovery, could also be reflective of a faster BM recovery after chemotherapy, allowing a shorter time to the first consolidation cycle and thus maintaining an optimal dose intensity (Behl et al., 2006).

Instead, even more recently, an interesting analysis was applied to AML patients in early relapse after induction chemotherapy. The evaluation involved recording at the time of early relapse both the ALC figure and also that of AMC.

Data in the literature on the role of monocyte count have always been highly controversial, monocytes having both a role in innate immunity but also potentially tumorogenic and immunosuppressive activity; moreover, recent studies had strengthened the idea that an elevated AMC could be a negative prognostic factor in all hematologic diseases. Lymphocytes, on the other hand, are essential for defense against severe infection during therapy and crucial in the context of immunosurveillance against cancer. In accordance with the known data on ALC, the study had proposed to combine the two parameters into a kind of surrogate comparative index of host's immunologic status at the time of relapse, and that this algorithm, correlated with long-term survival, could also influence the choice of therapeutic strategy, for example, between a second line of salvage intensive therapy versus a milder or conservative strategy in case of an inauspicious prognosis (Zhang et al., 2021). AMC and ALC data had been collected at the time of relapse in 57 patients, whose CR had lasted less than 6 months, including patients at low, intermediate and also high ELN risk. Established cut-offs for AMC were 120/mm^3 as

lower limit and 800/mm^3 as the higher one, while regarding ALC limit were 1100/mm^3 and 3200/mm^3.

In the study, it had been shown that early relapsing AML patients with normal AMC and normal or increased ALC had a reduced risk of death, both in univariate and multivariate analysis, compared with those who had low or elevated AMC and low ALC, with the predictive power strengthened by the combination of the two parameters compared with the impact of the same taken individually (Zhang et al., 2021).

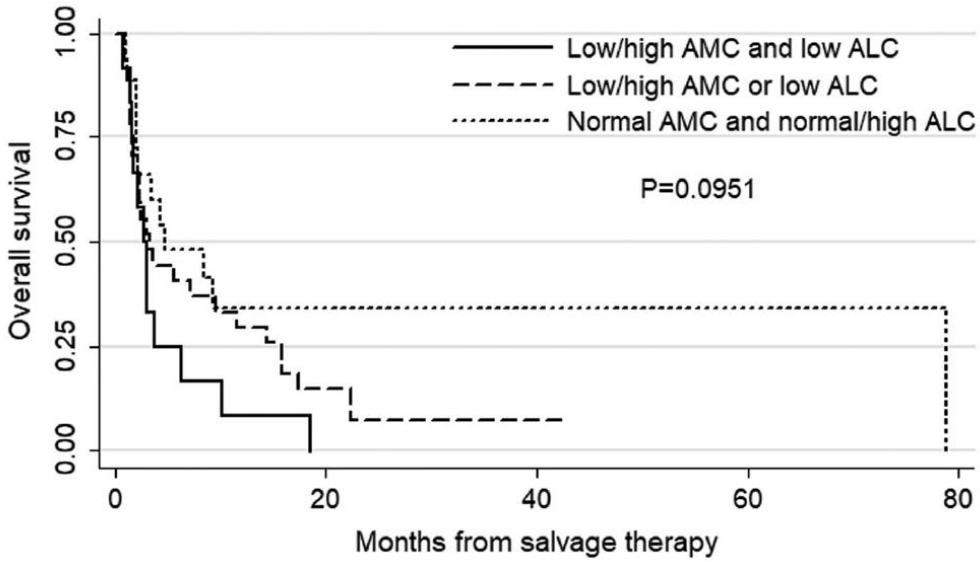


Fig 6.2 OS curve of AML patients in early relapse in corralation with AMC and ALC. From (Zhang et al., 2021)

Therefore, this study recently restated the correlation of high ALC, along with a normal AMC, with improved survival and can also be used as a prognostic marker in the setting of early relapsing AML (Zhang et al., 2021).

7. Methods ALC

7.1 Patients characteristics

The setting chosen for the retrospective analysis was adult AML treated with IC and who obtained CR after induction within the Seràgnoli Institute of Hematology and Oncology. The criteria for exclusion from the total group patients with new onset AML under study who were treated at the Institute were as follows: ineligibility for IC in relation to comorbidities, age at diagnosis, or fitness; treatment with supportive therapy only (e.g., hydroxyurea or mercaptopurine) or with palliation; patients who refused chemotherapy; patients treated with first cycle of induction at another center; early evidence of refractoriness to induction (due to reappearance or persistence of peripheral blasts or by BM aspirate at day 15) with need to undertake a second-line salvage therapy 15 or 21 days after induction; severe adverse event during induction therapy or during pancytopenia that had resulted in a transfer to other operating unit (Cardiology, ICU) or death in induction patient.

Therefore, considering the exclusion criteria listed above, we evaluated a total population of 188 AML patients from Bologna Seràgnoli Hematology Institute, who were diagnosed between 2007 and 2023 and resulted responsive to induction IC.

All patients, at the beginning of admission, had signed consent for the data to be used for clinical research studies.

The median age of patients at diagnosis was 55 years (within a range of 18 to 70 years); considering the cut-off of 60 years, 136 patients (72.3%) aged ≤60 years and 52 (27.7%) aged >60 years. One hundred out of 188 (53.2%) were female, while 88 (46.8%) were male.

7.2 Induction chemotherapy

Induction chemotherapy regimens in the study population were highly variable, due to treatment choices based on prognostic risk, changes in guidelines for induction therapy that have occurred over the years, and the availability of innovative target therapeutic options during the two decades considered.

In order to simplify the division of this large patient population, the study included two main treatment scheme groups, according to a criterion that ascribed the various options used to a common backbone. The two main backbones were: *"3+7" based* therapy with possible addition to the backbone of a third molecular specific target or changes in the number of days of chemotherapy administered;

fludarabine-based therapy, comprising mainly FLAI (fludarabine, cytarabine, idarubicin) schemes with addition of a third drug or reductions in the number of days of therapy.

Ninety-seven out of 188 patients (51.6%) were treated with *"3+7" based* backbone, comprising the therapies: "3+7" + gemtuzumab-ozogamicin (6 patients); "3+7" + quizartinib/placebo (3 patients); '3+7' + midostaurin (14 patients), while 3 patients received the reduced '2+5' + midostaurin scheme, also 1 patient treated with HDAC (high-dose cytarabine) + sorafenib; DAE (daunorubicin, cytarabine, etoposide) scheme in 29 patients; Vyxeos (3 patients); classic "3+7" in 25 patients; classic scheme reduced to "1+7" (9 patients); finally 3 cases treated with My-AIE (Mylotarg-citarabine, idarubicin, etoposide) and one case treated with "3+7" + SMO inhibitor. The subgroup treated with *fludarabine-based* chemotherapy includes 91 out of 188 patients (48.4%): the majority treated with FLAI-5 scheme (63 patients); another 8 patients with the addition of gemtuzumab-ozogamicin (My-FLAI-5); 11 patients according to a 3-day reduced scheme (FLAI-3); another 2 with the addition of Mylotarg (My-FLAI-3); lastly one patient treated with FLAIE scheme, one case with FLAN (fludarabine, cytarabine, mitoxantrone) and 5 patients with association of FLAI-5 and venetoclax.

7.3 Prognostic criteria

Since our population had involved patients in a long time span, starting from 2007, the study had chosen prognostic stratification of the totality of patients according to ELN 2010 risk classification, while only a portion of them, who were diagnosed in the last seven years, were also stratified according to the new ELN 2017 classification. As for the last updates of ELN 2022, only for few patients NGS panel was available.

The diagnostic characterization of acute onset leukemia consists of the assessments, on BM aspirate, of morphologic, immunophenotypic, cytogenetic, and molecular features. The panel of molecular investigations currently fielded at onset in order to obtain the essential informations, not much for prognostic reasons, but especially for therapeutic choices with target therapeutic options possible, involves the study of *NPM1, FLT3-ITD, FLT3-TKD, IDH1, IDH2*, and *TP53* genes. An additional tool, which complements the molecular data initially studied and completes the ELN 2017 risk stratification, is the in-depth study by NGS (Next

Generation Sequencing) panel of 30 myeloid genes commonly found in myeloproliferative syndromes.

Obviously certain molecular investigations among those listed were not yet available in daily diagnostic practice in the previous decade. Therefore, for this retrospective study, in order to achieve statistical significance with more homogeneous and consequently more numerous subgroups of patients, it was decided to consider for the totality of patients only those molecular alterations that constituted prognostic risk according to ELN 2010: *NPM1* and *FLT3-ITD* mutations.

Applying the ELN 2010 risk classification to the study population, there were 52 patients out of 188 (27.7%) belonging to favorable risk; 84 (44.7%) intermediate-1 risk; 17 (9%) intermediate-2 risk; and 35 patients (18.6%) considered to be at adverse prognostic risk.

Regarding the evaluation of the *FLT3* gene, the main information collected was the rate of mutated *FLT3-ITD* in the entire population, precisely 35 out of 188 patients (18.6%); while 153 patients were wild-type *FLT3-ITD*. To complete the characterization, it is fitting to report how 18 patients out of 188 were instead *FLT3-TKD* mutated; of note, 7 patients out of 188 were both *FLT3-ITD* and *FLT3-TKD* mutated.

NPM1 mutation had the following distribution in our case series: 66 out of 188 (36.3%) *NPM1* patients mutated at onset; 116 wild-type *NPM1* patients.

As for the cytogenetic study, in order to homogenize the totality of patients for statistical analysis into as few subcategories as possible, we considered the three risk categories according to ELN 2017 as the only subdivision: 22 patients out of 188 (11.7%) at low cytogenetic risk; the majority with 138 patients (73.4%) at intermediate risk; finally, 28 patients (14.9%) at high risk.

Our case series included 150 out of 188 (79.8%) patients with *de novo* onset and 38 (20.2%) forms of secondary AML, evolved from myelodysplastic syndrome (MDS), essential thrombocythemia (ET), idiopathic myelofibrosis (IMF) or chronic myelomonocytic leukemia (CMML), and therapy-related forms.

Considering the cut-off of white blood cells at onset of 30,000/mm^3, 127 patients out of 188 (67.6%) did not reach this cut-off, while 61 patients (32.4%) were hyperleukocytic at onset.

7.4 Allogeneic and autologous transplantation

Considering the total population, 124 out of 188 patients (66%) received consolidation with HSCT; 15 patients (8%) completed the treatment process with autologous stem cell transplantation; and 49 patients (26%) were not scheduled for consolidation with transplantation.

7.5 Response and outcome assessment

Complete response (CR) to induction chemotherapy was defined by a blast cell count of less than 5%, BM assessment performed at hematologic recovery after induction, and absence of extramedullary localization of AML. Patients refractory to chemotherapy had persistence of BM disease after induction or otherwise a reduction in BM blastic proportion of not less than 20%.

MRD was assessed at the end of the second cycle of therapy (after the first consolidation cycle) in responders or after salvage treatment in patient who further relapsed during the disease history. In accordance with guidelines in the literature, either the *WT-1/ABL* transcript data *x 10000* as a ratio, by PCR, or disease-specific transcripts, when available (*NPM1/ABL x 10000*; *RUNX1-RUNXT1*; *CBFB-MYH11*; *PML-RARA*) was used to assess MRD (Döhner et al., 2017).

As mentioned, according to our selection criteria, all 188 patients of our population had obtained a CR after induction. In 64 patients (34%) of the total, morphological disease relapse was documented during the course of treatment.

OS was measured from the date of treatment initiation until the date of death or last follow-up. RFS is defined as the period from treatment initiation to the date of documented frank relapse or disease progression from MRD positivity. One hundred eighteen out of 188 patients (62.8%) were currently alive, in CR at last follow-up; 70 patients (37.2%) died.

7.6 ALC assessment

We collected the blood count data from our patient population at the days 15, 21, 28 after induction begin and also before the consolidation course. Hence, in according to Porrata and colleagues' paper, we defined 4 ALC TPs: respectively at 15 (ALC-15), at 21 (ALC-21) and at 28 days (ALC-28) from IC start and before consolidation therapy (ALC-CC). ALC adopted cut-off was 500/mm^3. With the aim of survival analysis and correlation, we considered each TP singularly and also

different TPs together; whereby patients were grouped in those who obtained ≥500/mm^3ALC in all 4 TPs and those who had<500/mm^3ALC in at least one TP. Median follow-up was 51.4 months.

8. Statistical analysis

OS and RFS were analyzed using the method described by Kaplan and Meier.

Cox proportional hazards model was used for univariate analysis with regard to continuous variables and dichotomous variables. In addition, the same was used to evaluate ALC 500/mm^3 in each individual time-point with respect to the known risk variables and also considering this parameter in all TPs as a prognostic factor of OS and RFS, taking into account the other prognostic risk factors.

The logistic regression model was used to estimate the impact of ALC as an independent factor with respect to the known variables.

For all p values obtained from the analysis and transcribed, statistical significance was established for $p<0.05$.

9. Results ALC analysis

The objective of the second part of the research was to explore the impact of ALC on survival, in view of the known data in the literature, among our AML patients population, responsive to IC.

First, we considered the whole 188 patients population, thus including both induction therapy subgroups. When considering those patients who obtained ≥500/mm^{3}ALC in all 4 TPs in spite of those who had <500/mm^{3}ALC in at least one TP, no meaningful differences were spotted between those who recovered ALC and all the others as shown in the OS correlation curve (Fig 9.1). No significant differences regarding age, ELN risk, cytogenetics, HSCT consolidation rate were identified.

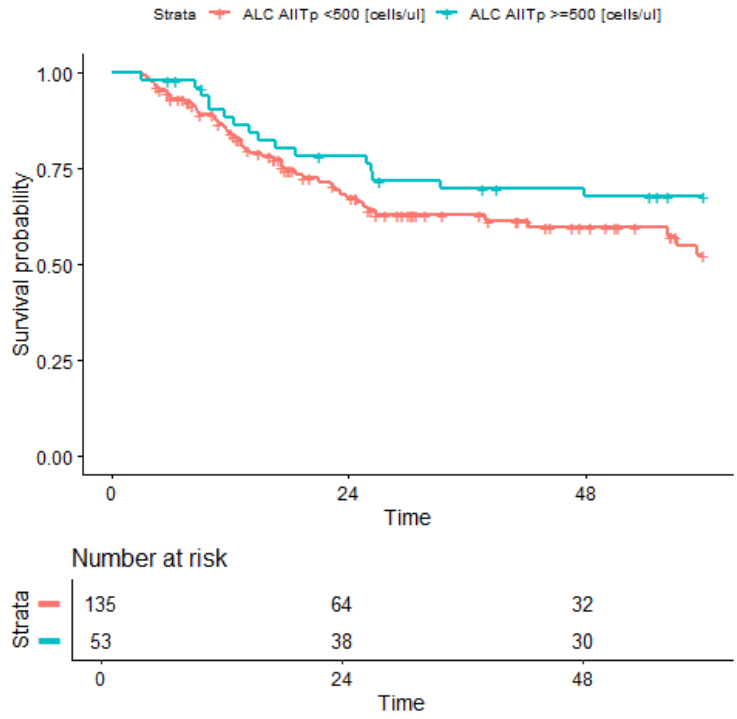


Fig 9.1 All patients correlation in OS at all TPs

Secondly, as there was no impact of ALC in whole population, we proceeded with the analysis in the subgroups. Among patients treated with "3+7-based" regimen (51,6%), at ALC-15 TP a trend as a benefit of patients with ≥500/mm^{3}ALC (31,9%) vs those under the cut-off (19,6%) was observed in a correlation with OS (p=0.10) (Fig 9.2). This finding had furthermore revealed to be statistically significant in patients with ALC≥500/mm^3 in all 4 TPs (25,5%) compared to those who had <500/mm^{3}ALC in at least one TP (26%)(p=0.02)(Fig 9.3).

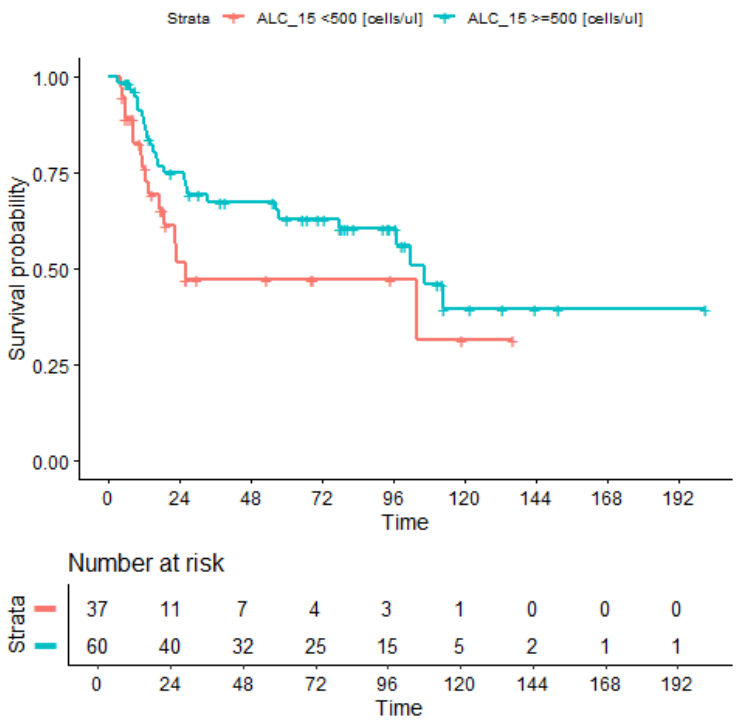


Fig 9.2 “3+7 based” patients correlation in OS at ALC-15 (p = 0.10)

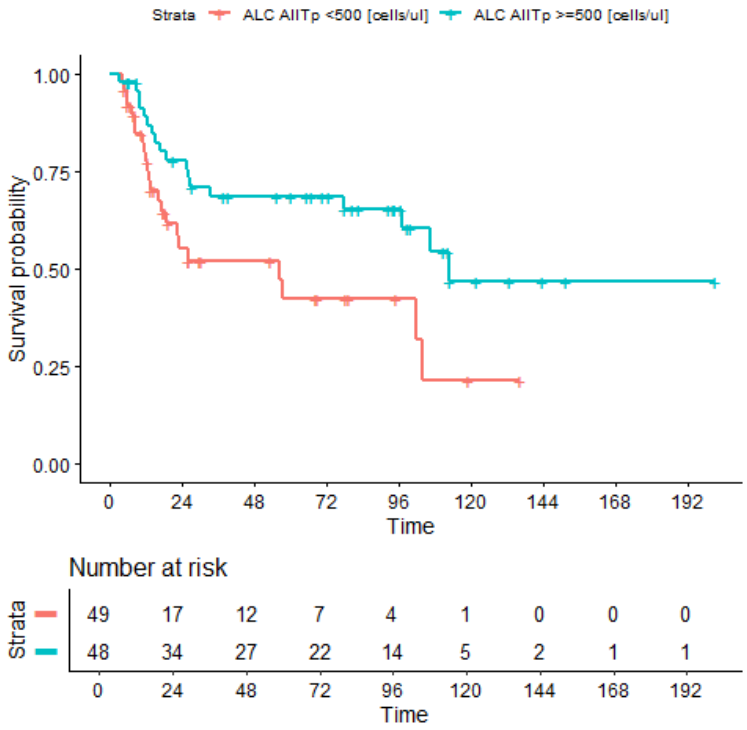


Fig 9.3 “3+7 based” patients correlation in OS at all TPs (p = 0.02)

In order to compare the two main regimen subgroups, we repetead the correlation in the other one. Conversely, in the "fludarabine-based" group (48,4%), ALC recovery showed a completely inverted correlation trend compared to “3+7” patients, both in RFS (Fig 9.4 and Fig 9.5) and in OS. Infact, in this cohort the patients who

had <500/mm^{3}ALC, both in each single TP and with all 4 of them, had taken the survival advantage compared to the other ones.

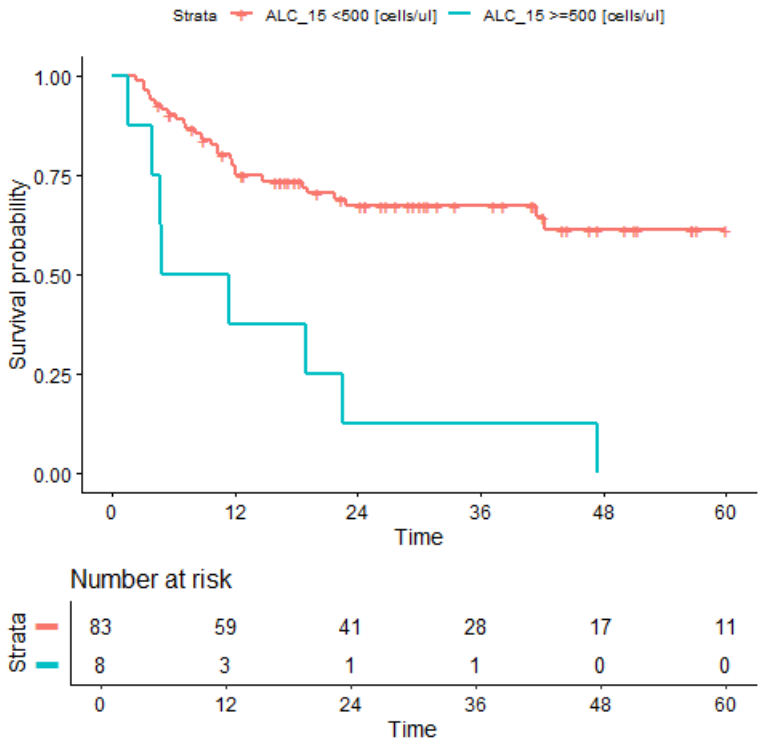


Fig 9.4 “FLAI-based” patients correlation in RFS at ALC-15 (p = 0.0001)

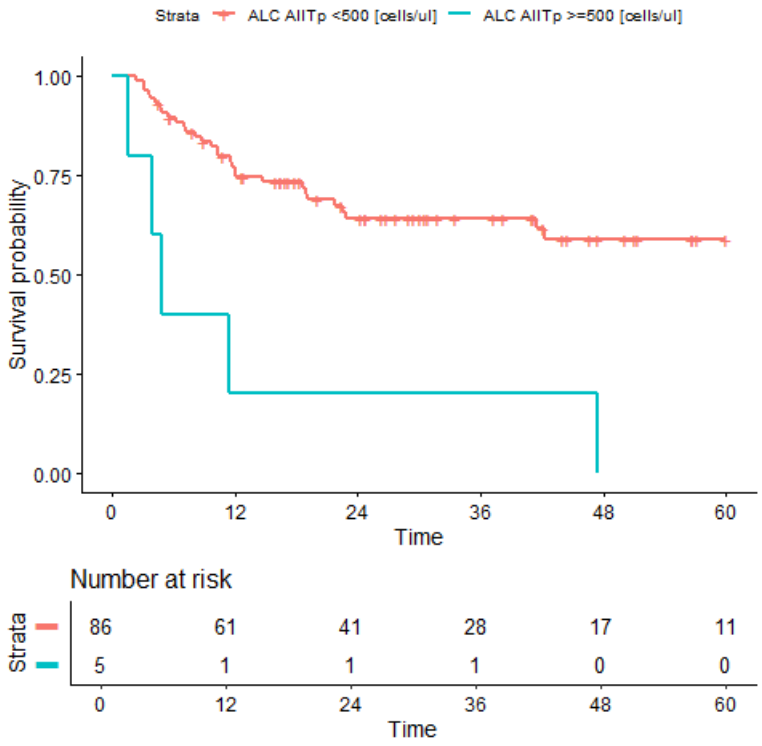


Fig 9.5 “FLAI-based” patients correlation in RFS at all TPs (p = 0.0007)

In further analysis, we assessed ALC impact in different subgroups, based on the molecular status. Our attention had focused mainly on *NPM1* mutated patients, on *FLT3-ITD* positive ones and on *FLT3-TKD* positive ones.

It is known and confirmed from the last ELN 2022 the impact of *FLT3-ITD* status as an intermediate risk and *NPM1* mutation as a favourable risk, despite several issue are emerging regarding the role of concomitant mutations or cytogenetic in *NPM1* AML and the possibility of risk redefinition in consideration of survival (Döhner et al., 2022).

In the *NPM1* mutated patients (36.3%) those who obtained ALC recovery in all 4 TPs (9.04%) were compared to those who missed recovery in at least one TP (26,06%) and it resulted not prognostically relevant (Fig 9.6); also in single TPs the trend was not significant. Regarding *FLT3-ITD* positive patients (14,89%), this subgroup included a small number of patients and ALC recovery showed an advantage in OS, without a significant correlation (Fig 9.7). A similar trend in correlation with RFS in all TPs was shown in the *FLT3-TKD* subgroup as shown in Fig 9.8.

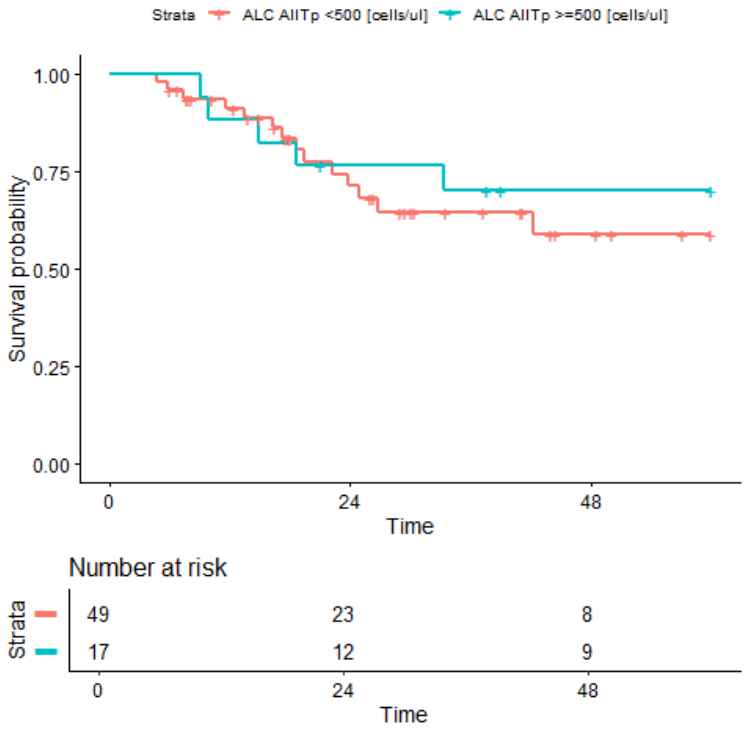


Fig 9.6 *NPM1* pos patients correlation in OS at all TPs (p = 0.57)

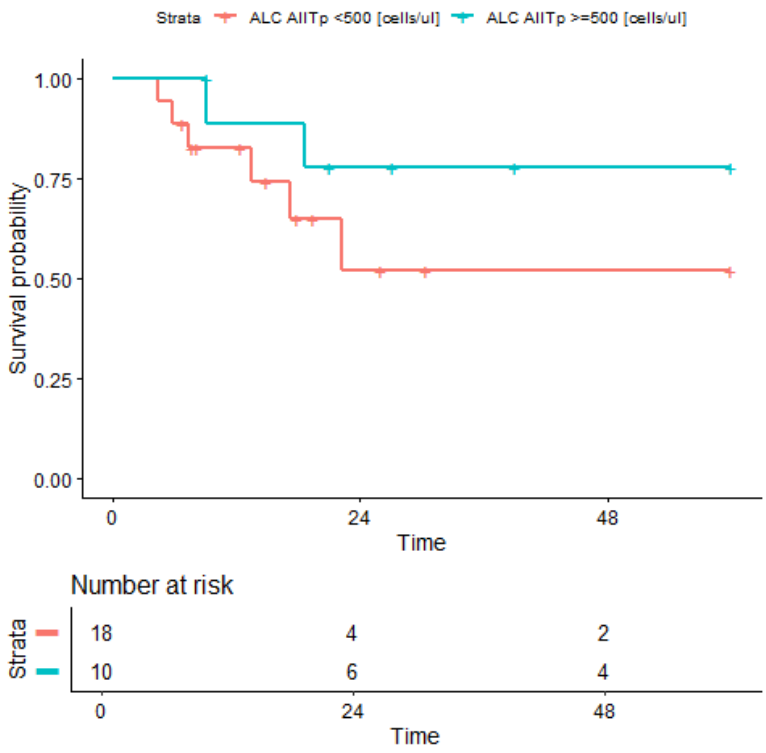


Fig 9.7 *FLT3-ITD* pos patients correlation in OS at all TPs (p = 0.25)

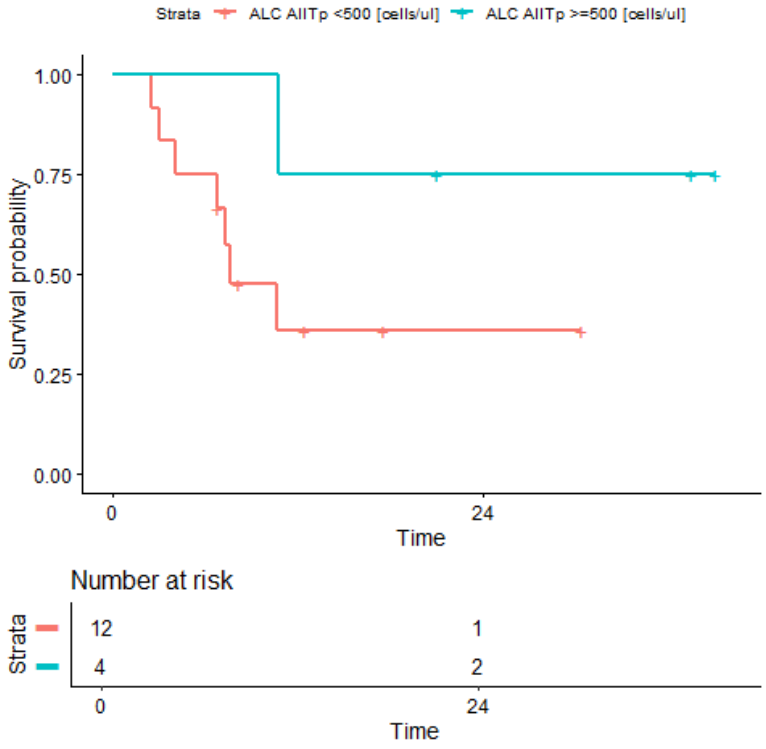


Fig 9.8 *FLT3-TKD* pos patients correlation in RFS at all TPs (p = 0.28)

Lastly, we evaluated ALC also in the subgroup with both *NPM1* and *FLT3-ITD* comutated. Twenty-six patients out of 188 resulted with this molecular pattern at diagnosis. Despite the small number, an ALC correlation with OS showed a trend, though not significant, with survival advantage for those who obtained ALC recovery in all 4 TPs (Fig 9.9).

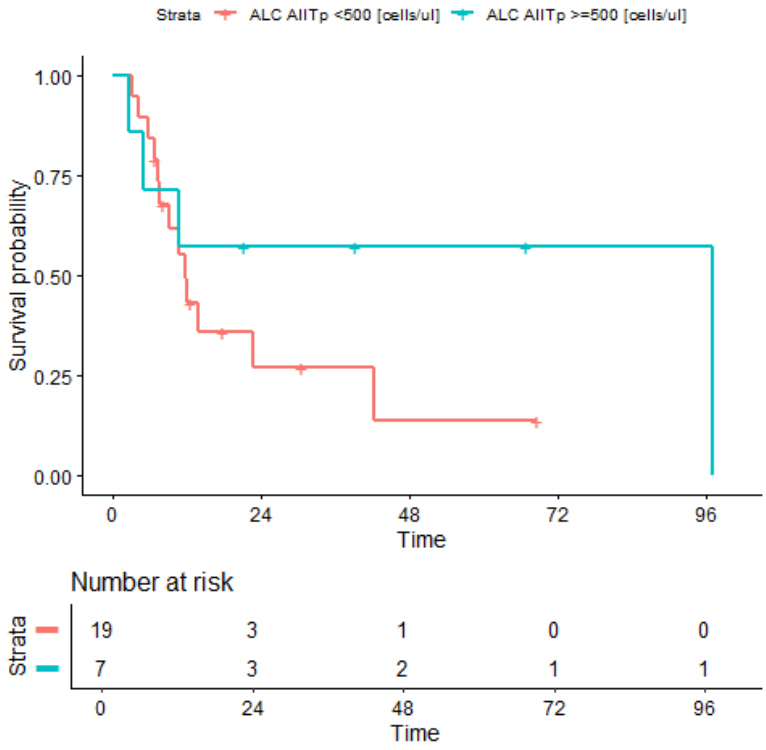


Fig 9.9 *NPM1 and FLT-ITD* comutated patients correlation in OS at all TPs (p = 0.28)

Beside the evaluation of ALC on molecular status, another main variable considered had been allogeneic transplantation. It is obvious to declare that a consolidation therapy with HSCT improves OS, with specific reference to non favourable ELN risk patients. Therefore we questioned if the ALC impact could match the transplantation prognostic fashion.

We analyzed ALC recovery, through the whole population, differentiating patients who received HSCT (66%) from those who did not (34%).

The result showed in Fig 9.10 is quite clear. As expected, the two curves representing transplanted patients (the blue and the purple ones) highlight a better survival, when compared to the other two curves (the red and the green ones) which correspond to those treated only with IC. However, more specifically, the blue and the red curves, from both subgroups of patients, show that ALC recovery at all TPs

may have conferred a better outcome versus each counterpart of patients who had ALC <500/mm^3 in at least one TP.

In particular, those who had <500/mm^3 in at least one TP and did not receive HSCT had the worst outcome (green curve); instead, interestingly, those who reached 500/mm^3 in all TPs despite not receiving HSCT (red curve) had a survival trend closer to the transplanted ones. We could assume that in whole population ALC might represent a prognostic factor that is independent from HSCT; therefore, HSCT was capable to abolish the negative prognostic effect of reduced ALC recovery, identifying a group of patients who may benefit from HSCT.

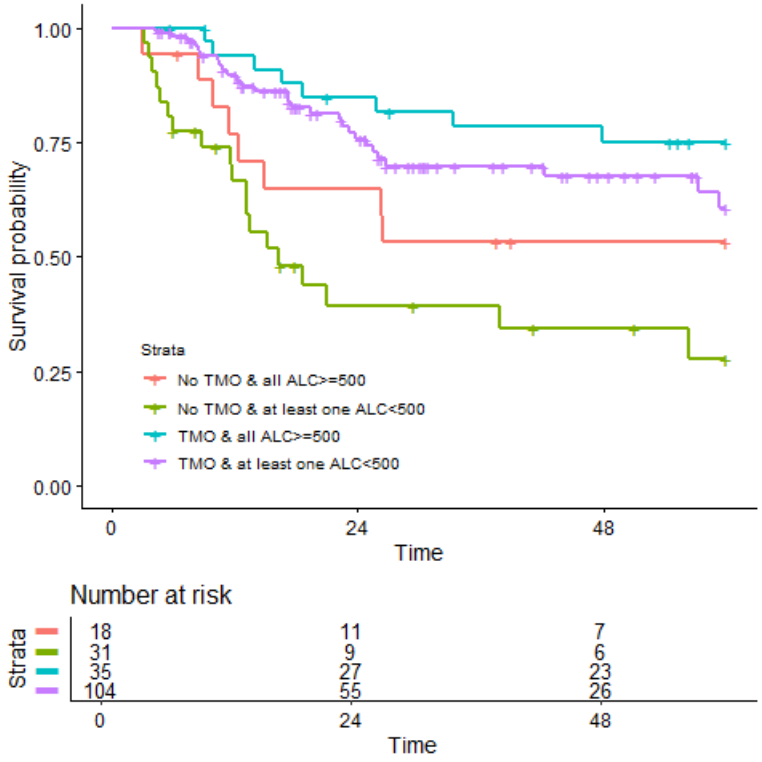


Fig 9.10 Correlation in OS between HSCT and ALC and impact of ALC recovery (p = 0.09)

We repeated this correlation analysis for all the four subgroups, defined according to ALC recovery and HSCT, subdividing each for the 4 ELN 2010 classification risk. Probably due to the small number of each ELN 2010 risk cohort in every subgroup, the value of this subanalysis should not be considered.

Finally, ALC recovery was correlated with MRD value. The MRD assessment as a independent survival prognostic marker in AML treated with IC is by now a strengthened evidence (Chea et al., 2024).

We decided to perform this subanalysis among “3+7-based” patients, where ALC seemed to show a higher impact. The “3+7-based” subgroup was then divided between MRD positive and negative patients, according to the value established as mentioned above at the post consolidation course BM assessment. When considering patients who obtained MRD negativity (n = 52), those who had ≥500/mm^{3}ALC in all ALC TPs (n = 29) and in ALC-15 (n = 35) resulted in a globally better OS as compared to patients with <500/mm^3 ALC at the two different evaluations (n = 23 and n = 17, respectively)(Fig 9.11 and Fig 9.12).

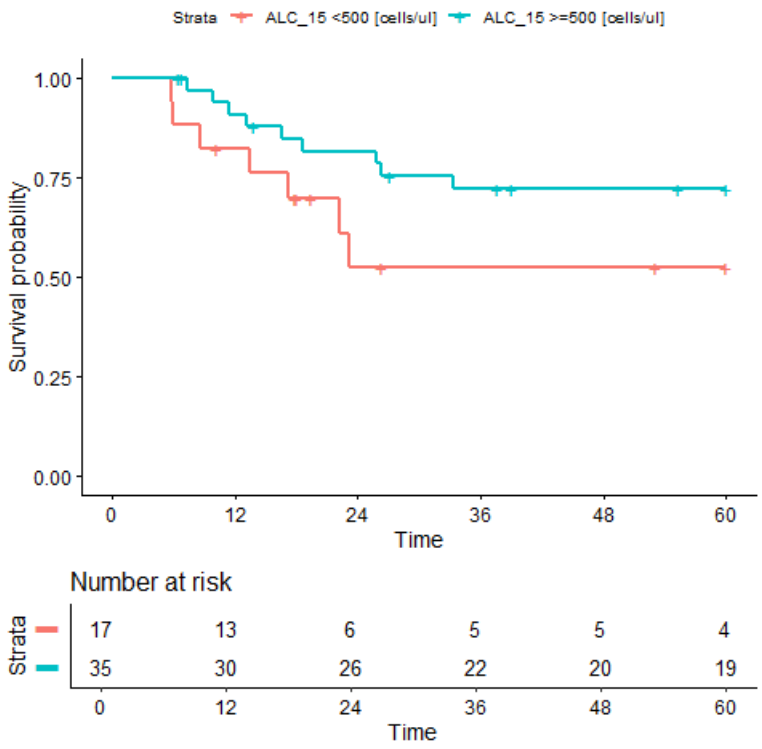


Fig 9.11 “3+7 based” patients MRD neg correlation in OS at ALC-15 (p = 0.14)

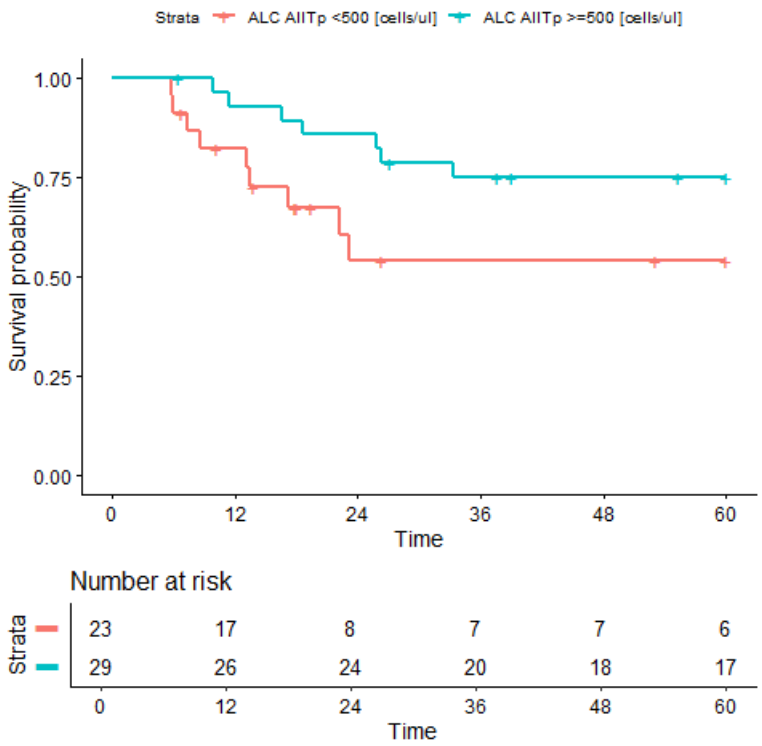


Fig 9.12 "3+7 based" patients MRD neg correlation in OS at all TPs (p = 0.07)

In a similar fashion, regarding patients who persisted as MRD positive (n = 44), although less significant, those who had ≥500/mm^{3}ALC in all ALC TPs (n = 19) and in ALC-15 (n = 24) had a better OS trend when compared to patients with <500/mm^3 ALC at the two different evaluations (n = 25 and n = 20, respectively)(Fig 9.13 and Fig 9.14).

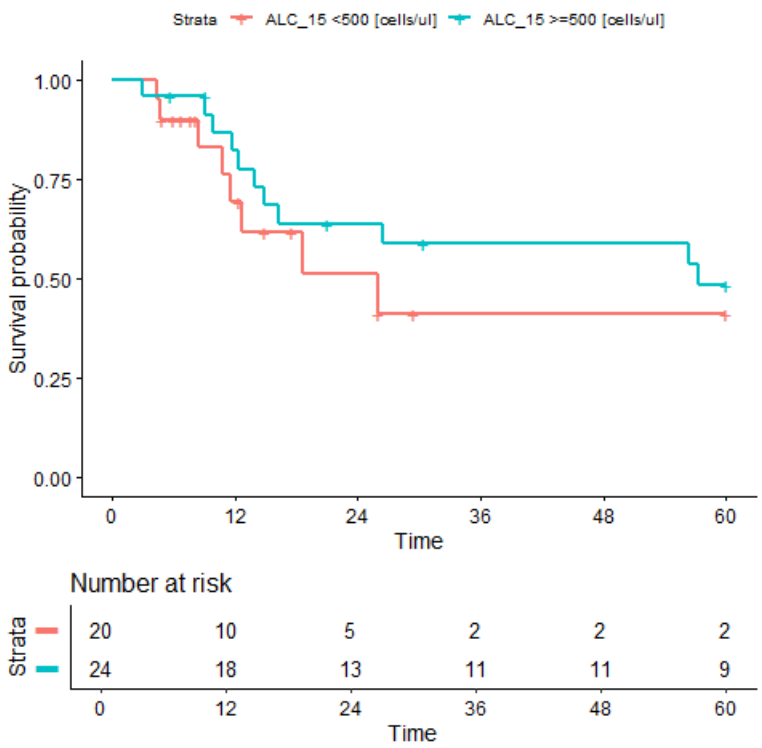


Fig 9.13 “3+7 based” patients MRD pos correlation in OS at ALC-15 (p = 0.4)

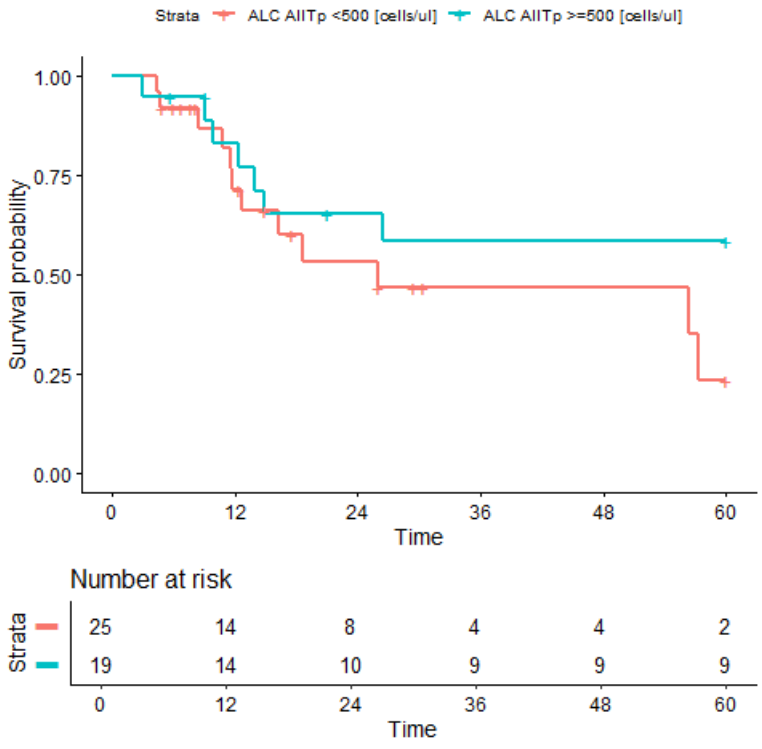


Fig 9.14 “3+7 based” patients MRD pos correlation in OS at all TPs (p = 0.19)

10. ALC recovery data from GIMEMA 1310

The results indicated in the previous paragraph, in terms of correlations with OS and RFS, regarding our study population of 188 AML responsive patients, have confirmed the potential prognostic impact of ALC recovery as an independent factor. In fact, the evaluation of ALC recovery role in different subgroups, subdivided according to IC therapy regimen, molecular status at diagnosis, consolidation with HSCT and MRD status after the first consolidation course, has shown a better survival trend patients who had recovered ALC≥500/mm^3 in all 4 TPs considered compared who those that did not achieve ALC recovery in one TP and also considering some singular TP. When focused in some subgroups, in particular in the *"3+7" based* regimen setting, the correlation with OS survival resulted to be statistically significant. The results of each subgroup, especially in terms of molecular status, would require an increase in number of population in order to pursue a significant survival advantage and further analysis.

Nevertheless, our 188 patient population, even when subdivided in subgroup, resulted pretty heterogenous, especially because of the wide variation among the two main regimen backbone, in terms of dose reductions and addition of a third drug to the backbone schemes (which led to the formation of the two main therapy subgroup for instance). Moreover, as mentioned, the MRD evaluation included different methods which were different according to specific disease transcript o flow cytometry.

However, the survival correlation among further molecular subgroups, because of small numbers, requires deeper investigations.

In order to compare data obtained in our population with a more homogeneous cohort of AML patient, we had to opportunity to apply, retrospectively, the same ALC impact analysis to all the patients who had previously been enrolled in a clinical trial mutuated by the italian hematological group GIMEMA (Gruppo Italiano Malattie EMatologiche dell'adulto). The trial known as GIMEMA1310 is a interventional multicenter italian clinical trial who had enrolled newly diagnosed AML adult patients. As its main feature, it proposed a risk-adapted MRD-directed

therapy, combining cytogenetics/genetics and post consolidation levels of MRD. After induction and consolidation, favorable-risk patients (FR) were led to receive autologous stem cell transplant (ASCT) and poor-risk patients (PR) to HSCT. Intermediate-risk patients (IR) were directed to receive ASCT or HSCT depending on the post consolidation MRD levels. DAE regimen were used as induction chemotherapy. The primary endpoint of the study was OS at 24 months from treatment start. Upfront evaluation included BM for morphology, cytogenetics, molecular biology, and multiparametric flow cytometry (MFC) analysis. The baseline MFC assessment was a necessary step, not only for diagnostic purposes but also to identify leukemia-associated immunophenotypes (LAIPs). Identification of baseline LAIPs was the essential requirement for monitoring MRD after therapy; at the established time point, BM MRD was determined by a high-sensitivity 8-color MFC assay; in subanalysis MFC MRD was compared also to RT-PCR specific transcripts when available.

Our choice of applying ALC evaluation to this cohort is based on the same IC regimen administered and on the homogeneous MRD status data of every patient included (flow cytometric method).

Among the whole population involved in the trial, 108 AML patients were evaluable for the ALC impact analysis. Patients' characteristics are resumed in Tab 10.1.

Characteristic	N = 108
Sex, n (%)	
Male	55 (51%)
Female	53 (49%)
Age, median (range)	50 (19 , 61)
Wbc, median (range)	14 (1 , 181)
Unknown	1
FLT3, n (%)	
Negative	85 (79%)
Positive	22 (21%)
Unknown	1
NPM1, n (%)	
Negative	67 (62%)
Positive	41 (38%)
Lymphocytes day+15, median (range)	0.32 (0.00 , 2.80)
Lymphocytes day+21, median (range)	0.40 (0.00 , 1.91)
Unknown	2
Lymphocytes day+28, median (range)	1.01 (0.00 , 11.30)
Unknown	2
Lymphocytes day+28 (survey complete), median (range)	1.02 (0.00 , 11.30)
Unknown	3
MRD post Consolidation, n (%)	
Negative	34 (52%)
Positive	31 (48%)
Unknown	43
Risk Category, n (%)	
Low Risk (final response - harvesting stem cells - autograft)	42 (39%)
Intermediate Risk (wait for MRD evaluation at the end of consolidation)	24 (22%)
High Risk (final response - allograft)	32 (30%)
LAIP not detected - final response – autograft	10 (9.3%)

Tab 10.1 Characteristics of GIMEMA1310 patients evaluated for ALC recovery

Of note, both responsive and refractory patients were included at first; median age was 50 years; 21% of patients was *FLT3* positive, while 38% was *NPM1* mutated; 39% was at favorable ELN risk at diagnosis, 31% of patients at intermediate risk and 30% adverse risk.

Regarding MRD status post consolidation, 34 patients out of 108 obtained MRD negativity, 31 patients resulted still MRD positive; 43 patient were not evaluable for MRD.

ALC cut-off was 500/mm^3; in this analysis the 3 TPs ALC-15, ALC-21 and ALC-28 have been used, alone or taken together.

The preliminary survival correlating results are shown in the following figures Fig 10.1 and 10.2.

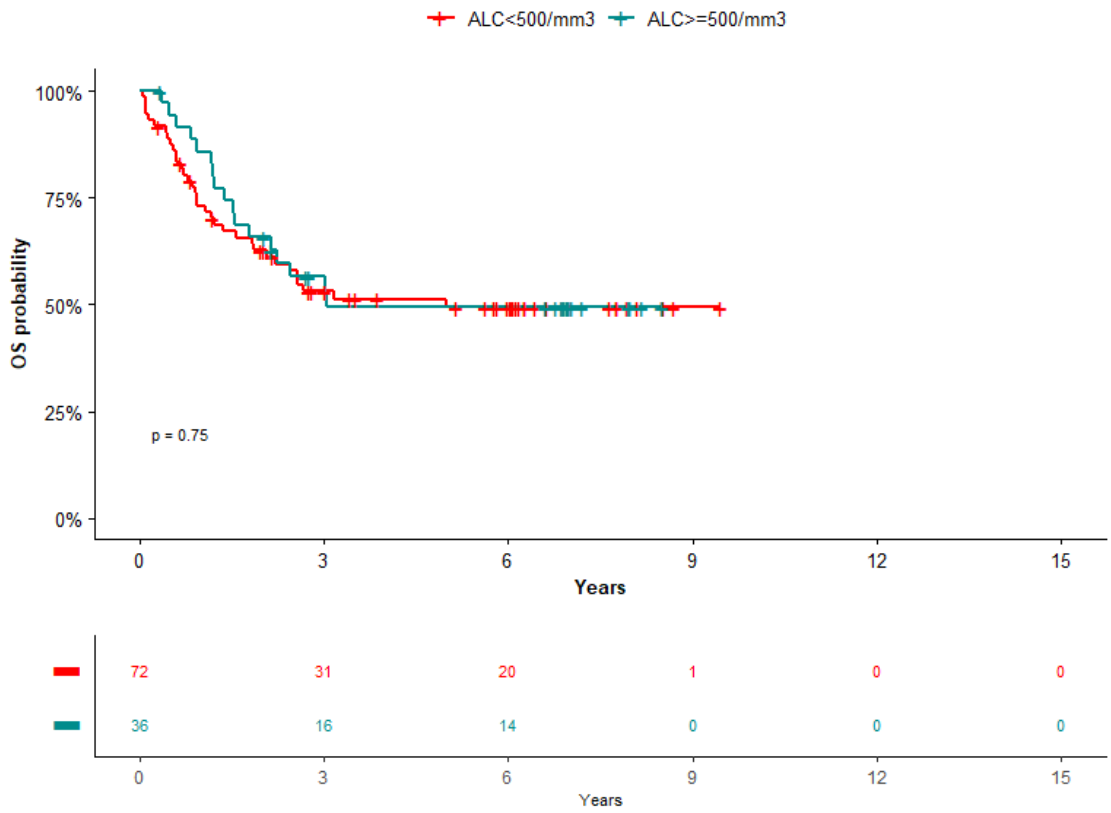


Fig 10.1 GIMEMA1310 patients correlated in OS at TP ALC-15

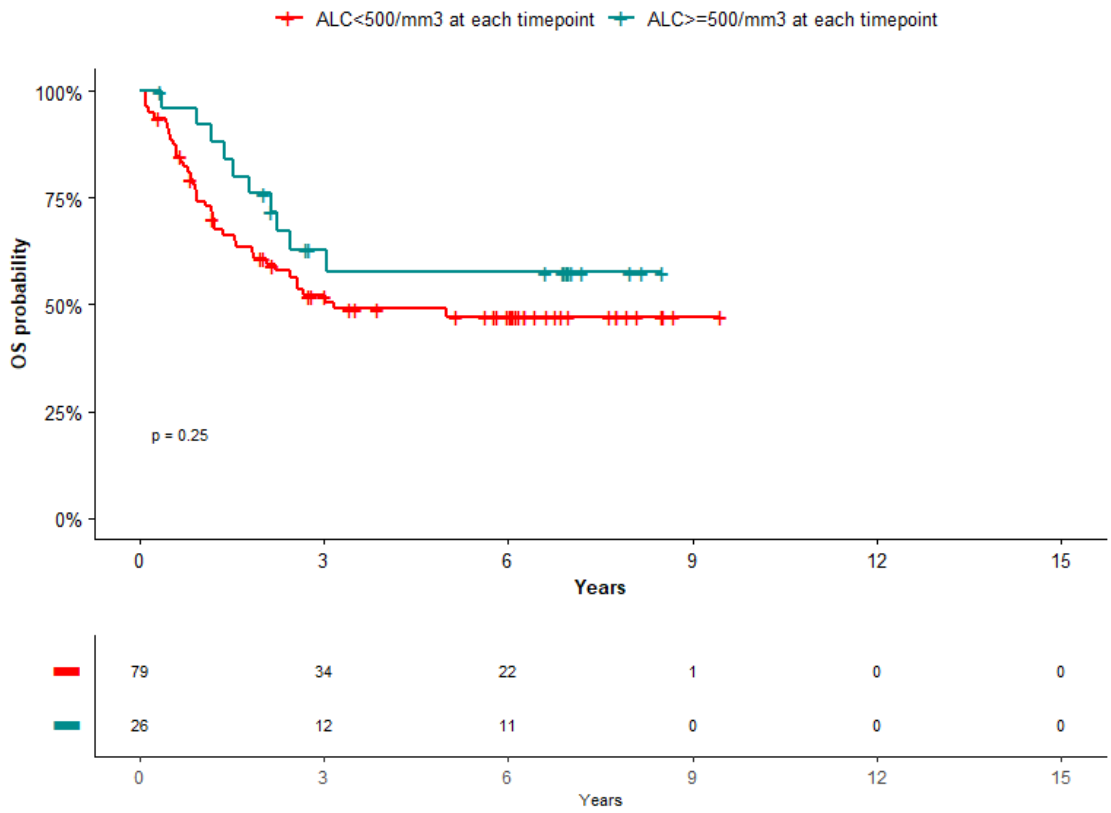


Fig 10.2 GIMEMA1310 patients correlated in OS at all TPs

By isolating single TPs in the analysis, as indicated in ALC-15 correlation, no survival advantage can be spotted. When considering patients who had ALC recovery in all 3 TPs versus those who had not, there is a better survival trend correlated with ALC, but still not statistically significant.

	MRD		
Characteristic	**Negative**, N = 33	**Positive**, N = 31	**p-value**[1]
ALC - Cutoff ALC 500/mm3 d+15, n (%)			0.48
ALC<500/mm3	24 (73%)	20 (65%)	
ALC>=500/mm3	9 (27%)	11 (35%)	
ALC - Cutoff ALC 500/mm3 d+21, n (%)			0.48
ALC<500/mm3	23 (70%)	19 (61%)	
ALC>=500/mm3	10 (30%)	12 (39%)	
ALC - Cutoff ALC 500/mm3 d+28, n (%)			0.67
ALC<500/mm3	4 (12%)	2 (6.5%)	
ALC>=500/mm3	29 (88%)	29 (94%)	
ALC - Cutoff ALC 500/mm3 each time point, n (%)			0.31
ALC<500/mm3 at each timepoint	27 (82%)	22 (71%)	
ALC>=500/mm3 at each timepoint	6 (18%)	9 (29%)	

[1]Pearson's Chi-squared test; Fisher's exact test

Tab 10.2 GIMEMA1310 MRD status in comparison to ALC TPs in patients evaluable

TabFig 10.2 indicates the comparison of MRD status after consolidation course, among the cohort of patients, who have been evaluable for MRD, matching the ALC recovery at the 3 TPs considered with the same cut-off.

Secondly, following our analysis strategy enlighted in paragraph 7, we excluded refractory patients from the analysis, focusing on the patients who had obtained CR after induction (90 patients out of 108). The two following figures show the correlation of ALC recovery at all 3 TPs with OS regarding CR patients (Fig 10.3) and also at TPs ALC-15 and ALC-21 taken together as the earliest TPs (Fig 10.4); despite not statistically significant, those patients who had ALC≥500/mm^3 in all 3 TPs and ALC≥500/mm^3 in ALC-15 and ALC-21 resulted to be prognostically favoured.

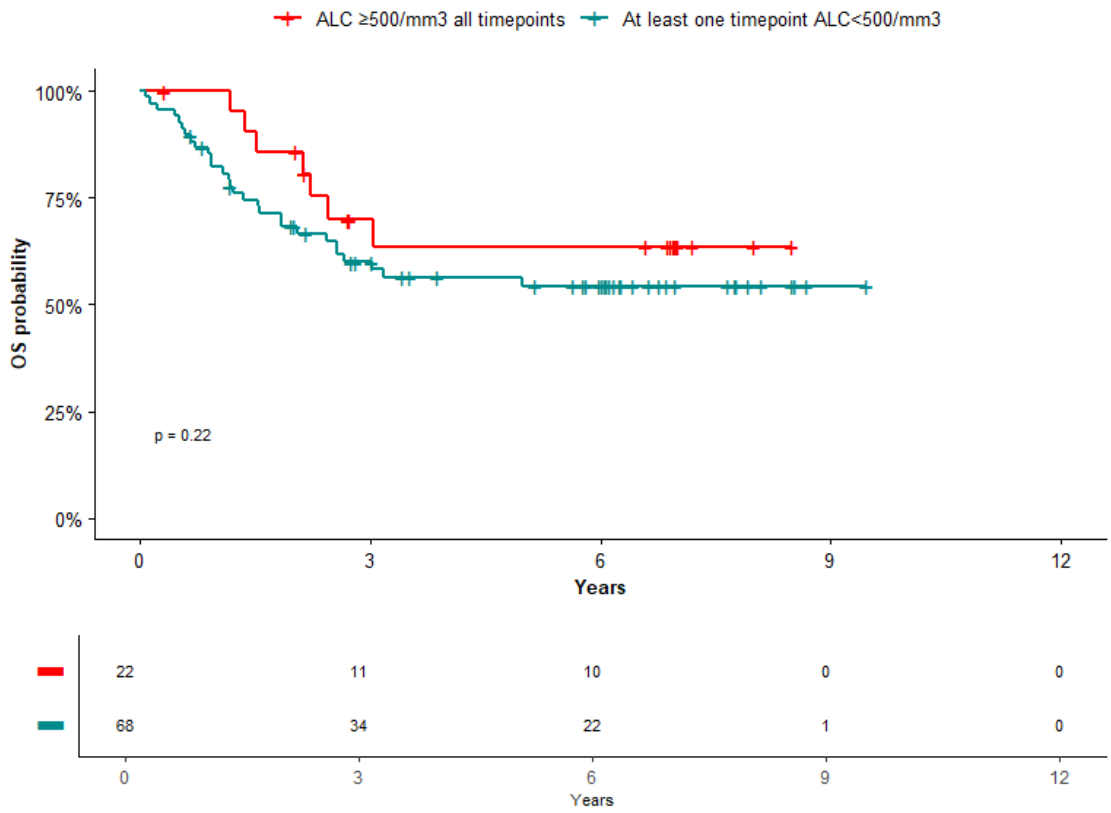


Fig 10.3 GIMEMA1310 CR patients correlated in OS at all TPs

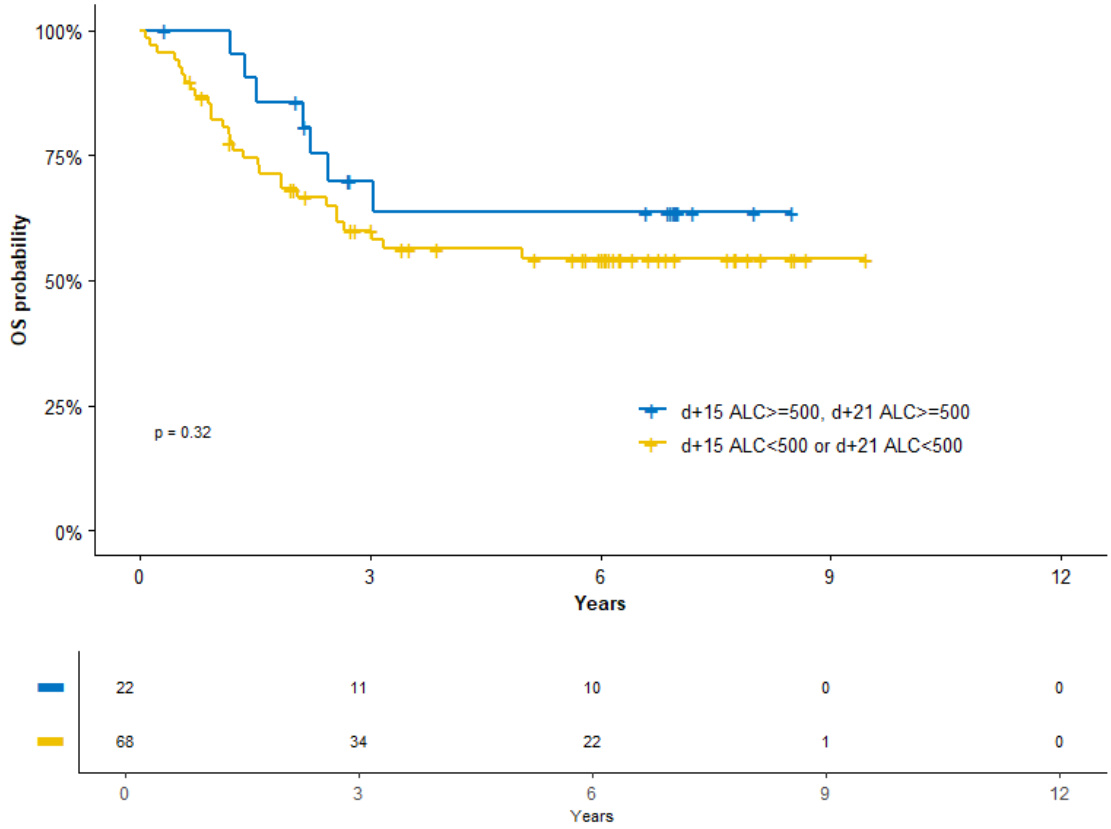


Fig 10.4 GIMEMA1310 CR patients correlated in OS at TPs ALC-15 and ALC-21

Lastly, among CR patients, we correlated ALC recovery impact with MRD status. The following figure and table indicate patients subdivision according to MRD quote (Fig. 10.5 and Tab 10.3).

In particular, in the graph of Fig 10.5 patients have been distinguished in 4 subgroup according to ALC recovery at ALC-15 and ALC-21 taken together and to MRD status post consolidation. In a similar fashion, without reaching a statistical significance though, the Fig 10.5 shows a trend of survival advantage in patients with good ALC recovery in the 2 TPs, both in patients resulted MRD positive than in MRD negative ones.

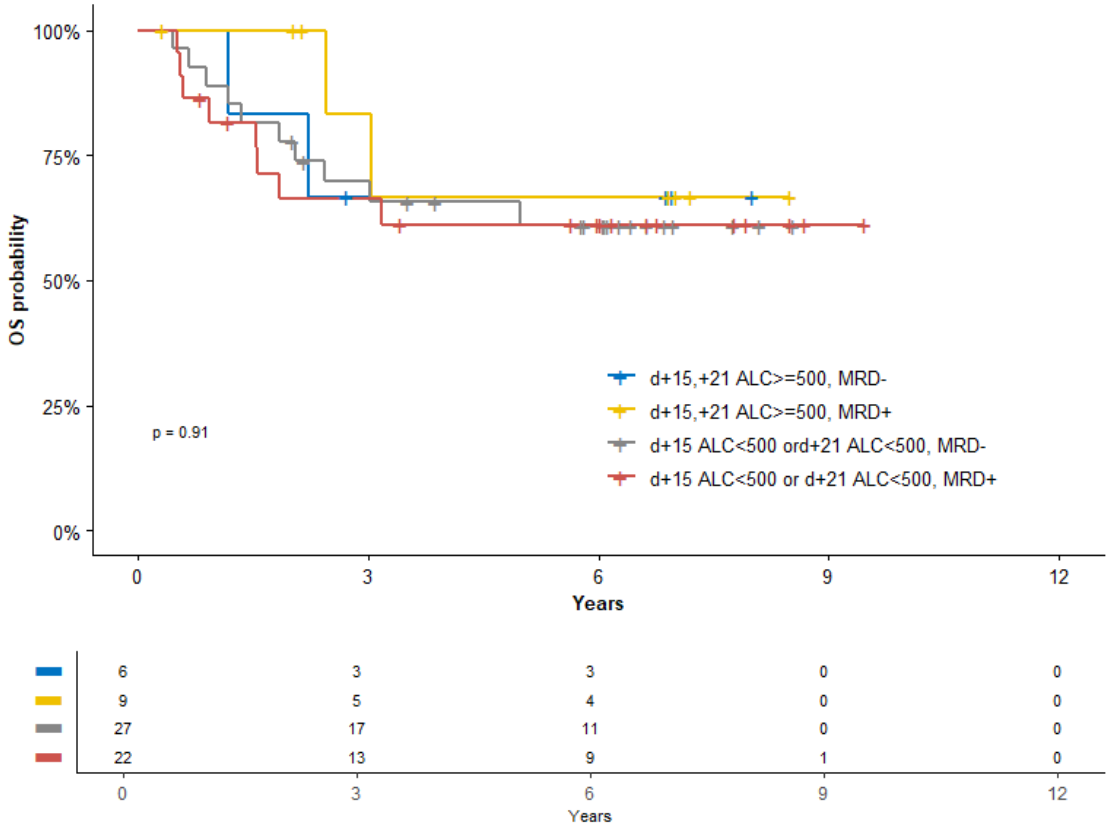


Fig 10.5 GIMEMA1310 MRD positive and negative CR patients subgroups correlated in OS at TPs ALC-15 and ALC-21

Characteristic	Overall, N = 64	MRD Negative, N = 33	MRD Positive, N = 31	p-value[1]
interaction d+15,d+21 for 500/mm3, n (%)				.31
d+15 ALC>=500, d+21 ALC>=500	15 (23%)	6 (18%)	9 (29%)	
d+15 ALC<500 or d+21 ALC<500	49 (77%)	27 (82%)	22 (71%)	

[1]Pearson's Chi-squared test

Tab 10.3 GIMEMA1310 MRD status in comparison to ALC-15 and ALC-21 TPs

11. ALC data and gut microbiota

During the chemotherapy administration and the following cytopenia period, the management of AML patients relies on different possible variables and complications, in terms of toxicities dueto cytopenias.

The gut microbiota (GM), which represents the largest human-associated assemblage of microorganisms residing in the gastrointestinal tract, is a key factor in modulation of immune system and global host health. Among others, GM is known to be directly involved in regulating hemopoiesis, affecting the host response to inflammation and infection (Manzo and Bhatt, 2015). It is therefore not surprising that an altered and dysbiotic GM profile has been associated with increased susceptibility to infection and inflammation-related disorders (from intestinal to metabolic, hepatic, respiratory, cardiovascular, neurologic and oncologic). Moreover, GM has also been shown to affect the outcome of several treatments, including anti-cancer immunotherapy. With regard to hematological malignancies, several studies have consistently described profound GM dysbiosis in both adult and pediatric patients, including those with AML (Galloway-Peña et al., 2020; Gopalakrishnan et al., 2018). Gut microbiota dysbiosis induced by antibiotic treatment accelerates murine AML progression, while faecal microbiota transplantation reverses this process. Butyrate produced by the gut microbiota (especially Faecalibacterium) significantly decreases in faeces of AML patients. Furthermore, we find that the intestinal barrier is damaged in mice with AML, which accelerates lipopolysaccharide (LPS) leakage into the blood. The increased LPS exacerbates AML progression in vitro and in vivo (Wang et al., 2022). In another recent study, it has been demonstrated that not only antibiotics but also the different AML chemotherapic regimens can differently influence GM. CPX-351, a new variant with "3 + 7" backbone, protected from gut dysbiosis, mucosal damage, and gut morbidity while increasing antifungal resistance. Mechanistically, the protective effect of CPX-351 occurred through pathways involving both the host and the intestinal microbiota, namely via the activation of the aryl hydrocarbon receptor-interleukin-22 (IL-22)-IL-10 host pathway and the production of immunomodulatory metabolites by anaerobes (Renga et al., 2024).

In consideration of this crucial role of GM in hemopoiesis and immune system regulation, our laboratory research group in IRCCS University Hospital of Bologna

had investigated its impact on post induction phase and on hematological recovery during AML induction therapy. In particular, we profiled the GM of 27 newly diagnosed AML patients using 16S rRNA amplicon sequencing and correlated it with key clinical parameters before and after induction therapy.

Stool samples from 27 AML patients at diagnosis before treatment were collected at IRCCS University Hospital of Bologna (Italy). Twenty-two patient received IC and 5 were treated with decitabine.

According to the same strategy mentioned in paragraph 7 and the known data in literature (Behl et al., 2006), ALC data, in association to absolute neutrophil count (ANC) and platelets count (PLT), were obtained at the 4 known established TPs. Hematological recovery was assessed using the following cut-off values: full recovery (FR) for PLT $\geq$50,000/mm^3 and ALC and ANC $\geq$500/mm^3; no recovery (NR) for PLT <50,000/mm^3 and ALC and ANC <500/mm^3. Due to prolonged neutropenia, 4 patients were administered subcutaneous granulocyte colony-stimulating factor until ANC recovery. Twenty-two out of 27 patients were evaluable for hematological recovery, none of whom received decitabine. The ALC value of 500/mm3 was reached only since day 28 in 12 patients, since day 21 in 8 patients and, remarkably, since day 15 in 5 patients. This early ALC recovery is known to be a strongest prognostic factor; in fact, these 5 patients achieved CR, which was maintained and then they succeded with HSCT consolidation. We could not directly correlate ALC recovery as a clinical biomarker to infection occurrence; nevertheless, in our cohort, patients who had achieved ALC recovery had not experienced prolonged fever episodes nor septic status and did not have microbiological isolates. As expected, the same considerations could be made for patients who rapidly recovered ANC. Regarding PLT recovery, on day 21, 10 out of 22 patients had already reached the 50,000/mm^3 cut-off without ANC recovery; all of them achieved CR after induction therapy, and most of them proved to be MRD negative after subsequent courses. Nine of them were alive and in CR at the time of writing, and only 2 achieved consensual ALC recovery at all TPs considered.

We found an association between GM diversity and composition at diagnosis and hematological (especially PLT and ANC) recovery at day 28 after therapy. In particular, patients who recovered PLT (RPs) had higher baseline alpha diversity

than those who did not (NRPs)(Wilcoxon test, $p = 0.002$). Moreover, PCoA based on both unweighted and weighted UniFrac distances showed a significant segregation between PLT RPs and NRPs (PERMANOVA, $p \leq 0.02$). Similar results were obtained for alpha (Wilcoxon test, $p = 0.05$) and beta diversity (PERMANOVA, unweighted UniFrac, $p = 0.04$; weighted UniFrac, $p = 0.1$) of patients stratified by ANC recovery, while no differences were found when considering ALC recovery ($p \geq 0.2$). GM alpha diversity was also positively correlated with both PLT and ANC recovery (point-biserial correlation rpb$\geq$0.47;$p \leq 0.03$). Taxonomically, PLT RPs showed increased proportions of the families *[Barnesiellaceae]*, *Prevotellaceae*, *S24-7* and *[Odoribacteraceae]*, and the genera *Ruminococcus*, *Blautia*, *Faecali bacterium*, *Butyricimonas* and *Prevotella* compared to NRPs (Wilcoxon test, $p < 0.05$). On the other hand, PLT NRPs patients were enriched in *Micrococcaceae*, including *Rothia*, and *Enterobacteriaceae* ($p \leq 0.05$). Partially overlapping results were obtained for ANC RPs, who were enriched in the families *[Barnesiellaceae]*, *S24-7*, *[Odoribacteraceae]* and *Alcaligenaceae*, and the genera *Faecalibacterium*, *Butyricimonas*, *Sutterella* and *Anaerotruncus* compared to ANC NRPs ($p < 0.05$). *Lachnospiraceae* and *Blautia*, together with *[Eubacterium]* were more represented in the baseline GM profile of ALC RPs compared to their NRP counterparts ($p \leq 0.03$). The above GM signatures were generally confirmed when considering integrated hematological recovery (*i.e.*, PLT, ANC and ALC together). In short, patients who fully recovered (FRs) tended to have higher alpha diversity than those who did not (NRs) (Wilcoxon test,$p = 0.079$), were significantly segregated in the unweighted UniFrac-based PCoA (PERMANOVA, $p = 0.021$), and were discriminated by higher proportions of *Blautia*, *Butyricimonas*, *[Eubacterium]*, *Odoribacter*, *Anaerotruncus*, *Dorea*, *Sutterella* and *Ruminococcus*, and lower proportions of *Rothia* and *Enterococcus* (Wilcoxon test, $p < 0.1$).

Our investigation revealed associations of interest between GM composition and crucial recovery indicators, including platelets, lymphocyte, and neutrophils counts, and identified early GM signatures predictive of improved hematological recovery. Despite certain study limitations, our findings suggest that evaluating GM features could serve as a predictive tool for hematological recovery.

12. Correlations between ALC and Nanostring

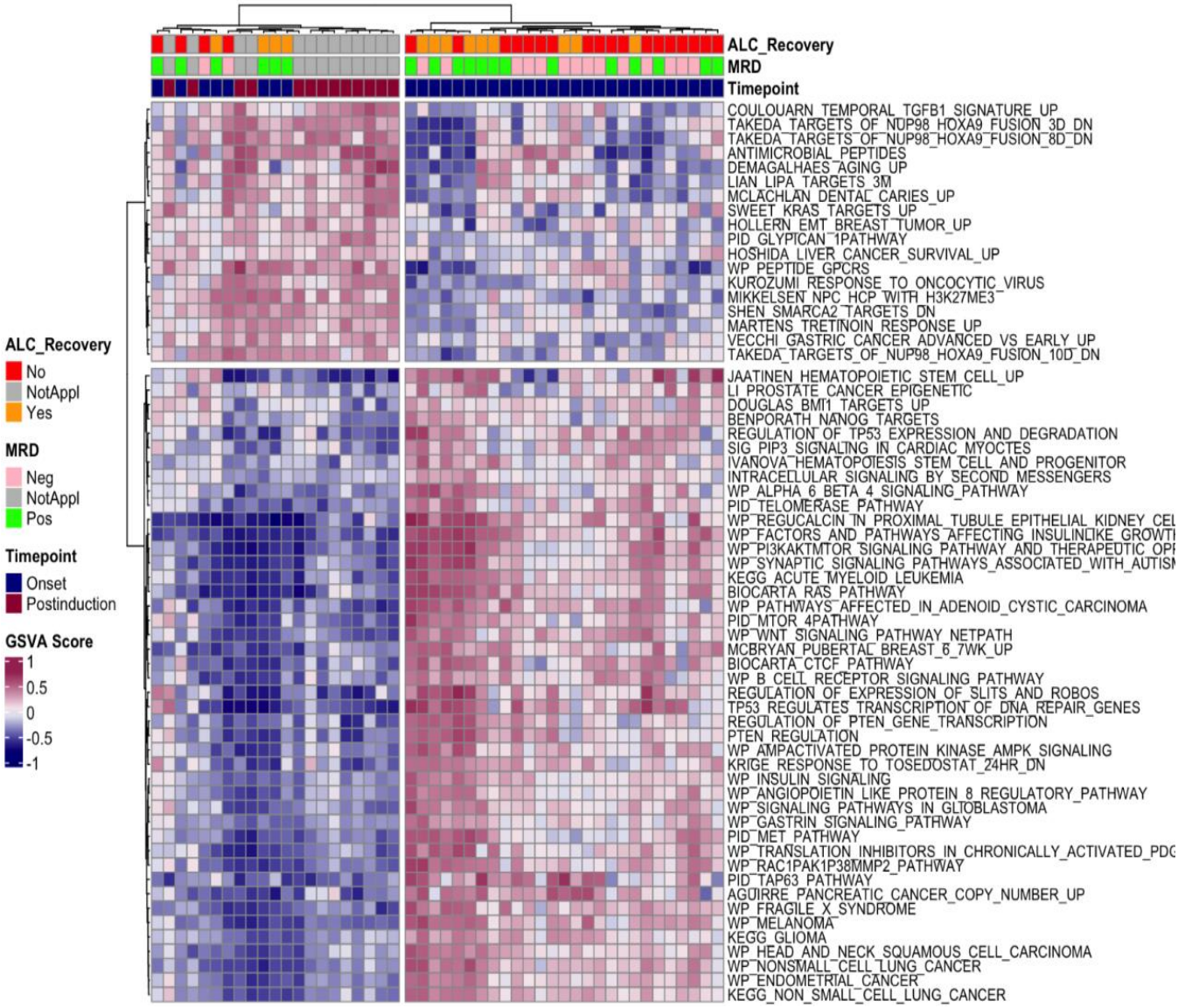
Through the heatmaps described above in paragraph 4, we have studied a population, represented by 35 patients treated with a *"3+7" based* backbone regimen, whose onset BM samples have been collected and for 13 of them also the post induction BM samples. The RNA extracted from these 48 BM samples have led to analysis using the *PanCancer IO-360* preconstituted gene panel by NanoString technology.

Considering the most significant gene pathways (Fig 4.2) and transcriptional factors (Fig 4.3), which belong to immune cells infiltration, immunologic changes in response to immunotherapy, tumor-specific antigens, cellular survival and proliferation, cellular cycle control and "*housekeeping*" genes, the two heatmaps indicate a quite evident difference in gene expression profile which separates onset samples from post induction ones, as the genes which resulted up-regulated in one TP were down-regulated in the other one and viceversa. Neverthless, this specular difference was not complete; in fact in both analysis some onset genetic profile resulted closer to post induction, as well as some post therapy sample resulted similar to onset profile, highlighting some heterogenous features, that need to be cleared up.

In paragraph 9, we have shown how ALC recovery, in accordance with the known data in literature, has been confirmed as an independent prognostic factor in our population of AML patients who obtained CR after IC. In fact, overall in the *"3+7" based* regimen subgroup, the advantage in terms of OS regarding patients who had recovered ALC≥500/mm^3 in all 4 TPs considered compared who those that did not achieve ALC recovery in one TP resulted statistically significant. However, the survival correlation among further molecular subgroups, because of the small numbers, requires deeper investigations.

Beyond the evidences which have been showed both in the biological and in the clinical sections, this last following heatmap (Fig 12.1) matches the results of the two different sections and fields, showing the same comparison between onset and post induction samples according to the most significant gene pathways, with the addition of the variables known as ALC recovery ALC≥500/mm^3 in all 4 TPs and the evaluation of MRD status after the first consolidation course.

Fig 12.1 Heatmap showing the most statistically significant gene pathways involved in the samples in study with the addition of the variables of ALC recovery and MRD status post consolidation. Each



sample is matched with every single lines of pathway defined. The level of expression of the pathways is represented by a different grade of color, from the "purple" which indicates down-regulated pathway to the "pink" which stand for up-regulated pathway. Focusing on the onset samples group, which can be subdivided for ALC recovery and MRD, the comparison according to these variables, in different gene pathways, highlights some differences in profiles.

As previous heatmaps, the columns represent every one of the 48 BM samples of the study, both onset and post therapy. Onset samples are represented in blue and are mainly located in the right side of the graph, while post induction ones in red and mainly represented in the left side of it. The horizontal lines identify the most significant gene pathways selected from the panel. The level of expression of every single pathway is represented by a different grade of color, from the "purple" which indicates down-regulated pathway to the "pink" which stands for up-regulated pathway.

Considering the 35 onset BM samples, 13 out of 35 corresponding patients had obtained ALC recovery in the chosen cut off at all 4 TPs after IC (colored in orange in the graph), while 22 patients did not reach that aim, having ALC <500/mm^3 at least in one out of 4 TPs (indicated in bright red). Regarding MRD status, 19 out of 35 patients still resulted MRD positive (according to the evaluation methods mentioned above) and 16 patients achieved MRD negativity (respectively shown in green and in bright pink in the graph).

Hence, we can obtain 4 subgroups according to these two parameters studied: 10 out of 35 patients had poor ALC recovery and MRD positivity after consolidation (bright red and green combination); instead 9 patients obtained ALC recovery but still were MRD positive (orange and green subgroup); viceversa 12 patients achieved MRD negativity but did not have a good ALC recovery (bright pink and bright red); lastly only 4 patients out of 35 belonged to the best match reaching proper ALC and MRD status (bright pink and orange).

Considering the different pathways shown in the heatmap, we still can assume there are clear differences between onset and post therapy setting, in terms of gene pathway expression.

Of note, focusing just on onset samples columns, on the right side of the graph, this heatmap can enlight some differences in expression profile considering ALC and MRD status variables and further subdividing patients in the 4 subgroup listed above. Here we show these differences focusing on some of the gene pathways, whose characteristics have been described previously in paragraph 4, indicated in the heatmap. Nevertheless, the numbers are very small in order to produce significant correlations.

Hence, regarding gene pathways which in most of onset samples resulted down-regulated (in the upper part of the heatmap), such as TGFB1 signature, antimicrobial peptides, KRAS targets, aging up genes, response to oncocytic virus, it is notable that these pathways result instead much up-regulated in those few patients who both had a good ALC recovery and MRD negativity (bright pink and orange), and further a different grade of expression also in those who obtained at least one of these two variables, when compared with samples in the same onset group who did not reach ALC and MRD negativity neither (bright red and green) .

Moreover, in a similar way, we considered some of the gene pathways which had mainly resulted up-regulated: hematopoietic stem cell; TP53 expression and degradation, stem cell and progenitor, alpha4beta6 signaling, PI3K-AKT-MTOR, acute myeloid leukemia, RAS pathway, MTOR, WNT signaling, CTCF, B-cell receptor signaling, PTEN transcription, AMP-activated protein kinase (AMPK) signaling. Specularly, the same differences spotted previously are notable also with these pathways, showing an evident less intensity grade of expression in patient of the “bright pink” and “orange” subgroup versus those ones with poor prognostic markers, and a different grade of expression when reaching ALC recovery or MRD negativity as well.

13. Discussion and future perspectives

A better and more-in-depth understanding of the interactions between AML and immune system is likely to enable the development of new therapeutic and prognostic scenarios.

Concurrently to the extensive characterization of AML blast cell, which has led to crucial changes and implications in AML prognostic evaluation, as shown in previous paragraphs, and to the addition of new targeted therapies to the armamentarium, in recent years the study and interest of ME in BM in which AML cells form and grow up has been rapidly raising.

This research project is closely focused, as a central theme, on the complex relationship between the immune system network and AML. In particular, our study explores the immune transcriptomic profile variation in AML patients treated with chemotherapy and, concurrently, the impact of a clinical prognostic parameter as ALC.

Therefore, in the context of this wide and complex issue, our research can be mainly divided in two stages. In the first one, a biological gene expression translational analysis has been performed using BM samples collected from a small portion (35 patients) of the population in study. In the second one, the impact of ALC recovery on disease survival has been evaluated in all patients of our population (188 patients), with appropriate subanalysis according to different clinical and molecular variables.

As for the first part of the research, RNA extracted from mononuclear cells of patients' BM samples was analyzed by NanoString technology using the *PanCancer IO-360* preconstituted gene panel. The aim was to assess potential differences in immunological gene expression and different gene pathways involved in AML patients treated with IC. Hence, the comparison have been conducted between BM samples at diagnosis and samples collected after induction therapy. Regarding the 35 AML patient, BMs were collected at onset and for 13 of them also after therapy. In total, 48 BM samples used for NanoString analysis. All these 35 patients belong to the *"3+7" based* therapy group; moreover, it is worth remembering that all 35 patients, in line with the inclusion criteria for the AML patient whole population

selection in the study, obtained CR after induction chemotherapy. Based on this, the 13 BM post induction samples collected refer to patients in CR.

The Volcano plot graph shown in Fig 4.1 indicates the gene expression distribution among the whole 48 BM samples from AML patients, subdivided in 35 onset samples and 13 post therapy ones (without considering every single patient) among the immunological network genes of major interest included in the NanoString panel. Observing the red-marked genes, which correspond to the statistically significant ones, the Volcano plot highlights remarkable gene expression differences between the onset samples and the post induction ones. In particular, if more genes regarding cell survival, cell cycle regulated, proliferation enhancers were much more represented in the onset group, conversely, in the post induction samples, genes involved in the immune system activation were more expressed, as expected when evaluating post chemotherapy BMs collected from responsive patients.

In addition to this comparison, similar differences have been highlighted in the heatmap representations. Considering the different gene pathways shown in the first heatmap (Fig 4.2), there is quite a straight separation in terms of gene pathway expression between onset and post therapy samples. In fact, as indicated in the upper part of the graph, all the pathways which are up-regulated (towards pink) in the post induction (collected from patients who obtained CR) result down-regulated (towards purple) in the onset samples; instead, specularly, all the pathways which are up-regulated at onset disease correspond to a down-regulated expression of the same pathway in the post induction phase. These differences match again the expected BM transcriptomic profile at AML onset in comparison with the post therapy.

Considering the transcriptional factors shown in the second heatmap (Fig 4.3), the comparison between a large portion of the onset samples and the post induction ones exhibits a clear and specular distinction in transcriptional factors gene expression also in this representation. Of note, there is a third column on the right side which points out no clear data and much more heterogenous gene expressions result; this column includes, according to genomic profile, samples of both TPs. The heterogeneity of expression distribution in this patients portion make us assume that necessarily other variables must be involved. In order to explain the reason of these

exceptions and this behavior of expression at onset which results similar to remission phase (and viceversa), further subanalysis are needed and other variables are ought to be found.

In the second part of the PhD research, according to data known in literature, we applied the ALC recovery tool as a clinical prognostic marker in our population, thus investigating its impact on survival. We collected data of 188 newly diagnosed AML patients treated at Bologna Seràgnoli Hematology Institute, who all resulted responsive to treatment. Then we stratified patients into different subgroups according to factors such as the type of chemotherapy regimen, the molecular status at diagnosis, the use of consolidation with HSCT and the level of MRD status after consolidation. We defined the 4 ALC time-points (TPs) from IC start and an ALC cut-off of 500/mm^3.

ALC recovery did not significantly impact on survival in the whole population. Hence, we decided to repeate the survival correlation analysis on single subgroups to evaluate the impact. Among patients treated with "3+7-based" regimen (51,6%), a relevant correlation with OS of patients with ≥500/mm^3ALC (31,9%) vs those under the cut-off (19,6%) was observed at ALC-15 alone. This finding resulted further statistically significant in patients with ALC≥500/mm^3 in all 4 TPs (25,5%) compared to those who had <500/mm^3ALC in at least one TP (26%)(p=0.02). These curves suggested that ALC recovery in all 4 TPs is a significant prognostic marker in "3+7-based" patients.

The explanation of this result was evident when the analysis was applied to the fludarabine-based subgroup. The correlation with OS of patients who reached ALC ≥500/mm^3 in all TPs vs those who did not obtain 500/mm^3 in at least one TP was completely inverted compared to "3+7-based" subgroup, with a better survival of those who had a poor ALC recovery. It is plausible to speculate that probably the lymphocytolitic effect of fludarabine had a remarkable influence for this evaluation and that apparently the better survival despite the inadequate ALC recovery could have been tainted by the subsequent HSCT used in the FLAI-based group.

In the molecular subgroups (*NPM1* and *FLT3*), a trend of better survival can be spotted among patient who achieved ALC recovery, but it was not statistically

significant, suggesting the ALC, according to our data, cannot modify molecular prognostic impact.

Of note, the discussion regarding the role of transplantation is worthwhile. As can be shown in the figure, which indicated the 4 subgroup base on ALC and HSCT from the whole population in study, patients belonging to the transplanted groups had globally a better survival. For instance, an ALC $\geq 500/mm^3$ in all 4 TPs determined a better survival regardless the use of HSCT. In fact, for example, patients who obtained a complete recovery, despite not receiving HSCT, resulted to have a better OS, closer to the two subgroups who had been transplanted. Therefore, we can assume that, given that an insufficient ALC recovery constitutes a poor prognostic factor, the use of HSCT could revert the worse prognosis of these patients, in a similar way to well-known consolidated parameters of ELN prognostic criteria.

Lastly, ALC recovery was also correlated with post consolidation MRD value. Among "3+7-based" patients who obtained MRD negativity (n = 52), those who had $\geq 500/mm^3$ALC in all ALC TPs (n=29) and in ALC-15 (n=35) resulted in a globally better OS as compared to patients with $<500/mm^3$ ALC at the two different evaluations (n=23 and n=17, respectively). Results were similar among MRD positive patients. These outcomes are not statistically significant; data clearly need higher number to conclude if ALC could represent an independent prognostical factor.

Moreover, according to our findings reported in terms of GM composition among AML patients treated with IC, ALC, as long as other single recovery data, can been further associated with other immune related variables, such as early GM signatures data themselves.

In a third part of the research, we combined the results obtained from the biological part (paragraph 4) and the clinical data of ALC, pursuing the correlation between NanoString data and prognostic factors such as ALC or MRD.

We used another gene pathways heatmap, which had let the distinguishing in 4 subgroups according to MRD status post consolidation therapy and ALC recovery, intended as recovered ALC in the same known cut-off $500/mm^3$.

Considering the 35 onset BM samples, 13 out of 35 patients had obtained ALC recovery at all 4 TPs after IC (colored in orange in the graph), while 22 patients did

not reach that aim, having ALC <500/mm^3 at least in one out of 4 TPs (indicated in bright red). Regarding MRD status, 19 out of 35 patients still resulted MRD positive and 16 patients achieved MRD negativity (respectively shown in green and in bright pink in the graph). If we take the two parameters studied, 4 different groups can be established, as mentioned above in paragraph 12.

Considering the different pathways shown in the heatmap, we still can assume there are clear differences between onset and post therapy setting, in terms of gene pathway expression. Nevertheless, focusing just on onset samples, on the right side of the graph (Fig 12.1), this heatmap can enlight further unequal expression profiles when considering ALC and MRD status variables, alone or taken both. Here we show these differences focusing on some of the gene pathways, whose characteristics have been described previously in paragraph 4, indicated in the heatmap. Nevertheless, the numbers are very small in order to produce significant correlations, which for instance has to be interpreted cautionally and conclusive analysis are ought to be performed, regarding the evaluations for every single pathway or single gene, in order to add validity to this finding.

Pursuing new biological and clinical prognostic tools in management of AML, it has been demonstrated that ALC recovery after IC can be a promising independent survival predictor, in AML patients who achieve CR. Its impact is different according to the used IC regimen type (3+7 versus fludarabine-based). Importantly, ALC recovery seems to emphasize an OS difference among MRD negative patients and may represent a predictive biomarker to identify a group of responding patients who may benefit from HSCT.

In order to explore ALC potentiality as a prognostic predictor, we should further subdividing AML patients in more groups, considering the use of a third drug added to a backbone (such as FLT3 inhibitors, gemtuzumab-ozogamycin, venetoclax) or the differentiation of categories according to ELN risk, or including a more precise molecular characterization. Along with these subdivisions, studies with larger cohorts of patients are needed and highly warranted, in order to consolidate the results.

In a scenario where cancer immunotherapy is rapidly changing the therapeutic armamentarium available to treat human solid and hematological malignancies, a new era of immunotherapy has also been initiated in the AML setting.

As a future perspective, once consolidated, the ALC could be incorporated as an independent prognostic factorinto a multivariable matched prognostic score, which therefore might consider both biological and clinical factors and thus lead, hypothetically, to ELN risk category redefinition for a certain amount of patients.

In this setting, biological data such as the complete transcriptomic characterization of AML ME, in the context of increasing the knowledge of immune system behavior in AML, could help finding strategies to improve the outcome of patients with AML. This would be helpful especially in high-risk patients, whose prognosis is still largely disappointing and who represent an unmet medical need. Conversely, these tools could be also applied to patients with a favorable ELN risk, where the molecular status could hide a non-prognostic favorable immune ME and a poor quantitative and qualitative ALC recovery.

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