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**CHARACTERIZATION OF T CELL DYSFUNCTION IN AML PATIENTS
DURING TREATMENT WITH HYPOMETHYLATING AGENTS PLUS
VENETOCLAX**

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Abstract

In AML there is an adaptive immune dysfunction, involving above all T lymphocytes, with a prevalence of Tregs and Th17 producing IL-10; than T cells in AML exhibit features of senescence, exhaustion and anergy.

This study project aims to provide comprehensive insights into longitudinal immune landscape in AML, focusing on characterization of T cell dysfunction in patients candidates for treatment with Azacitidine or Decitabine, alone or in combination with Venetoclax, both in first line and in the relapsed/ refractory setting, using different methods.

Firstly, we have collected retrospective data about patients affected by newly diagnosed AML, candidates for Azacitidine single agent. For those patients, peripheral blood and bone marrow samples, have been analyzed using flow cytometry, in order to characterize T cells (as T cell subpopulations, and as their expression of the immune checkpoints). We have found that PD1 expression by T cells resulted to be high, leading to an impaired T cell function and to tumor escape; furthermore, in our cohort of treatment naïve AML patients, the mean concentration of Tregs in peripheral blood was 18% of CD4⁺ T cells. Using the same flow cytometry characterization, in a small cohort of patients responsive to hypomethylating agents plus Venetoclax, we found a low expression of all the immune checkpoints and a lower concentration of immunosuppressive Tregs in peripheral blood (about 7% of CD4⁺ T cells), compared to treatment naïve patients.

Because of cell sorting by flow cytometry has some limitations, we then used a RNA sequencing for the prediction of immune cell subtypes abundances, in patients candidate to first line or salvage therapy with Venetoclax plus an hypomethylating agent. With CYBERSORT, we found that, in AML patients, immune cells (both T helper and natural killer cells) were mostly in a rest functional state, and this could contribute to immune evasion.

Background

1. Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is a disease arising from uncontrolled proliferation of clonal hematopoietic cells. AML is diagnosed mainly at older age, with a median age at diagnosis of 68 years old and has an estimated 5-year OS of 32% (up to 50% in young patients and less than 10% in patients older than 60 years) (*Shimony S, AJH 2025*).

1.1 Diagnosis

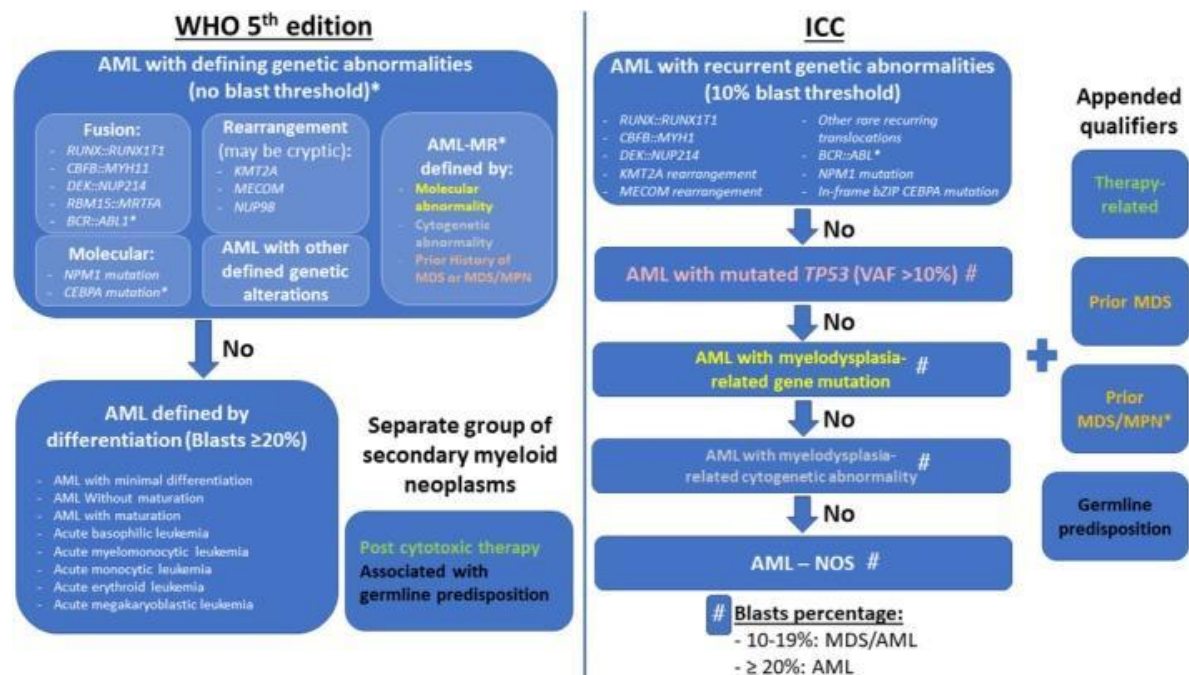
The WHO 5th edition and the International consensus criteria (ICC) have different criteria for AML diagnosis, as illustrated in figure 1.

According to WHO 5th edition, there is no minimum threshold of blasts for AML with defining genetic abnormalities (that include AML with RUNX::RUNX1T1, CBFB::MYH11, DEK:NUP214, RBM15:MRTFA, AML with rearrangement of KMT2A, MECOM, NUP98, and AML with NPM1 mutation), whereas 20% of blasts are required for the diagnosis of AML with BCR::ABL1 and CEBPA bZIP mutation, and for the diagnosis of AML without defining genetic abnormalities. Then the WHO recognizes the category of AML myelodysplasia related (AML-MR), which includes a clinical history of MDS or MDS/MPN, or cytogenetics typical of MDS, or molecularly defined based on eight secondary ontogeny defining mutation. Finally, the separate group of secondary myeloid neoplasms include those with a prior cytotoxic therapy and those with a germline predisposition.

Different from the WHO, the ICC requires at least 10% of blasts in the bone marrow or in the peripheral blood for diagnosis of AML with recurrent genetic abnormalities, with the exception of AML with BCR::ABL1 or with prior MDS/MPN, that require 20% of blasts for the diagnosis. Then the ICC introduces the category of MDS/AML comprising those with 10-19% of blasts. As regards secondary AML, the ICC includes two separate entities, which are molecularly defined and cytogenetically defined. A prior history of MDS or MDS/MPN, or prior cytotoxic exposure, or a germline predisposition, do not define separate diagnostic category of AML but are only qualifiers or annotation according to ICC classification.

So, the WHO describes the entities of AML, one defined by genetic abnormalities, and the other by differentiation, whereas the ICC classification uses a hierarchical model for the diagnosis of AML (Shimony S, AJH 2025).

Figure 1



*>=20% of blasts are required for AML definition

Shimony S, AJH 2025

1.2 Risk stratification

Risk stratification in AML is based on European Leukemia Network (ELN) guidelines. The ELN 2022 risk classification has been developed from intensively treated patients up to 60 years, and it has a limited prognostic role for older patients who are treated with less intensive therapies, mainly Venetoclax based. The ELN 2024 risk classification has been developed and validated for patients with AML treated with lesser intensive therapies.

Focusing on the ELN 2024 risk classification, this recognizes three prognostic groups: those with TP53 mutations are included in a lower benefit group; those without TP53 mutations, but harboring a FLT3-ITD or NRAS or KRAS mutations are intermediate; those without any of these four mutations are favorable (*Döhner H, Blood 2022, Döhner H, Blood 2024*).

Table 1 and table 2 illustrates ELN 2022 and ELN 2024 risk classification respectively.

Table 1: ELN 2022 risk classification (adapted from *Döhner H, Blood 2022 and Shimony S, AJH 2025*)

<u>Risk category</u>	<u>Genetic abnormality</u>
Favorable	t(8;21)(q22;q22.1)/ <i>RUNX1::RUNX1T1</i>
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22)/ <i>CBFB::MYH11</i>
	Mutated <i>NPM1</i> , without <i>FLT3</i> -ITD
	bZIP in-frame mutated <i>CEBPA</i>
Intermediate	<i>FLT3</i> -ITD (irrespective of allelic ratio or <i>NPM1</i> mutation)
	t(9;11)(p21.3;q23.3)/ <i>MLLT3::KMT2A</i>
	Cytogenetic and/or molecular abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23.3;q34.1)/ <i>DEK::NUP214</i>
	t(v;11q23.3)/ <i>KMT2A</i> -rearranged (excluding <i>KMT2A</i> -PTD)
	t(9;22)(q34.1;q11.2)/ <i>BCR::ABL1</i>
	t(8;16)(p11.2;p13.3)/ <i>KAT6A::CREBBP</i>
	inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2)/ <i>GATA2</i> , <i>MECOM(EVI1)</i>
	t(3q26.2;v)/ <i>MECOM(EVI1)</i> -rearranged
	−5 or del(5q); −7; −17/abn(17p)
	Complex karyotype, monosomal karyotype
	Mutated <i>ASXL1</i> , <i>BCOR</i> , <i>EZH2</i> , <i>RUNX1</i> , <i>SF3B1</i> , <i>SRSF2</i> , <i>STAG2</i> , <i>U2AF1</i> , and/or <i>ZRSR2</i>
	Mutated <i>TP53</i> (variant allele frequency ≥10%)

Table 2 ELN 2024 risk classification for patients treated with less intensive chemotherapy (adapted from *Döhner H, Blood 2024 and Shimony S, AJH 2025*)

Risk category	Genetic abnormality
Favorable	Mutated <i>NPM1</i> (<i>FLT3</i> -ITD ^{neg} , <i>NRAS</i> ^{wt} , <i>KRAS</i> ^{wt} , <i>TP53</i> ^{wt})
	Mutated <i>IDH2</i> (<i>FLT3</i> -ITD ^{neg} , <i>NRAS</i> ^{wt} , <i>KRAS</i> ^{wt} , <i>TP53</i> ^{wt})
	Mutated <i>IDH1</i> (<i>TP53</i> ^{wt})
	Mutated <i>DDX41</i>
	Other cytogenetic and/or molecular abnormalities (<i>FLT3</i> -ITD ^{neg} , <i>NRAS</i> ^{wt} , <i>KRAS</i> ^{wt} , <i>TP53</i> ^{wt})
Intermediate	Other cytogenetic and molecular abnormalities (<i>FLT3</i> -ITD ^{pos} and/or <i>NRAS</i> ^{mut} and/or <i>KRAS</i> ^{mut} ; <i>TP53</i> ^{wt})
Adverse	Mutated <i>TP53</i>

An important role in defining the prognosis of AML patients belongs to the minimal or measurable residual disease (MRD) also for patients treated with less intensive regimens. Among patients who have been treated with Azacitidine and Venetoclax in the VIALE-A trial, in patients who achieved complete remission, the achievement of undetectable MRD was associated with better outcomes compared to those who failed to have responses at such a deep level (*Pratz KW, AJH 2024*).

1.3 Treatment

The therapeutic landscape in AML has undergone a major change in last years with the approval of 12 new drugs or combination regimens.

For patients with newly diagnosed AML (ND-AML) age <60 years, eligible for intensive induction, the backbone of the treatment consists of intensive chemotherapy (3+7, FLAG-IDA and other regimens), alone or in combination with Gemtuzumab ozogamicin (a CD33-directed antibody drug conjugate) or a FLT3 inhibitor (Midostaurin or Quizartinib). Then CPX-351, a liposomal formulation of daunorubicin and cytarabine, was approved for AML with myelodysplasia related changes and therapy related-AML. For patients with TP53 mutated AML, the treatment algorithm suggested a chemo free approach, based on a hypomethylating agent (HMA) plus Venetoclax (*Shimony S, AJH 2025*).

Older patients unfit for intensive chemotherapy had a dismal prognosis in past years, when the treatment was based on hypomethylating agents (HMAs) alone (Azacitine or Decitabine) or best supportive care.

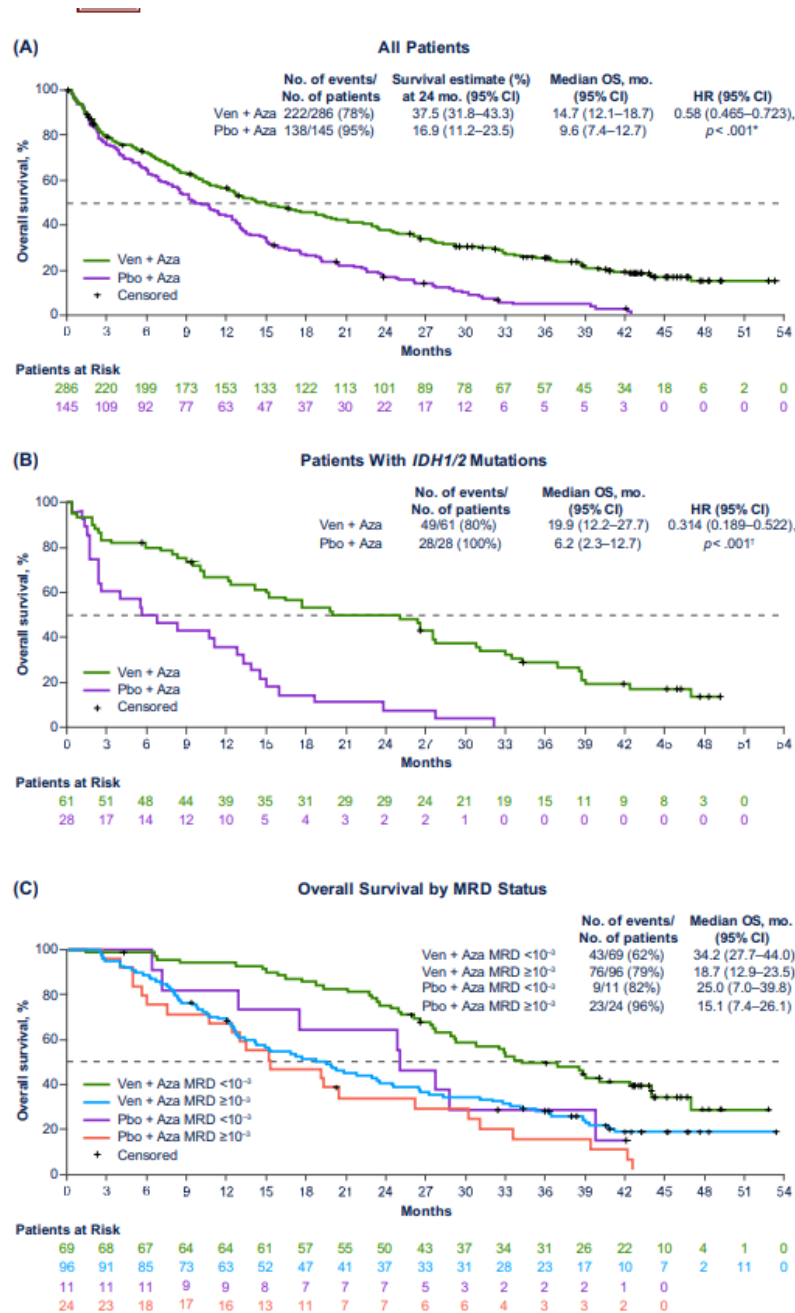
The approval in the last years, of Venetoclax plus Azacitidine or Decitabine (VEN+HMA) revolutionized the treatment paradigm for AML patients unfit for intensive chemotherapy. Venetoclax, a BH3 mimetic, that selectively inhibits the proapoptotic BCL2 protein, and induces apoptosis in AML cells, was approved in combination with HMAs or low dose AraC (LDAC) for newly diagnosed (ND) AML patients, unfit for induction with intensive chemotherapy (based on age $\geq$ 75 years old, or younger but unfit because of significant comorbidities), according to the results of the phase III VIALE-A and VIALE-C trials. So, the treatment algorithm for ND-AML in patients older than 75 years old or younger but unfit, is mainly based on HMA+ VEN, with the addition of Ivosidenib in IDH1 mutated patients, or Gilteritinib in FLT3 mutated patients (*Shimony S, AJH 2025*).

In the phase III VIALE-A trial, Azacitidine added to Venetoclax improved responses and overall survival (OS) versus Azacitidine alone in patients >75 years old or those with significant comorbidities. In the study, ND-AML patients, who were ineligible for intensive chemotherapy, were randomized 2:1 to receive Venetoclax- Azacitidine or placebo- Azacitidine. The primary endpoint was OS. The trial enrolled 431 patients.

In the final analysis, performed at 43.2 months of median follow-up, median OS was 14.7 months with VEN-AZA vs 9.6 months with placebo-AZA, and the estimated 24-months OS rate was 37.5% vs 16.9% respectively. The rate of CR was 38.8% vs 17.9% with VEN-AZA vs placebo-AZA, with a median time to first CR of 1.3 months and a median duration of response (DOR) of 22.1 months in the VEN-AZA arm.

Analysis of baseline characteristics associated with response, MRD outcomes and survival, indicated that VEN –AZA provides benefit over AZA alone, across all patient subpopulations. A higher proportion of patients with poor-risk cytogenetics, IDH1/2, NPM1 or TP53 mutations, achieved undetectable MRD with VEN-AZA versus AZA alone. Attainment of MRD negativity was associated with longer survival also in the VEN-AZA arm, supporting the prognostic role of MRD response even with non – cytotoxic therapy, as mentioned before. AZA-VEN demonstrated benefits also in subgroups of patients with poorer outcomes to conventional therapies. IDH1 and IDH2 mutated AML patients experienced improved OS when treated with VEN-AZA vs AZA alone, and in particular IDH2 mutation was associated with longer OS than IDH1 mutation in both treatment arms. Poor-risk cytogenetic and TP53 mutations were associated with poor outcomes to conventional therapies. In TP53 mutated patients, AZA-VEN demonstrated a benefit in terms of survival vs AZA alone, but different therapeutic strategies are still necessary for these resistant genomic subgroups, according to the low rate of survival at 2 years. Figure 2 illustrates OS in patients treated with Venetoclax- Azacitidine or placebo- Azacitidine, in the entire study population, and in patient subgroups, defined by IDH1/2 mutation status, and according to MRD status (*Pratz KW, AJH 2024*).

Figure 2



Pratz KW, AJH 2024

2. Immune dysfunction in AML

In AML, as in other cancers, there is a severe adaptive immune dysfunction. Blasts cells are capable of creating an immunosuppressive milieu, where both innate and adaptive immune responses are deregulated (*Tang L, Clin Cancer Res 2020*).

T cells are a crucial part of the adaptive immune system and potent effector cells in targeting leukemia. Different studies showed that both CD8⁺ cytotoxic T cells and CD4⁺ T helper cells, exhibit features of senescence, exhaustion and anergy. Senescence is the natural age-related process, which occurs with a shortening of telomeric ends. T-cell exhaustion is a poor responsive status, with upregulated expression of inhibitory receptors, decreased production of effective cytokines, and reduced cytotoxic activity. Circulating T cells from patients with AML demonstrate signatures of terminal senescence (CD28^{low}CD57^{high}IFN γ ^{high}TNF α ^{high}) and exhaustion (PD-1^{high}IFN γ ^{low}TNF α ^{low}) simultaneously (*Tang L, Clin Cancer Res 2020*). Furthermore, T cells in AML have an abnormal gene expression profile and are unable to form effective immune synapses with AML blasts (*Le Dieu R, Blood 2009*). T-cell dysfunction/exhaustion is present at diagnosis and T-cell functional status correlates with disease phase and treatment. A significant immune evasion mechanism is the expression of immune checkpoint ligands by leukemic blasts: T cells from AML patients express higher levels of immune checkpoint molecules (such as cytotoxic T-lymphocyte associated protein 4, CTLA4, programmed cell death protein 1, PD-1, T cell immunoglobulin and mucin domain 3, Tim-3, and lymphocyte activating-3, LAG-3) and their expression increases with disease progression (*Barakos GP, Diseases 2022*).

CD4⁺ T helper (Th) lymphocytes are essential for the regulation of immune responses, as they have the ability to modulate the function of CD8⁺ cytotoxic T lymphocytes (CTLs), B cells, natural killer (NK) cells, macrophages, and dendritic cells. Following triggering of their T cell receptor (TCR), and in the presence of appropriate costimulatory signals and specific cytokines, naïve CD4⁺ T lymphocytes differentiate into various effector or regulatory cells, characterized by distinct functions and specific cytokine production profiles.

For many years, it has been believed that the expression of two mutually exclusive differentiation programs led to the polarization of naïve CD4⁺ T cells towards either Th1 or Th2 lymphocytes. Terminally differentiated Th1 cells are characterized by the expression of the transcription factor *Tbet* and the production of IFN γ . Th1 cells activate CTLs, macrophages, and are required for the elimination of intracellular pathogens. Th1 cell lineage commitment is primarily triggered by IFN γ and IL-12. Th2 lymphocytes, defined by transcription factor GATA3 expression, and by the secretion of IL-4, IL-5, IL-10, and IL-13, play an essential role in B cell-mediated humoral responses against extracellular pathogens, and can inhibit Th1-dependent cellular immunity (Alizadeh D, *Clin Dev Immunol* 2013). On AML patients, there is a Th1/Th2 imbalance, with a prevalence of Th2 on Th1, demonstrated by increased value of IL-4 and by a decreased value of IFN γ in bone marrow microenvironment (Sun YX, *Hum Immunol* 2014).

Recently, several subsets of CD4⁺ T helper cells have been described, including Tregs and Th17 cells, and also Th22 cells.

Regulatory T lymphocytes (Tregs) may be generated during T cell development in the thymus (naturally occurring, nTregs), or may be induced in the periphery from naïve CD4⁺ T cells (induced/adaptive, iTregs). Tregs generation essentially depends on TGF β , together with TCR, costimulatory signals, and IL-2. The transcription factor *FoxP3* is fundamental for the development and function of Tregs. Tregs efficiently suppress effector T lymphocytes; they are, therefore, essential components of the regulatory networks controlling autoimmunity, infection, or cancer (Alizadeh D, *Clin Dev Immunol* 2013). Tregs are defined with the special phenotype of CD4⁺ CD25⁺ and the transcriptional factor *Foxp3*. Tregs secrete IL-10 and IL-35, that are regulatory cytokines with anti-inflammatory and immunosuppressive activity (Sun YX, *Hum Immunol* 2014). In AML patients, several studies found that there is an increased frequency of immunosuppressive CD4⁺ CD25^{high} CD127^{low} Tregs, compared to healthy volunteers, above all in bone marrow. Tregs' frequency seems to correlate also to treatment response, as a reduction in Tregs' frequency has been observed in patients who achieved a complete remission, whereas an increase of Tregs has been found in relapses (Shenghui Z, *Int J Cancer* 2011). So the accumulation of Tregs in bone marrow and peripheral blood of AML patients, not only facilitates the development and advancement of AML but also amplifies treatment resistance and the likelihood of

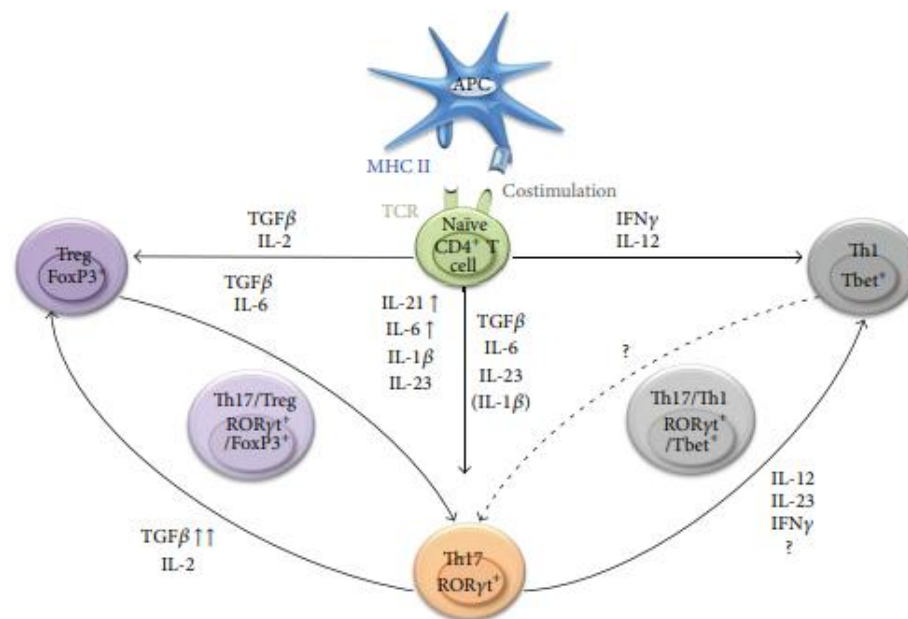
relapse. Different mechanism led to Tregs accumulation in AML patients: extracellular vesicles, derived from blasts, play a pivotal role in augmenting the expression of *Foxp3* and the effector phenotype in Tregs; furthermore elevate expression of Indoleamine 2,3-dioxygenase (IDO) in patients with AML may contribute to an increase in the number of Tregs; additionally AML cells also generate significant quantities of $\text{TNF}\alpha$ which has the potential to induce the proliferation of Tregs (Liu M, *Front Immunol* 2025).

In recent years, T helper 17 lymphocytes (Th17) have emerged as a new distinct effector CD4⁺ T helper cell subset, prompting revision of the Th1/Th2 paradigm. Th17 are a subtype of CD4⁺ T cells, which play a regulatory role in inflammation, autoimmune diseases and tumors (Sun YX, *Hum Immunol* 2014). They release their signature cytokine IL-17A, together with IL-17F, IL-21, IL-22, IL-26, and, depending on the differentiation/environmental conditions, they secrete also $\text{TNF}\alpha$ and $\text{IFN}\gamma$. IL-17 has pro- and anti-tumor actions. IL-21 amplifies the differentiation of Th17 cells in an autocrine manner, as well as control T cell-dependent humoral responses (Abousamra NK, *Med Oncol* 2013). Th17 express the transcription factor *ROR γ t*, which seems to act as a molecular determinant for their polarization. $\text{TGF}\beta$, IL-6, IL-23 and IL-21 are the cytokines involved in Th17 cell lineage commitment (Alizadeh D, *Clin Dev Immunol* 2013).

As regards the role of Th17 in cancer, it is still a matter of debate, with conflicting reports related to the pro-versus anti-tumoral effects of these cells. This discordance may be explained by different cytokine signature profiles, inherent in the high degree of plasticity of these cells. In fact, Th17 showed remarkable epigenetic plasticity, and the ability to transdifferentiate into Th1-like cells (secreting $\text{IFN}\gamma$ and showing tumor suppressor activity) or Treg-like cells (secreting IL-10, with immunosuppressive functions and probably tumor promoter activity). $\text{TGF}\beta$ appears as a master regulator of the balance between Th17 and suppressive Treg differentiation: large amounts of $\text{TGF}\beta$ primarily promote the development of cells endowed with immunosuppressive activity, while low concentration of $\text{TGF}\beta$, in combination with the proinflammatory cytokines IL-6 or IL-21, drives primarily the differentiation of naive CD4⁺ T cells into Th17 (Alizadeh D, *Clin Dev Immunol* 2013).

Figure 3 illustrates the differentiation of specialized T helper lymphocytes driven by specific cytokines.

Figure 3



Alizadeh D, *Clin Dev Immunol* 2013

In AML there are few studies focusing on Th17 cells. Some studies found that circulating Th17 cells are increased in patients with AML respect to healthy controls, and that Th17 cells are reduced significantly in patients who achieve complete remission after induction therapy. So Th17 cells can exert an anti- tumor effect, and in light of these results, elevation in Th17 cells in AML patients may be explained as a protective reaction of the immune system in certain stages of the disease. Furthermore, the reduction of Th17 in patients who achieved a response, could suggest that circulating Th17 cells are related to tumor burden and could be valuable for monitoring response to therapy. Serum levels of IL-17 and IL-21 correlated positively with levels of circulating Th17 cells (*Abousamra NK, Med Oncol* 2013). In other studies Th17 levels resulted to be normal in AML patients.

Tregs and Th17 cells have a reciprocal relationship. However, few researches have been done about Th17/Treg imbalance in AML, especially in bone marrow microenvironment (*Sun YX, Hum Immunol* 2014). In a study evaluating T helper subsets

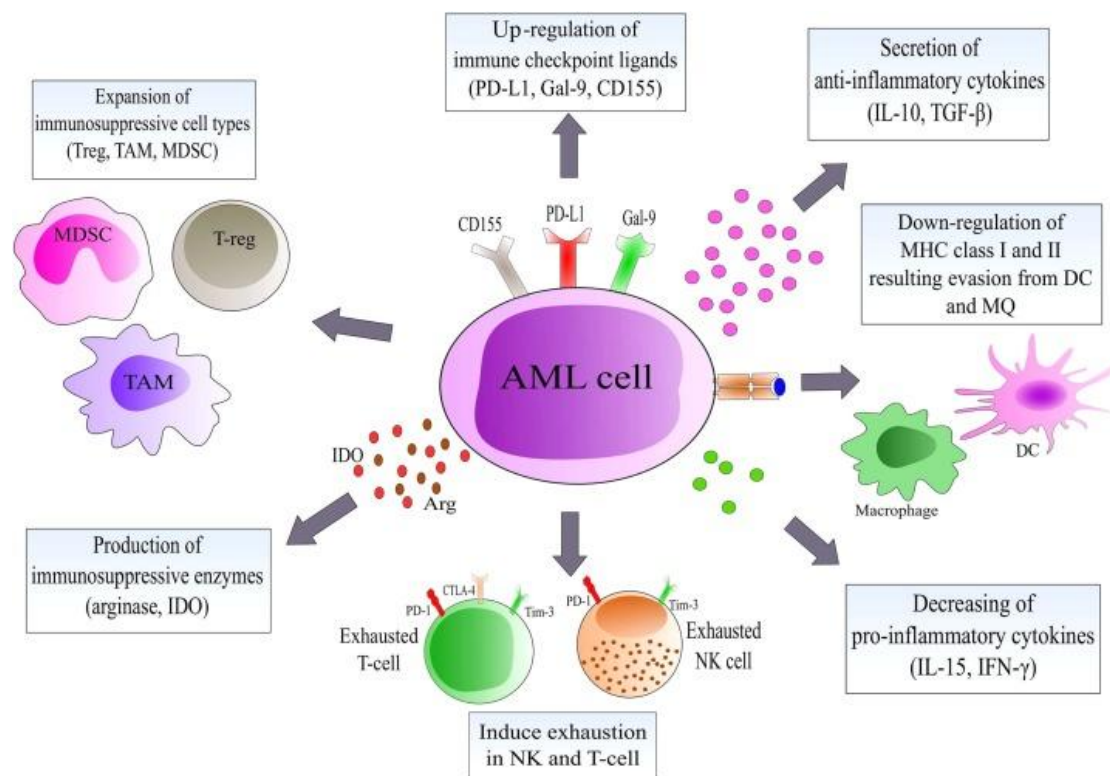
in AML patients, a higher frequency of Th17 and of Tregs has been found respect to healthy people. This study performed a more in-depth investigation into the ability of the increased Th17 cells to simultaneously produce other cytokines, different from IL-17, above all IFN γ and IL-10: a higher increase in the frequency of CD4⁺ IL-17/IL-10 double secreting cells has been observed in AML patients, whereas the frequency of CD4⁺ IL-17/ IFN γ double secreting cells was not increased. Finally, this study suggested that the imbalance between IL-17/IL-10 producing cells and IL-17A/IFN γ producing cells, may act as an additional immunosuppressive factor, promoting leukemia progression and favoring infections (Musuraca G, *J Transl Med* 2015).

Finally, Th22 cells are a novel type of Th cells, characterized by secreting IL-22 and TNF α , but not IFN γ , IL-4 or IL-17, and they are therefore distinct from Th1, Th2, and Th17 cells. Accumulating evidences uncovered that IL-22 play an important role in the progress of autoimmune diseases and in the defense against bacterial, viral, and fungal infection. However, so far there have been few studies on whether IL-22 was involved in tumorigenesis or tumor progression. In AML, IL-22 levels in bone marrow, are significantly reduced, but the role of Th22 and IL-22 in AML need to be clarified (Sun YX, *Hum Immunol* 2014).

Immune dysfunction in AML dos not involve only T cells. Dendritic cells have been reported to be diminished or even absent in AML patients and NK cells resulted to be impaired in their function contributing to immune evasion. Moreover, leukemia cells have the ability to reprogram macrophages toward a leukemia promoting phenotype (Barakos GP, *Diseases* 2022).

Figure 4 illustrates different mechanisms of immune evasion in AML.

Figure 4



Taghiloo S, Crit Rev Oncol Hematol. 2021

Treatment with hypomethylating agents could change the immune microenvironment in AML patients: different studies showed that HMAs possess immunomodulatory functions, such as upregulation of epigenetically silenced tumor antigens and interferon signaling to promote T cell proliferation. During treatment with HMAs, a reduction in Tregs' frequency has been reported, but HMAs have also immunosuppressive functions, with upregulation of immune checkpoints, such as PD-L1 and inhibition of T cell activation (Grimm J, Blood Cancer J 2022).

Not only hypomethylating agents, but also Venetoclax could modulate immune response. Venetoclax could enhance T-cell effector function by increasing reactive oxygen species (ROS) generation, through inhibition of respiratory chain super complexes formation. In addition, Azacitidine renders AML cells more susceptible to T cell-mediated cytotoxicity. Thus this immune-mediated mechanism of action for Venetoclax plus Azacitidine could contribute to the efficacy of this combination (Lee JB, Blood 2021).

Research project

Aim

This study project aims to provide comprehensive insights into longitudinal immune landscape in AML, focusing on characterization of T cell dysfunction in patients candidates for treatment with hypomethylating agents, Azacitidine or Decitabine, alone or in combination with Venetoclax, both in first line and in the relapsed/ refractory setting, using different methods. According to literature data, we hypothesize that, although T cells recognize myeloid blasts, cancer cells could evade immune control, through the creation of an immunosuppressive tumor microenvironment. We also hypothesize that treatment with hypomethylating agents and Venetoclax could change these features, enhancing the immune response.

Part 1 of the research project using FLOW CYTOMETRY before treatment with AZACITIDINE single agent

Aim

In the first two years of the project, the research activity focused on quantification of some T cell subpopulations (CD4+ T cells, CD8+ T cells and Tregs) and on evaluation of their immune checkpoints' expression (PD1, LAG3 and TIM3), both in peripheral blood, and in bone marrow samples, in newly diagnosed AML patients, before starting treatment with Azacitidine single agent (AZA); then after 48 hours from the first administration of AZA, another peripheral blood sample has been collected for any early changes induced by the therapy.

Methods

Study population

The study population included a cohort of 46 newly diagnosed acute myeloid leukemia (ND-AML) patients, referred by eight hematologic units in Italy (Novara, Castelfranco

Veneto, Treviso, Udine, Brescia, Genova, Rimini, Ravenna), and candidate to treatment with Azacitidine alone. All patients included in the study signed a written informed consent.

Laboratory methods

Peripheral blood (PB) samples and bone marrow (BM) samples (50 µl for each one) were collected from a large cohort of ND-AML patients, candidates for treatment with AZA alone. PB and BM samples were both collected before starting treatment with AZA, and PB samples were collected also after 48 hours from the first administration of AZA. All samples were centralized in IRST laboratory for analysis.

All the samples were examined by flow cytometry. After adding antibodies, the samples were incubated for 10 minutes at 4°C. Following the incubation, 900 µl of Lysis Buffer were added to each sample. Next the samples were incubated again, at room temperature, until the lysis was not completed (about 15-20 minutes). Finally we added 1 ml of peripheral blood sample, and we centrifugated the samples at 1200 rpm for 5 minutes, before re-suspending them in peripheral blood sample (250-500 µl).

For a basic T cells characterization, two tubes were used:

- *Basic T cells tube* which includes evaluation of CD3, CD4, CD8, PD1, LAG3, TIM3;
- *Tregs tube* which includes evaluation of CD4, CD25, CD127, PD1, LAG3, TIM3.

These retrospective data were analyzed using FlowJo software (*FlowJo V10.8.1*).

In the gating strategy, a single cell gate based on FSC (the forward scatter)-A and FSC-H was used to exclude doublets and cell aggregates. Then a nucleated cell gate based on FSC-A and SSC (the side scatter)-A was set to remove debris from further analysis. So with these two physic parameters, we selected only single and live cells.

After defining single and live cells, using FSC and SSC parameters, T lymphocytes were identified using the *tube Basic T cells*, through the expression of CD3. T cells were further examined for the expression of CD4 and CD8, which resulted in two main populations: CD4⁺ T cells and CD8⁺ T cells (a minority of T cells resulted double positive or double negative for CD4 and CD8). Each of the T cell populations exhibited

a different expression of the immune checkpoints (PD1, LAG3 and TIM3). Figure 5 shows this gating strategy on one sample.

Next we identified regulatory T cells (Tregs), among CD4⁺ lymphocytes, using only two cell surface markers, CD25 and CD127, contained in the *Tregs tube*: we know that Tregs show an high expression of CD25, whereas CD127 is absent or low. Furthermore, using this tube, we examined the expression of PD1, LAG3 and TIM3 on this population (*Stankovic B, Front Immunol 2019, Santegoets SJ, Cancer Immunol Immunother 2015*).

Figure 6 illustrates the gating strategy used, after selection of single and live cell, on one sample.

Figure 5 Gating strategy for Basic T cells tube

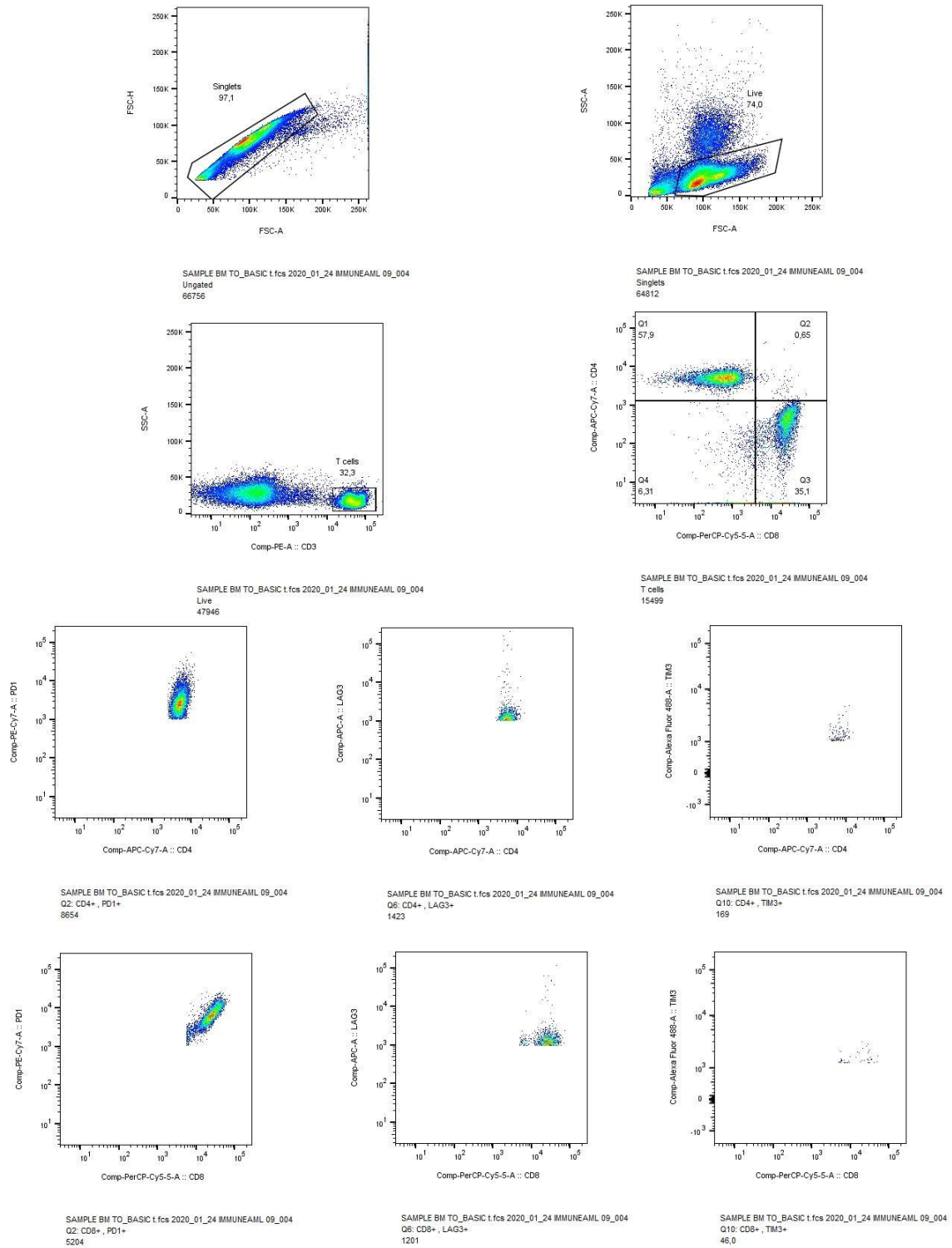
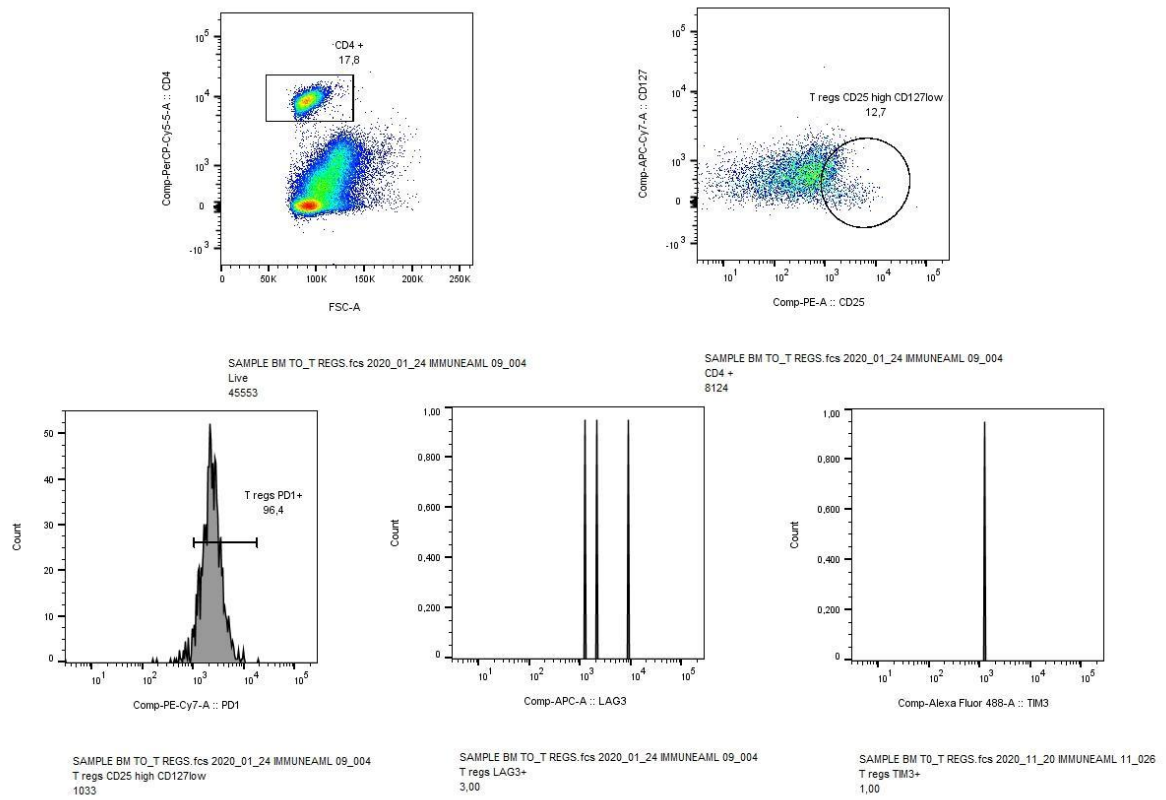


Figure 6 Gating strategy for Tregs tube



Results

Baseline

Peripheral blood samples

We have collected PB samples from 46 patients with ND-AML, before starting treatment with AZA; for 41 of them a BM sample was also available for cytofluorimetric analysis.

At baseline in PB, CD3⁺ T cells constituted on average 36% of single and live cells. Among CD3⁺ T cells, CD4⁺ lymphocytes were the most frequent, representing on average 60% of T cells (range 17-88%), followed by CD8⁺ lymphocytes, on average 30% of T cells (range 6-80%), whereas the CD4⁻/CD8⁻ and the CD4⁺/CD8⁺ T cells amounted to a small portion of the CD3⁺ population in PB. Then we explored the checkpoint inhibitors' expression in T cells. First of all, PD1 expression by T cells in PB resulted to be very high, both in the CD4⁺ subset and in the CD8⁺ subset, respectively on average 69% and 89%. Instead LAG3 expression was very variable in T cells, greater in the CD4⁺ subset (mean 51 %, range 0.33-99%), than in the CD8⁺ subset (mean 17%, range 0-89%). Finally, except for two PB samples, the percentage of TIM expression on CD4⁺ and CD8⁺ T cells resulted to be under 10%, and in most cases was about 1% or less.

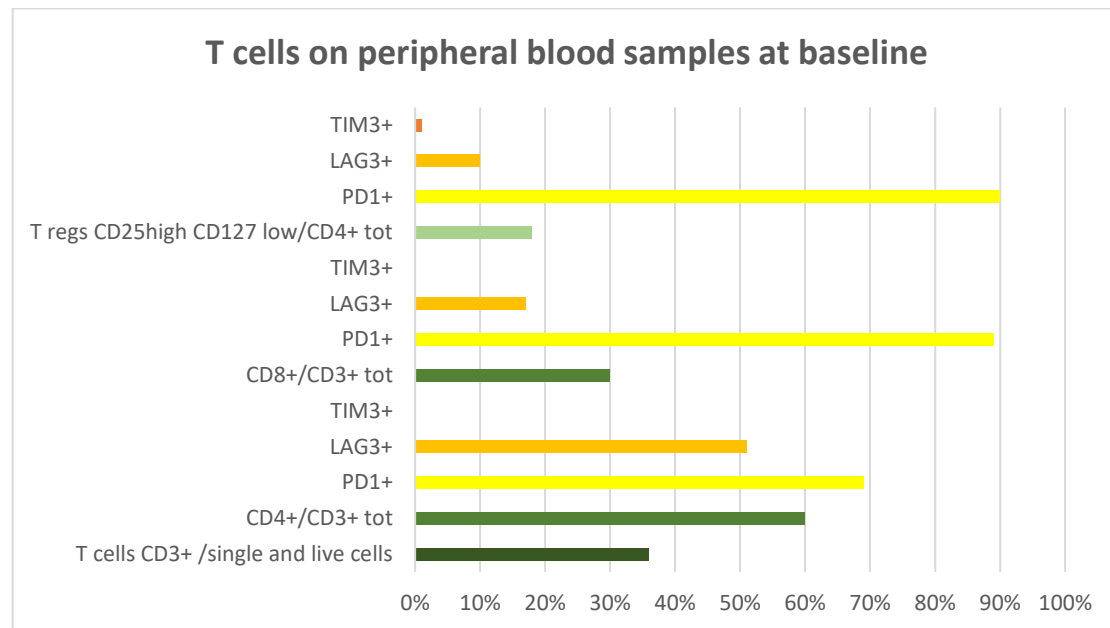
Using CD25 high expression and CD127 low expression, we found that the mean concentration of Tregs was 18% of the total number of CD4⁺ T cells in PB. So AML patients in this cohort had a greater CD4⁺ CD25^{high} CD127^{low/-} Tregs percentage in PB at diagnosis, compared to healthy people, according to literature data (percentage 5-10%, *C. Uston et al. Blood 2011*). Furthermore, on Tregs, we assessed the expression of the immune checkpoints. PD1 expression was >90% of Tregs in 41 of the 46 PB samples, 50-90% in 3 samples, not evaluable in 2 samples, because of absence of CD4⁺ T cells. The expression of LAG3 and TIM3 on Tregs resulted to be very lower: for LAG3, the expression was <10% of Tregs in all except one sample; for TIM3 the expression exceeded 1% of Tregs in only 5 of the 46 PB samples.

Table 3 and figure 7 sum up the results obtained on peripheral blood samples at baseline.

Table 3: Results on peripheral blood samples at baseline

T cells CD3+ /single and live cells	36%
CD4+/CD3+ tot	60%
PD1+	69%
LAG3+	51%
TIM3+	low
CD8+/CD3+ tot	30%
PD1+	89%
LAG3+	17%
TIM3+	low
Tregs CD25high CD127 low/CD4+ tot	18%
PD1+	>90%
LAG3+	<10%
TIM3+	<1%

Figure 7



Bone marrow samples

As regards BM samples, we found that CD3+ T cells constituted on average 25 % of the total amount of bone marrow cells; among CD3+ T cells, also in this case, CD4+ T cells were the most frequent, representing on average 58 % of all T cells (range 21-83%), followed by CD8+ T cells, on average 33% (range 13-74%); double negative or double positive elements represented a minority of the T cell population in BM.

Also in the bone marrow, most of T cells resulted to express PD1, above all the CD8+ T cells (CD4+ PD1+ were on average 63% of all CD4+, CD8+ PD1+ were on average 93% of all CD8+). Among the other inhibitory receptors, LAG3 was expressed on average in 53 % of CD4+ subset and on average in 15 % of CD8+ subset. BM T cells, as PB T cells, showed a low expression of TIM, generally <1%, and only in few cases >10%.

Using the *Tregs tube*, we found that the amount of Tregs in BM samples was on average 21% of all CD4+ elements. Moreover, most of Tregs in BM were PD1+: in details in 35 samples, PD1 was positive in >90% of Tregs, in 5 samples was positive in 50-90% of Tregs, and the last sample was not evaluable for the absence of Tregs. LAG3 resulted to be positive in less than 10% of Tregs in 38/41 samples, and in more than 10% only

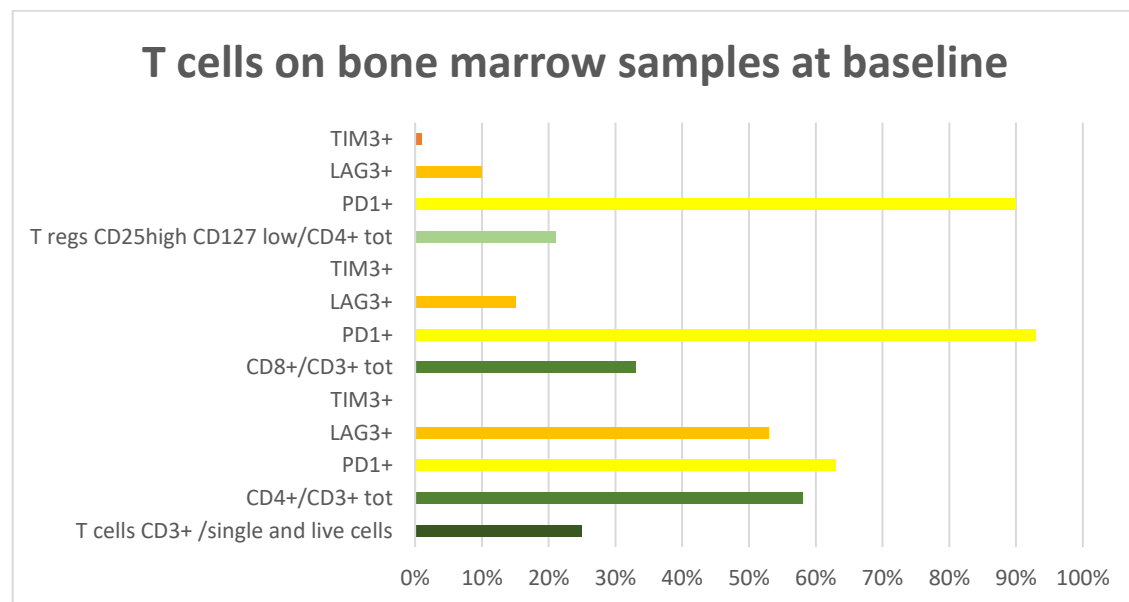
in 3 samples. Finally, TIM3 was expressed in less than 1% of Tregs in 28/41 samples, and in 1-20% of Tregs in 11 samples.

Table 4 and figure 8 sum up the results obtained on bone marrow samples at baseline.

Table 4: Results on bone marrow samples at baseline

T cells CD3+ /single and live cells	25%
CD4+/CD3+ tot	58%
PD1+	63%
LAG3+	53%
TIM3+	low
CD8+/CD3+ tot	33%
PD1+	93%
LAG3+	15%
TIM3+	low
Tregs CD25high CD127 low/CD4+ tot	21%
PD1+	>90%
LAG3+	<10%
TIM3+	<1%

Figure 8



48 HOURS

After 48 hours from the first administration of AZA we collected and analyzed a PB sample from 31 patients.

At 48 hours after the first dose of AZA, the mean percentage of T cells in PB did not change significantly from baseline (31 %), always with a prevalence of CD4⁺ T cells (on average 60%) over CD8⁺ T cells (on average 29%). Moreover, also at this time point, we found a tolerant immune microenvironment, due to a high expression of PD1 both on CD4⁺ T cells and on CD8⁺ T cells (on average 63% and 94% respectively). As regards the other two checkpoint inhibitors, the expression of LAG3 resulted to be significantly higher on CD4⁺ elements rather than on CD8⁺ ones (48% and 12%), whereas TIM3⁺ was little expressed on T cells, both CD4⁺ and CD8⁺ ones.

Finally, at 48 hours after AZA, the mean percentage of Tregs on total CD4⁺ T cells did not result to be different respect to baseline (21 %), and most of Tregs showed a high expression of PD1: in detail, in 25 samples a percentage from 90 to 100% of Tregs resulted PD1⁺, and in 3 samples Tregs PD1⁺ were 50-90% of all the Tregs. LAG3 and

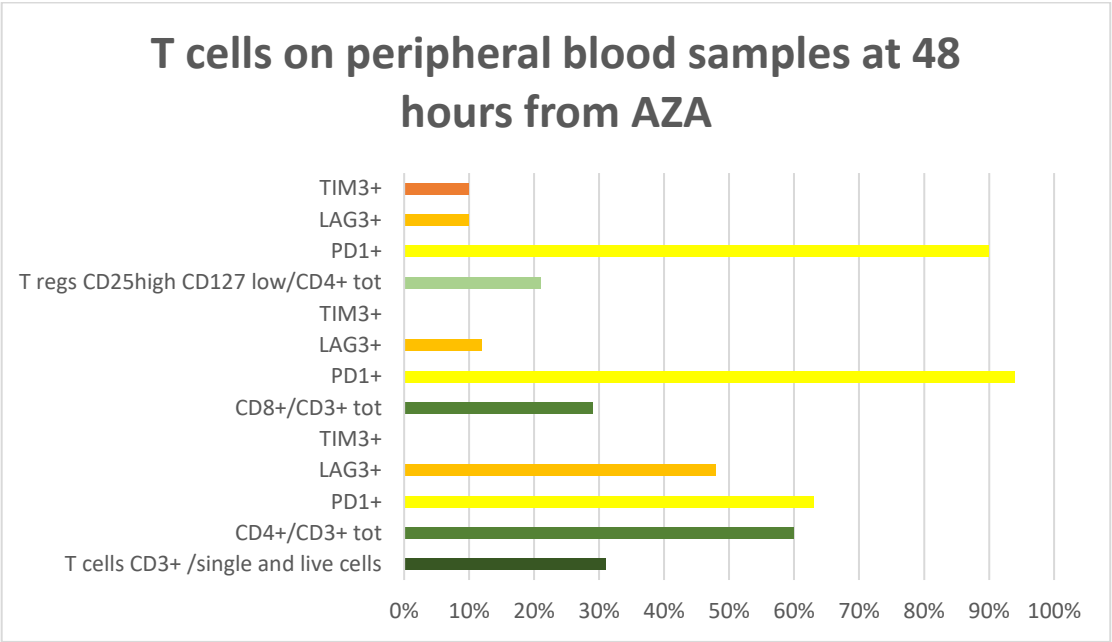
TIM3 were positive in few samples (Tregs LAG3+>10% of all the Tregs only in two samples, Tregs TIM3+>10% only in one sample).

Table 5 and figure 9 sum up the results obtained on peripheral blood samples at 48 hours from the first administration of AZA.

Table 5: Results on peripheral blood samples at 48h

T cells CD3+ /single and live cells	31%
CD4+/CD3+ tot	60%
PD1+	63%
LAG3+	48%
TIM3+	low
CD8+/CD3+ tot	29%
PD1+	94%
LAG3+	12%
TIM3+	low
Tregs CD25high CD127 low/CD4+ tot	21%
PD1+	>90%
LAG3+	<10%
TIM3+	<10%

Figure 9



Part 2 of the research project using FLOW CYTOMETRY during treatment with HMA+ VEN

Aim

In the third year of the research project, we explored T cell subpopulations through flow cytometry, in a cohort of patients responsive to treatment with HMA plus Venetoclax, for any change in immune microenvironment induced by the treatment. A peripheral blood sample has been collected for flow cytometry analysis, in order to quantify T cell subpopulations (CD4+ T cells, CD8+ T cells and Tregs) and their immune checkpoints' expression (PDL1, LAG3 and TIM3).

Methods

Study population

The study population included a cohort of 9 newly diagnosed- AML patients, followed in our hematological units (IRST in Meldola and Cesena), during treatment with an hypomethylating agent (Azacitidine or Decitabine) plus Venetoclax. All patients included in the study signed a written informed consent. Clinical data of the study population were collected.

Laboratory methods

Laboratory methods are the same illustrated in the part 1 one the study project, but the analysis has been performed only on PB samples and not in BM samples. IRST laboratory performed the analysis. The same tubes were used for T cells characterization, through flow cytometry:

- *Basic T cells tube* which includes evaluation of CD3, CD4, CD8, PD1, LAG3, TIM3;
- *Tregs tube* which includes evaluation of CD4, CD25, CD127, PD1, LAG3, TIM3

These data were analyzed using FlowJo software (*FlowJo V10.8.1*).

We use the same gating strategy, illustrated in the part 1 of the study project.

Results

Of the 9 patients enrolled in this part of the study, 6 were male and 3 were female; median age at diagnosis of AML was 76 years old (range 71-80). Three patients had an AML secondary to myelodysplastic syndrome; one patient had an AML secondary to myelofibrosis, whereas the other five patients had been diagnosed with de novo AML.

All patients included in this analysis had newly diagnosed AML and all pts received HMA+ Venetoclax, as first line of therapy for AML. As regards risk assessment, using ELN2022 risk stratification, three patients have been classified as favorable because of NPM1 mutation (2 pts) or CEBPA mutation (1 pts), other two patients had an adverse prognosis for SF3B1 and complex karyotype, whereas the remaining four patients had an intermediate risk. Using ELN2024 risk stratification, none of the pts had an adverse prognosis, one had an intermediate risk score because of FLT3 ITD mutation, and the remaining eight pts had been classified as favorable prognosis.

The analysis was performed during treatment with HMA (above all Azacitidine); five of those pts received this treatment for more than 2 years, so they are long responders to the treatment, compared to the VIALE-A study population.

All patients had a complete response as best response to the treatment (with a negative MRD in two patients). One patient had recently relapsed.

Table 6 reported baseline features of the study population and data about the treatment administrated and the patients' outcomes.

Table 6 Study population: baseline features and treatment response

Sex	
M	6
F	3
Age (years)	
median	76
range	71-80
AML	
de novo	5
secondary	4
ELN 2022 risk category	
favorable	3
intermediate	4
adverse	2
ELN 2024 risk category	
favorable	8
intermediate	1
adverse	0
Therapy	
AZA VEN	8*
DEC VEN	10*
n° months of treatment	
median	25
range	2-44
Best response	
CR MRD+	7
CR MRD-	2

**one patient received Dec Ven e then was shifted to Aza Ven*

Flow cytometry analysis

We have collected PB samples from these 9 patients with ND-AML, during treatment with HMA plus Azacitidine or Decitabine.

The median percentage of CD3+ T cells resulted to be 8% of single and live cells (range 1.4%-24.3%). Among CD3+ T cells, CD4+ lymphocytes were the most frequent, representing on average 57% of T cells (range 33-66%), whereas the median percentage of CD8+ lymphocytes was 29% (range 14-56%).

Then we explored the checkpoint inhibitors' (CKi) expression in T cells, finding a low expression of all the CKi (PD1, LAG3 and TIM3) both in CD4+ T cells than in CD8+ T cells. Specifically, in the CD4+ T cells PD1 was positive in a median of 15% (range 6-24%), whereas LAG3 was positive on average 11% (range 6-16%), and TIM3 was positive in a median of 11% (range 7-20%). In the CD8+ T cells subset, the median expression of PD1 was 11% (range 6-42%), whereas LAG3 was positive only in a median of 2% (range 0.4-7%), and TIM3 was positive only in a median of 3% (range 0.5-12.6%).

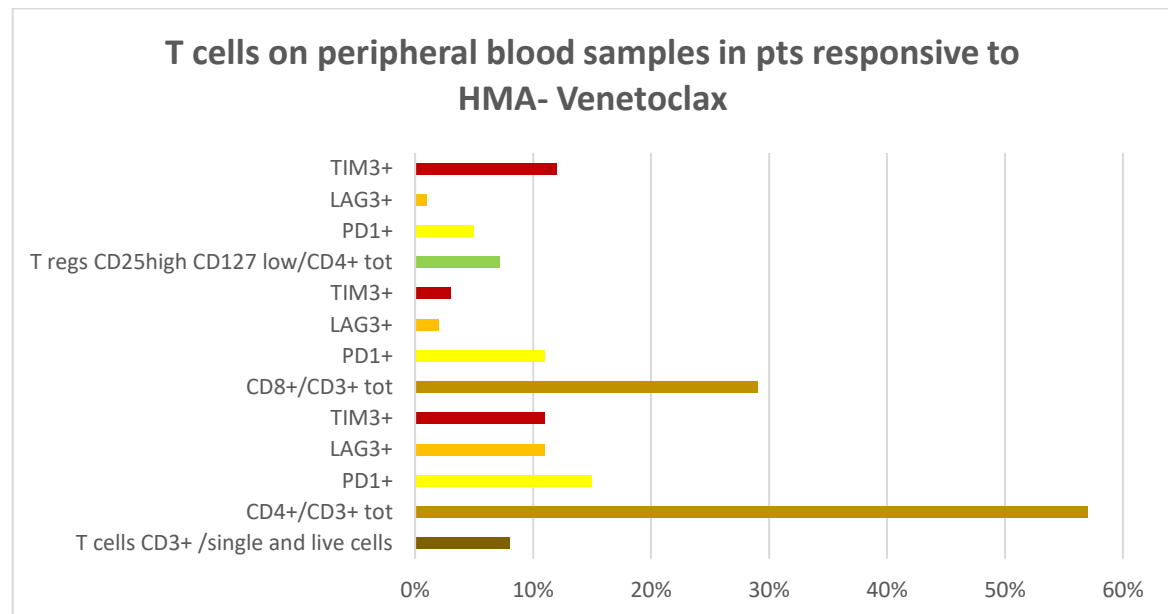
Using CD25 high expression and CD127 low expression, we found that the mean concentration of Tregs was 7.2% (range 4.2-13.2%) of the total number of CD4+ T cells in peripheral blood samples of pts responding to HMA-VEN. Also on Tregs, we assessed the expression of the immune checkpoints, finding a low expression of PD1 (median 5.1%, range 0-8.9%) and a low expression of LAG3 (median 1.4%, range 0-13.3%), whereas the median expression of TIM3 was 12.1% (range 0-18.15%).

Table 7 and figure 10 sum up the results obtained on peripheral blood samples in this cohort of patients.

Table 7: Flow cytometry results in HMA-VEN responders

T cells CD3+ /single and live cells	8%
CD4+/CD3+ tot	57%
PD1+	15%
LAG3+	11%
TIM3+	11%
CD8+/CD3+ tot	29%
PD1+	11%
LAG3+	2%
TIM3+	3%
Tregs CD25high CD127 low/CD4+ tot	7,2%
PD1+	5.1%
LAG3+	1.4%
TIM3+	12.1%

Figure 10



Part 3 of the research project using CYBERSORT before treatment with HMA+ VEN

Aim

In the last year of the study project, we used a computational approach for the prediction of immune cell subtypes abundances, based on the readily available gene expression data on transcriptomes, in patients candidates to treatment with HMA+ Venetoclax.

In particular, the aim in this part of the research project is to assess immune cell subtypes, with a focus on T cell lymphocytes, in blood or in bone marrow samples collected from patients with AML candidates for treatment with Venetoclax plus HMA, using the computation algorithm CIBERSORT.

Methods

Study population

The study population consists of a cohort of 39 patients with newly diagnosed (ND) or relapsed/ refractory (R/R) AML, before starting treatment with a hypomethylating agent (Azacitidine or Decitabine) plus Venetoclax. Of those 39 patients, 12 were followed in our hematological units (IRST in Meldola and Cesena), whereas 27 have been referred by hematological unit in IOV in Castelfranco Veneto. All patients included in the study signed a written informed consent. Clinical data of the study population have been collected.

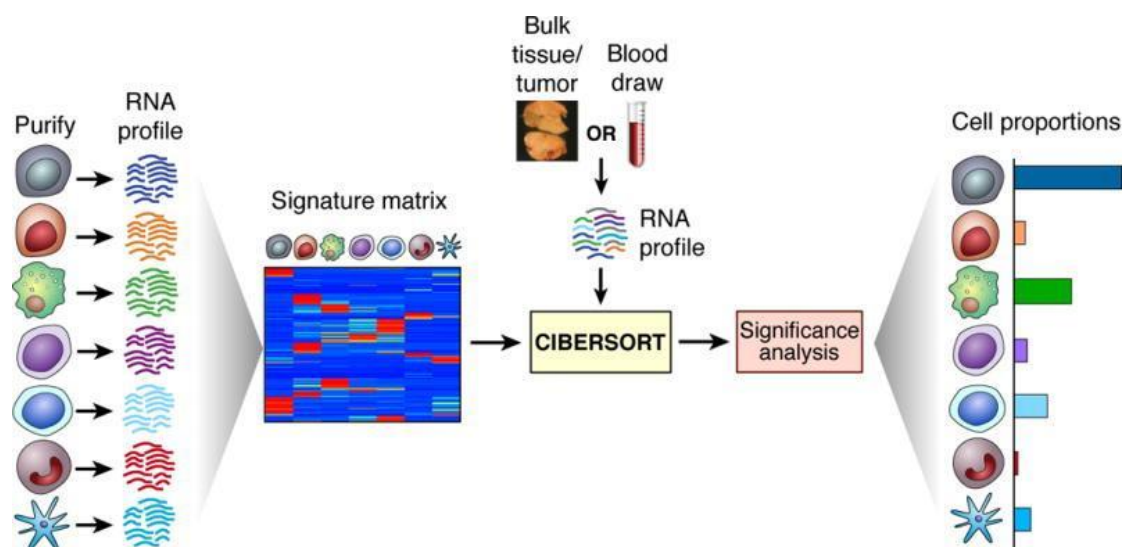
Laboratory methods

Bone marrow and/or peripheral blood samples were collected before starting treatment with hypomethylating agent plus Venetoclax, and centralized in IRST laboratory for analysis. On those samples, CIBERSORT was used as a computation algorithm, for characterizing cell composition of the tissue from cells' gene expression profiles (GEPs). This method led to cell-type identification by estimating relative subsets of RNA transcripts (RNA-sequencing). CIBERSORT requires an input matrix of reference gene expression signatures, collectively used to estimate the relative

proportions of each type of interest (expressed as a fraction). We used a designed and validated leukocyte gene signature matrix, termed LM22, which includes 547 genes, that distinguish 22 human hematopoietic cell phenotypes (including naïve and memory B cells, plasma cells, seven T cell subtypes, natural killer cells, monocytes, macrophages subtypes, dendritic cells, mast cells, neutrophils and eosinophils), reported in literature (Newman AM, *Nat Method* 2015, Chen B, *Methods Mol Biol.* 2018). This leukocyte gene signature matrix had been used for leukocyte monitoring during immunotherapy. As regards sensibility, CIBERSORT detected cellular fractions down to 0.5% in mixtures containing $\leq 50\%$ tumor content, and down to 1% in mixtures containing $>50\%$ tumor content.

Figure 11 illustrates CIBERSORT.

Figure 11



Chen B, Methods Mol Biol. 2018

Results

Of the 39 patients enrolled in the study, 31 were male and 8 were female; median age was 74 years old (range 29-83). About an half of the study population had a secondary AML, in particular 10 pts had a AML secondary to myelodysplastic syndrome (MDS), 5 pts had a prior history of myeloproliferative neoplasm (MPN), whereas 4 pts had a therapy- related AML. Eight patients were relapsed/refractory to the first line of therapy, whereas the other 31 were newly diagnosed AML pts. Prior treatment included intensive chemotherapy with Daunorubicin and Citarabine, plus Gemtuzumab ozogamicin or Sorafenib, in 3 pts, and of CPX351/Vyxeos in 4 pts; one patient received Decitabine alone. As regards risk assessment, 12 pts had a complex karyotype and 7 pts had a TP53 mutation. According to ELN2022 risk stratification, 24/39 pts had an adverse prognosis, whereas, according to ELN2024 risk assessment, only the 7 pts with TP53 mutation were included in a lower benefit group. Table 8 illustrates features of the study population.

Treatment included combination of Venetoclax with a hypomethylating agent (18 pts received Azacitidine, whereas 20 pts received Decitabine, and the last one received both the association). Median number of cycles was 3 (range 1-25).

Five patients proceed to allotransplantation, after Venetoclax-HMA.

Of the study population, 32 patients were died at the time of the analysis, most of all due to progressive disease (20 pts). Other causes of death included infection in 5 pts, complications of allotransplantation in 2, worsening of clinical conditions in other 2, a second malignancy in one patient, whereas in the last 2 cases the definitive cause of death has not been reported.

Table 9 reported data about the treatment administrated and the patients' outcomes.

Table 8 Study population: baseline features

Sex	
M	31
F	8
Age (years)	
median	74
range	29-83
AML	
de novo	20
secondary	15
therapy- related	4
AML	
ND	31
R/R	8
ELN 2022 risk category	
favorable	1
intermediate	14
adverse	22
unknown	2
ELN 2024 risk category	
favorable	19
intermediate	10
adverse	7
unknown	3

Table 9 Study population: Treatment and outcome

Therapy	
AZA VEN	18+1*
DEC VEN	20+1*
n° cycles	
median	3
range	1-25
best response	
CR	2
CRp	1
PR	1
bridge to alloSCT	5
death	
yes	32
no	2
unknown	5
cause of death	
PD	20
infection	5
alloSCT complications	2
2nd malignancies	1
other	2

**one patient received both Azacitidine Ven than Decitabine Ven*

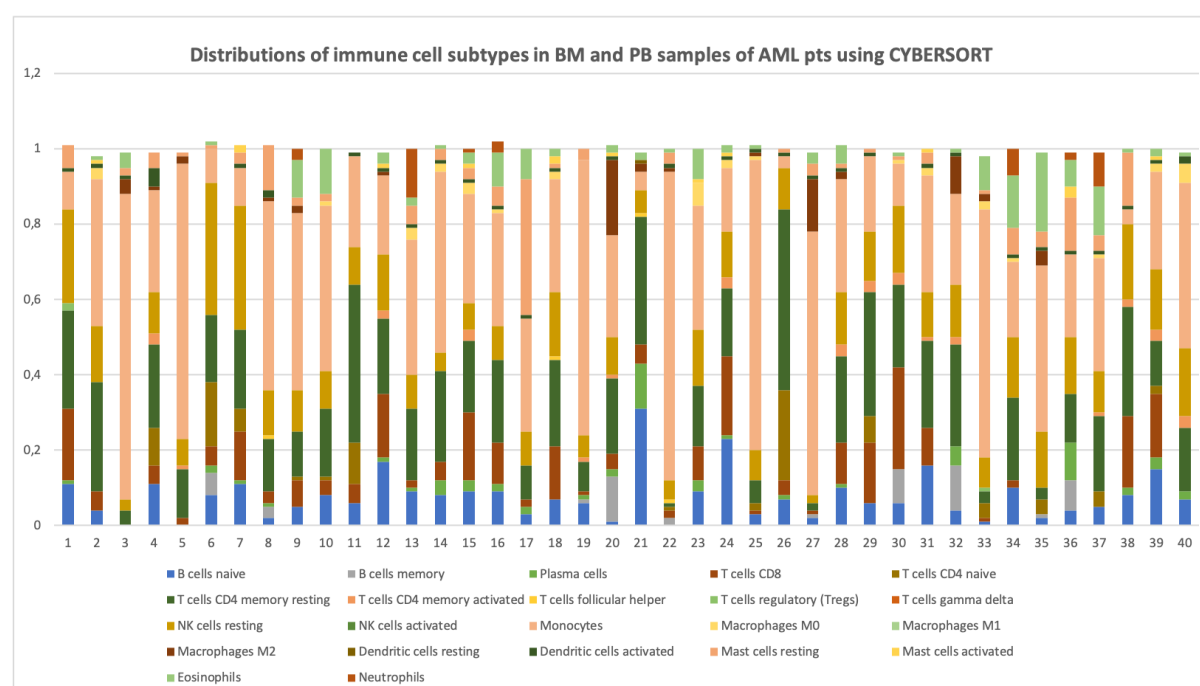
Clusters of immune cell subtypes

Figure 12 shows the relative abundances of each immune cell subtypes in bone marrow and peripheral blood samples (40 samples collected from 39 pts). Most of the cell subtypes had very low abundances.

Cells subtypes with relatively high abundances were monocytes (median value 0.30; range 0.00-0.82), T cells CD4 memory resting (median value 0.195; range 0.01-0.48) and NK cells resting (median value 0.115; range 0.02-0.35).

B cells naïve and T cells CD8 had a relatively intermediate abundances (B cells naïve median value 0.065 with the range 0-0.31; T cells CD8 median value 0.05 with the range 0-0.27).

Figure 12

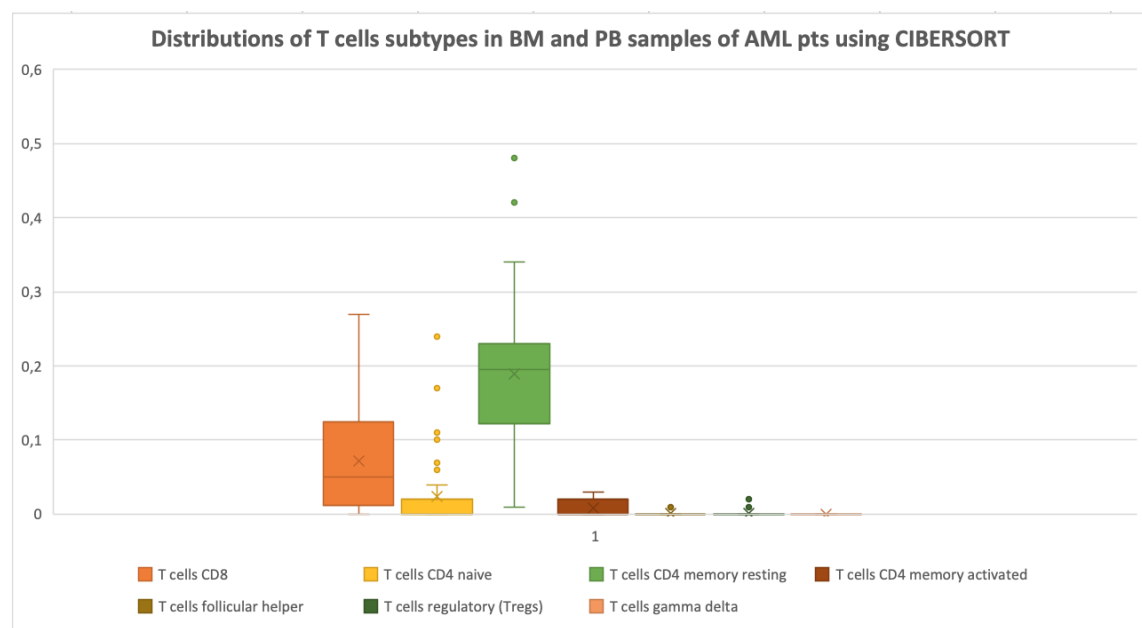


Focusing on T cells subtypes (as shown in table 10 and figure 13), T cells CD4 memory resting had the higher levels (median value 19.5%), whereas T cells CD4 memory activated were almost absent (median value 0%). Moreover we found a high abundance of T cells CD8 (median value 5%), whereas T cells CD4 naïve, T cells follicular helper, T cells regulatory (Tregs) e T cells gamma delta were almost absent.

Table 10

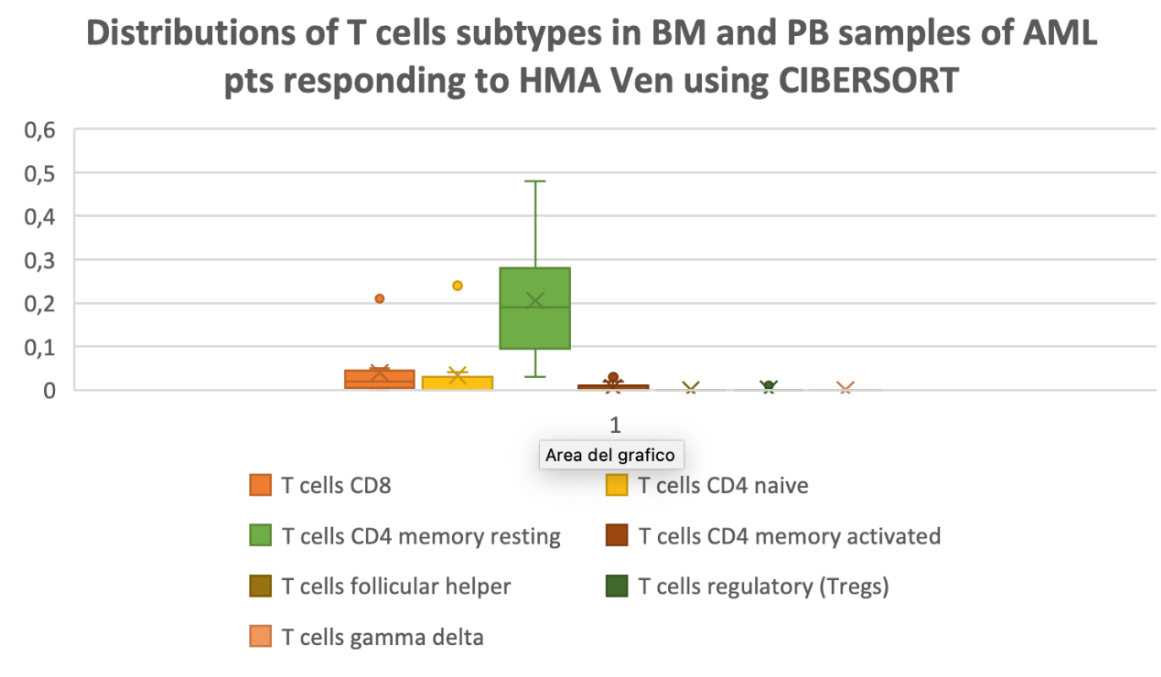
T cells subtypes	Median value	Range
T cells CD8	0,05	0-0,27
T cells CD4 naive	0	0-0,24
T cells CD4 memory resting	0,195	0,01-0,48
T cells CD4 memory activated	0	0-0,03
T cells follicular helper	0	0-0,01
T cells regulatory	0	0-0,02
T cells gamma delta	0	0

Figure 13



Analyzing only the 9 patients that had a response to the treatment (including CR, CR with incomplete platelets recovery, PR and those who proceed to allotransplantation), we found that T cells CD4 memory resting had the higher levels (19%), as in the entire study population, whereas T cells CD8 were less abundant respect to the whole study population (median value 2%), as shown in figure 14.

Figure 14



Discussion

Immune system dysfunction is now a well-known mechanism that contributes to the onset of cancer, but also to treatment resistance and to disease recurrence. Cancer cells can evade immune surveillance through different strategies, leading to the so-called “immune evasion”. A better understanding of the immune microenvironment, in cancer, has the potential to guide therapeutic strategies, in order to ameliorate the patients’ outcome.

In acute myeloid leukemia, cancer cells (myeloid blasts) are capable of creating an immunosuppressive milieu, where both innate and adaptive immune systems are deregulated. T lymphocytes, above all T helper CD4+, are the backbone of the adaptive immune system, as they modulate the function of the other immune cells, including B lymphocytes, but also natural killer cells, macrophages and dendritic cells. So, it is not surprising that T cells’ dysfunction plays a key role in the onset and progression of AML. Literature data report that both CD8+ cytotoxic and CD4+ T helper cells exhibit features of senescence, exhaustion and anergy, and that T cells are unable to form effective immune synapses with blasts. T cells in AML express high levels of immune checkpoint molecules (PD1, TIM3 and LAG3). Moreover, the immune microenvironment in AML is unreached of T cells that have immunosuppressive functions, including Tregs, leading to a higher secretion of anti-inflammatory cytokines, above all IL-10 and TGF β . On the other hand, a lower release of pro-inflammatory cytokines, above all IFN γ , has been observed in AML. The accumulation of Tregs in bone marrow and in peripheral blood of AML patients, not only facilitates the onset of leukemia, but also contributes to treatment resistance and correlates to the likelihood of relapse. In AML a higher concentration of immunosuppressive enzymes, such as IDO, contributes to the higher frequency of Tregs. In recent years, Th17 lymphocytes have emerged as a new T helper subtype; their role in cancer is still a matter of debate, with conflicting studies reporting pro- versus antitumoral effect. Th17 have a high degree of plasticity, that allows these cells to transdifferentiate into Th1-like cells (secreting IFN γ) or to Tregs-like cells (secreting IL-10), depending on the environment conditions.

Treatment could change the immune microenvironment in AML. Venetoclax combined with hypomethylating agents, Azacitidine or Decitabine, represents the first line therapy in newly diagnosed older and/or unfit patients, and a good therapeutic strategy

in the relapsed/refractory setting. Hypomethylating agents could promote T cell proliferation through epigenetic mechanisms, and could lead to a reduction in Tregs' frequency. Venetoclax could enhance T-cell effector function by increasing ROS generation.

This research project aims to provide comprehensive insight into immune microenvironment in AML, focusing on T cell dysfunction.

Firstly, we used flow cytometry to quantify T cells' frequency in blood and bone marrow samples, distinguish CD4⁺ from CD8⁺ T cells, and to quantify Tregs (defined as T CD4⁺ CD25^{high} CD127^{low}). Then we analyzed the expression of the immune checkpoints, PD1, LAG3 and TIM3, on T cells. The study population is represented by 46 newly diagnosed AML that will be treated with Azacitidine single agent. After 48 hours from the first administration of Azacitidine, a peripheral blood sample was collected for any early changes induced by therapy.

We found a high percentage of CD3⁺ T lymphocytes among all live cells (36% in peripheral blood and 25% in bone marrow). T CD4⁺ were prevalent on T CD8⁺ (60% vs 30% in peripheral blood, 58% vs 33% in bone marrow). Among immune checkpoints, PD1 is strongly positive in all T cell subpopulations, both in peripheral blood (69% in CD4⁺, 89% in CD8⁺) and in bone marrow (63% in CD4⁺, 93% in CD8⁺). The high expression of PD1 found in these samples confirmed literature data, that reported high levels of immune checkpoint molecules, leading to an impaired T cell function and to tumor escape in newly diagnosed AML. As regards the other immune checkpoint molecules, we did not find a role for TIM3, whose expression resulted to be very low in all T subtypes and in all samples, whereas LAG3 could have a role in T CD4⁺ dysfunction, because it is strongly positive in T CD4⁺ cells (about 50% both in peripheral blood and bone marrow).

Then using CD25 high expression and CD127 low expression, we found that the mean concentration of Tregs was 18% of the total number of CD4⁺ T cells in peripheral blood. So, AML patients in this cohort had a greater Tregs' percentage compared to healthy people (according to literature data, percentage 5-10%). In bone marrow Tregs resulted to be on average 21% of all CD4⁺ cells. So, we confirmed previous published data that showed an immunosuppressive microenvironment in AML, unreached from Tregs.

Moreover, we did not find significant differences in T cells composition in blood and in bone marrow. Finally, we did not find significant changes in immune composition

after only 48 hours from the first administration of Azacytidine: at this time point we still found an immune dysfunction, characterized by high expression of PD1 on T cells, and a prevalence of Tregs.

Then we used the same flow cytometry analysis to explore T cell subpopulations in peripheral blood samples in a small cohort of patients (9 pts) responsive to treatment with HMA plus Venetoclax (2 patients obtained a CR MRD-), for any change in immune microenvironment induced by the treatment. In this cohort of responders, we found a low expression of all the immune checkpoints, including PD1, then the T lymphocytes would have lost their exhausted profile respect to treatment naïve patients. Moreover in this subset of patients we found a lower concentration of immunosuppressive Tregs in peripheral blood (about 7% of CD4+ T cells), compared to treatment naïve patients (18%), according to literature data, which reported that during treatment with HMAs, a reduction in Tregs' frequency has been observed.

Because of cell sorting by flow cytometry has some limitations, including processing challenges and subjective evaluation, in the last year of the study project, we used a computational approach for the prediction of immune cell subtypes abundances, based on the readily available gene expression data on transcriptomes (RNA sequencing). The computational approach used was CIBERSORT, which requires a designed signature matrix. We used a validated leukocyte gene signature matrix, termed LM22.

Study population consisted of 39 patients with newly diagnosed or relapsed/refractory AML; peripheral blood samples and/or bone marrow samples have been collected before starting treatment with Venetoclax plus Azacitidine or Decitabine. The study population consisted above all of older patients; as regards risk stratification, according to ELN2024, 7 patients had an adverse prognosis, related to the presence of TP53 mutation.

Using this approach, we could establish the relative abundance of each immune cell subtype in blood or in bone marrow samples. LM22 distinguish 22 immune cell subtypes (including naïve and memory B cells, plasma cells, seven T cell subtypes, natural killer cells, monocytes, macrophages, dendritic cells, mast cells, neutrophils and eosinophils). Furthermore, CIBERSORT allows to distinguish functional phenotypes for each immune cell subtypes (resting or activated).

In these AML patients, immune cell subtypes with relatively high abundances were monocytes (30%), T cells CD4+ memory resting (19.5%) and NK cells resting (11.5%). B cells naïve and T cells CD8+ had a relatively intermediate abundances (B cells naïve

6.5%; T cells CD8+ 5%). Most of the cell subtypes had very low abundances, quite absent with this method. Among those, there were the other T cell subtypes, which included T cells CD4+ naïve, T cell follicular helper, Tregs and T gamma delta.

In literature CIBERSORT algorithm, with LM22 signature matrix, was used to evaluate the infiltration of immune cells, especially in solid tumors. In breast cancer, for example, *Wang Z et al.* found a high abundance of memory B cells, follicular helper T cells, gamma delta T cells, and M0 and M1 macrophages, whereas less abundant cell type included naïve B cells, resting CD4+ memory T cells, resting NK cells, M2 macrophages, and resting mast cells (*Wang Z., Sci Rep 2023*).

CIBERSORT has been used also in hematological malignancies. In myelodysplastic syndromes (MDSs), the immune dysregulation in bone marrow microenvironment plays a role in the progression to AML. Using CIBERSORT on bone marrow samples collected from 316 MDS patients, *Wang YH et al.*, found that a lower fraction of unpolarized M0 macrophages and higher fractions of M2 macrophages and eosinophils were significantly associated with inferior survival (*Wang YH, Blood Adv. 2021*).

When applied to blood and bone marrow samples, collected from AML patients, the LM22 validated matrix may be inaccurate to estimate the immune cell composition, because of the resemblance between normal monocytes and myeloid blasts. In our cohort of patients, we found a high abundance of monocytes, both in peripheral blood and in bone marrow samples, which can be explained with the high concentration of myeloid blasts in these tissues. This resulted in a lower concentration of other cell subtypes, for example Tregs, that resulted to be quite absent in our cohort of patients, in contrast with literature data and in contrast with prior illustrated results obtained with flow cytometry. So, we suggest the use of other reference matrixes, different from LM22; in this manner, the estimated relative proportions of using CIBERSORT could reflect the real proportions of each cell type in the sample.

However, while flow cytometry performs poorly at capturing functional phenotypes, CIBERSORT allows to distinguish these phenotypes for each immune cell subtypes (resting or activated). In AML patients, we found a prevalence of T cells CD4+ memory resting, whereas T cells CD4+ memory activated were almost absent, and a prevalence of NK cells resting, with NK cells activated quite absent. So, in AML patients, immune cells (both T helper and natural killer cells) were mostly in a rest functional state, and this could contribute to immune evasion. In literature, we found that in gastric cancer, resting CD4+ memory T cells and activated CD4+ memory T cells had opposite effects

on overall survival. Specifically, lower levels of resting CD4+ memory T cells and higher levels of activated CD4+ memory T cells were associated with better prognosis (Sun Y, *Frontiers Onc* 2023).

In conclusion, this study supports that an in-depth immunological evaluation of T cells in patients with AML, in addition to the study of blasts, could represent a key point in diagnostic understanding, in response monitoring, and possibly, in determining the therapeutic strategies more or less available to the various therapies currently available. So the pre-therapy immunological signature and the immunological response, as well as the genetic characterization of the blasts and the MRD calibrated only for leukemic cells, must be integrated in monitoring AML patients.

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