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Drug Sensitivity and Resistance Profiling: a platform to identify new therapeutic strategies in Relapsed/Refractory Acute Leukemia

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Abstract

Precision Medicine with N-of-1 personalized approach represents an intriguing tool to treat one patient at a time and can have interesting clinical applications in rare malignancies or relapsed/refractory (R/R) diseases. In this context, Drug Sensitivity and Resistance Profiling (DSRP) helps to detect compounds with selective effectiveness in a variety of diseases: preclinical studies on Acute Myeloid Leukemia (AML) and Acute Lympoblastic Leukemia (ALL) provided evidence of efficacy of this approach with potential of clinical translation.

In our study, we have applied DSRP by performing ATP-cell viability assay on primary bone marrow blasts samples of AML and T-ALL patients aimed to obtain in AML a better selection of patients sensitive to alternative salvage therapies such as Venetoclax-containing regimen and in T-ALL a possible combinatorial strategy in high risk ETP-ALL category with R/R disease.

In a small population of ETP-ALL our approach has been validated by a broader Drug Sensitivity Profile performed in collaboration with the laboratories of the Division of Pediatric/Oncology, University Children's Hospital of Zurich and Hematology and Bone Marrow Unit of University of Perugia. The results have led to a clinical application with a DSRP-driven salvage strategy in 3 patients with chemorefractory disease.

Our study supported the approval of a biological trial promoted by the GIMEMA group to providing a DSRP oriented approach to treat R/R T-ALL. Furthermore we expect that DSRP on large scale will generate information about mechanisms of drug resistance in leukemia and will identify personalized treatment based on *ex vivo* drugs sensitivity.

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1 Introduction

1.1 Drug Sensitivity and Resistance Profiling in Relapsed/Refractory Acute Leukemia

Individualized systems medicine (ISM) approaches represent an emerging and important tool to optimize cancer therapy [1] based on different steps: i) molecular profiling and *ex vivo* drug sensitivity and resistance testing (DSRT) of cancer cells to several compounds; ii), application of *ex vivo* results and implementation of therapies based on sensitivity test; iii) studying second samples from relapsed diseases, to understand the mechanism of resistance mediated in the majority of patients by increased number of genetic alterations, which can be attributed to disease progression, clonal selection and/or DNA-damaging induced by chemotherapy [2].

An interesting study by Pemovska et collegues describes drug sensitivity and resistance profiling (DSRP) as an alternative approach that could identify innovative strategies to treat chemorefractory/relapsed acute myeloid leukemia (R/R AML)[3]. It's well known that recurrent genetic alterations link to tractable drug targets (e.g. FLT3 inhibitors in FLT3 mutated AML, as recently approved [4 5]); however, AML genetic testing has not yet results in effective personalized or stratified therapies in the majority of patients. The aim of this study was to initially identify cancer cells' drug dependency based on *ex vivo* DSRP on 28 bone marrow (BM) samples from 18 AML patients using a library of clinically available drugs and then, in a second step, to verify DSRP predictions *in vivo* by treating selected patients with off-label drugs **(Fig.1).** Furthermore, the authors identified the molecular mechanisms of cancer progression and performed analyses with genomic and transcriptomic profiling to treat resistant disease. On the basis of BM samples analyzed, they created a database to define the efficacy of several compounds, identifing a

functional taxonomic classification of AML based on the sensitivity to different classes of drugs: in particular all five identified groups showed increased sensitivity to BCL-2 inhibitors, HSP90 inhibitors and histone deacetylase (HDAC) inhibitors. Group I demonstrated a strong selective response to BH3 mimetic navitoclax; group II showed a potential inflammatory signal-driven phenotype with a specific sensitivity to immunosuppressive drugs (steroids), Janus-activated kinase (JAK) family kinase inhibitors, and MEK inhibitors. Group III, IV, and V were selectively sensitive to a broad range of Tyrosine kinase inhibitors and to HSP90, HDAC, and phosphoinositide 3-kinase (PI3K)/mTOR inhibitors, indicating that they were driven or addicted to these pathways. Group IV AML were especially sensitive to MEK and PI3K/mTOR inhibitors, whereas group V AML showed responses to receptor TKIs targeting ABL, VEGF receptor (VEGFR), platelet derived growth factor receptor (PDGFR), FLT3, KIT, PI3K/mTOR **(Fig.2)**

This functional classification and identification of emerging resistance during the course of the disease led to a real-time treatment, with optimization and increased efficiency of therapies, specific for each patient and with a broad spectrum of applicability. In conclusion the role of DSRP can be clinically relevant if we have a desease with specific characteristics, if a distinct leukemia response pattern is identificable, if potential effective compounds are available without significantly delaying the treatment and if there is no standard approved alternative therapy.

Drug profiling-oriented strategy showed its potential benefit also in Acute Lymphoblastic Leukemia (ALL) setting. Indeed, although the progress in ALL genomics provides unprecedented insight into potentially individualized targets, a vast majority of patients who may benefit from innovative therapies are still identified on the basis of the persistence of minimal residual disease (MRD) or failure of remission induction therapy [6]. An important study by Frismantas et collegues was conducted using BM samples from

ALL patients with high risk or relapsed/refractory (R/R) disease [7]. The aim of the study was to obtain *in vitro* a platform with the information of interpatient drug response heterogeneity, using patients derived leukemia cells; the goal was to identify a drug sensitivity profile regardless of the information about genetic lesions or activated pathways. The authors tested 60 preclinical and clinical compounds on ALL patients samples based on persistence and relapsed/refractory disease and demonstrated a strong sensitivity in these patients for drugs that are generally not considered as active in ALL. Furthermore, with this drug activity profiles they identified different phenotypes and target therapies **(Fig.3)**, but they did not find any stright link between drug response and specific genetic lesion, although drug profiling identified leukemia intrinsic differences in mechanism of cell proliferation and survival. On the basis of heterogenic characteristics of survival cells, the authors identified differences in cell proliferation and in physiological cell death across BM ALL samples. Finally, they validated drug sensitivity patterns obtained *ex vivo* in patient-derived leukemia xenograft models. Another result of this study was that patients with R/R ALL are commonly more resistant to classic agents used for induction therapy in ALL, such as dexamethasone, cytarabine, and antracycline, whereas unexpected responses were observed to venetoclax and dasatinib in some T-ALL patients. Venetoclax, tested in combination with standard therapies typically used in this subset as vincristine, dexamethasone, asparaginase, and anthracycline, induced progression delay of leukemia with the same results in *in vivo* as well as in *in vitro* studies. Furthermore biomarkers for venetoclax sensitivity were identified as members of B-cell lymphoma-2 (BCL2) family, but in this study *in vitro* response to venetoclax was not closely related to level of *BCL2* expression. The authors also noticed unexpected responses to dasatinib in some patients, but they didn't found recurrent mutations or gene fusions by exome and transcriptome sequencing that could be associated with dasatinib sensitivity in T-ALL; they identified hyperexpression of SRC pathway on dasatinib sensitive

samples. In conclusion this seminal study recognized drug response profile as a useful platform to identify alternative therapeutic strategies in selected subgroups of ALL patients. This model also increased the possibilities for multidimensional approach. Effects can be analyzed not only on target leukemia cells but also on non-hematopoietic microenvironmental cells and additional markers can be used to indicate metabolic state, distinct differentiation processes, or signaling activity. Another important concept is that DSRP is a dinamic approach, because drug resistance can appear during target treatment and sensitivity profile can be modified during the course of the disease in relation to leukemia progression or drug-induced clonal selection.

1.2 Venetoclax as a treatment option in AML

AML is the most common acute leukemia in the adult and older population with a median age at diagnosis of over 65 years. The standard management for patients elegible for intensive treatment is antracycline-cytarabine based chemotherapy regimen, whereas older and unfit population have limited curative options and unsatisfactory outcome. First line approaches for this population are represented by less intensive chemotherapy, supportive care or hypomethilating agents (HMA) suchs as 5-azacytidine (AZA) or Decitabine (DAC) [8].

Since we are entering in the genomic era in AML, the depeer knowledge of specific genetic lesions and discover of drugs inspired by genomic, leads to the possibility of innovative therapeutic options: the major example in this category is represented by FLT3 inhibitors and IDH1-2 inhibitors[9]. However, this kind of approach is feasible in a minority of patients because of the relatively low incidence of targettable mutations in this population. Thus the identification of alternative activated pathways susceptible of target therapy regardelss of leukemia mutational status is desirable.

One common property of malignancies is the dysregulation of apoptotic programs, so BCL2-family proteins represent ad appealing target to consider also in hematologic cancers [10].

We know that the BCL-2 family includes multiple proteins sharing BCL-2–like homology domains (BH1-BH4), each playing a specific role in the intrinsic apoptotic pathway. These roles can be divided into 4 main functions: suppressors (BCL-2, BCL-XL, BCL-W, BCL2-A1, and MCL-1), that prevent apoptosis, activators (BIM and PUMA), effectors (BAX and BAK), and sensitizers (NOXA) that promote the inhibtion of suppressors and the activation of apoptotic cell death program. The pro-apoptotic cascade converges on the creations of pores in the mitochondria membrane, with subsequent release of cytochrome C and activation of caspase 9, ultimately leading to proteolytic cell death **(Fig. 4)** [11 12].

Since its identification, more than 30 years ago, BCL2 has been considered an attractive therapeutic target in hematologic cancers, expecially in lymphoid malignancies. For this reason the first in class BCL2-specific inhibitor ABT-199 (Venetoclax) was tested and succesfully approved in 2016 for the treatment of Chronic Lymphocytic Leukemia (CLL) [13] and is actually under investigation in other lymphoproliferative diseases [14].

BH3 profiling studies, performing by measure of mithocondrial membrane permeabilization in presence of known concentration of different BH3 proteins, demonstrated how different type of cancers have dependency in their survival from different anti-apoptotic pathway [15]. We also know that AML cell lines are BCL2-dependent in their survival (e.g. MOLM-13) and express high level of BCL2 [16]. These considerations led to the developement of trials to test preclinical and clinical efficay of BCL2 inhibitors in AML and in particular Venetoclax, because its oral bioavailability, better tolerability than other inhibitors (e.g. ABT-263 Navitoclax) and specific action of selective BCL2 inhibition[17].

The first clinical trial of Venetoclax in AML patients was a phase 2 single-agent study on R/R population. This study showed a modest ORR of 19%, with a median duration of

remission of 48 days [18]. These results led to investigate the possiblity of combinatiorial approaches, expecially as upfront strategy **(Fig.5)**. In AML patients unelegible for intensive treatment the standard of care is represented by supportive care, low dose chemotherapy (e.g. low dose Cytarabine, LDAC) and HMA. The limited efficacy of current treatment in this population made interesting in assessing venetoclax as frontline approach in combination with backbone therapies (HMA and LDAC), given the synergistic activity seen in preclinical studies[19 20]. In a phase I open-label dose escalation trial, previously untreated elderly patients ineligible for induction chemotherapy were treated with venetoclax plus HMA (AZA or DAC) at the standard dose and schedule[21]. There were 3 target doses of venetoclax (400, 800, and 1200 mg), and patients assigned to AZA and DAC were enrolled into each of the 3 cohorts, for a total of 6 distinct groups of patients. A total of 45 patients were enrolled in the first part of the study: 22 received azacitidine and 23 received decitabine.

Since the higher incidence of adverse events (in particular gastrointestinal) in the 1200mg groups, the cohorts with AZA/DAC plus 400-800mg were expanded for a total of 80 patients. The ORR was 83%; the complete remission (CR) rate was 37%, and the CR with incomplete hematologic recovery (CRi) rate was 30%. At a median follow-up of 15.1 months, the median overall survival (OS) for all groups was 17.5 months. Atter 2 years follow up the median OS was similar for different doses cohorts **(Fig.6)** [22].

Another study investigated efficacy of Venetoclax with LDAC: in this open-label, multicenter phase IIb trial, previously untreated patients with AML >60 years and ineligible for standard induction regimen were treated with venetoclax and LDAC[23]. Among 82 patients who received 600 mg, the CR/CRi rate was 54%. Thirty-three patients had had prior HMA exposure (post MDS AML previous treated with AZA); the CR/CRi rate for this group was only 33%, whereas the group of patients who were treatment naive had a

CR/CRi rate of 62%. The median OS was 10.1 months, and the median duration of response was 8.1 months.

In conclusion results of Venetoclax-based trial in untreated AML patients inelegible for standard intensive treatment compare favorably with clinical trial of the backbone therapies in terms both of tolerability and clinical response [24] [25] . Another advantage of the addition of venetoclax to HMA may be the rapidity of responses. With single-agent HMAs, median time to response was 4 cycles, whereas median time to response with the combination of venetoclax-HMA was 1.2 months. Furthermore Venetoclax-based approaches appear to have activity in all cytogenetic and genomic category, although prior HMA treatment and TP53 mutations result in lower response rates [22].

Results obtained from phase I-II studies led to a phase III clinical trial (VIALE-A) that provided further information about clinical efficacy of the combination HMA-Venetoclax. The study, recently published by DiNardo and collegues, randomly assigned newly diagnosed AML not elegible for intensive chemotherapy to receive azacitidine plus either Venetoclax (at target dose of 400mg daily) or placebo [26]. At a median follow up of 20.5 months the results confirmed superiority of the combinatorial approach both in terms of OS (14.7 vs 9.6 months) and incidence of CR (36.7% vs 17.9%). Adverse events were similar in both groups excpet for febrile neutropenia, that was higher in Venetoclax group.

These data led to the recent FDA-EMA-AIFA Venetoclax approval as first line therapy in combination of HMA in previous untreated AML inelegible for induction regimen.

In R/R setting results about Venetoclax efficacy are less known: in 2018 DiNardo and the MD Anderson Cancer Center (MDACC) group published first results about a real life experience of Venetoclax in combination with salvage regimen in R/R AML, myelodysplastic syndrome (MDS) and blastic plasmacytoid dendritic cells neoplasms (BPDCN) [27]. Forty-three patients received Venetoclax at maximum dose of 400mg/daily in association with HMA (31/43), LDAC (8/43), other chemotherapy (2/43) or target therapies

(midostaurine 2/43, sorafenib 1/43, ruxolitinib 1/43). A median of 2 cycles of treatment were administered (range 1-4): ORR was 21% (9/43), including 2 CR and 3 CRi; the median OS was 3 months in the whole population and 4.8 months in reponder patients. Estimated 6 months survival was 24%.

This study was the first to analyze the efficacy and tolerability of Venetoclax-based regimen in R/R myeloid neoplasms and, in a heavily pretreated population (median prior therapy 2 or more), demonstrated that HMA/LDAC in combinaton with Venetoclax represents a viable salvage option. Notably better response rates were observed in patients with intermediate cytogenetics, IDH1, IDH2, and/or RUNX1 mutations. Furthermore, as recently published, the VEN-AZA regimen showed activity and potential applicability as a bridge strategy to allogenic stem cells tranplantation (HSCT) in selected patients, producing objective response also in highly pretreated population[28].

1.3 Venetoclax is effective in a subset of T-ALL: role of BCL-2 inhibition in Early T- Cell Precursor ALL

Acute Lymphoblastic Leukemia/Lymphoblastic lymphoma (ALL/LBL) is the most common cancer in childhood, whereas is less frequent in adult population. T-ALL phenotype constitutes approximately 15- 20% of all ALLs, is more common in adults than in children and unfortunately is associated with an increased risk of induction failure and early relapse[29].

Immunophenotypic characterization defines each stage of intrathymic differentiation of T lymphocytes by the expression pattern of cluster of differentiation (CD) antigen and historically T-ALL diagnosis relies on cytoplasmic and/or membrane CD3 positivity, while other T-cell antigens, such as terminal deoxynucleotidyl transferase (TdT), CD7, CD2, CD5, CD1a, CD3, CD4, and CD8, are variably expressed during each step of

development from pro-T to pre-T, cortical-T, and mature-T cell **(Fig.7)**[30] . More recently a novel subtype of T-ALL was recognized, characterized by the oncogenic transformation of Early T-Cell Precursor and recognized by the 2016 revised WHO classification as a distinct provisional entity [31]. ETP-ALL is postulated to derive from a stem cell precursor migrated from the bone marrow to the timus but not yet irreversible committed to T-cell lineage and retaining potential for myeloid/dendritic differentiation. ETP-ALL was first described in 2009 by Coustan-Smith et al. in a study that analyzed leukemic cells from 239 patients with T-ALL enrolled at St. Jude and in the Italian national study AIEOP ALL-2000 by gene expression profiling, flow cytometry and single nucleotide polymorphism array. ETP-ALL is characterized by a distinct immunophenotype with various expression of lymphoid and myeloid markers: CD7 is expressed, but CD8 and CD1a are lacking. Blasts express cytoplasmic or in rare cases surface CD3, may express CD2 and CD4 while CD5 is weak (<75% of blasts population). Myeloid markers are common positive: CD34, KIT (CD117), CD13, HLA-DR, CD33, CD11b and CD65. By definition MPO is negative because leukemia with ETP phenotype that express MPO meet the criteria for T/myeloid mixed phenotype acute leukemia [32]. Compared to all the other patients, subjects with ETP-ALL had inferior remission after the first phase of induction therapy and higher level of MRD after induction and consolidation with a dismal prognosis and significantly inferior outcome than classic T-ALL **(Fig.8)**[33].

First line therapeutic approaches in all subtypes of T-ALL elegible patients are represented by intensive chemotherapy protocols based on the combination of steroids-antracyclines-vincristine-asparaginase following by multiple consolidation cycles and HSCT in high risk or minimal residual disease (MRD) positive patients[29] [34]. Despite remarkable improvements in T-ALL cure rate achieved during the last years, a significant proportion of patients still relapse. Multiple mechanism of resistance to conventional drugs have been described, such as NT5C2 mutations, Murine Double Minute 2 (MDM2) disregulation,

Polycomb Repressive Complex 2 (PRC2) mutations, disregulation of IL7-R and PI3K-AKT-mTORs pathways[35] and several compounds are under investigation with the purpose to overcome chemoresistance in T-ALL (e.g. NOTCH1, PI3K-AKT, and Cyclin D3:CDK4/6 inhibitors) **(Fig. 9-10)**[36].

Altough several experimental approaches, few options are currently available in R/R disease and the only drug FDA approved since 2005 is Nelarabine, that provided limited results in this setting with overall response rate of 41-46% and 1-year OS in responder patients of 24-28% [37].

As above described in AML setting, also in T-ALL apoptosis pathway represents an appealing target to overcome mechanisms of resistance in R/R disease. Several preclinical studies tested BH3-mimetics in ALL models: for example Navitoclax (ABT-263) and Venetoclax (ABT-199) were tested in B-ALL cell lines and demonstrated the ability to suppress leukemia proliferation[38]. Furthermore, Navitoclax was tested in vivo in xenografts models obtained from 31 pediatric patients with T-ALL, B-cell precursor ALL, or MLL-rearranged ALL and showed in vivo efficacy [39]. Peirs et al. analyzed the BCL2-expression level in 64 human T-ALL samples to investigate the potential activity of selective BCL-2 inhibition in this setting. The results of this study showed that distinct T-ALL subtypes have different levels of *BCL-2* expression, with immature phenotype presenting the higher level, whereas mature TAL1 T-ALL express low levels of *BCL-2*[40]. Further experiments confirmed this observation and showed that *BCL2*-expression correlates with the stage of blast differentiation suggesting that more undifferentiated T-ALL cells express greater levels of *BCL-2*. Moreover, the immature, ETP-like, T-ALL cell-line LOUCY and its orthotopic mouse model displayed the greatest sensitivity toward Venetoclax treatment. The study published by Chongaille et collegues demonstrated, using a mitochondrial BH3 profiling assay, that ETP-like T-ALL cell lines and pediatric ETP lymphoblasts were more dependent on BCL-2 compared to other T-ALL subtypes that are generally more BCL-XL

dependent for their survival **(Fig. 11)**. Consistent with this data, ETP cells and ETP-xenograft models showed greater sensitivity to Venetoclax than classic T-ALL[41]. In conclusion, all these studies underline that BCL-2 expression and response to BCL-2 suppression depend on T-ALL maturation stage, with ETP phenotype showing the higher levels of expression of *BCL-2* and BCL-2 dependence in its survival: these observations establish the possibility of Venetoclax as potential therapeutic strategy in this high risk subtype of T-ALL.

1.4 Strategy to overcome Venetoclax resistance in hematologic malignancies and rationale for combinatorial approaches

Overexpression of *BCL-2* is implicated in sustaining the survival of neoplastic cells in several hematological malignancies, therefore targeting BCL2 has long been an attractive strategy to treat relapsed/chemorefractory diseases. The discovery of *BCL-2* overexpression in AML and in a T-ALL subtype that result in BCL2-dependent survival led to the observation of selective BCL2 inhibitor Venetoclax as a promising candidate to treat chemoresistant leukemias[16 41]. In AML limited results in terms of efficacy were observed with Venetoclax in monotherapy in R/R patients, whereas there were good results when Venetoclax is used as first line approach in combination with HMA[18 26], probably because BCL2-inhibitor sensitize relatively resistant AML cells to HMA, expeciallly AZA[42]. An interesting correlation was observed in AML between good clinical response to Venetoclax and some genetic groups, for example AML with IDH1/2 mutations. In this Venetcolax-sensitive category, drug efficacy is probably related to 2-hydroxyglutarate-mediated disruption of the mitochondrial electron transport chain. These findings indicate that IDH1/2 mutation status may identify patients that are likely to respond to BCL-2 inhibition and raise rational basis for combining agents that may help to overcome resistance of

IDH1/2-mutant AML cells to IDH1/2 inhibitors[43]. However, use of Venetoclax in clinical trials and real-life experiences led to the observation of emerging resistance in AML setting. Different mechanisms have been demonstrated to contribute to venetoclax resistance. One of the main determinants is the upregulation of other anti-apoptotic BCL-2 family proteins, including MCL1, BCL2L1 (BCL-xL) and BCL2L2. Another major factor related to drug sensitivity is the disruption of mitochondrial structure and metabolic-related pathways. It is also known that venetoclax-resistant cells show increased levels of phosphorylated ERK (pERK) and phosphorylated AKT, suggesting that the upregulation of MAPK and AKT pathways may lead to venetoclax resistance[44] [45]. Furthermore p53 pathway is involved in mechanisms that lead to cell survival, thus targeting simultaneously BCL2 and p53 could overcome resistance to apoptosis of AML cells[46].

A recent study recognized mechanisms of Venetoclax resistance in AML using a platform based on the integration of clinical parameters, whole-exome sequence data and RNA-seq data[47]: the results showed that some clinical parameters are associated with BCL-2 inhibitor resistance such as low blasts count, monocytic/mielomonocytic phenotype[48] and secondary AML. Subsequently the study investigated the relationship between Venetoclax sensitivity and common AML mutations, observing that samples with KRAS, PTPN11 and SF3B1 mutations were relatively resistant to Venetoclax. Other factors associated with low Venetoclax-sensitivity in cell-lines models was *BCL2A1* overexpression, that conferred relative resistance to the majority of BH3 mimetics, but not to HMA or chemotherapy alone. Furthermore, high expression of *CLEC7A* or *CD14* predicts venetoclax resistance. Finally, knowing that more common mechanisms of Venetoclax resistance are related to the overexpression of other antiapoptotic proteins, primarily MCL1, the authors provided evidence in a xenogratf model that the combination of MCL1-inhibitor (AZD5991) with Venetoclax can overcome resistance and obtain therapeutic effect in AML.

In T-ALLs the question of Venetoclax resistance is also relevant and, in preclinical models, is predominantly related to a transcriptional feedback mechanism to escape from BCL-2 dependency[49 50]. Indeed, as seen in AML setting, the downregulation of BCL-2 reflects the loss of addiction to this protein for cell survival, and simultaneously the compensatory upregulation of other anti-apoptotic proteins (like BCL-XL and MCL1) not targeted by BCL2-selective inhibitor. Consequently, combination strategies will be required to circumvent this limitation. These can follow two different way: empiric combinations, for example, with standard cytotoxic agents, or rational combinations with other target agents based upon molecularly-based interactions. Recently MDAAC Group published preliminar results of a phase 1 study of Venetoclax in association with standard chemotherapy (mini-HyperCVD) in older untreated ALL patients[51]: dose level 1 of 400mg and level 2 of 600mg were administered in two separeted cohorts: the dose of 600mg were the phase 2 recommended. Among 18 enrolled patients the CR and MRD negative rates were exceptionally high and the therapeutic regimen was particularly well tolerated so that the authors conclude that addition of venetoclax may represent a major advance in older newly diagnosed ALL. In R/R setting reports of combinatorial approaches both with conventional chemotherapeutic agents [52] and HMA[53] are described.

In the context of "target" approaches recently, Li et al. developed a new BH3-mimetic selective for MCL1, S63845, which was demonstrated to be active in the majority of T-ALL in preclinical models. Interestingly, in T-ALL expressing high levels of MCL1, S63845 appears able to revert resistance to Venetoclax, as shown by analysis of drugs synergy[54]. However, not only new compounds could help to overcome selective BCL-2 inhibitor acquired resistance, but a solution may come from relatively "old" drugs. We know that proteasome inhibitor Bortezomib downregulates MCL1 through the induction of NOXA protein with a mechanism that finally lead to mytochondrial membrane depolarization **(Fig.12)**[55 56]. Bortezomib, in a combination approach, represents the standard treatment in

multiple myeloma[57] and is under investigation in preclinical studies in T-ALL[58], where it seems able to cause cellular death in a majority of T-ALL cell lines at sub-nanomolar concentrations, to suppress the expression of Notch and its target genes (*HES1*, *GATA3*, and *RUNX3*) and, as we known, to blockade NF-Kb activity. As for other malignancies Bortezomib synergizes with several other drugs. In the citated study the combination of bortezomib and dexamethasone lead to almost complete remission of xenografted T-ALLs. A further mechanism of venetoclax resistance, as observed in other malignancies, has been described in B-cell lymphoma and could has implications in ALL. Indeed, developement of Venetoclax resistance during treatment in a diffuse large B-cell lymphoma cell line seems to be related to expression of high level of phosphorylated AKT and PTEN suppression, leading to enhanced AKT pathway activation and concomitant susceptibility to PI3K/AKT inhibition. The authors conclude that high AKT activation in Venetoclax resistant cells lead to potential high sensitivity to PI3K/AKT inhibition, offering potential mechanisms to overcome acquired and intrinsic Venetoclax resistance through PI3K/AKT inhibitors **(Fig.13)**[59]. Furthermore the combinatorial approach of JAK2 inhibitor Ruxolitinib and BCL-2 inihbitors demonstrated to reduce disease burden in an IL7-R mutant T-ALL mouse model[60]. In conclusion, all these studies support the hypotesis that BCL-2 pathway may represent a promising target in T-ALL, expecially in more immature phenotype such as ETP. At the same time the combination of Venetoclax with other drugs is necessary to prevent and overcome emerging of resistant clone during treatment. However Venetoclax-based strategies could lead to significant progresses in T-ALL therapy and improve survival in patients with R/R disease.

2 Aim of the study

Individualized Systems Medicine represents a challenging approach to improve cancer treatment one patient at a time. In this scenario, DSRP on primary leukemia samples provides useful information to assist clinical decisions through the identification of actionable targets and biomarkers also regardless of the mutational status of leukemic cells.

An alternative strategy to directly determine drug dependency of leukemic cells based on ex vivo DSRP showed potential benefit in R/R AML setting with some examples of clinical translation. As Pemvoska et collegues demonstrated, drug profiling helps to provide a functional taxonomic classification of AML based on sensitivity to different category of molecules (tyrosin Kinase inhibitors, JAK2 inhibitors, FLT3 inhibitors, BH3 mimetics)[3].

Among several compounds the BCL2 inhibitor BH3 mimetic Venetoclax (ABT199) showed efficacy in preclinical models and resulted in significant inhibition of AML progression and extension of survival[17].

The first Phase II trial of Venetoclax was as a single-agent for R/R AML and showed limited efficacy in this population. For patients with prior HMA exposure, the CR/CRi rate was higher (25%) compared to other patients (CR+CRi 19%)[18].

Conversely, DiNardo et al. recently published results of a phase III clinical trial (VIALE-A) reporting efficacy of the combination Azacitidine-Venetoclax (AZA-VEN) in previously untreated AML ineligible for intensive treatment with an advantage in the Venetoclax group in terms of complete remission rate and overall survival[26].

On the basis of these data Venetoclax was approved as first line therapy for AML patients unfit for chemotherapy in combination with hypomethilating agents.

The limited efficacy data of single-agent venetoclax in high-risk and R/R AML has encouraged the understanding of mechanisms of resistance to BCL2-inihbition and the

investigation of rationally designed combination approach to increase its activity in R/R AML population altough toxicity profile may limitate clinical benefit and selection of patients plays a critical role. In this setting DSRP could helps to identify patients who may benefit of BCL2 inhibitor by testing ex vivo BM blasts viability in the presence of Venetoclax.

In ALL genomic provides new insight into potentially actionable targets and DSRP is an interesting tool that may provide crucial information to guide ALL treatment, especially in the relapsed/refractory (R/R) disease, that still represents a clinical challenge, and in the high-risk subtypes with extremely chemoresistant phenotype.

In T-ALL setting there are few therapeutic options in the R/R patients and Nelarabine, a purine nucleoside analogue, is the only FDA approved treatment since 2005 with an overall response rate less than 50% and a CR rate of 25%[37].

Among T-ALL, early T cell precursor (ETP), a subtype recently recognized by the 2016 revised WHO classification and believed to have originated from thymocytes that retain stem cell-like features, represents a very high risk disease with aggressive clinical course and dismal prognosis[33]. ETP-ALL patients have a survival rate significantly inferior to patients with other T-ALL subtypes, because of the intrinsic resistance to conventional chemotherapy, with high rate of induction failure and MRD positivity: thus, novel treatment strategies are needed to improve the outcome of these patients.

BH3 profiling studies have demonstrated that T-ALL with ETP phenotype are BCL-2 dependent and sensitive both in vitro and in vivo to BH3 mimetics, especially Venetoclax[41].

Dysregulation of apoptosis signaling via overexpression of the Bcl-2 family members (Mcl-1, BIM) is crucial in the pathogenesis of several hematologic malignancies and in particular in lymphoproliferative disorders.

It has been hypotized that dual inhibition of anti-apoptotic proteins BCL-2 and MCL-1, by using Venetoclax in combination with agents that disrupt Mcl-1-promoted cell-survival, like

proteasome inhibitor Bortezomib[56], could result in a synergistic activity. This approach could overcome mechanisms of drug-resistance and could be translated clinically.

On the basis of these data, the aim of the study was to:

1. Assess *ex vivo* drug sensitivity on primary bone marrow blasts of AML patients testing compounds with potential activity in this setting and in particular Venetoclax, considering its therapeutic potential in a selected population, expecially in combinatorial approach.
2. On the basis of *ex vivo* results and in according to Local Ethic Committee use Venetoclax in association with hypomethilating agents in an off label setting in R/R AML patients Venetoclax-sensitive and ineligible for intensive chemotherapy or other approved drugs or clinical trial.
3. Assess drug-sensitivity of R/R T-ALL patients through a DSRP approach and, in a small population of ETP/near ETP-ALL, test a broad selection of compounds (chemotherapeutic agents, FDA approved compounds or in clinical trial) on primary bone marrow blasts samples.
4. Assess the clinical response to a combination therapy identified from a DSRP approach.

3 Materials and methods

In our study we enrolled patients > 18 years with confirmed diagnosis of AML and T-ALL by morphology and flow citometry, referred to Haematology and Bone Marrow Transplant Unit of University Hospital of Parma between January 2018 and June 2020.

Samples were obtained after written informed consent under an approved protocol at the Parma University Hospital (n.18249/18/05/2017) and according to the declaration of Helsinki guidelines for the protection of human rights.

From 8 to 10 mL of bone marrow sample was drowned in Na citrate tubes, concomitantly to other lab work-ups for disease staging/follow up at different timepoints (diagnosis, re-evaluation, chemoresistance, relapse).

Patient's clinical data (age, gender, bone marrow morphology and flow cytometry, mutational status, karyotype, complete blood cells count at diagnosis and at the moment of samples collection, previous therapies) were collected in a dedicated database.

DSRP was performed:

- In all leukemia samples using Cell-viability test on primary cell coltures and ATP-assay to measure the sensitivity of blasts obtained from patients bone marrow samples to compounds potentially active in the disease, regardeless of leukemia mutational status. ATP-assay is an helpful strategy to hightlight drug sensitivity, based on the measurement of intracellular concentration of ATP as an indicator of cell viabity in the presence of increasing concentrations of drug.
- In 3 ETP-ALL patients a more complete profiling was performed using a small molecule library of 85 compounds (chemotherapeutic agents, target therapy in clinical trial or clinical development) tested in multiple concentrations in cell-coltures. The sensitivity of blasts to every drug was assessed by automated microscopy at different timepoints. This Drug response test was performed in the

Laboratories of the Division of Pediatric/Oncology, University Children's Hospital Zurich, Zurich, Switzerland. In this small population data obtained from DSRP were validated by Cell viability assay (described belowe) and qRT PCR was used to evaluate level of BCL2 expression in ETP ALL patients.

3.1 Compound sources for cell-viability test

We obtained the compounds for this study from the following sources: Bcl-2 inhibitor Venetoclax (ABT-199, GDC-0199, #S8048), BCR/ABL inhibitors Ponatinib (AP24534, #S1490) and Dasatinib (BMS-354825, #S1021), HDAC inhibitor Entinostat (MS-275, #S1053) and FLT3 inhibitor Gilteritinib (ASP2215, #S7754) (Selleckchem Houston, TX USA) reconstituted in DMSO (dimethyl sulfoxide) to a final concentration of 10 mM. Bortezomib (PS-341, LDP-341, MLM341, #217785) and Quizartinib (AC220, #6788) (Santa Cruz Biotechnologies, CA USA) reconstituted in DMSO to a final concentration of 50 mM.

3.2 ATP-based Cell Viability and Proliferation

Mononuclear cells were isolated by density gradient centrifugation using LSM-lymphocyte separation medium (Cappel^{TM} MP Biomedicals, LLC, Ohio, USA #50494) and cultured for a short time. Cells were seeded in 384-well plates (Corning Life Sciences Plastic, Bedford MA, USA, #3570) at the final concentration of 0.2 x 10^6/mL per condition. Drugs were added with a nanometric dispenser Tecan D300e (Tecan Trading AG, Switzerland). Cell viability was assessed after 72 hours of drug treatment using a CellTiter-Glo ATP assay (Promega Corporation, Madison, WI, USA, #G7573). IC_{50} was calculated using GraphPad Prism software (La Jolla, CA, USA).

3.3 Cell coltures

In this study, we used the following cell lines. T-ALL cell lines: ALL/SIL, DND41, Jurkat, Loucy, MOLT16, PF382, RPMI8402, SKW3 and SUPT1. AML cell lines: HL60, HNT34, MOLM1, MUTZ3, NOMO1, TF1, THP1, UCSD/AML1. All cell lines were grown in RPMI 1640 medium (1x) (#31870025) supplemented with 10 or 20% Fetal Bovine Serum (FBS, #10270-160), 100 U/ml penicillin and 100 µg/ml streptomycin (#150070-063) and L-Glutamine (#25030081) at 37°c, in a humidified atmosphere under 5% CO2.

3.4 Cell viability assay

Effect of 24 hours of the vehicle (dimethyl sulfoxide), 10 mM hydrogen peroxide (H2O2), venetoclax and bortezomib on cellular viability was measured by a functional profiling, based on lab-on-a-chip technology, which allows testing the response of live tumor cells exposed to anticancer drugs in a automated process developed by CellPly. ETP- ALL cells were labeled with a 7-amino-4-chloromethylcoumarin (CMAC) dye (blue), an anti-HLA-DR (green), and an anti-CD34 antibody (yellow) for leukemia cells identification. High-content time-lapsed imaging was performed for 24 hours, every 12 hours on cells in 480 microwells (75um diameter) at a 1px- 1um resolution at each time points/conditions tested. Cell death was quantified by imaging HLA-DR^+/$CD34^+$ cells positive to the propidium iodide (PI) staining (red). Results are expressed as the percentage of PI^+/HLA-$DR^{+/}CD34^+$ positive cells relative to the negative population

3.5 Immunodetection

Protein lysates for western blotting were incubated with Bcl-2 (124) Mouse mAb (#1507), Mcl-1 (D35A5) Rabbit mAb (#5453), Caspase 3 (#96645) and Cleaved PARP(Asp214) antibody (human specific) (#9541) (Cell Signaling, Beverly, MA, USA). Loading controls were performed with antibodies specific for actin (#ACTN05, Thermo Scientific, Waltham, MA, USA), HSP90 (# sc-69703 (4F10)), Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were developed using species specific fluorescent antibodies obtained from LI-COR (Biosciences, Lincoln, NE, USA) such as IRDye 680LT Goat anti-Mouse IgG (#925-68020); IRDye 800CW goat anti-rabbit IgG (#925-32211); IRDye 680RD goat anti-rabbit IgG (#925-68071).

3.6 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Expression of *BCL2* in ETP-ALL samples obtained from our patients was assessed by quantitative real-time polymerase chain reaction (qRT-PCR). Data were analyzed using the ΔΔCT method.

4 Results

From January 2018 to June 2020 91 AML, 25 ALL (12 B-ALL, 13 T-ALL), 3 acute leukemia of ambigous lineage (2 Mixed Phenotype Acute Leukemia -MPAL- and 1 Acute Undifferentiated Leukemia -AUL-) patients were admitted to Hematology and BMT Unit of University Hospital of Parma and, after written informed consent, 126 AML, 43 ALL and 8 MPAL/AUL BM samples were collected at different timepoints, in particular at diagnosis, at relapse and in case of chemorefractory disease with the aim to perform drug sensitivity test in our Laboratory. Furthermore 5 T-ALL BM samples were sent to our Laboratory from University of Perugia to perform DSRP in patients with chemorefractory disease.

DSRP was performed using compounds chosen on the basis of their potential activity in the specific disease setting, regardeless of leukemia genetic/mutational status.

Among all samples referred to our Laboratory sensitivity profiling to different compounds using ATP-cell viability test was performed for a selected patients population as below:

- BH mimetics and in particular Venetoclax was tested in 34 AML, 3 MDS, 12 T-ALL and 4 MPAL samples
- FLT3 inhibitors Quizartinib and Gilteritinib were tested in 6 FLT3-negative AML samples
- Tyrosin Kinase inhibitor (TKI) Dasatinib was tested in 5 AML, 10 T-ALL and 3 MPAL/AUL samples; Ponatinib was tested in 3 AML and 3 T-ALL samples
- HDAC inhibitor Entinostat was tested in 7 AML samples

Clinical and biological characteristics of AML/MDS, ALL and MPAL/AUL patients tested for DSRP are showed in **Table 1-3.**

4.1 BH3 mimetics, TKI and HDAC inhibitors show *ex vivo* activity in AML

ATP cell viability tests were performed on 31 AML and 3 MDS samples from 30 patients at different timepoints. In our experience primary BM blasts demonstrate sensitivity to a specific compound when IC50 was <3μM as compared to results obtained in AML cell lines tested for drug profiling.

Venetoclax sensitivity was analyzed in the entire population, whereas a small number of samples were tested for other drugs. Other compounds were: HDAC inhibitor Entinostat, FLT-inhibitors Quizartinib and Gilteritinib and TKI Dasatinib and Ponatinib. All drugs exhibited a median IC50 compatible with potential sensitivity in a subset of samples without correlation with AML mutational status (ex: all 3 FLT3 inhibitors-sensitive samples were AML FLT3 negative). **Table 4** showed results of viability assay for each drugs with mediani IC50. Three/six FLT3 negative AML samples tested for FLT3 inhibitors were sensitive to Gilteritinib; TKI and in particular ponatinib showed efficacy in 3/5 AML (1 myeloid blast-phase of CML, 1 AML inv(16) c-KIT unmutated and 1 R/R AML normal karyotype FLT3/NPM1 negative). HDAC inhibitor Entinostat was tested for 7 samples demonstrating efficacy on 6/7 AML (1 newly diagnosed inv(16) AML c-KIT unmutated,1 newly diagnosed AML with normal karyotype NPM1/FLT3 negative, 1 R/R AML with complex karyotype, 1 R/R AML with normal karyiotype and 2 sAML).

Among patients tested for Venetoclax sensitivity 7 samples were newly diagnosed AML, whereas 26 were R/R AML/MDS and one sample lacked for clinical information. BCL2 inhibitor demonstrated efficacy in the majority of patients, except for 8 samples resulted refractory with IC50 between 3,629 and 100. The results of Venetoclax sensitivity test are discussed below.

4.2 Primary BM blasts from AML patients show significant sensitivity to Venetoclax at different timepoints

In our population Venetoclax was tested in 34/34 BM samples and resulted as the most active compound showed a median IC50 of 0,4705 (range 0,005054-100).

Among newly diagnosed AML samples median IC50 was 0,4222 (range 0,0782-4,24), while among R/R AML/MDS was 0,5188 (range 0,005054-100).

IC50 was not influenced by karytotype, mutational status or disease status: in particular Venetoclax showed a good level of efficacy also in R/R AML with high risk features, such as MDS-related disease and complex Karytotype (**table 5**), demonstrating a large spectrum of *ex vivo* efficacy in AML population as single agent, with potential applicability in clinical practice.

On the basis of the *in vitro* results and of the data of the literature about Venetoclax efficacy in combinatorial approach with hypometilating agents, we selected 14 R/R AML patients, in some cases heavly pretreated, who were *ex vivo* Venetoclax-sensitive, with active disease after intensive treatment and inelegible for chemotherapy, other approved drugs or clinical trial.

Venetoclax use was authorized by Local Ethic Committe in a off-label setting.

4.3 Venetoclax in combination with HMA represents a therapeutic option in highly refractory AML patients selected on the basis of *ex vivo* sensitivity test

From May 2018 to December 2019 14 R/R AML patients were considered elegible for Venetoclax-based salvage regimen as single agent or in association with 5-Azacitidine (AZA) or Decitabine (DAC) and started treatment after Venetoclax off-label use approval. *Ex vivo* sensitivity data indicated a median IC50 for this group of 0,2384 (range 0,005054-

11,54) versus 0,4705 of the whole AML population tested.

Patients median age was 74 (range 30-83), male/female 8/6. Clinical characteristics, as described in **table 6** were the following: 4 were de novo AML, 8 sAML (post-MDS, post-CMML, myeloid BP-CML), 2 MDS in leukemic evolution. Karyotype was normal for the majority of patients (9/14), whereas only 2 patients had complex karyotype.

Mutational status, investigated by QT-Rt PCR for NPM1, FLT3 ITD/TKD, IDH1-2 mutations displayed the presence of FLT3-ITD mutations in 2 patients and IDH2-R140Q mutation in 1 patient. Genetic risk according to ELN2017 classification for AML was high in 4 patients and intermediate in 8. Two MDS patients, with features of leukemic evolution were stratified according to IPSS and resulted as int-2 risk category.

All patients were relapsed or refractory after at least one prior therapy and 8/14 patients received at least 3 lines of therapy before Venetoclax-based treatment.

First line regimen was for the majority of patients (10/14) HMA (7 patients AZA/ 3 patients DAC) and median number of cycles administered were 13 (range 4-69). Best response obtained was CR in 4 cases and SD in 6 patients. Median time to progression was 13 months (range 4-69).

Three patients received intensive chemotherapy as first line treatment: 2 were chemorefractory after at least 2 cycles (D3A7, FLAI) and 1 patient relapsed 6 months after D3A7 induction and HD-AraC consolidation with CNS and BM involvement.

One patient received as first treatment TKI for AP-CML, rapidly evolved to myeloid blastic phase refractory to intensive chemotherapy and with disease progression after a short course of Ponatinib.

Venetoclax was administered as single agent during 2018 in 2 patients with heavly pre-treated sAML (1 post-CML and 1 post-CMML). The first patient was refractory to TKI and intensive chemotherapy, also in combination; the second patient was higly chemorefractory and relapsed 3 months after allogenic HSCT. Both patients received

Venetoclax with rapid rump-up at the final dose of 800mg daily po and experienced a rapid peripheral blood blasts clearance and an hematological improvement with a time to progression of 2 months.

Between August 2018 and December 2019 Twelve R/R AML patients started on Venetoclax in association with HMA, according to the following schedule: 5-Azacitidine 75mg/m2 sc day 5-2-2 or Decitabine 20mg/m2 ev day 1-5 every 28 days. Venetoclax final dose was 100mg/m2 po daily after a rapid rump up. Venetoclax maximum dose of 100mg/die was identified to limitate hematologic toxicity in a frail and often heavly pretreated population.

Median cycles administered was 6 (range 1-12): 5 complete remissions were obtained (3 CR+2 CR without complete hematologic recovery); one patient achieved a PR with a reduction of BM blasts >50% and one patient had a stable disease for 6 months. Median progression free survival for entire population was 2.5 months (range 1-12). Median OS was 5 months (range 1-15 months).

Three patients experieced a long-term remission of almost 12 months and 1/3 is still on treatment and in hematologic CR. 2/3 responder patients were AML with MDS related changes and all patients had a story of long term remission with HMA as first line therapeutic approach (median TTP 29 months, range 12-70). Results of Venetoclax based strategy in this population are illustrated in **Table 7**.

4.4 T-ALL showed sensitivity to different compounds that could represent an alternative therapeutic approach in R/R setting

During the study period 12 BM samples from 10 T-ALL patients at different timepoints were sent to our Laboratory to perform DSRP. Six patients were referred to Hematology and BMT Unit of University of Parma, 4 patients were treated in Hematology and BMT Unit of University of Perugia.

ATP-cell viability assay was perfomed for these patients using BH3 mimetic Venetoclax, TKI Dasatinib and Ponatinib and Bortezomib as MCL1-inhibitor. Results of drug sensitivity test with IC50 for each compound are shown in **table 8**. Eight/ten T-ALL patients had a relapsed or refractory disease after one or more lines of therapy: for all these patients Bortezomib showed a very low IC50 with high *ex vivo* efficacy. Median IC50 for Venetoclax was 0,9777 (range 0,01005-100) and confirmed its potential activity in this category of ALL as reported in several studies.

4.5 ETP phenotype is the most sensitive to Venetoclax among T-ALL and ETP patients could benefit from a Venetoclax-based treatment approach

Among 12 T-ALL BM samples tested for Venetoclax, 6 showed an Early T-precursor phenotype and all ETP patients had a R/R disease treated with at least 2 lines of prior therapy at the moment of evaluation.

Venetoclax median IC50 for ETP samples was 0,8729 (range 0,01005-38,52) versus 1,3825 (range 0,1081-100) for T-ALL samples with no ETP phenotype, confirming higher susceptibility of this subset of T ALL to BCL2 inhibition.

This data is supported by the detection of higher expression of BCL2 through Western Blot (WB) analysis in ETP-ALL cell lines compared to typical T-ALL as indicated in **fig.14**.

On the basis of ATP-cell viability test performed in our Laboratory and in collaboration with Jean Pierre Bourquin and collegues at University Children's Hospital Zurich and Hematology and BMT Unit of University of Perugia we performed a broad DSRP on 3 highly refractory ETP-ALL patients using 85 compounds to validate the results of our vialibility assay and identify the most active drugs in this high risk setting as described below. Results of ATP-cell viability tests for Venetoclax and Bortezomib in these patients are showed in **fig.15.**

4.6 Venetoclax-Bortezomib as a therapeutic strategy in R/R ETP ALL

To identify an effective strategy we tested the blasts obtained from BM samples of 3 ETP ALL patients with a library of 85 small molecules including conventional chemotherapeutic drugs, FDA approved or yet in clinical trial compounds **(Fig.16).** DSRP revealed insensitivity to almost all conventional chemotherapeutic drug as Cytarabine, Methotrexate, Vincristine and Mercapropurine, confirming the profile of chemorefractory of ETP-ALL. Clinical and biological data of our patients are showed in **table 2** (UPR1, UPR2, UPG3).

Among different molecules tested in DSRP we confirmed a certain homogeneity of response to BH3 mimetics (Navitoclax, Venetocax) and proteasome inhibitors, such as Carlfizomib and Bortezomib **(Fig.17).**

We therefore validated the data of Drug Profiling for Venetoclax and Bortezomib using cell colture analysis through the CellPly platform based on microwell for the study of cell viability in the presence of increasing concentrations of drugs and a different time frame. We identified the leukemic cells labeled with HLA-DR and CD34 and the apoptotic cells (red), in the presence of veichle, H2O2, Venetoclax and Bortezomib. We showed the percentage of $CD34^{+}$/$HLADR^{+}$ that undergo cell death increased linearly with increasing of

drug concentrations **(Fig.18).**

Then we aimed to identify the expression of BCL2 on BM samples from ETP patients and confirm data demonstrated in ETP-ALL cell lines and reported in published studies. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to tested *BCL-2* expression and evidenced high level of BCL-2 in all three patients compared to T-ALL with other phenotypes, as showed in **fig.19.**

Given the evidence of chemotherapy resistance, in the absence of approved alternative therapeutic options and thanks to the results of DSRP and validation analysis it has been hypotized that dual inhibition of anti-apoptotic proteins BCL-2 and of MCL-1 with Venetoclax and Bortezomib could result in a synergistic activity.

After obtaining local Ethic Committee approval, our 3 patients were started on a personalized target-therapy: the combination of Venetoclax-Bortezomib (VEBO) was obtained in a off-label setting in highly resistant disease and was administered as an outpatient regimen with rapid Venetoclax rump-up to a maximum dose of 800mg/day po and sub-cutaneos Bortezomib bi-weekly 1.3mg/m2. Rasburicase and steroids were used during the first three days of treatment to prevent tumor lysis syndrome.

The administration of VEBO was overall well tolerated and no major adverse effects or toxicity related to the combination have been reported, except a grade 4 neutropenia in one patient (UPR2) and a grade 2 peripheral neuropathy bortezomib-related in UPG3, characterized by plantar pain controlled by analgesic and pregabalin. UPG3 experienced also a grade 2 erythematous rash extended to the trunk and the face during the first week of venetoclax treatment that non-required pharmacological intervention.

All three patients completed almost a cycle of treatment (4 weeks). In all patients we highlighted a clinical response with haematological recovery, and the bone marrow evaluation performed after the first cycle revealed a significant bone marrow blasts percentage reduction with 2 partial response and one complete remission **(Fig.20).** The

two elegible patients (UPR1 and UPG3) received an allogeneic hematopoietic stem cell transplantation as consolidation strategy achieving a stable cytogenetic remission (MRD negativity by FISH, (**Fig.21**) with a 10 months follow up.

The combinatorial regimen was effective also for residual extramedullary diasease in UPR1 as showed in **fig.22.**

Patient 2 (UPR2) after only one cycle of target therapy achieved a complete remission with MRD positivity at flow cytometry and hematological normalization that remained stable for 4 months.

5 Discussion

Personalized approaches are desiderable options in Acute Leukemia treatment, especially in relapsed or chemorefractory diseases and in case of lack of approved drugs. In this scenario, DSRP represents a strategic tool to identify potential active compounds in highly refractory leukemias[3 7]. During the last years, preclinical examples were published both in AML and ALL setting, with some clinical translation; these studies underlined the emergency of potential effective molecules identified by drug profiling with therapeutic activity regardless of mutational status of leukemia and with potential efficacy also in a combinatorial approach. In this context, targeting apoptosis pathway appears as a promising option with a broad applicability[12]. In particular, BH3 mimetic selective BCL-2 inhibitor Venetoclax is a candidate as a salvage drug in several settings.

In AML, Venetoclax was recently approved as first line treatment in combination with HMA[26], but some clinical experiences in R/R patients seems to be effective[27], although the emergency of resistant clones represents a serious issue and Venetoclax is not able to induce satisfactory clinical response in relapsed patients when it is used as single agent[18]. Resistance to BCL2 inhibitors in AML is probably mediated by different mechanisms and several clinical and genetic variables are associated with increased probability of Venetoclax resistance[50]. Neverthless, the main mechanism of escape from BCL2 inhibition is the overexpression of other anti-apoptotic protein such as BCL-XL and MCL1[50].

The study published by Frismantas et collegues[7] demonstrated how a subtype of T-ALL shows a selective sensitivity to Venetoclax and BH3-profiling studies identified that more immature phenotype of T-ALL, so called ETP-ALL, are BCL2 dependent in their survival and more sensitive to Venetoclax activity in preclinical models than classic T-ALL[41].

Even in the T-ALL context, combinatorial approaches are preferable in order to overcome the emergency of Venetoclax resistance mechanisms: clinical examples of association

with standard chemotherapy both in frontline as in R/R setting and HMA are either described or ongoing in clinical trial[61 51 62], but the possibility of target combination direct against other antiapoptotic proteins could be equally effective with a lower toxicity profile. Although some preclinical studies described the potential efficacy of MCL1 inhibition in T-ALL subset[63], drugs already known such as Bortezomib could represent a valid option in clinical translation: indeed, Bortezomib is a strong selective inhibitor of MCL1[55], that we know it is often overexpressed at relapse during BCL2-inibitors treatment in hematologic malignancies.

Based on these data, we performed drug sensitivity test for a study period of almost two years, using an ATP-cell viability assay on 34 AML, 3 MDS, 12 T-ALL and 4 MPAL samples, collected at different moments during disease staging and treatment.

FLT3 inhibitors, HDAC inhibitor and TKI were tested in a small population of AML samples, confirming the effectiveness of these compounds in this setting, regardless of leukemia cytogenetic and mutational status. TKI Dasatinib and Ponatinib were tested in T-ALL setting, confirming data known by the literature of a certain TKI sensitivity in this subset.

Venetoclax was tested in the whole AML population and showed significant efficacy in *ex vivo* model in most of samples with low IC50. On the basis of the drug sensitivity test, we selected 14 patients with R/R AML who could benefit from a Venetoclax-based salvage regimen. Two patients with highly refractory leukemia received Venetoclax as single agent and manifested a rapid peripheral blood blasts clearance. This result confirms BCL-2 inhibition as a strategic target also in advanced disease, although resistance appeared early during treatment, as we observed disease progression. Twelve patients were treated with Venetoclax in combination with HMA (AZA or DAC): the results demonstrated, even in a heavily pretreated population, that a Venetoclax-based strategy could lead to hematologic remission in a significant proportion of patients (5/12, 41.6%), with some long term response (TTP>12months) in patients with previous durable remission with single

agent HMA, although median TTP for the whole population was <3months, confirming data known by prior studies. The short median PFS is probably related to the presence of many patients with very advanced disease, who experienced a rapid progression during the first course of HMA-Venetoclax, without achieving benefit from combinatorial DSRP-based approach. Neverthless, Venetoclax plus HMA confirmed its efficacy as an alternative therapeutic approach also in R/R AML, with some excellent results in patients selected by *ex vivo* sensitivity profile. This represents an interesting platform to help clinicians to choose the more active salvage regimen in refractory disease, maybe also as a bridge to HSCT (as described in some clinical experiences)[28].

In T-ALL population, ex vivo drug sensitivity tests confirmed the higher Venetoclax-sensitivity of ETP/ETP-like phenotype. On the basis of laboratoristic results and of the broad DSRP performed on 3 R/R ETP-ALL, we treated our three patients with a total DSRP-oriented approach, using a combination of drugs targeting apoptosis pathway: Venetoclax and Bortezomib. After only one cycle of therapy, the results were impressive, carrying out 2/3 CR and one partial remission in highly refractory diseases. This approach was feasible and well tolerated also as outpatient regimen and allowed to perform HSCT in two elegible patients, obtaining sustained remission.

During the last year, results of our DSRP-approach in ETP-ALL were published on JCO Precision Oncology[64] and GIMEMA group (Gruppo Italiano Malattie Ematologiche dell'Adulto) approved a clinical trial for R/R T-ALL providing a DSRP oriented salvage treatment with the use of a broad library of small molecules FDA/EMA/AIFA approved and potential active to be administered in clinical practice, basing on Drug Sensitivity Profiling results. This approach will provide new insights in a subset of leukemia with small therapeutic option currently available and will lead us to better understand mechanisms of leukemogenesis.

We hope that results of this trial can help us to identify new molecules active in T-ALL setting both as salvage treatment and as bridge to HSCT.

6 Tables and Figures

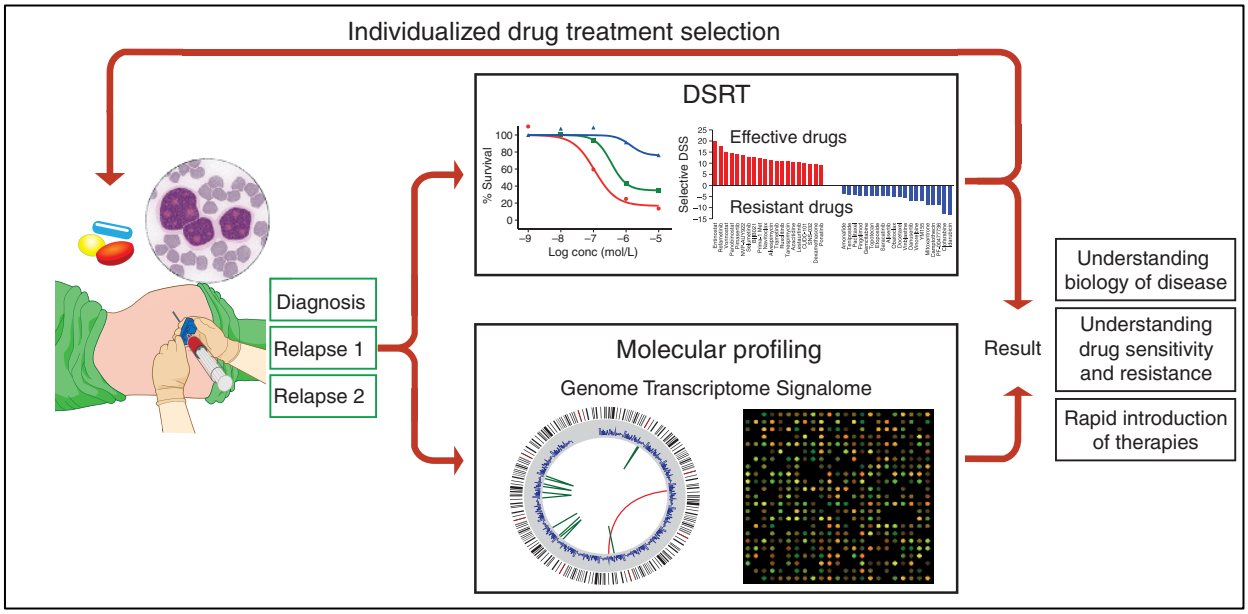

Fig.1: An example of individualized platform for improved AML therapy. DSRP using 187 approved and investigational compounds was performed in *ex vivo* primary cells from AML samples during different moments of the disease, while disease progression and clonal evolution were monitored by deep molecular profiling. Integration of drug sensitivity, next-generation sequencing, and clinical follow-up can lead to rapid introduction of novel therapies to the clinic[3].

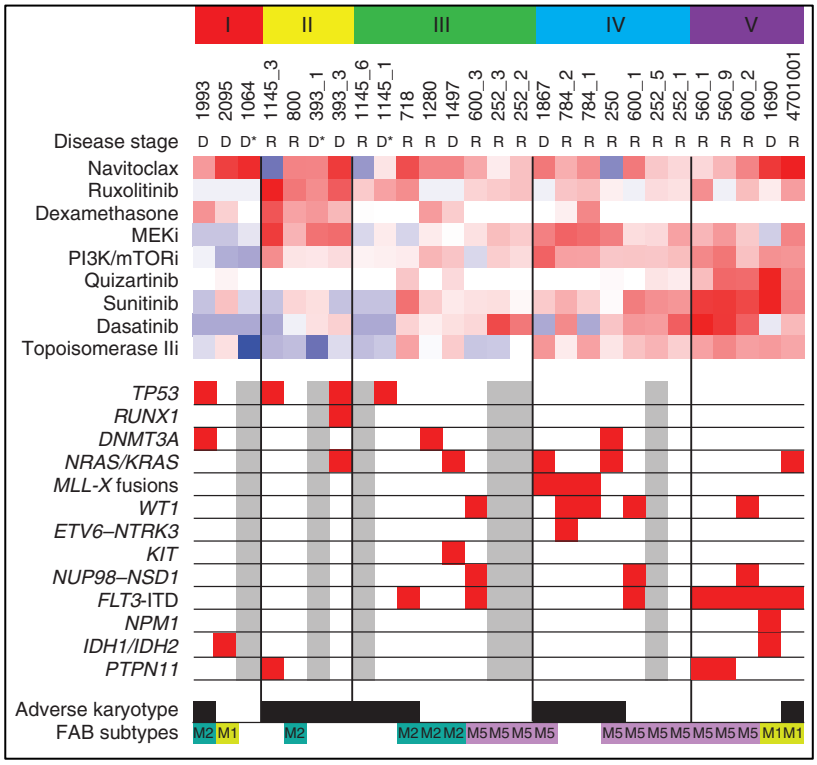

Fig.2: Functional taxonomic AML classification in 5 groups based on DSRP results in the first preclinical experience in AML setting[3].

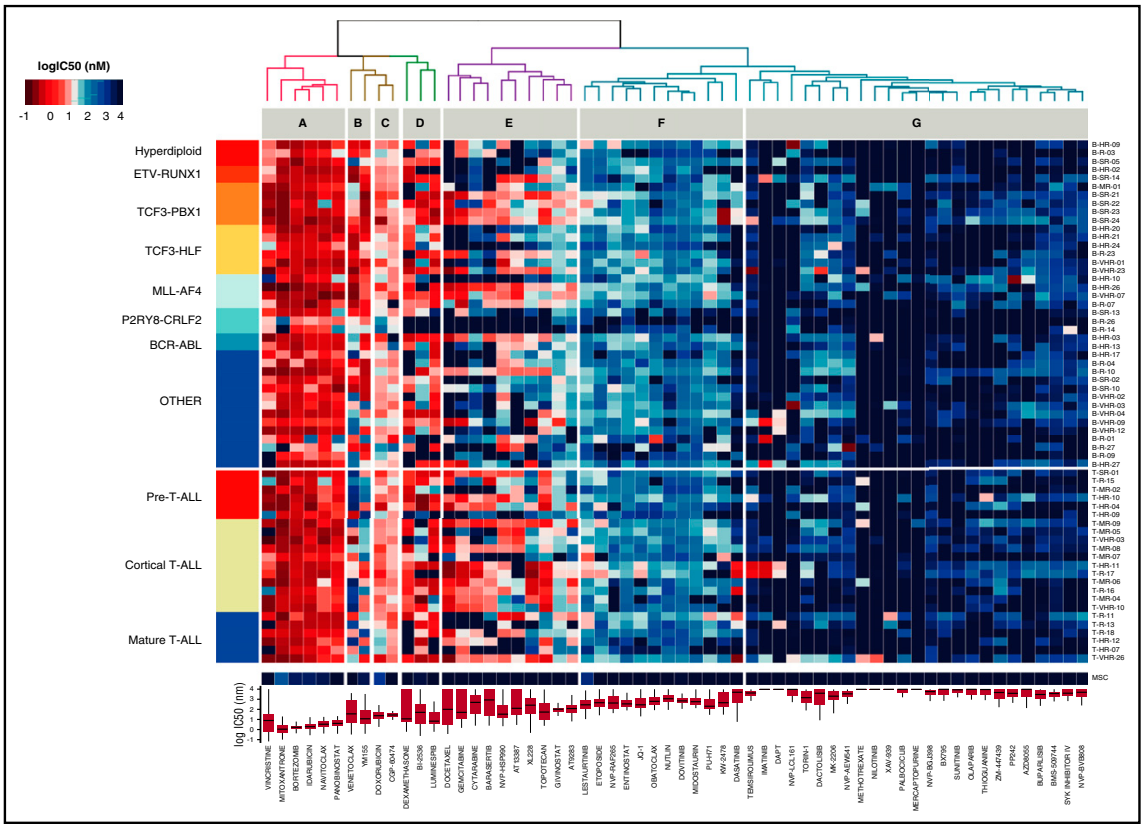

Fig.3: Drug response profile in B-ALL and T-ALL performed in the study published by Frismantas et. al. Heatmap indicates the response of each subtype of ALL to 60 compounds and sensitivity is represented by IC50 values. Generally active drugs, mean IC50 values<10 nM, were represented in red boxes[7].

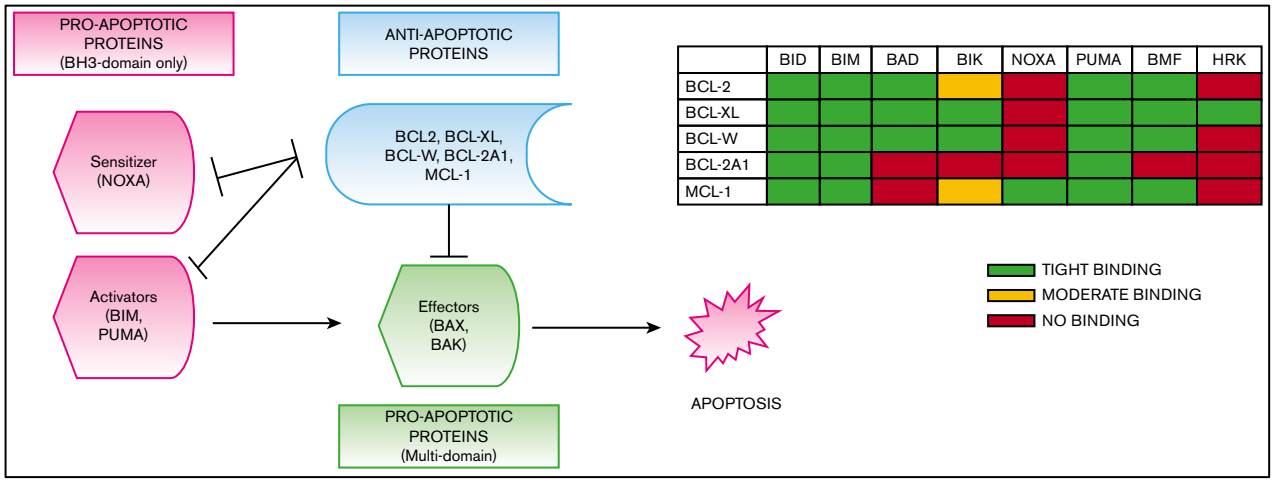

Fig.4: Apoptosis pathway is regulated by BCL2-family protein with 4 funtions: BCL2-like proteins (e.g. BCL-2, BCL-XL,MCL1) are suppressors that prevent apoptosis, BH3 only proteins BIM and PUMA are activators, BAX and BAK are effectors, and protein like NOXA are sensitizers that promote the inhibition of BCL-2 like proteins and the activation of apoptotic cell death program. The activation of pro-apoptotic cascade leads to depolarization of mytochondrial membrane, release of cytochrome-C and activation of caspase 9, ultimately leading to proteolytic cell death. BH3 profiling indicates the selective binding of BH3 peptides to specific antiapoptotic proteins [12 17]

Drug name	Drug class
Chemotherapy	
Daunorubicin[35]	Anthracycline
Cytarabine[35]	Nucleoside analog
Epigenetic regulators	
Azacitidine[36-38]	Hypomethylating agent
Panobinostat[39]	Histone deacetylase inhibitor
Targeted agents	
Alvocidib (flavopiridol)[40]	CDK9 inhibitor
MLN4924 (pevonedistat)[41]	NED inhibitor
GDC-0980[42]	PI3K/mTOR inhibitor
RG7388 (idasanutlin)[43,44]	MDM2 antagonist
GDC-0973 (cobimetinib)[45]	MEK inhibitor
A-1210477[46]	MCL-1 inhibitor
VU661013[47]	MCL-1 inhibitor
AMG 176[48]	MCL-1 inhibitor
Quizartinib[49]	FLT3 inhibitor
Enasidenib[50,51]	IDH2 inhibitor

Fig.5: Compounds that showed preclinical evidence of synergistic effect with Venetoclax[17]

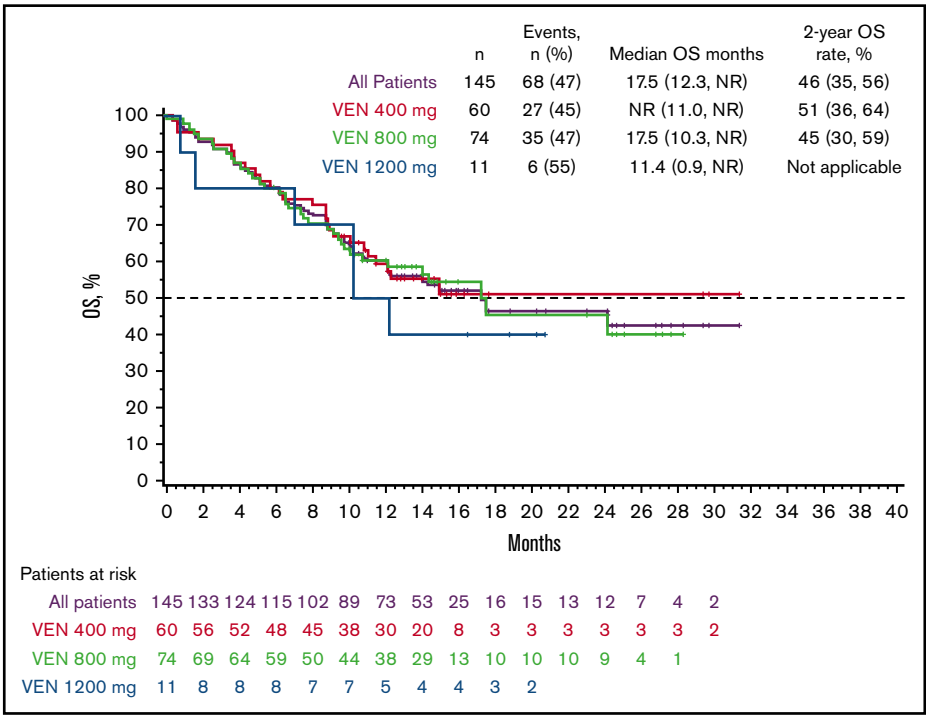

Fig.6: 2 years OS rate of patients enrolled in phase I trial of Venetoclax in association with HMA in previous untreated AML. OS is divided into the 3 different doses of Venetoclax used in the phase Ib trial[22].

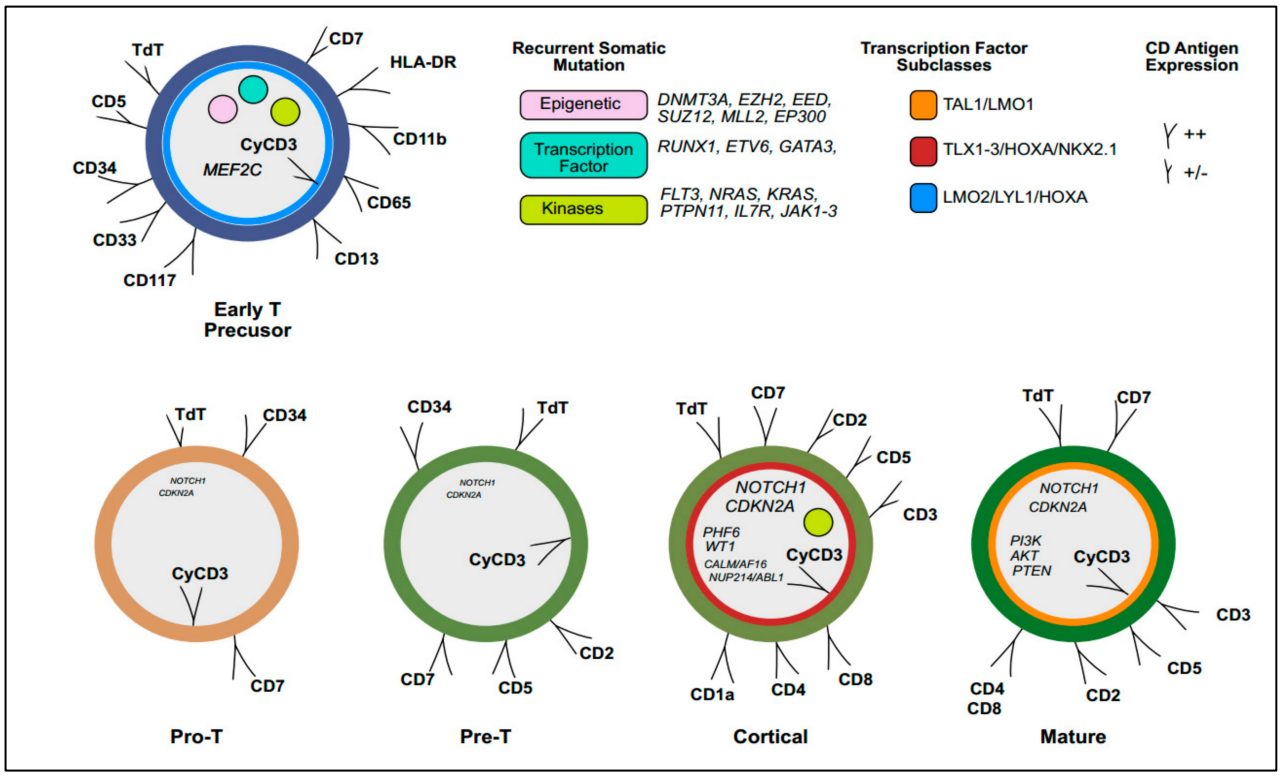

Fig.7: schematic representation of surface markers and molecular signature of ETP ALL compared with other T-ALL phenotypes. ETP-cells display a genetic signature similar to myeloid stem cells, with a high frequency of mutations in genes involved in epigenetic regulation, kinase signaling, transcription factors whereas T-ALL is divided into transcription factor subgroups, LYL1/LMO2, HOXA, TLX1, TLX3, NKX2-1 and TAL1/LMO1. NOTCH1 and CDKN2A mutations rarely occur in ETP while they are frequent in cortical subtype. Other common mutations in cortical-T ALL are PHF6, WT1, CALM-AF1, NUP214-ABL1, and IL7R-signaling. PI3K, AKT, and PTEN are frequently mutated in mature T-ALL cells[35].

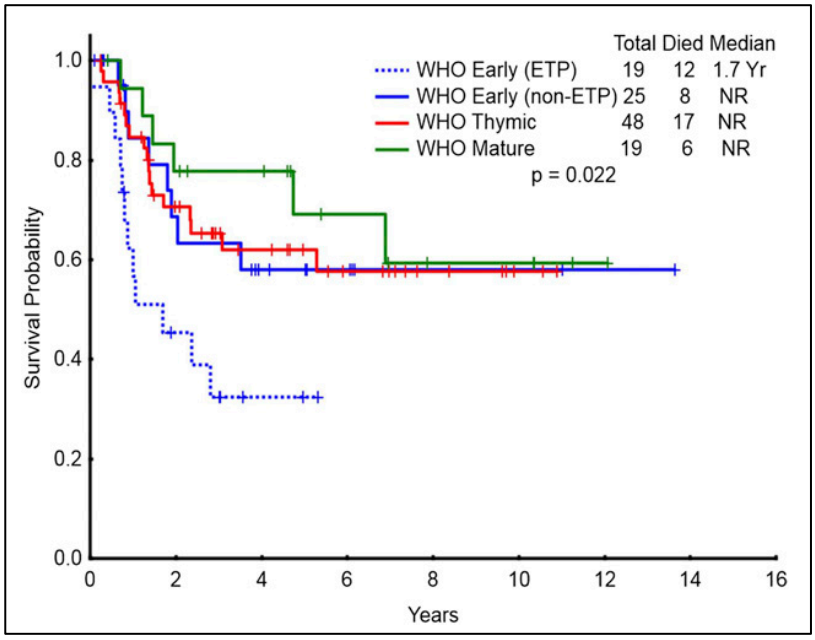

FIg.8: OS of ETP-ALL compared to other T-ALL subgroups[33]

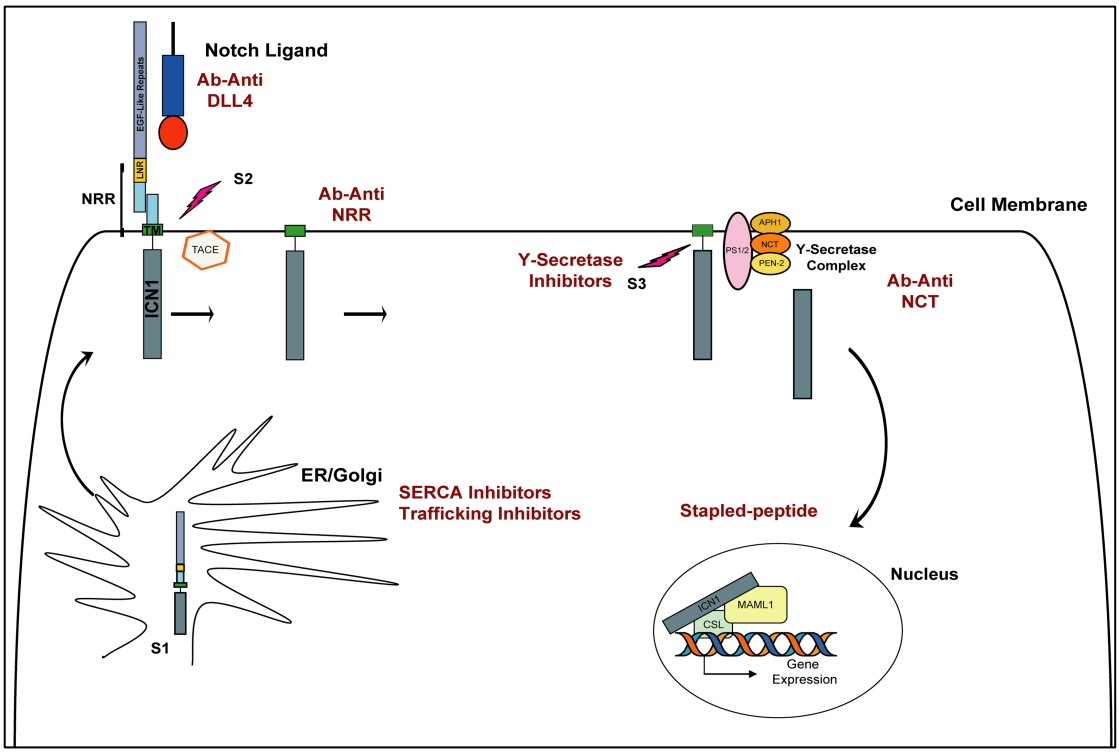

Fig.9: schematic representation of NOTCH signaling and therapeutic options to target NOTHC in T-ALL[36]

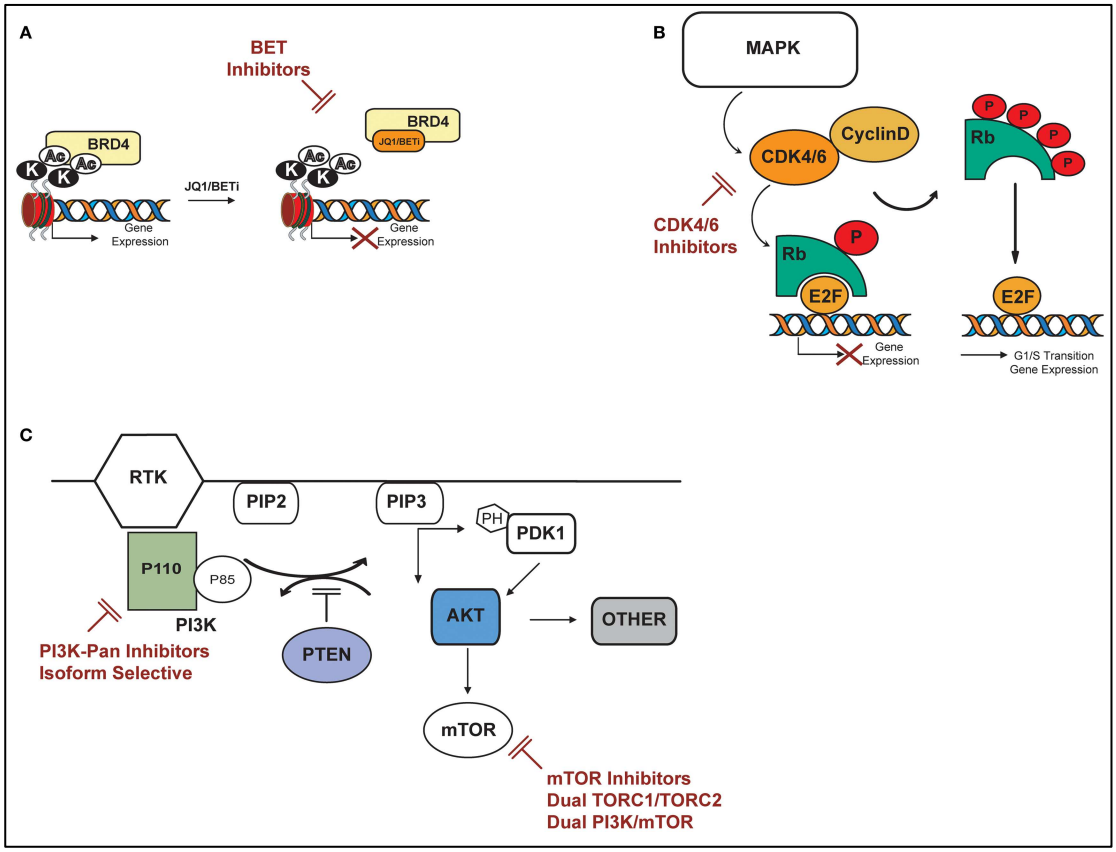

Fig.10: alternative therapeutic target in T-ALL. (A) BET inhibitors displace BET bromodomain, such as BRD4, from chromatin and lead to the repression of transcriptional targets. (B) CDK4/6 inhibitors block retinoblastoma (Rb) protein phosphorylation and prevent G1-S-phase transition, arresting the cell cycle. (C) inhibition of P3K-AKT-mTOR pathway prevents cell-cycle progression and survival of T-ALL cells[36]

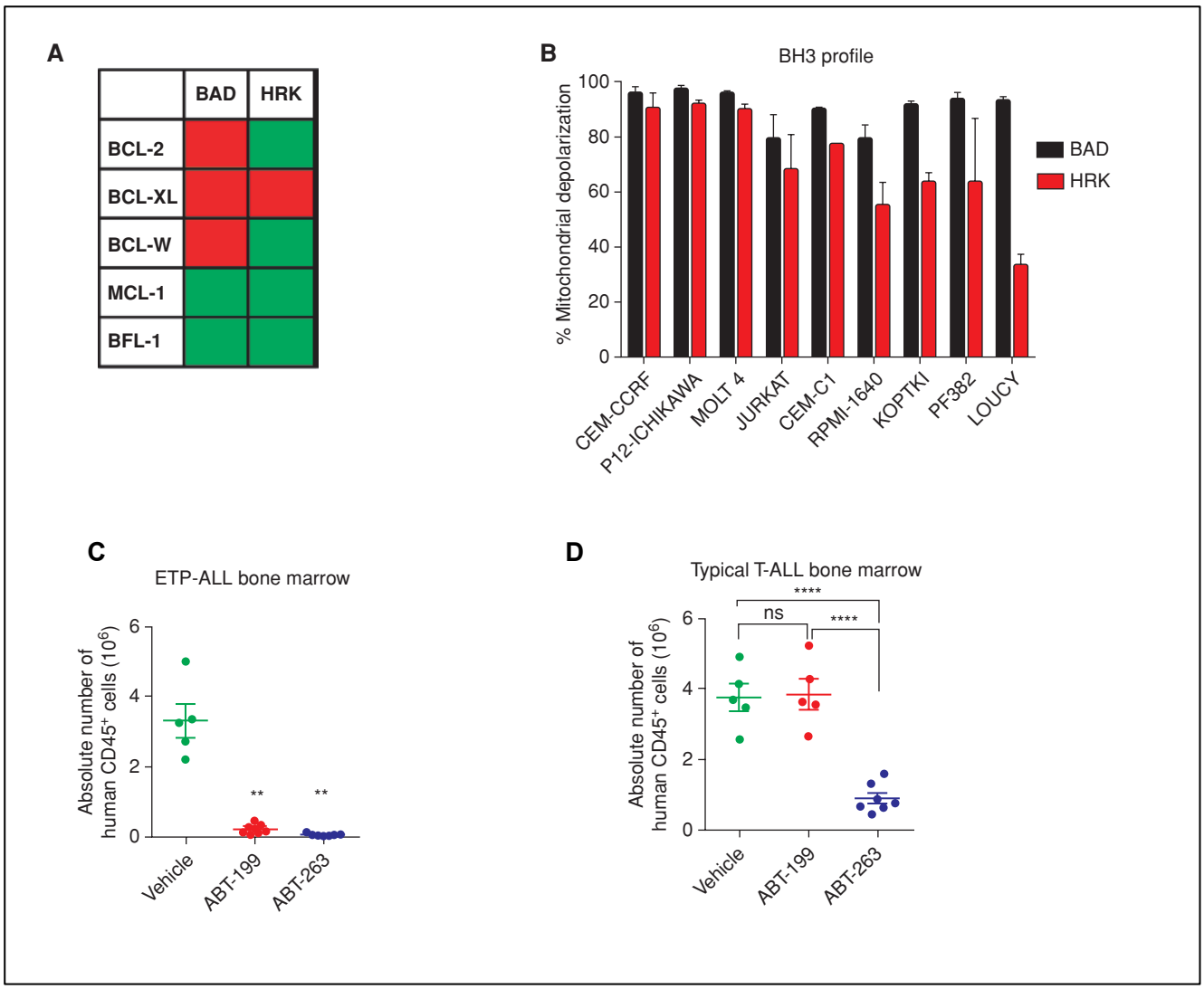

Fig.11: BH3 profiling study and potential therapeutic application. (A) binding affinities of BH3 peptides BAD and HRK for the antiapoptotic BCL-2 family (high affinity in red) tested by fluorescence polarization assay. (B) Expressione of BAD and HRK peptides in T-ALL cell lines. ETP-like cell line LOUCY displays high expression of BAD, confirming BCL-2 dependency in its survival. (C-D) Potential clinical appication of BH3 profiling: ETP-ALL cell lines show higher sensitivity to BH3 mimetics ABT-199 e ABT-263 compared to typic T-ALL cell lines. *Adapted from Chongaile et al.*[41]

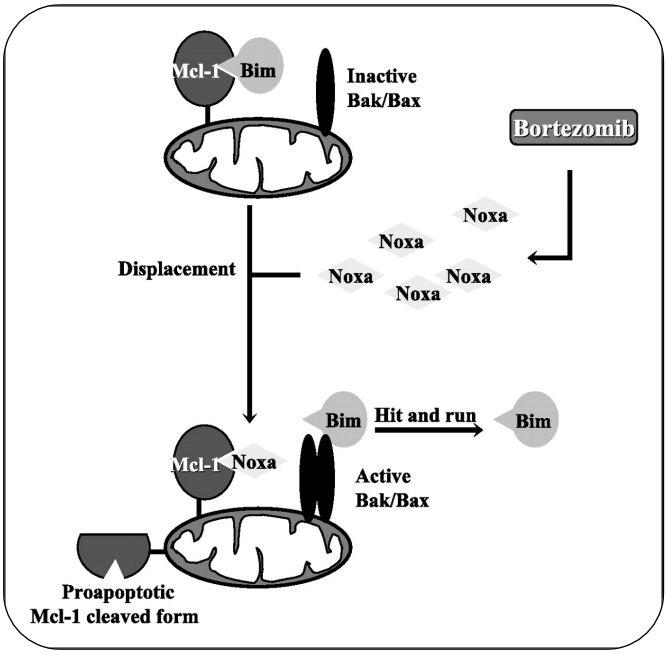

Fig.12: Bortezomib inhibits MCL1 by induction of NOXA, that lead to displacement of Bim and activation of the apoptosis cascade, through Bak/Bax activation[56]. This mechanism of action of Bortezomib represents rationale for combinatorial approach to overcome BCL-2 selective inhibitor resistance due to overexpression of MCL1 in hematologic malignancies[56].

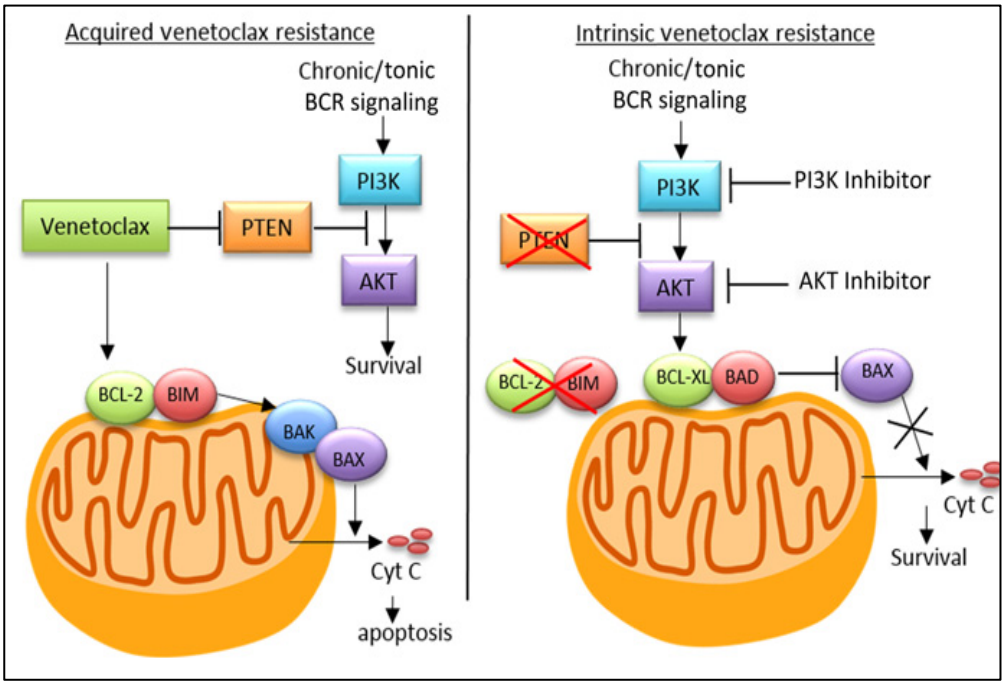

Fig.13: mechanism of intrinsic and acquired Venetoclax resistance in B aggressive lymphoproliferative diseases through tha activation of PI3K-AKT pathway and rationale for use of PI3K inhibitor in association with BCL-2 inhibitor[59]

AML tot. 27 MDS tot. 3	WHO subtype	prior MDS/MPN	Karyotype	mutations	ELN 2017
	MDS EB-2 3	Yes 9	Normal 12	NPM1 1	Fav 2
de novo 7	AML MDS related 15	No 17	Complex >3 7	FLT3 ITD 2	Int 6
R/R 22	AML rec.gen.abn. 3		Complex <3 1	IDH2 R140Q 1	High 11
UNK 1	NOS 7		Trisomy 8 2		Not evaluable 7
	myeloid BP CML 1		Inv(16) 2		*IPSS int-2 3
			t(9;22)(q34;q11) 1		
median age 74			Other 2		
(range 29-83)					

Table 1: Clinical and biological characteristics of AML/MDS patients included in the study (available data)

Patient	Age	Phenotype	Karytotype	FISH	Mutations	Extramedullary
UPR1	26	near-ETP	t(X;3;10) (p11;?;p13)	DDX3X-MLLT10	NOTCH1 ex27, NRAS	mediastinum
UPR2	80	ETP	47,XX,+4	BCL11B translocation	FLT3-ITD	no
UPG3	61	ETP	46, XY	ETV6-CDKN1B	no	spleen liver lymphonodes
UPR3	56	tymic	46, XY, add(7q)	TRB-HOXA	CDKN2A-B, FBXW7, NOTCH1	spleen liver lymphonodes
UPR4	36	tymic	not evaluable	TCRAD-TLX1, del CDKN2A, del BCL11B	no	lymphonodes
UPR5	36	tymic	46, XY, del9(p21), t(14q11;?)	TCRAD-TLX1, del CDKN2A	no	spleen liver lymphonodes

Table 2: Clinical and biological characteristics of T-ALL patients included in the study (available data)

Patient	Age	phenotype	Karyotype	FISH	Mutations	Extramedullary
UPR6	55	MPAL T/myel.	46,XY,t(7;14)(q21;q32)	CDK6-BCL11B	FLT3-ITD	no
UPR7	35	AUL	46,XY,del(14)(q21?),add(10)(p12?)	PICALM-MLLT10	FLT3-ITD	lymphonodes, spleen

Table 3: Clinical and biological characteristics of Acute Leukemia of ambigous lineage patients included in the study (available data)

Disease	Drug	Median IC50	Range IC50	N^ samples
AML/MDS	**TKI**			
	Dasatinib	6,983	0,8874-49,55	5
	Ponatinib	0.2928	0,03673-0,4752	3
	FLT3 inh			
	Quizartinib	13,51	9,62-100	6
	Gilteritinib	10,514	0,3204-19,12	6
	HDAC inh			
	Entinostat	0,364	0,07693-100	7
	BH3 mimetics			
	Venetoclax	0,4705	0,005054-100	34

Table 4: Results of ATP-cells viability assay in AML/MDS samples with median IC50 for every compound tested in the drug profiling

New AML 7	**WHO Subtype**		**Prior MDS/MPN**		**Karyotype**		**NPM1,FLT3, IDH1-2**		**ELN 2017**	
median IC 50 0,3947	RecGenAlt	2	Yes	1(PV)	Normal	3	Neg	7	Fav	1
(range 0,0782-4,24)	MDS rel	2	No	6	Complex	3	Pos	0	Int	2
	NOS	3			Other	1 (inv16)			High	3
									UNK	1
R/R AML-MDS 26	**WHO Subtype**		**Prior MDS/MPN**		**Karyotype**		**NPM,FLT3, IDH 1-2**		**ELN 2017**	
median IC 50 0,5188	EB-2	3	Yes	11	Normal	11	Neg	7	Fav	1
(range 0,005054-100)	RecGenAlt	1	No	15	Complex	7 (>3abn)	Pos	3 FLT3 ITD	Int	5
	MDS rel	13			other	7		1 NPM1/FLT3 ITD	High	11
	NOS	7			ND	(1)		1 IDH2	UNK	9
	other (BP CML)	2					ND	14		
UNK 1										

Table 5: Distinct features of newly diagnosed AML and R/R AML/MDS and sensitivity to BCL2 inhibitor BH3 mimetic Venetoclax (IC50)

Venetoclax pts	**14**		
Sex	M F	8 6	
Age	Median	74	Range 30-83
Subtype	AML de novo sAML MDS	4 8 2	
BM blasts%	median	56	Range 10-90
Karyotype	Normal Complex Other *	9 2 3	* del 20q, +8, t(9;22)
Mutations **	FLT3-ITD IDH2	2 1	**(FLT3 ITD/TKD, NPM1, IDH1-2)
Risk (IPSS/ELN2017)	Int. ELN2017 High ELN2017 Int-2 IPSS	8 4 2	
First Line	**HMA**	**10**	**(AZA 7, DAC 3)**
	Median cycles	13	Range 4-69
Best Response	CR SD	4 6	
TTP (months)	Median	13	Range 4-70
	CHT	**3**	
Best Response	CR NR	1 2	
	Other (TKI)	**1**	
Line of therapy	2^line 3^line >3^line	6 3 5	
PFS from last therapy	Median	12	Range 1-70

Table 6: Clinical features of R/R AML elegible for Venetoclax single agent or in association with HMA

Venetoclax pts	**14**		
Single Agent		2	
With HMA		12	
BM blasts%	median	65	Range 6-90
N^ Cycles	median	6	Range 1-12
	CR	3	
	CRi	2	
	PR	1	
	SD *	3	
	PD	5	
PFS	median	2.5	Range 1-12

Table 7: Results of Venetcolax-based treatment strategy in R/R AML selected on the basis of DSRP approach**.** *One stable disease and two hematological improvement.

T-ALL	TKI	Median IC50	Range IC50	N^ samples
	dasatinib	5,6975	1,868-100	10
	ponatinib	0,34	0,34-100	3
	Venetoclax	0,9777	0,01005-100	12
	non ETP	1,3825	0,1081-100	6
	ETP	0,8729	0,01005-38,52	6
	Bortezomib	0,007269	0,002036-0,01272	10
	non ETP	0,01083	0,005-0,01272	6
	ETP	0,0061655	0,002036-0,007269	4

Table 8: Results of ATP-cells viability assay in T-ALL samples with median IC50 for every compound tested in the drug profiling

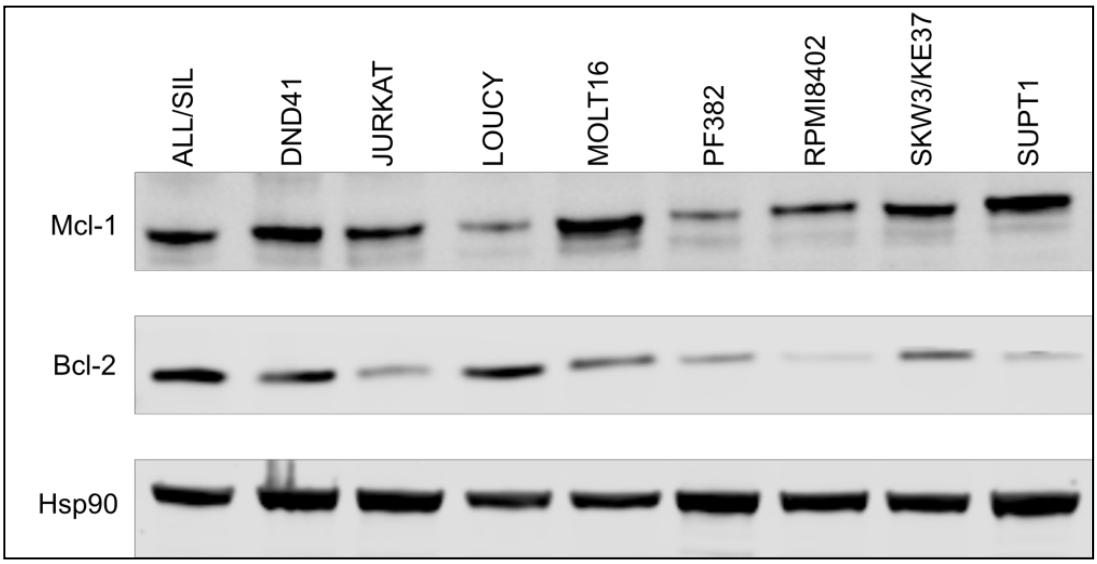

Fig.14: Western Blot analysis on T-ALL cell lines shows higher expression of BCL2 in ETP-like cell lines ALL/SIL and LOUCY compared to typical T-ALL

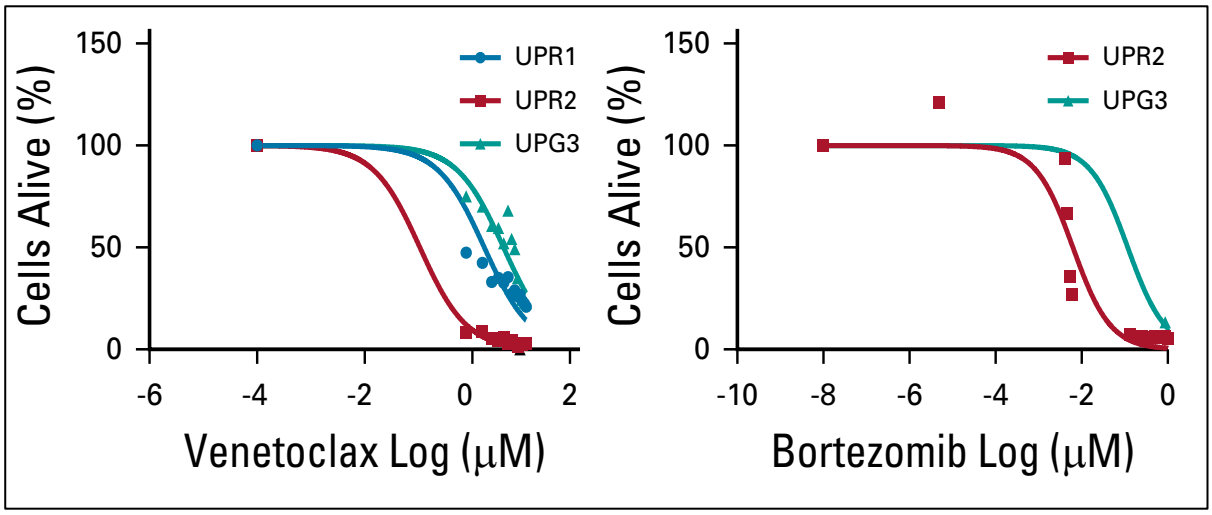

Fig.15: Effect of Venetoclax or Bortezomib on cell viability after 72 hours of treatments in available ETP-ALL cells as assesses by ATP-based luminescence viability assay[64]

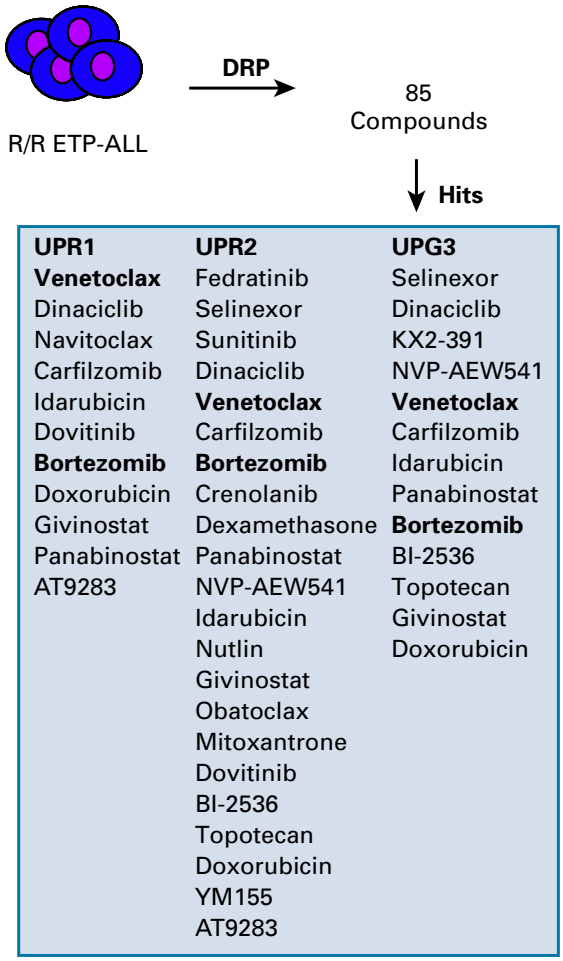

Fig.16: Identification of active compounds in three ETP-ALL patients on a library of 85 small molecules. Dose-response curves were generated as using five-point drug dilutions in duplicate. A list of top hits was generated according to the criteria[64]

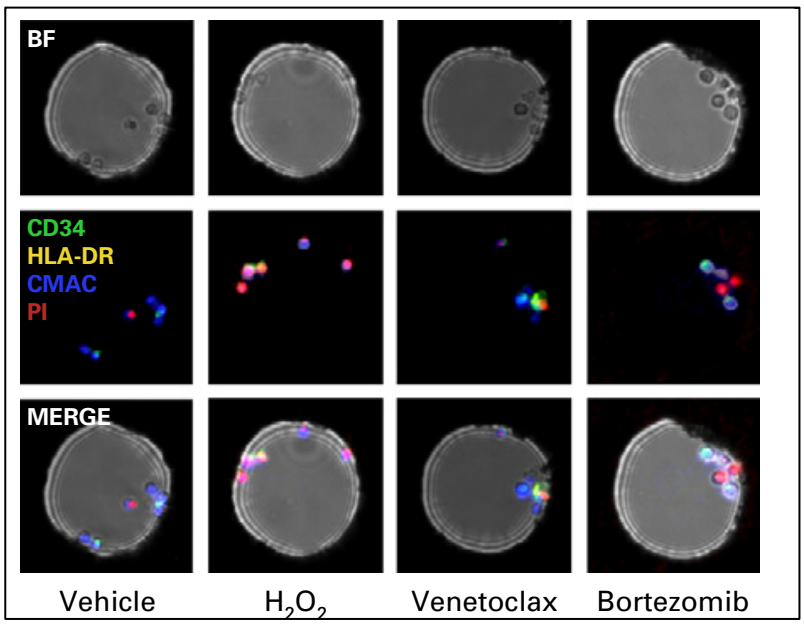

FIg.17: Effect of 24 hours of the vehicle (dimethyl sulfoxide), 10 mM hydrogen peroxide (H_2O_2), venetoclax, and bortezomib on cellular viability as measured by a functional profiling obtained with an automated process developed by CellPly. Details are reported in Results Paragraph.

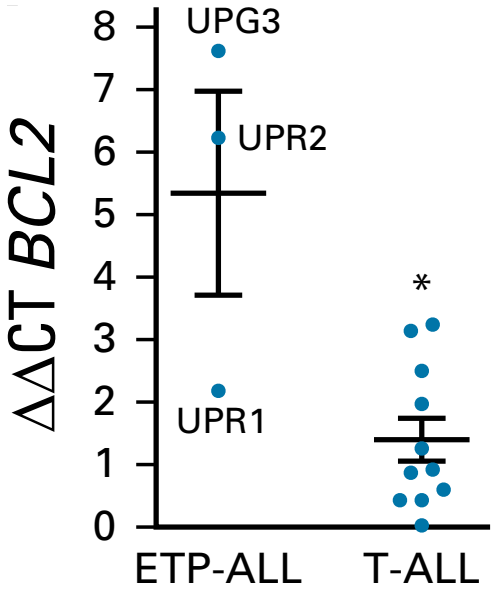

Fig.18: BCL-2 Expression in ETP ALL and non-ETP ALL (11 cases tested at Hematology and BMT Unit University of Perugia) as assessed by quantitative real-time polymerase chain reaction[64]

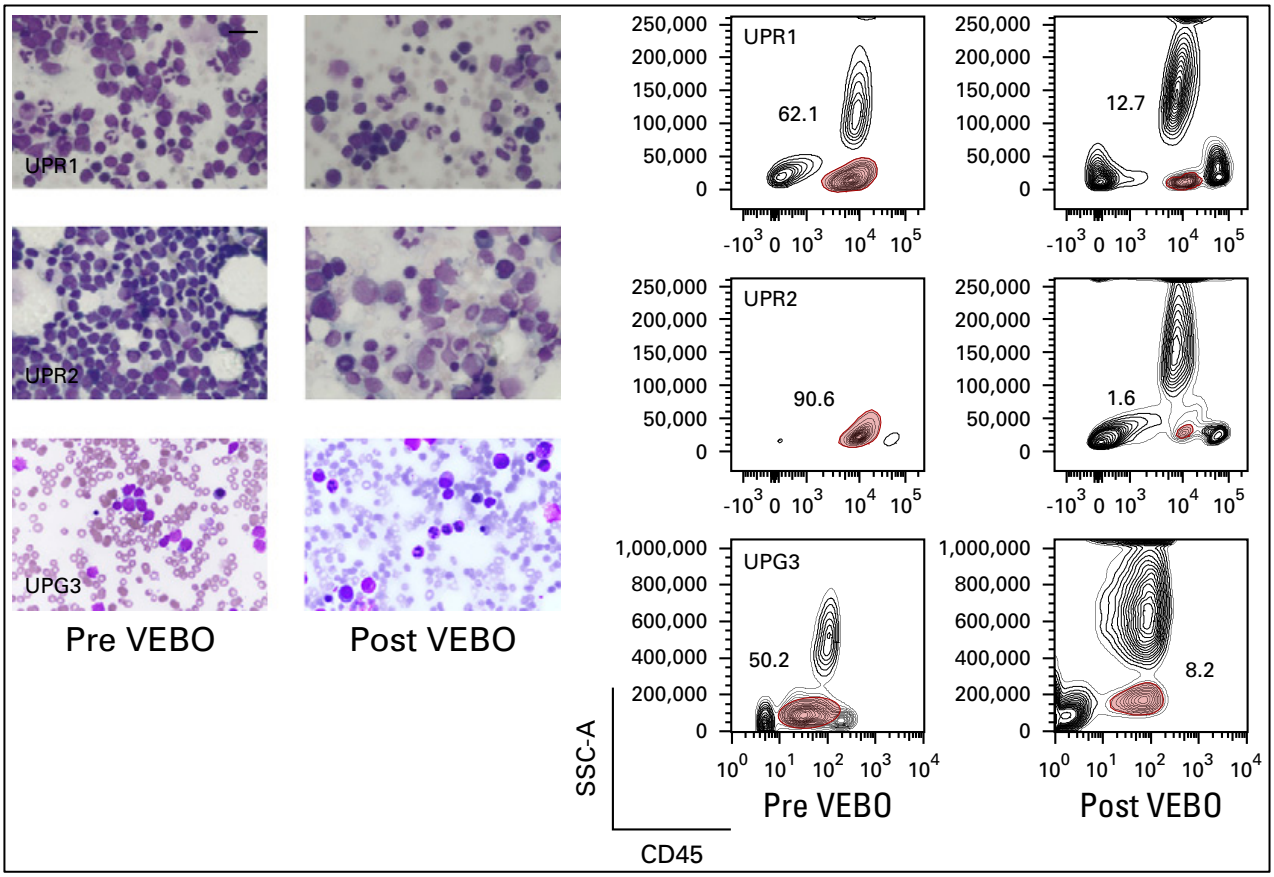

Fig.19: BM cytology and flow cytomety pre-VEBO and after one cycle of treatment (May-Grunwald-Giemsa staining at 63×). BM contains 50%(UPR1), 90%(UPR2) and28% (UPG3) of blasts before treatment, which decreased rapidly after therapy[64]

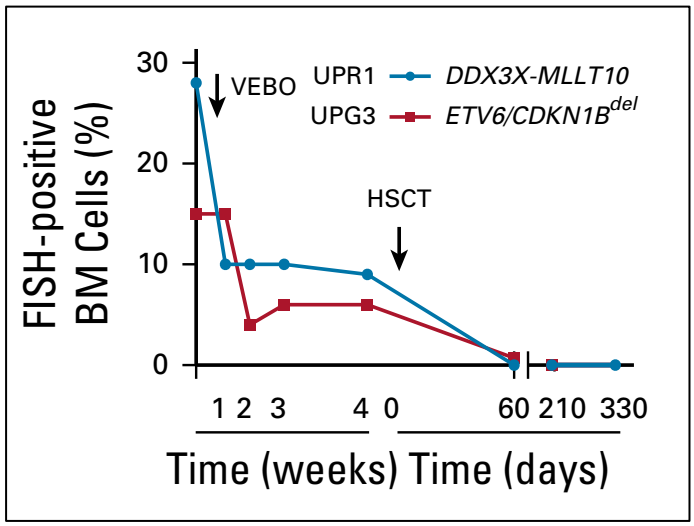

Fig.20: BM misurable residual disease assessed by FISH for UPR1 (DDX3X-MLLT10) and UPG3 (ETV6/CDKN1Bdel) during VEBO treatment and after HSCT. The analysis was performed on 500 to 1,000 interphase nuclei; the cutoff for DDX3X-MLLT10 fusion was 2% and for ETV6/CDKN1Bdel was 3%[64]

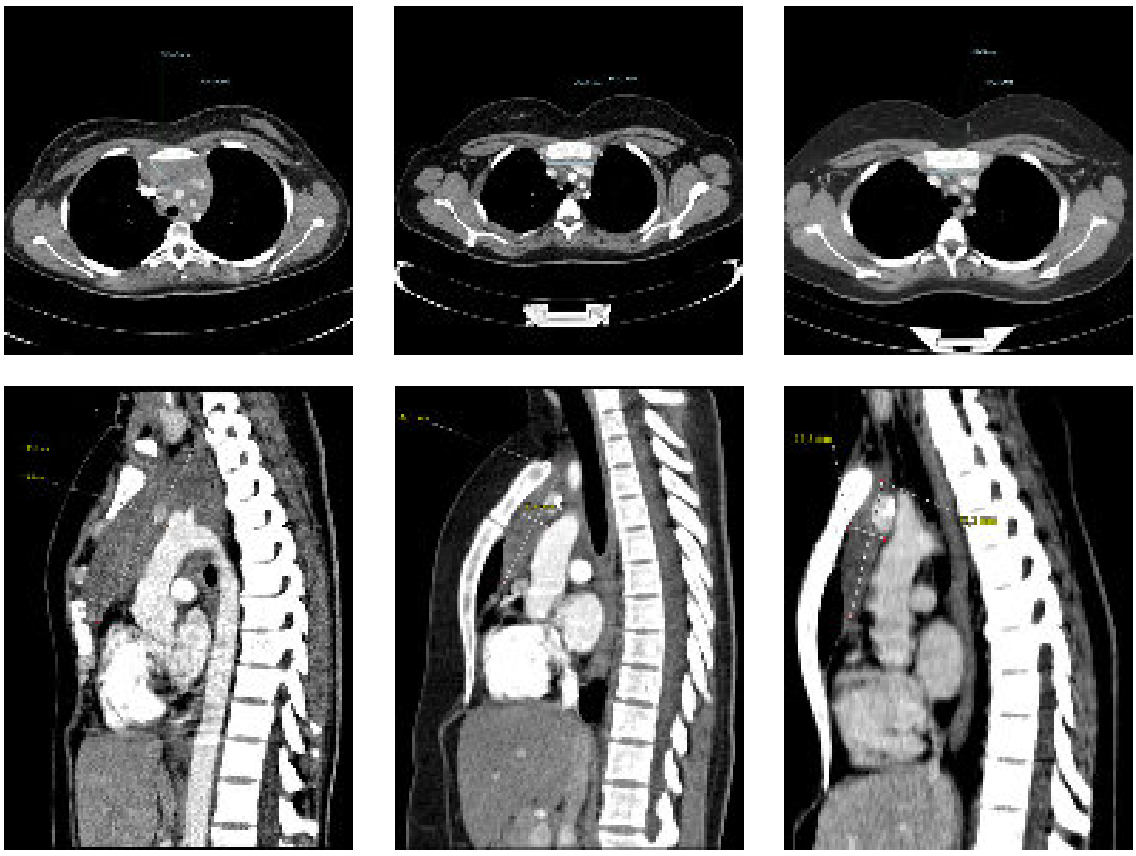

Fig.21: VEBO was an effective strategy also for residual extramedullary involvement in UPR1. The patient had a bulky disease in the mediastinum at the onset that persisted after chemotherapy. VEBO led to a significant reduction of the lymphonodes, as showed in pre-HSCT, post VEBO chest TC evaluation

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