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Doctoral Programme in Clinical Research

INDIVIDUALIZING THERAPY FOR ACUTE LEUKEMIA

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Academic dissertation

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ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

I: Pemovska T*, **Kontro M***, Yadav B, Edgren H, Eldfors S, Szwajda A, Almusa H, Bespalov MB, Ellonen P, Elonen E, Gjertsen BT, Karjalainen R, Kulesskiy E, Lagström S, Lehto A, Lepistö M, Lundán T, Majumder MM, Martí JML, Mattila P, Murumägi A, Mustjoki S, Palva A, Parsons A, Pirttinen T, Rämet ME, Suvela M, Turunen L, Västrik I, Wolf M, Knowles J, Aittokallio T, Heckman CA, Porkka K, Kallioniemi O and Wennerberg K.
Individualized Systems Medicine (ISM) strategy to tailor treatments for patients with chemorefractory acute myeloid leukemia. *Cancer Discovery* 2013; 3(12):1416-29.

II: **Kontro M***, Kuusanmäki H*, Eldfors S, Burmeister T, Andersson EI, Bruserud Ø, Brümmendorf TH, Edgren H, Gjertsen BT, Itälä-Remes M, Lagström S, Lohi O, Lundán T, Martí JML, Majumder MM, Parsons A, Pemovska T, Rajala H, Vettenranta K, Kallioniemi O, Mustjoki S, Porkka K and Heckman CA.
Novel activating *STAT5B* mutations as putative drivers of T-cell acute lymphoblastic leukemia. *Leukemia* 2014; 28**:**1738–1742.

III: **Kontro M**, Kumar A, Majumder MM, Eldfors S, Parsons A, Pemovska T, Saarela J, Yadav B, Malani D, Fløisand Y, Höglund M, Remes K, Gjertsen BT, Kallioniemi O, Wennerberg K, Heckman CA and Porkka K.
HOX gene expression predicts response to BCL-2 inhibition in acute myeloid leukemia. *Leukemia.* 2017; 31:301-309

IV: Vesterinen T, **Kontro M**, Aranko K, Gesterberg A, Heckman CA, Koskenvesa P, Miettinen T, Mustjoki S, Pitkänen K, Saarela J, Strömberg A, Tiitinen S, Wahlfors T and Porkka K.
Finnish Hematology Register and Biobank (FHRB) – a new resource for hematological research. Submitted

*The authors contributed equally to this work.

ABBREVIATIONS

2-HG	Two hydroxyglutarate
A	Adenine/alanine
ABL	Abelson murine leukemia viral oncogene homolog
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APL	Acute promyelocytic leukemia
ASXL1	Additional sex combs like transcriptional regulator 1
ATRA	All-trans retinoic acid
AUC	Area under the curve
BAD	BCL2 associated agonist of cell death
BAX	BCL2 associated X
BCL-2	B-cell lymphoma-2
BCL-XL	B-cell lymphoma-extra large
BCR	Breakpoint cluster region
BH-3	Bcl-2 homology domain 3
BM	Bone marrow
C	Cytosine/cysteine
CBF	Core binding factor
CCLE	Cancer Cell Line Encyclopedia
CD	Cluster of differentiation
CEBPα	CCAAT/enhancer binding protein alpha
CGP	Cancer Genome Project
CHIP	Clonal hematopoiesis of indeterminate significance
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
COSMIC	Catalogue of somatic mutations in cancer
CR	Complete remission
CRi	Complete remission with incomplete blood recovery
CSF1R	Colony-stimulating factor 1 receptor
D	Aspartic acid
del	Deletion
DNA	Deoxyribonucleic acid
DNMT3A	DNA methyltransferase 3A
DMSO	Dimethylsulfoxide
DSRT	Drug sensitivity and resistance testing
DSS	Drug sensitivity score
E	Glutamic acid
EFS	Event-free survival
ELN	European Leukemia Net
EMA	European Medicines Agency
FAB	French-American-British
FAH	The Finnish Hematology Association
FDA	Food and Drug Administration
FHRB	The Finnish Hematology Registry and Biobank
FIMM	Institute for Molecular Medicine Finland
FLT3	Fms-like tyrosine kinase 3
FRCBS	Finnish Red Cross Blood Service
FBS	Fetal bovine serum
G	Guanine/glycine
H	Histidine/histone
HOX	Homeobox
HSCT	Hematopoietic stem cell transplantion
HSPC	hematopoietic stem/progenitor cells
HSP90	Heat shock protein 90
IC50	Half-maximal inhibitory concentration
IDH	Isocitrate dehydrogenase
ISM	Individualized systems medicine
ITD	Internal tandem duplication
JAK	Janus-activated kinase
K	Lysine
KIT	KIT proto-oncogene receptor tyrosine kinase
KMT2A	Lysine methyltransferase 2A
L	Leucine
LOH	Loss of heterozygosity
LSC	Leukemic stem cell
LDAC	Low-dose cytarabine
NGS	Next generation sequencing
NRAS	Neuroblastoma RAS viral oncogene homolog
NPM1	Nucleophosmin gene 1
M	Methionine
MAPK	Mitogen-activated protein kinase
MCL-1	BCL2 family apoptosis regulator (myeloid cell leukemia sequence 1)
MCM	Mononuclear Cell Media
MED12	Mediator complex subunit 12
MDS	Myelodysplastic syndrome
MLL	Mixed lineage leukemia (currently KMT2A)
MNC	Mononuclear cell
N	Asparagine
NOTCH1	Notch (Drosophila) homolog 1, translocation-associated
mTOR	Mechanistic target of rapamycin
NCI	National Cancer Institute
NSD1	Nuclear Receptor Binding SET Domain Protein 1
NUP98	Nucleoporin 98
OS	Overall survival
p	Short arm of a chromosome
P	Proline
PB	Peripheral blood
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PI3K	Phosphatidylinositide 3-kinase
PIM	Pim-1 Proto-Oncogene, Serine/Threonine Kinase
PLK	Polo-like kinase
q	Long arm of a chromosome
Q	Glutamine
R	Arginine
RIN	RNA integrity number
RNA	Ribonucleic acid
RUNX1	Runt-related transcription factor
RQ-PCR	Reverse transcriptase-polymerase chain reaction
S	Serine
SD	Standard deviation
SH2	SRC homology 2
STAT	Signal transducer and activator of transcription
T	Thymine/threonine
T-ALL	T-cell acute lymphoblastic leukemia
TA	Total area
TCGA	The Cancer Genome Atlas
TKI	Tyrosine kinase inhibitor
TKD	Tyrosine kinase domain
TMM	Trimmed mean of M-values
TP53	Tumor protein P53
Y	Tyrosine
V	Valine
VAF	Variant allele frequency
VEGFR	Vascular endothelial growth factor receptor
W	Tryptophan
WT	Wild type
WHO	The World Health Organization

Gene names and symbols are italicized in the text.

ABSTRACT

Therapeutic success achieved for chronic myeloid leukemia (CML), a disease driven by the *BCR-ABL1* fusion gene and targeted by specific inhibitors of the ABL1 kinase, has encouraged the cancer field to seek analogous effective targeted therapies. Disappointingly, direct translation of cancer-specific genomic information into effective personalized-therapeutic strategies for acute leukemias and other more complex malignancies has proved difficult. Thus, with only a few exceptions for successful targeted therapies, current therapies for acute leukemia remain similar for all patients.

This study aims to utilize novel tools for the detailed characterization of genomic, transcriptomic, and functional aberrations in acute leukemia to gain an understanding of disease pathology and guide individualized therapies. The main methods used were *ex vivo* drug sensitivity and resistance testing (DSRT), whole exome sequencing, and transcriptome (RNA) sequencing, all of which were facilitated by extensive biobanking.

In study I, we developed a cancer pharmacopeia-wide DRST platform performed *ex vivo* on primary leukemic cells for the rapid identification of effective patient-specific drugs. We quantified leukemia-selective responses to a comprehensive library of clinical and investigational drugs in 28 acute myeloid leukemia (AML) patient samples and five healthy control samples. Exome and transcriptome sequencing of serial samples from individual patients were used for the molecular characterization and to understand the mechanisms of acquired resistance. Although AML samples exhibited unique DSRT profiles, responses could be clustered into five distinctive groups. AML-selective responses were seen for several approved agents such as dasatinib (33% of patient samples), temsirolimus (29%), trametinib (21%), and sunitinib (21%). Individualized treatment of refractory patients with DSRT-guided therapy resulted in responses in three of six patients (one complete remission with incomplete blood recovery, two morphological leukemia-free states).

In study II, we studied molecular drivers for relapsed T-cell acute lymphoblastic leukemia (T-ALL) and found three different mutations in the SH2 domain of *STAT5B*. DSRT was performed on primary blasts from the index patient to assess drug sensitivity. Functional studies with transiently transfected HeLa cells demonstrated that the N642H mutation induced the constitutive phosphorylation of STAT5B and led to an enhanced transcriptional activity. *STAT5B* mutated primary blasts showed elevated BCL-XL expression and were sensitive to the BCL-2/BCL-XL inhibitor navitoclax. No sensitivity to the inhibitors of the upstream targets of the IL7r-JAK-STAT pathway was observed. Targeted sequencing of 67 additional T-ALL samples revealed activating *STAT5B* mutations in 6 of 68 patients.

In study III, we aimed to identify robust biomarkers for sensitivity to novel apoptosis modulators. We first evaluated the *ex vivo* BCL-2 inhibitor sensitivity of fresh leukemic cells from 28 newly diagnosed and 45 relapsed/refractory AML patient samples. We then systematically assessed whether these responses correlated with specific mutations or gene-expression signatures determined by exome and RNA sequencing. AML samples exhibited variable responses to BCL-2 inhibition: 32% of samples were resistant, whereas highly leukemia-selective responses were observed in 15% of the AML samples. BCL-2 inhibitor sensitivity was associated with mutations in *IDH1/IDH2* and *WT1,* as well as with aberrations in chromatin modifiers. Importantly, the overexpression of a specific set of *HOX* genes predicted highly selective responses to BCL-2 inhibition.

In study IV, we developed a national hematological biobank to allow researchers to access high-quality samples with accompanying clinical annotation data, thus also enabling the research community in general to deepen its understanding of the pathophysiology of hematological malignancies and advance diagnostics and treatment. The biobank samples are board-informed and consent-compliant as per the Finnish Biobank Act, and samples from three collection time-points—diagnosis, potential remission, and relapse—are available. For this study, we also evaluated the quality of stored samples and demonstrated that extracted DNA and RNA remain usable for high-quality, demanding down-stream experiments.

Taken together, we evaluated and developed novel techniques for individualizing therapy for acute leukemia patients. First, we demonstrated that combining data from different platforms enables the repurposing of targeted therapies for both individual AML patients as well as for distinct disease subgroups. These data also allow to study of each individual disease and possible mechanisms of sensitivity and resistance in a comprehensive manner. Second, we used these methods to study the prevalence of *STAT5B* mutations in T-ALL. We validated that these mutations were recurrent events and demonstrated mutations to be activating. We presented a hypothesis of possible vulnerability of *STAT5B* mutated blasts for BCL-XL inhibition. Third, we showed that extensive data from various platforms enable the isolation and discovery of biomarkers to inform individualized therapy. We found that a specific *HOX* gene-expression pattern serves as a robust biomarker for venetoclax sensitivity *ex vivo*. Our results identified a BCL2 inhibitor-sensitive AML subgroup for validation in upcoming clinical trials. Finally, we developed a nation-wide biobank containing high-quality sample material accompanied with annotated clinical data enabling research for individualizing therapy for acute leukemia.

INTRODUCTION

Detailed knowledge of the molecular pathobiology behind hematological malignancies has revolutionized cancer research and resulted in new, effective, targeted therapies in few hematological disorders. This progress is best illustrated in chronic myeloid leukemia (CML), a disease defined by the fusion of the BCR and ABL1 genes through a translocation of chromosomes 9 and 22 (the Philadelphia chromosome). Treatment with drugs that inhibit BCR-ABL1 proved that understanding the mutations driving the cancer can lead to effective targeted nontoxic therapies.[1-3]

Encouraged by these results, the general cancer field hoped to witness similar advances in treatment of other malignancies. Unfortunately, nearly all other hematologic malignancies unraveled to be significantly more complex than CML, with various driving genetic lesions interacting together to form complicated puzzles of networks. While we are beginning to understand the detailed biology and drivers of individual cancers, major obstacles have prevented the implementation of such advances through current therapies. For example, the therapy for most acute myeloid leukemia (AML) patients has remained relatively unchanged for four decades, despite intensive trial activity exploring new agents and an impressive number of approved anti-cancer drugs in general. This lack of progress is due to both inter-patient and intra-tumor heterogeneity. That is, AML is not an overarching disease, but a cluster of innumerous, dynamically evolving disease entities. Thus, paradoxically, the dynamic complexity of individual cancer remains the major hindrance to individualizing therapies.

The aim of this study was to utilize novel tools for the detailed characterization of genomic, transcriptomic, and functional aberrations in leukemia to gain a deeper understanding of its pathogenesis and guide individualized therapies. The main methods used were *ex vivo* drug sensitivity and resistance testing (DSRT), whole exome sequencing, and transcriptome (RNA) sequencing, all of which were aided by extensive biobanking.

LITERATURE REVIEW

1. ACUTE MYELOID LEUKEMIA (AML)

1.1. Background

Acute myeloid leukemia (AML) is a hematological malignancy characterized by infiltration of the bone marrow by clonal, proliferative, abnormally or poorly differentiated cells of the hematopoietic system.[4,5] In addition to hindering the normal functions of hematopoiesis, these cells can escape to the blood and may have the ability to infiltrate other tissues such as the lungs, central nervous system, lymph nodes or the spleen.[6]

The current World Health Organization (WHO) classification recognizes disease entities by focusing on significant cytogenetic and molecular genetic events characterized by specific drivers of disease progression.[7] According to the minimum WHO criteria, 20% of myeloid lineage blasts must be detected either in bone marrow (BM) or blood to meet the criteria for AML diagnosis.[7] The incidence of AML has increased, whereby AML now represents the most common diagnosed leukemia among adults, with nearly 20,000 new cases diagnosed annually in the US (2015)[8] and 224 in Finland (2014, Finnish Cancer Registry)[9]. The leading reasons for this increase are the ageing of the population together with increased incidence of therapy related AML following therapy of another cancer (e.g. breast cancer and non Hodgkin lymphomas).[5,10,11]

1.2. Molecular and clonal heterogeneity of AML

The cytogenetic heterogeneity of AML has been recognized for more than 30 years.[5] Recurrent chromosomal structural variations are well recognized both as diagnostic and prognostic markers, suggesting that acquired genetic abnormalities have an essential role in AML pathogenesis.[12,13] However, as nearly 50% of AML cases have a normal karyotype, and our understanding of molecular heterogeneity has lagged behind that of CML, AML treatment protocols have remained largely unchanged for more than 30 years.[14]

The understanding of molecular heterogeneity originated in the recognition of recurrent hotspot mutations using targeted sequencing in *FLT3, NPM1, KIT, CEBPA,* and *TET2.*[15-17] Nevertheless, the massive parallel sequencing truly enabled us to gain understanding of AML's enormous molecular heterogeneity. This era began in 2008, when the Washington University group sequenced the first whole AML genome, which was, in fact, the first cancer genome sequenced.[18]

1.2.1 The origin of leukemia

Welch and co-workers demostrated that normal self-renewing human hematopoietic stem/progenitor cells (HSPCs) accumulate random somatic variants as a function of age. The number of variants is lowest in cord-blood samples, and they increase as the donor ages. For example, at the age of 40, a single HSPC has gained, on average, from four to seven somatic variants, whereas cord-blood samples have none or only one[19]. These pre-excising (or "background" mutations) are generally considered to be irrelevant for AML pathogenesis and are probably benign. Yet, they are consequently present in all AML cells, further reflecting the stability of the AML genome.[19]

Subsequently, several other investigators, including Majeti and Shlush and their groups, unraveled the nature of leukemia-initiating events in AML pathogenesis. These researchers demonstrated that highly purified HSPCs contain recurrent early mutations, typically in epigenetic regulators (e.g., *DNMT3A*, *TET2, IDH 1/2*) at high variant allele frequency (VAF) (**Figure 1**).[20-22] Importantly, these cell populations lacked the concurrent late mutations present in AML blasts and were also detected in mature blood cell fractions in AML patients.[20] Early *DNMT3A* mutated HSPCs demonstrated a multilineage repopulation advantage over non-mutated HSPCs.[20] Researchers assumed that these early pre-leukemic cells resist chemotherapy and can expand during remission.[5,20,22] The effect of persistence of pre-leukemic clones in AML prognosis remains controversial, although a few reports have shown that *DNMT3A* clone persistence does not adversely affect outcomes.[23,24] Thus, the existence and possible survival of the pre-leukemic clones does not currently entail clinical consequences.

Leukemia-originating cells are thought to arise from healthy hematopoietic stem and progenitor cells. Gene-expression studies on healthy human hematopoiesis demonstrate that *HOX* gene expression is largely restricted to hematopoietic stem and progenitor cells[25,26] and, in AML, *HOX* expression appears to be regulated. In fact, the similarity between normal and leukemic expression patterns suggests that most *HOX* expression in AML reflects a normal stem cell state that is "captured in the transformed cells."[27]

The occurrence of early mutations does not inevitably lead to the development of leukemia. Several recent studies showed that somatic mutations in epigenetic modifiers can also be detected in healthy individuals' blood samples.[28,29] This phenomenon, termed the clonal hematopoiesis of indeterminate significance (CHIP), increases as individuals age and is detected in approximately 10% of individuals aged 70 to 79.[28] Furthermore, CHIP is associated with an increased risk of hematologic cancer and cardiovascular mortality, although that risk is relatively low. Currently, these findings have not yet affected on treatment or diagnostics. [5,28,30]

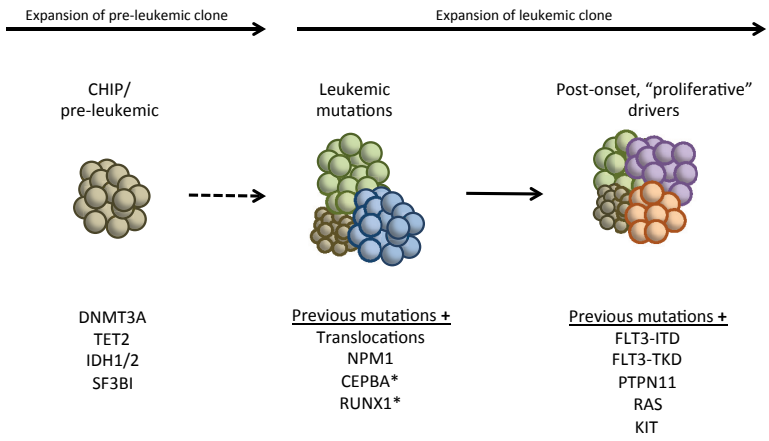


Figure 1. Clonal evolution leading to AML.

CHIP/pre-leukemic phase: Mutations in genes that are commonly involved in epigenetic regulation (e.g., *DNMT3A*, *ASXL1*, *IDH2* and *TET2*) are present in pre-leukemic hematopoietic stem cells. These ancestral, pre-leukemic stem cells are capable of multilineage differentiation and hematopoiesis.[19-21, 31]

Leukemic phase: Upon diagnosis, pre-leukemic cells will have gained additional aberrations, and AML is characterized by clonal heterogeneity with the presence of both a founding clone and at least one subclone.[19, 31, 32]

"Later" leukemic phase: Post-onset drivers are not required for AML but, when present, may have a significant effect in AML pathogenesis and phenotype. These mutations typically affect signaling pathways (e.g. MEK-ERK, AKT-mTOR) .[22, 31]

*If not in the germ-line.

1.2.2. The genomic landscape and complexity of AML

To decode the genomic heterogeneity of AML, in 2013 The Cancer Genome Atlas (TCGA) Research Network analyzed 200 clinically annotated adult cases of *de novo* AML, by means of either whole-genome sequencing (50 cases) or whole-exome sequencing (150 cases), along with DNA methylation, and RNA and microRNA sequencing.[33] Compared to most solid tumors, AML turned out to have significantly fewer mutations. On average, the AML genome had 13 nonsynonymous somatic mutations; importantly, most of these were passenger mutations and, on average, only five mutations occurred in genes that are recurrently mutated in AML. A total of 23 genes were significantly (with a prevalence higher than expected) mutated, while additional 237 were mutated in 2 or more samples.[33] The genes that were significantly mutated were further functionally divided into nine categories (**Table 1**).

Functional group	Prevalence in TCGA data	Function	Examples/prevalence in TCGA data (if available)
Transcription factor fusions	18%	Transcriptional deregulation and impaired hematopoietic differentiation	t(15;17);*PML-RARA* (9%) inv (16);*MYH11-CBFB* (6%) t(8;21);*RUNX1-RUNX1T1* (4%)
Myeloid transcription factors	22%	See above	*CEBPA* (6%) *RUNX1* (10%)
Tumor suppressor genes	16%	Transcriptional deregulation and impaired degradation through MDM2 and PTEN	*TP53* (8%)
DNA-methylation-related genes	44%	*DNMT3A, TET2* and *IDH1/2* mutations, (through the 2-hydroxyglutarate) leading to the deregulation of DNA methylation	*DNMT3A* (26%) *TET2* (8%) *IHD1/2* (20%)
Chromatin-modifying genes	30%	*ASXL1* and *EZH2*: deregulation of chromatin modification (e.g., methylation of histones on lysine residues K79, K27, and K119, respectively). *KMT2A–MLLT3* fusion: impair function of other methyltransferases such as DOT1-like histone H3K79 methyltransferase.	*ASXL1* (3%) *EZH2* (2%) *TET2* (9%) *KMT2A–MLLT3* (9%)
Activated signaling	59%	Proliferative advantage through different signaling pathways (e.g. RAS–RAF, JAK–STAT, and PI3K–AKT)	*FLT3-ITD, TKD* (20%) *NRAS/KRAS* (12%) *KIT* (4%)
Nucleophosmin	27%	Aberrant cytoplasmic localization of NPM1 and NPM1-interacting proteins	*NPM1 (27%)*
Spliceosome-complex genes	14%	Deregulated RNA processing	*SRSF2* *SF3B1*
Cohesin-complex genes	13%	Impair precise chromosome segregation and transcriptional regulation	*STAG2* *RAD21*

Table 1. The nine functional categories of commonly mutated genes in AML.

Modified from Döhner, Weisdorf and Bloomfield: Acute myeloid leukemia. *N Engl J Med* 2015[5] and The Cancer Genome Atlas Research Network: Genomic and epigenomic landscapes of adult *de novo* acute myeloid leukemia. *N Engl J Med* 2013.[33]

Note: The current gene symbol for *MLL, KMT2A* (lysine [K]-specific methyltransferase 2A) has been applied.

To understand how this genetic diversity defines the pathophysiology of AML and its possible clinical implications, a recent study evaluated somatic-driver mutations retrospectively among more than 1500 AML patients.[31] Using the patterns of co-mutation, the cohort was divided into 11 classes, each with distinct diagnostic

features and clinical outcomes. In addition, researchers identified further information on the cooperation of key mutations exemplified by the interaction of three common mutations: *NPM1*, *DNMT3A*, and *FLT3* internal-tandem duplication (ITD). This combined genotype was the most frequent three-gene co-occurrence, identified in 6% of subjects. The adverse effect of *FLT3-ITD* was most clinically relevant in patients with concomitant *NPM1* and *DNMT3A* mutations. When present with either *NPM1* or *DNMT3A*—or with neither of these other genes—the survival effect of *FLT3-ITD* was less evident.[31] These findings illustrate the complexity of gene-to-gene interactions affecting both disease biology and prognosis, and they underscore the necessity of further studies to establish novel, specific prognostic classifications. In addition, the RNA sequencing data from TCGA's study illustrated the co-operation of specific mutations. Despite the substantial molecular heterogeneity described above, an unsupervised cluster analysis of mRNA sequencing data clustered samples to only seven groups. To some extent, differences among the groups also correlated with different outcomes.[33]

1.3. Risk classification of AML

Recently, the European LeukemiaNet (ELN) updated the risk classification of AML (**Table 2**).[14,34] Disease risk is assessed based on the leukemic blast karyotype and six specific molecular markers. Previously, only three of these (*NPM1*, *FLT3-ITD*, and biallelic *CEBPA*) were used in clinical practice to classify the disease risk. The newly added markers (*TP53*, *ASXL1,* and *RUNX1*) are all associated with worse outcomes.[34] The new classification also takes into account the allelic burden of *FLT3-ITD*. Although the prognostic significance of *DNMT3A* remains controversial, it is likely dependent on the co-existence of other mutations, and this is still excluded.[31, 34-36] *IDH2* R172 mutations may present a favorable outcome[31], but additional studies are needed before including this in risk classification.

Risk Category	Genetic Abnormality
Favorable	t(8;21)(q22;q22); *RUNX1-RUNX1T1* inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11* Mutated *NPM1* without *FLT3-ITD* or with *FLT3-ITD* (VAF <0.5) Biallelic mutated *CEBPA*
Intermediate	Mutated *NPM1* and *FLT3-ITD* (VAF ≥0.5) Wild-type *NPM1* without *FLT3-ITD* or with *FLT3-ITD* (VAF <0.5) (without adverse risk genetic lesions) t(9;11)(p22;q23); *MLLT3-KMT2A* Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(9;22)(q34.1;q11.2); *BCR-ABL1* inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *GATA2-MECOM* (*EVI1*) t(6;9)(p23;q34); *DEK-NUP214* t(v;11)(v;q23); *KMT2A* rearranged −5 or del(5q); −7; abn (17p) Complex karyotype*, monosomal karyotype** Wild type *NPM1* and *FLT3-ITD* (VAF ≥0.5) Mutated *RUX1* (if not associated with favorable risk subtype) Mutated *ASXL1* (if not associated with favorable risk subtype) Mutated *TP53*

Table 2. Current stratification of molecular genetic and cytogenetic alterations in AML.

Modified from ELN recommendations (Döhner et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel[34]).

*Complex karyotype is defined as three or more chromosomal aberrations in the absence of one of the designated recurring translocations or inversions, i.e., t(8;21), inv(16) or t(16;16), t(9;11), t(v;11)(v;q23.3), t(6;9), inv(3) or t(3;3); AML with BCR-ABL1.

** Defined by the presence of one single monosomy (excluding loss of X or Y) in association with at least one additional monosomy or structural chromosome abnormality (excluding core-binding factor AML)

Abbreviations: abn: abnormal; del: deletion; inv: inversion

2. AML THERAPY – TOWARDS TARGETED THERAPY

2.1. Current AML therapy

As pointed out by Prasad and Gale, "Leukemias were among the first cancers cured by hematologists, but we have reached a plateau in AML."[37] Although the AML prognosis has improved over the recent decades, currently only 35% to 40% of adult patients 60 years of age or younger and 5% to 15% of patients older than 60 are cured.[5] Therapeutic results for AML remain particularly dismal for older patients unfit for intensive therapies and for relapsed or refractory patients. Very few of these patients survive beyond two years.[38] Chemotherapy, which has remained largely unchanged over four decades,[39] is stagnantly based on a combination of cytarabine and anthracycline (most commonly idarubicin or daunorubicin). Among fit patients, the standard combination is 7+3, with a seven-day continuous infusion

of cytarabine at a 100–200 mg/m2 dose per day on days one through seven and daunorubicin at 60–90 mg/m2 per day or idarubicin 8–12 mg/m2 per day for three days.[38] The optimal combination therapy with cytarabine has been explored in several trials[40, 41] comparing daunorubicin to idarubicin, but all have failed to demonstrate the superiority of either. Standard strategies after achieving remission include conventional intermediate to high-dose cytarabine-based chemotherapy as well as hematopoietic stem cell transplantation (HSCT).[5,38] Patients with comparatively low risk of relapse should not receive HSCT in first remission, whereas HSCT offers survival benefit for patients with intermediate or high risk.[5,38] The improvement in AML survival (apart from acute promyelocytic leukemia (APL)) can be primarily attributed to the development of supportive care (e.g., new antibiotic and antimycotic drugs, fractionated and safer blood products, anti-emetics, etc.), facilitating survival by mitigating periods of severe pancytopenia caused by high-dose cytotoxic therapy.[39,42]

By contrast, almost no advances have been made for patients unfit for standard intensive treatment, specifically patients with co-morbidities or elderly patients.[38] A recent randomized phase III trial evaluated treatment options for newly diagnosed elderly AML patients (>65 years). Hypomethylating agent azacitidine was compared to conventional care regimens (intensive chemotherapy, low-dose cytarabine (LDAC), or supportive care only) in 488 patients.[43] Patients were randomized to receive either a preselected (conventional) therapy or azacitidine. Median overall survival (OS) increased to 10.4 months with azacitidine, whereas OS reached 6.5 months under conventional care; however, the difference was not statistically significant. Notably, patients preselected to receive intensive chemotherapy and randomized to receive azacitidine therapy had OS similar to patients receiving conventional chemotherapy (13.3 and 12.2 months, respectively).[43] Interestingly, a recent study demonstrated responses to a hypomethylating agent decitabine to be more prevalent among patients with unfavorable-risk cytogenetic profiles, including *TP53* mutation.[44]

Because treatment outcomes among older AML patients remain dismal, additional research is necessary. Hypometylating agents may offer a feasible basis for studies with patients ineligible for conventional chemotherapy.

2.2. The effect of therapy to clonal composition

Several studies have shown that at diagnosis, AML is characterized by clonal heterogeneity, with the occurrence of a founding clone and at least one subclone derived from the founding clone.[32,33,45] At least two major clonal evolution patterns may lead to relapse after initial chemotherapy: (1) the founding clone in the primary AML gains additional mutations, or (2) a subclone that survived the initial therapy gains new mutations and expands to a relapse major clone (**Figure 2**).[32] Additionally, the leukemic clones may arise directly from an ancestral clone; in such cases, additional new mutations may result in a genetically dissimilar leukemic relapse.[21] At the time of relapse, AML genomes also typically acquire transversions, probably caused by cytotoxic chemotherapy in analogous to treatment-related AML.[32]

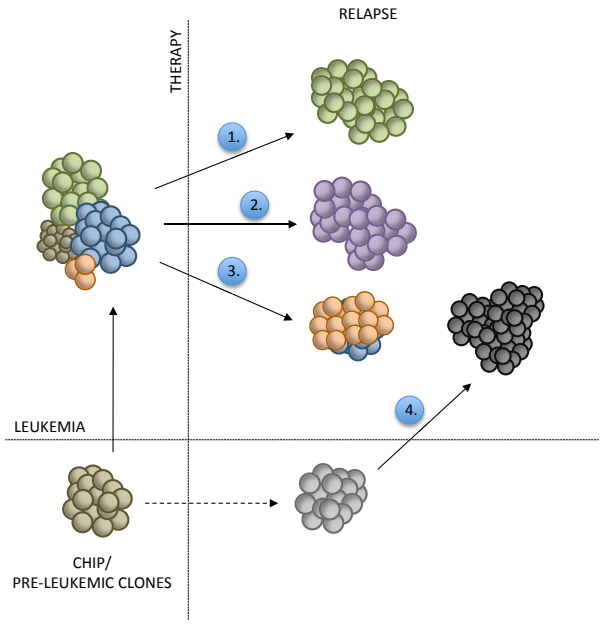


Figure 2. The effect of therapy to the clonal composition of AML.

The clonal hematopoiesis or other pre-leukemic clone is an ancestor clone for potentially following AML. This state is frequently monoclonal (represented by the brown group). At diagnosis, the disease is typically polyclonal, including at least the founding clone and one subclone, but several subclones (represented by the colored groups) also can be detected. The relapse can be caused either by **(1)** the re-occurrence of the initial clone, **(2)** the founding clone with additional mutations, **(3)** the expansion of a sub-clone with possible additional mutations, or **(4)** by a clone arising from an ancestral clone with later divergent mutations.

Adapted with permission from Macmillan Publishers Ltd: *Oncotarget*, Jan et. Majeti. Clonal evolution of acute leukemia genomes,[46] copyright 2013.

Furthermore, novel highly sensitive ultra-deep amplicon resequencing methods demostrated that the leukemic subclones may be present at the diagnosis with a very small allele frequency, and thus remain non-detectable for exome sequencing.[47] Some of these subclones may expand during therapy or relapse, thus providing a resistance mechanism for both targeted and conventional chemotherapy.[47]

2.3. The rocky road of targeted therapies in AML – lessons learned from FLT3 inhibitors

A powerful illustration of targeted therapy in leukemia and, moreover, cancer treatment in general, is the use of BCR-ABL1 inhibiting tyrosine kinase inhibitors (TKIs) in CML.[1,3] Correspondingly, the inhibition of FIP1L1-PDGFRα-induced myeloproliferative diseases and inhibition of the Bruton tyrosine kinase (BTK), as well as the phosphatidylinositol 3-kinases (PI3K) in chronic lymphocytic leukemia (CLL), have all shown paradigm-shifting clinical efficacy.[48-51]

In AML, *FLT3* is frequently mutated: 17% to 34% of AML patients have *FLT3* internal tandem duplication (ITD), and approximately 7% have activating mutations in the tyrosine kinase domain (TKD).[33,51,52] *FLT3-ITD* mutations are associated with poor prognosis.[52] At the beginning of the millennium, cancer research witnessed the successful emergence of targeted therapies led by imatinib in CML[1,3] and trastuzumab in HER2-positive breast cancer.[53] Thus, FLT3 inhibition provided great hope for paving the way for targeted AML therapies. This potential was attractively illustrated by an article title in Cancer Cell in 2002: "Finding the next Gleevec: FLT3 targeted kinase inhibitor therapy for acute myeloid leukemia."[54] Like several other targeted therapies, FLT3 inhibition presented roadblocks, as illustrated by subsequent titles: "FLT3 as a therapeutic target in AML: still challenging after all these years."[55] (2010) and "FLT3 inhibition: a moving and evolving target in acute myeloid leukaemia."[56] (2013). More recently, FLT3 inhibitor's ability to fulfill expectations has been questioned: "Will FLT3 inhibitors fulfill their promise in acute myeloid leukemia?"[57] (2014).

Fortunately, the several clinical trials that failed facilitated further research by producing comprehensive data on kinase inhibition in AML and, thus far, several mechanisms of resistance have been identified. Because FLT3 inhibitors act through the competitive inhibition of ATP-binding sites in the FLT3 receptor kinase domain,[56] mutations occurring in the ATP-binding site or activation loop (kinase domain mutations: D835V/Y/F/H, D839G and Y842C/H; FLT3 gatekeeper mutation: F691L/I) appear to disturb the binding of FLT3 inhibitor leading to mutation-site-dependent resistance to the individual FLT3 inhibitors.[51,58,59] Secondly, resistance to FLT3 inhibition can be mediated through non-mutational mechanisms. The up-regulation of pro-survival pathways such as MEK/ERK, PI3K/AKT, and STAT5/PIM can circumvent the cell dependence of FLT3 signaling, thus leading to loss of sensitivity.[51,59,60] Correspondingly, the up-regulation of both the FLT3 receptor and

the FLT3 ligand can cause resistance[51,59] and interestingly, in *FLT3* mutated AML patients the conventional chemotherapy appears to induce the overexpression of the FLT3 ligand.[61] Furthermore, in cell lines and primary patient samples with *FLT3* mutation, the microenvironment and stroma may mediate resistance through CXCL12/CXCR4 signaling which can be circumvented by the inhibition of CXCR4.[62] Also, in primary *FLT3* mutated blasts the activation of anti-apoptotic proteins (BCL2, MCL1) may enhance resistance.[63] Thirdly, although initially supposed, FLT3 inhibitors do not actually cause direct apoptosis of *FLT3* mutated blasts. Instead, FLT3 inhibition leads to terminal myeloid differentiation of *FLT3* mutated primary blasts, which can be accompanied by a similar differentiation syndrome observed in patients with APL.[64] Correspondingly, culturing *FLT3* mutated primary blasts with FLT3 inhibitor quizartinib leads to induced differentiation and cell-cycle arrest instead of apoptosis.[64]

The effectiveness of FLT3 inhibition itself can be questioned. Previous generations of FLT3 inhibitors (lestaurtinib, sorafenib, midostaurin, and quizartinib) target only the inactive conformational state of FLT3, while the next-generation FLT3 inhibitors, including crenolanib, instead target both the inactive and active conformational states, perhaps leading to better results.[51] Moreover, to achieve durable remission or a cure, therapies must eradicate the early preleukemic clones present at diagnosis and which persist throughout therapy, ultimately giving rise to relapse (**Figure 1**).[45] As *FLT3* mutations are late events, targeting only these mutations may lead to insufficient long-term response.

Lastly, ATP-binding cassette (ABC) proteins ABCB1 (P-glycoprotein) and ABCG2 (breast cancer resistance protein) mediate the drug-efflux of several conventional chemotherapeutic agents and tyrosine kinase inhibitors.[65] Yet, the influence of the drug-efflux on most FLT3 inhibitors remains inadequately understood. Quite intriguingly, midostaurin, sorafenib, and quizartinib have all inhibited ABC proteins in cell lines[66, 67] and primary *FLT3* mutated primary blasts[68], whereas crenolatinib is only a substrate with no ability to inhibit the transport function.[69]

Despite intensive research, these agents have thus far failed to demonstrate a survival benefit. Thus, recent results from the international randomized prospective multi-center phase III trial (RATIFY) were eagerly awaited. Intriguingly, that study demonstrated that adding the FLT3 inhibitor midostaurin to standard chemotherapy (i.e. cytarabine and anthracycline) together with one year of maintenance, significantly improved OS in younger patients (aged 18 to 60) with *FLT3* mutated AML (either TKD or ITD, regardless of allele burden).[70] Still, it is unclear whether these results are due to FLT3 inhibition alone. FLT3 inhibitors midostaurin and sorafenib inhibit also multiple other kinases as well. Therefore, results from a recent phase II trial (SORAML) with a very similar set-up among non-selected patients 60 years or younger (both *FLT3*-mutated and wild-type [WT]), are of particular interest.

Notably, this study demonstrated that sorafenib increased event-free survival in combination with standard chemotherapy,[71] leaving the question on the importance of FLT3 inhibition unanswered.

2.4. Upcoming targeted therapies

While the idea of inhibiting activated signaling has been inspiring, it remains unlikely that the inhibition of a single pathway will result in durable responses in a heterogeneous disease with multiple other abnormalities potentially capable of driving disease progression.[72] Furthermore, multiple clones or subclones might depend on entirely different oncogenic pathways.[72] Thus, agents targeting earlier events in leukemogenesis might be associated with better outcomes in AML.

The *IDH1* and *IDH2* mutations have been identified in up to 20% of patients with normal karyotype AML. These mutations lead to the production of oncometabolite 2-hydroxyglutarate (2-HG), which interrupts epigenetic functioning, promotes hypermethylation, and may be sufficient independently to cause AML.[73,74] In phase I trials, the inhibition of *IDH1* by AG120 or *IDH2* by AG221, demonstrated reduced 2-HG production translating to complete response (CR) rates of 15% and 16%, respectively.[75] These responses occurred progressively over continuous therapy and were associated with the terminal differentiation of leukemic blasts.[75]

Polo-like kinase 1 (PLK1) is a cell-cycle kinase that plays a central role in the regulation of centrosome maturation, spindle formation, and cytokinesis during mitosis.[76] PLK1 is inhibited by volasertib, a small-molecule kinase inhibitor. *In vivo*, this inhibition leads to cell-cycle arrest in prometaphase due to impaired spindle formation, ultimately causing apoptosis.[77] In the phase II trial, unfit elderly patients were randomized to receive either LDAC or LDAC in combination with volasertib. The two-drug combination led to a higher remission rate (CR or complete remission with incomplete blood recovery [CRi]) and also to improved median OS (8 months versus 5.2 months).[78] The Phase III trial (NCT01721876) is estimated to be completed by 2017 (https://clinicaltrials.gov).[79]

SGI-110 (guadesitabine) is a second-generation hypomethylating compound, in which deoxyguanosine is incorporated into decitabine to reduce its degradation by cytidine deaminase, thus increasing the *in vivo* exposure of decitabine.[75,80] In the phase II trial, guadecitabine was administered at either 60 or 90 mg/m^2 per day subcutaneously for 5 days in 28-day cycles in newly diagnosed elderly (≥ 65 years) AML patients who were not eligible for standard chemotherapy. CR was observed in 19 of 51 (37%) patients and CRi occurred in 10 of 51 (20%) treated patients.[81] A phase III trial in patients applying similar inclusion criteria is now fully recruited, and the study should be completed in December 2017 (https://clinicaltrials.gov).[82]

Antibody therapy for several hematological malignancies, including AML, is experiencing a renaissance.[5] Strategies using the anti-CD33 monoclonal antibody

drug conjugate gemtuzumab ozogamicin (GO) combined with chemotherapy have demonstrated a significant survival benefit for patients without adverse cytogenetic characteristics, thus validating CD33 as an important target in AML.[83-86] Because GO has been withdrawn from the US market due to liver toxicity, several other compounds are being developed.[75] Vadastuximab talirine (SGN-33A) carries a potent DNA-crosslinking toxin, pyrrolobenzodiazepine dimer. Since this toxin is not a substrate for a drug-efflux pump, it is hypothesized to carry lower toxicity.[87] In the phase I trial for older treatment-naïve patients with CD33-positive AML, vadastuximab talirine monotherapy resulted in CR or CRi in 15 of 26 (58%) patients.[88] In a similar patient cohort, a combination of vadastuximab talirine with hypomethylating agent was well tolerated and resulted in CR or CRi among 35 of 49 (73%) patients.[89] Currently, this conjugate is being explored both as a single agent and in combination therapies in phase II and III trials.[75]

Another novel approach targets leukemia-specific antigens using bi-specific antibodies. This method has been widely explored in lymphoid disease with promising results.[90] The bi-specific antibody has two motifs. First, in the case of AML, the other recognizes the tumor-specific antigen CD33. The second recognizes the T-cell receptor CD3. This connection leads to T-cell activation and recruitment of the host immune system to recognize and eliminate leukemia blasts through cell-mediated cytotoxicity.[91] Phase I trials utilizing bi-specific antibody AMG-330 in AML are currently ongoing.[75]

Finally, several novel strategies aim to target other common antigens expressed on leukemic stem cells, such as CD123, the alpha subunit in the interleukin-3 receptor. CD123 is currently being studied as a target for chimeric antigen receptor T-cell engineered cellular therapy.[92] Furthermore, other approaches to active the host immune system are also being studied. For example, the PD-1 antibody nivolumab is being explored in AML in phase II trials, both as a single agent or in combination therapies.[93]

3. BCL-2 INHIBITORS AS A NOVEL TARGETED THERAPY

3.1. Rationale for targeting the BCL-2 protein family in cancer

Apoptosis failure is a hallmark of the development of several cancers and as well as a frequent cause of refractoriness in cytotoxic therapies.[94] In general, two major signaling pathways can activate apoptosis: (1) the extrinsic, or death receptor pathway; and (2) the intrinsic or mitochondrial pathway. The extracellular binding of death ligands from the tumor necrosis factor family (e.g., Fas, CD95) activates the extrinsic pathway leading to the activation of caspases and, ultimately, to apoptosis.[95] Correspondingly, the dynamic balance of BCL-2 family proteins is the

key regulator of the intrinsic pathway. BCL-2 proteins can be divided into three important subfamilies: (1) BCL-2, BCL-XL, and MCL-1 are anti-apoptotic proteins; (2) BH3-only proteins (BIM, BID, BAD, and PUMA) function as initiator proteins; and (3) BAK and BAX are pro-apoptotic cell death mediators. Anti-apoptotic proteins promote survival by blocking BH3-selective initiators and their pro-apoptotic effectors (BAX and BAK), thus preventing them from triggering mitochondrial outer membrane permeabilization, release of cytochrome c, and apoptosis (**Figure 3**).[96,97]

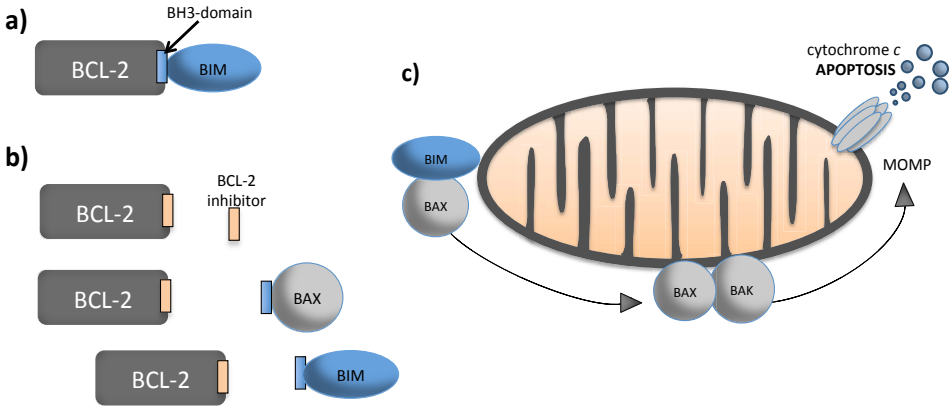


Figure 3. Mode of action of BCL-2 inhibitors.

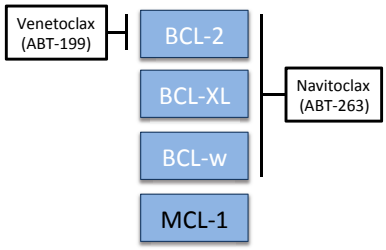
(a) In a normal healthy cell the balance between pro-apoptotic and anti-apoptotic proteins maintains anti-apoptotic equilibrium. **(b)** BCL-2 inhibitors (e.g., navitoclax and venetoclax), as BH-3-mimetics, dislodge pro-apoptotic proteins (i.e., BAK and BAX) from their binding to BCL2 to initiate apoptosis. **(c)** Once activated, BAX and BAK trigger permeabilization of the outer mitochondrial membrane. Permeabilization leads to release of factors such as cytochrome c, leading to activation of caspases and mitochondrial damage, ultimately leading to apopotosis.[95] Abbreviation: MOMP: mitochondrial outer membrane permeabilization

Adapted from *Cancer Discovery*, 2016, Volume 6/Issue 10, 1106-1117, Konopleva et al.: Efficacy and biological correlates of response in a phase II study of venetoclax monotherapy in patients with acute myelogenous leukemia,[98] with permission from AACR.

Low levels of reactive oxygen species have been shown to be a metabolic feature of leukemic stem cells (LSCs), and such cells aberrantly overexpress BCL-2. Thus, high levels of BCL-2 are considered a possible defining LSC characteristic and, intriguingly, BCL-2 inhibition selectively induced apoptosis in the LSC.[99] Similarly, BCL-2 inhibition induces cell death in progenitor cells of patients with high-risk myelodysplastic syndromes (MDS) or secondary AML.[100] By contrast, however, in normal healthy hematopoiesis, stem cell survival depends primarily on MCL-1, and it has been hypothesized that BCL-2 inhibition response might be rather selective for AML cells.

Small-molecule BCL-2 homology domain 3 (BH-3) mimetic drugs bind to the common BH-3 domains of anti-apoptotic proteins and liberate the pro-apoptotic proteins to initiate apoptosis (**Figure 3**).[101,102] Earlier investigational BH-3 mimetic compounds were found to bind efficiently to multiple anti-apoptotic proteins, including BCL-2, BCL-XL, and MCL-1 (**Figure 4a**). In clinical trials on lymphatic malignancies, inhibition of BCL-XL by navitoclax (ABT-263) resulted in severe, dose-dependent thrombocytopenia, which ultimately relegated the progression of navitoclax to clinical use.[103] This sparked development of second-generation BH3 mimetic agent venetoclax (ABT-199), a structurally redesigned navitoclax compound. Venetoclax, despite a five-fold greater binding affinity for BCL-2, has a >800-fold lower affinity for BCL-XL and, thus, minimal effects on thrombopoiesis (**Figure 4b**).[102]

a)



b)

Target binding K_i	Navitoclax	Venetoclax
BCL-2	<1 nM	<1 nM
BCL-XL	<1 nM	≈ 50 nM
MCL-1	>500 nM	>400 nM

Figure 4. Selectivity of BCL-2 family inhibitors.

(a) Navitoclax inhibits BCL-2 and BCL-XL, as well as BCL-w. Venetoclax selectively inhibits BCL-2. Neither agent inhibits MCL-1. **(b)** Target binding profiles of BH-3 mimetic compounds. Lower K_i values represent tighter binding to the target.

Adapted from: M.A. Anderson, D. Huang, and A. Roberts. Targeting BCL2 for the treatment of lymphoid malignancies. *Seminars in Hematology*, 2014.[94]

3.2. Clinical trials of BCL-2 inhibitors in lymphoid malignancies

The BCL-2 protein is commonly expressed in hematologic malignancies and has been shown to be involved in tumor survival and chemoresistance.[104] Thus far, the specific orally bioavailable BCL-2 inhibitor venetoclax has been primarily explored in lymphoid diseases. Single-agent therapy proved highly efficacious and tolerable in the phase I trial in chronic lymphocytic leukemia (CLL)[105] and led rapidly to a phase II trial. This trial recruited patients who relapsed or presented with refractory CLL as well as treatment-naïve patients with *17p* deletion. The therapy was well-tolerated with an overall response rate of 79% (85/107 patients).[106] Based on these results, venetoclax was recently granted accelerated FDA approval for patients with CLL who lack part of chromosome 17 and are refractory to standard treatment.[107] Furthermore, venetoclax is currently being explored in combination with other

targeted therapies such as anti-CD20 monoclonal antibodies (e.g., rituximab, ofatumumab, and obinutuzumab).[108] Phase I and II clinical trials are also being conducted in several other lymphoid malignancies (e.g., diffuse large B-cell lymphoma, follicular lymphoma, multiple myeloma and mantle cell lymphoma) for patients with relapsed and/or refractory disease.[108] Despite its downgrade due to thrombocytopenia mediated by BCL-XL inhibition, navitoclax is also being explored as treatment for non-Hodgkin's lymphomas in both phase I and II trials. Additionally, navitoclax is being investigated in T-cell lymphomas (e.g., cutaneous and peripheral T-cell lymphomas).[108]

3.3. BCL-2 inhibitors in myeloid malignancies

In AML, several studies demonstrate the expression and protein levels of anti-apoptotic proteins BCL-2, BCL-XL, and MCL-1 to be variable. To some extent, this reflects the prognosis.[109-111] BCL-2 inhibitors have been explored in AML cell lines and primary patient cells, and the protein levels of BCL-2, BCL-XL, and MCL-1 have been correlated with venetoclax sensitivity.[112] A study by Pan et al. found that venetoclax carries a single-agent cytotoxic activity in 6 of 12 AML cell lines and 20 of 25 patient samples with diploid cytogenetics and mutations in *FLT3*, *NRAS*, and *NPM1* genes.[112] By contrast, AML patient samples with complex cytogenetics, t(8;21), and *JAK2* mutations were largely insensitive. Resistance to a BH-3 mimetic was observed following the activation of escape routes: amplified BCL-XL expression, together with low expression of BCL-2, led to a dimished of venetoclax sensitivity.[112] Investigators postulated that the BCL-2 protein level and short-term exposure of AML cells to venetoclax predicted the BCL-2 inhibitor response. Moreover, the response could be predicted by BH-3 profiling, a method that functionally assesses the capacity of the BH-3 peptides to induce mitochondrial depolarization and, ultimately, apoptosis.[112,113] Mutations in *IDH1* or *IDH2* have been shown to induce venetoclax sensitivity by the (R)-enantiomer of 2-hydroxyglutarate ([R]-2-HG) mediated inhibition of cytochrome c oxidase (COX) activity in the mitochondrial electron transport chain.[114] COX inhibition promotes a lower mitochondrial threshold, thus sensitizing blasts to venetoclax.

Recently, Konopleva and co-workers reported their results from a phase II trial exploring venetoclax monotherapy in patients unfit for standard chemotherapy with relapsed and/or refractory AML. In total, 26 of 32 patients underwent at least four weeks of therapy. Venetoclax monotherapy resulted CR or CRi in 6 patients.[98] Although this comprises only 19% of treated patients, such results have not been previously observed using any other oral monotherapy for AML.[115] Because these monotherapy results appear promising, combinations are also being explored. Initial combinational studies are ongoing, both combining venetoclax with low-dose cytarabine (NCT02287233) and azacytidine (NCT02203773).[116,117] Preclinical studies of RNA-interference sensitizer screens demonstrate that the inhibition of

anti-apoptotic BCL-2 family proteins enhances the antileukemic activity of azacytidine.[118] This hypothesis is further supported by results from other ongoing trials. For instance, in the azacytidine trial, the overall response rate (CR or CRi) is 24 of 34 (71%) patients, and the median time to CR or CRi was 29.5 days.[116] Because the toxicity profile appears acceptable, these results are considered highly encouraging.[115]

3.4. Proof of concept – secondary mutations mediating venetoclax resistance

Mutations in the BH-3 domain are infrequent events. Previously, 353 patients with untreated lymphomas were screened for *BH-3* mutations, and only one carried a mutation in the BH-3 domain.[119,120] Interestingly, BCL-2 expressing murine lymphoma cells that are continuously exposed to venetoclax may develop resistance. In resistant cells, two missense mutations (F101C and F101L) within the BH-3 domain have been identified. Both mutations impair the binding of venetoclax to the BH-3 domain, thus hindering apoptosis.[119] This infrequent prevalence in treatment-naïve patients strongly indicates that mutations in the BH-3 domain may result from prolonged venetoclax exposure. Similarly, in a resistant human lymphoma cell line, a missense mutation in the transmembrane domain of pro-apoptotic *BAX* (G179E) has correspondingly been described.[119] This mutation prevented BAX anchoring to mitochondria, consequently hindering venetoclax-induced apoptosis.

4. T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA (T-ALL)

4.1. Background

T-cell acute lymphoblastic leukemia (T-ALL) is a rare aggressive leukemia that can present with leukemic and/or lymphomatous manifestations.[121] T-ALL represents approximately 20% of adult cases of ALL.[121] T-ALL arises in the thymus from an immature thymocyte that has accumulated, stepwise, both genetic and epigenetic aberrations.[122] These aberrations lead to the accumulation of clonal, immature hematopoietic cells in bone marrow, blood and tissues, particularly mediastinal lymph nodes.[121] The WHO continues to classify T-ALL and T-cell lymphoblastic lymphoma (T-LL) together, despite differences in clinical presentation. T-LL frequently occurs as a bulky mediastinal mass and is distinguished from T-ALL by a rather arbitrary cut-off: the presence of <20% BM blasts.[121]

Most patients with T-ALL (and T-LL) are treated with protocols adapted from pediatric care relying on complex multiagent chemotherapy and frequent intrathecal chemotherapy. HSCT consolidation is used for selected patients with high-risk features, including those with persistent minimal residual disease.[123] Patients achieving negative minimal residual disease (MRD) status have good prognosis. In

the German Multicenter Trials for Adult ALL (GMALL) study, the molecular response appeared only variable with multivariate analysis indicating a significant prognostic impact. As such, patients with a negative MRD had a significantly better OS (80% versus 42%).[124]

4.2.The T-ALL genomic landscape

As mentioned above, T-ALL results from a multistep transformation process. During progression from a healthy thymocyte to a leukemic blast, the cell accumulates mutations, leading to disrupted cell cycle, differentiation, survival, and growth.[125] NOTCH1 activation stands as a hallmark in T-ALL pathogenesis. Activating mutations and mutations involving negative regulatory domains controlling NOTCH1 signaling are observed in up to 60% of T-ALL patients.[125-127]

In addition, the genetic landscape of T-ALLs includes translocations in the T-cell receptor gene, leading to the aberrant expression of transcription factors (e.g., *TAL1, LMO1/2, LMO2,* and *TLX1*). Similarly, mutations in *IL7r, JAK1,* and *JAK3* activate the IL7R/JAK-STAT pathway, whereas loss of the *PTEN* tumor suppressor gene drives aberrant PI3K-AKT signaling. In addition, the loss of transcription factors (e.g., *WT1, RUNX1,* and *ETV6*), cell-cycle inhibitors (e.g., *CDKN2A* and *CDKN1B*), and epigenetic tumor suppressors (e.g., *EZH2, SUZ12* and *PHF6*), together with chromosomal rearrangements and consequential fusion proteins (e.g., NUP214-ABL1, CALM-AF10, and MLL1-ENL) contribute to leukemogenesis of T-ALL.[121, 125, 128, 129]

Based on the immunophenotypic maturation status, the disease can be characterized as early cortical, late cortical, or mature T-ALL. These subgroups can also be distinguished by oncogenic events and gene expression signatures.[125] Likewise, early T-cell precursor ALL (ETP-ALL) has a distinct phenotype, but interestingly is characterized by a mutation spectrum similar to that of myeloid tumors. These aberrations disrupt hematopoietic development, histone-modifying genes, and activating mutations in genes regulating cytokine receptors and RAS signaling. Furthermore, the global transcriptional profile of ETP-ALL resembles that observed in normal and myeloid leukemia hematopoietic stem cells.[130]

4.3. The IL7r-JAK-STAT5 pathway in T-ALL

IL-7 signaling induces phosphorylation and activation of both JAK1 and JAK3, which subsequently activate the STAT5 transcriptional regulator to drive increased cell proliferation and survival.[131] Approximately 9% of childhood T-ALL patients display activating mutations in *IL-7r*,[132] while activating mutations in *JAK1* and *JAK3* have been reported in approximately 10% of T-ALLs.[133, 134] In non-leukemic naïve T cells, the stimulation of the JAK–STAT5 or PI3K pathway results in expression of several anti-apoptotic factors, including BCL-2 and MCL-1, while pro-apoptotic BCL-2 family members are inhibited.[135]

STAT5 plays a key role in many hematologic cancers, but constitutive activation typically remains a secondary event to up-stream mutations.[136] *STAT5B* mutations and their functional significance were originally unraveled in large granular lymphocytic (LGL) leukemia[137] and, subsequently in several other hematological T-cell malignancies. These include T-cell acute lymphoblastic leukemia, hepatosplenic gamma-delta T-cell lymphomas, enteropathy-associated T-cell lymphoma, and T-cell prolymphocytic leukemia.[138-141] The mutations located in the Src homology 2 (SH2) domain of *STAT5B* promotes constitutive phosphorylation of the mutant protein, which is believed to stabilize the parallel conformation of STAT5 dimers, thus increasing transcriptional activity and activation of downstream target genes.[142]

5. INDIVIDUALIZED THERAPY

5.1. Methods for individualized therapy

5.1.1. In vitro drug testing

The ability to predict anticancer therapy responses in the laboratory prior to clinical treatment—analogous to the success of antibiotic sensitivity testing in microbiological diseases—has long been the goal of leukemia and cancer research in general.[143] After initial successes in cancer medicine in the 1940s, the scope of cancer treatment, in general, was adjusting doses and finding suitable combinations and timing to allow most patients to tolerate and benefit from therapy.[144] From the late 1970s, enthusiasm for *ex vivo* drug sensitivity testing resulted in hundreds of publications seeking correlation between *in vitro* drug sensitivity and clinical outcomes (reviewed in[145-148] and exemplified in[149-151]). Although these studies produced varied results, three major limitations hampered the translation of *in vitro* drug testing results into the clinical settings. First, testing often remained limited to a quite small number of compounds, typically those implemented as therapy regardless of results. Second, while a correlation with resistance or sensitivity could be established in some cases, these studies failed to introduce new therapy options in a systematic manner. Third, the response assessment for most conventional chemotherapeutics relied on *in vitro* proliferation. Thus, results from mostly non-proliferative primary patient samples remained difficult to both interpret and translate into clinical care.

5.1.2. Next-generation sequencing

Next-generation sequencing (NGS), or high-throughput sequencing, stands as a catch-all term used to describe several modern sequencing technologies that enable several downstream applications, such as genome sequencing, transcriptional profiling (RNA-sequencing), and the high-throughput survey of DNA-protein

interactions (ChIP-Seq) and of the epigenome.[152-154] NGS allows the sequencing of millions of nucleotides in parallel, yielding extensively more throughput. Thus, the demand for fragment-cloning methods needed for Sanger sequencing is minimized. The samples are sequenced with high coverage, known as deep sequencing, enabling even a small number of cells to be sequenced at a very high resolution.[155] Due to their lower costs, these methods have now driven our understanding of many diseases, including cancer.[153,156]

Several next-generation genome-sequencing techniques exist. Targeted sequencing involves the analysis of selected parts of the genome, and appears suitable for sequencing known disease genes. It may also be used for targeted gene panels as well as for the validation of mutations. In exome sequencing (referred to occasionally as whole exome sequencing), the protein-coding part of the genome (i.e., the exome), constituting 1% to 2% of the human genome, is sequenced. This application enabled the discovery of novel disease genes as well as evolutionary patterns in cancer. Whole-genome sequencing refers to the analysis of the entire genome, including non-coding and regulatory DNA.[152,155,157-159]

5.1.3 Biomarkers

The National Cancer Institute (NCI) defines a biomarker as "a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease."[160] In cancer, biomarkers are frequently used to evaluate the disease prognosis, to predict the therapeutic response, and to monitor the relapse or progression of disease. [161, 162]

According to Teutcsh and coworkers, the discovery and development of a biomarker from initial finding to everyday practice can be divided into three separate phases: assessing the analytic validity, the clinical validity, and the clinical utility of a potential biomarker.[163] First, a cohort of samples is analyzed to identify or test a specific potential new biomarker. Subsequent analysis with an independent sample cohort may validate the original findings and may facilitate a detailed evaluation of possible confounding factors. Finally, the capability of aiding clinical decision-making together with improving treatment outcomes will validate the value of the potential biomarker.[161,163] Currently, phase I trials increasingly use biomarker-driven patient selection, thus attempting to validate a biomarker for a specific therapy and improve treatment outcomes.[164] In AML, biomarkers were initially used to assess the risk classification and MRD detection. As discussed previously, several existing phase I, II, and III trials are exploring the effect of biomarker-guided targeted therapies.

5.1.4 Biobanking

Although translational research is undergoing a renaissance, the gap between laboratory research and clinical care ("the valley of death") remains wide.[165] To shorten the time for translation of basic research, a well-organized system of patient-derived material, i.e., a biobank, may serve as an essential instrument. Biobanks can be classified by purpose into population-based biobanks and disease-oriented biobanks.[166] Typically, the healthy population, or an unselected population with chronic illnesses, forms the foundation for population-based biobanks.[166] Disease-oriented biobanks are typically integrated within healthcare organizations.[167] Whereas population-based biobanks customarily collect blood or DNA together with data on lifestyle, family history and environmental exposure, disease-oriented biobanks typically store more extensive sample collections (e.g., tumor tissue, cells, blood, and other body fluids) with a variable range of clinical data.[166]

The broad and robust collection of patient-derived primary material paired with relevant clinical data may accelerate translational research by providing an available resource of high-quality patient samples, thus complementing research performed on cell lines and animals.[168] As recommended by ELN in the 2017 AML guidelines, informed consent should allow for a range of laboratory studies along with analysis of germline DNA.[34] Pretreatment leukemic marrow, processed to DNA, RNA, and viable cells, as well as plasma samples, a methanol/acetic acid-fixed cell pellet, and frozen cell pellets and blood should be stored within a biobank. According to the ELN recommendations, sampling should include specimens from various time points during and after treatment (CR, remission, and relapse), as well as samples for MDR monitoring.[34]

5.2. Attempts to individualize therapy

The Latin phrase *divide et impera*—divide and rule—refers to politics or war, where breaking up large and complex opponents into smaller units renders them more manageable and, thus, surmountable. As highlighted by Cortes and Kantarjian, this strategy might also apply to the treatment of AML.[169] Broadening our knowledge base regarding the disease allows us to identify multiple distinct entities rather than a single overarching disease.[169] Given our current understanding of genomics, we can accurately estimate which aberrations are most harmful, which can putatively be targeted, and what is the biology of an individual patient's disease.[169] Regrettably, putting these diverse data to use in everyday practice remains challenging and elusive. Each patient's tumor typically features several driver mutations forming numerous possible combinations, where only few driver genes represent straightforward therapeutic targets.[170,171]

Currently, the major dilemma in treating fit AML patients centers around whether they should receive allogeneic stem-cell transplantation or chemotherapy alone.[172] Current guidelines are based on risk grouping characterized by single mutations.[34] Recent studies developed a knowledge bank, an approach that utilizes information on matched comprehensive genomic and clinical data from more than 1500 AML patients to support clinical decision-making. Presently, this approach is limited to evaluating the effect of one therapy option; in the future, however, it may provide the foundation for more comprehensive decision-making.[171]

The German-Austrian AML Study Group (AMLSG) has made one of the first attempts to comprehensively integrate genomic data into treatment decision-making. AMLSG has launched investigator-initiated trials where therapy is based on the molecular subtyping of the disease. AML patients are assessed for aberrations within 48 hours of diagnosis. Patients are then stratified into placebo-controlled trial groups based on driver mutations (CBF-positive patients to the dasatinib-trial, FLT3-positive to the trial with midostaurin or the crenolanib trial, NPM1-positive to trials with GO/ATRA, and high-risk patients to the volasertib trial). All patients receive conventional chemotherapy combined with these agents. Although this study offers a platform for identifying targeted therapies for a given subgroup, the study is not designed to estimate the strength of using more precise disease characterization to improve survival.[173]

Quite recently, the Leukemia & Lymphoma Society (LLS) launched its Beat AML master trial (Beat AML 2.0) in which patients 60 years and older with newly diagnosed AML are eligible for participitaion.[174,175] Each patient will undergo genomic screening at diagnosis, with results available in seven days. Based on the results, patients are assigned to personalized therapy based on one of several sub-studies, each with arm-specific endpoints. Despite varying endpoints, all therapies have the common goal of improving patient outcomes.[174,175] The trial differs from previous AMLSG-initiated trials in four major ways. First, all patients receive active therapy (without placebo). Second, conventional chemotherapy is reserved only for those patients shown to benefit from it. Third, the trial protocol includes algorithms to implement targeted therapies for common situations with several targetable mutations. Finally, this umbrella trial design will be assessed for its overall survival benefit. The Beat AML 2.0 trial will explore an expanded set of drugs and novel combinations.[174]

During the last decade, NGS methods have extended our understanding of AML pathophysiology, ultimately also leading to the expansion of our anti-leukemic armory. Continuous work towards unraveling novel tools to enable therapeutic individualization together with innovatively designed trials will gradually improve our competence in subdividing patients' diseases into well-defined—and thus defeatable—subgroups.

AIMS OF THE STUDY

The overall aim of this study was to utilize novel techniques (e.g., exome and transcriptome sequencing, *ex vivo* drug sensitivity, and resistance testing) to individualize therapy for patients with acute leukemia. In addition, these platforms for were used for predictive biomarker discovery.

The specific aims were:

- To evaluate whether an *ex vivo* drug testing platform can be used for functional classification of AML and as a predictor for *in vivo* therapy responses. (I)
- To determine the prevalence of *STAT3* and *STAT5* mutations in T-cell acute lymphoblastic leukemia and to evaluate potential sensitivity for targeted therapies. (II)
- To evaluate biomarkers for BCL-2 inhibitor sensitivity in AML. (III)
- To develop a hematology-based national biobank to inventory samples for future research. (IV)

MATERIAL AND METHODS

1. STUDY SPECIMENS

1.1.Study patients and consents (I-III)

We collected bone marrow (BM) aspirates or peripheral blood (PB) samples (leukemic cells) and skin biopsies (non-malignant cells for germline genomic information) from patients after they signed informed consent forms (permit numbers 239/13/03/00/2010 and 303/13/03/01/2011, Helsinki University Hospital Ethics Committee) in accordance with the Declaration of Helsinki. The consent forms included the possibility of using DSRT data to guide therapies after all other treatment options were exhausted. In addition, we obtained BM aspirates from healthy donors. Finally, we obtained approval to use archival samples from deceased patients for study II from the National Supervisory Authority for Welfare and Health

Study I evaluated the drug sensitivity and molecular profiles of 28 fresh BM samples from 18 AML and high-risk MDS patients. In addition, we analyzed 7 BM samples from healthy individuals, which served as controls. We collected 10 samples from newly diagnosed patients, whereas 18 samples were collected from relapsed or refractory or both types of patients.

Study II investigated the prevalence of *STAT* mutations in T-ALL, by sequencing 64 adult and 4 pediatric T-ALL patients. The bone marrow samples from *STAT5B* mutated index patient were evaluated for drug sensitivity and *BCL-2* family gene expression.

Study III evaluated the biomarkers for venetoclax and navitoclax sensitivity in 73 AML BM aspirates and PB samples from 57 AML patients. In addition, BM aspirates from healthy donors (12 for navitoclax testing and 7 for venetoclax), and 3 CLL patients were obtained. We obtained 28 samples from newly diagnosed patients, whereas 45 samples were collected from relapsed or refractory AML patients or both.

1.2. Sample processing (I-III)

Mononuclear cells were isolated from fresh samples using Ficoll density gradient (GE Healthcare, Little Chalfont, UK), washed, counted, and suspended in Mononuclear Cell Medium (MCM) (PromoCell, Heidelberg, Germany) supplemented with 0.5 µg/ml gentamicin. Possible excess cells were stored in liquid nitrogen in fetal bovine serum (FBS) and 10% dimethylsulfoxide (DMSO) for future use.

For study I, a sample from patient 393 with secondary AML after MDS with 20% myeloblasts, was enriched for the CD34+ cell population using paramagnetic beads

according to the manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). For study II, CD3+ cells were enriched from the PB mononuclear cell (MNC) fraction of a healthy author using the Easy Sep Human CD3 Positive Selection Kit (StemCell Technologies, Vancouver, Canada) following the manufacturer's protocol.

2. ASSESSMENT OF DRUG SENSITIVITY

2.1. Drug sensitivity and resistance testing (DSRT) (I-III)

The oncology compound collection covers the active substances from the majority of FDA/EMA approved anti-cancer drugs as well as a wide range of emerging investigational and pre-clinical compounds. These compounds were obtained from the National Cancer Institute Drug Testing Program (NCI DTP) and commercial chemical vendors. Initially, in 2011 the collection consisted of 187 compounds; the latest library (version FO4B) contains 525 compounds. (**Figure 1**)

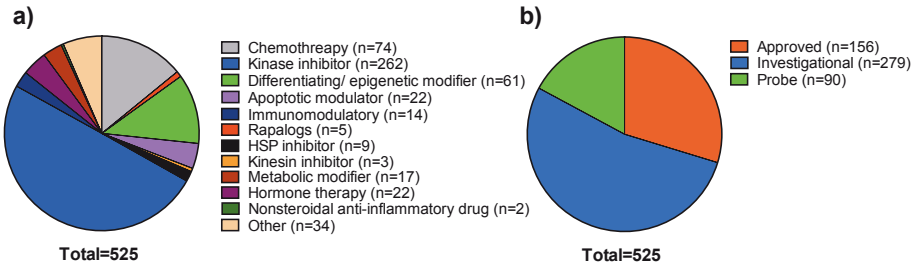


Figure 1. Composition of the current drug sensitivity and resistance testing (DSRT) library. Partitioned according to the mode of action **(a)** and the clinical phase **(b)**.

We performed *ex vivo* DSRT on freshly isolated primary patient MNCs as well as MNCs derived from healthy donors using a 72-h culture on multiwell plates. The compounds were dissolved in 100% DMSO and dispensed on tissue culture treated 384-well plates using an acoustic liquid handling device Echo 550 (Labcyte Inc., Sunnyvale, CA, USA). The compounds were plated in five different concentrations in 10-fold dilutions covering a 10,000-fold concentration range (e.g. 1-10,000 nM). The pre-drugged plates were stored in pressurized StoragePods (Roylan Developments Ltd., Fetchmam, UK) under inert nitrogen gas until needed. The compounds were dissolved using 5 µl of MCM. Then, 20 µl of a single cell suspension (10,000 cells) from either primary patient or control cells was transferred to each well using a MultiDrop Combi (Thermo Scientific Inc., Waltham, MA, USA) peristaltic dispenser.

The plates were incubated in a humidified environment at 37°C and 5% CO_2 and after 72 h cell viability was measured using the CellTiter-Glo luminescent assay (Promega, Madison, WI, USA) according to the manufacturer's instructions with a Molecular Devices Paradigm plate reader. Data were normalized to the negative control (wells preprinted with DMSO only) and positive control wells (wells containing a final concentration of 100 µM benzethonium chloride, which effectively kills all cells).

2.2. Analysis of DSRT data (I-III)

We used Dotmatics Studies software (Dotmatics Ltd., Herts, UK) to calculate normalized survival for each data point and to generate the dose response curves. The dose response curves were fitted based on a four parameter logistic fit function defined by the top (a) and bottom asymptote (d), the slope (b) and the inflection point (EC50) (c). The non-linear curve fitting equation is given by: $y = d + ((a-d)/(1+10^{b(c-x)})$. In the curve fitting, the top asymptote of the curve was fixed to 100% viability, while the bottom asymptote was allowed to float between 0 and 75%. Thus, the drugs causing less than 25% inhibition were considered inactive.

In order to quantitatively profile individual patient samples, we developed the drug sensitivity score (DSS) as a single measure.[176] The curve fitting parameters were used to calculate the area under the dose response curve (AUC) relative to the total area between 10% threshold and 100% inhibition (TA). Furthermore, to favor on-target responses over toxic off-target responses, the integrated response was divided by a logarithm of the top asymptote (a). The DSS was calculated as follows: DSS= (100 *x* AUC)/(TA *x* log *a*). We scored for differential activity of the drugs in blast cells in comparison to control cells (MNC fraction from healthy BM) using the selective DSS (sDSS) (**Figure 2**).

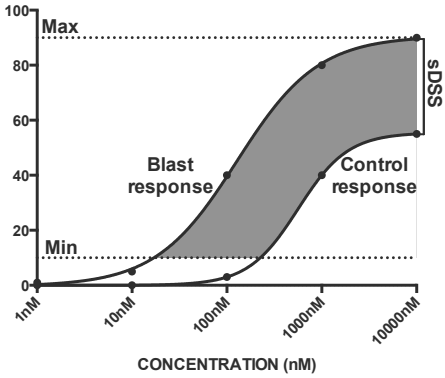


Figure 2. Curve fitting and selective drug sensitivity score (sDSS) calculation.
sDSS reflects the difference in blast cell response compared with the median response in healthy donor BM MNC fraction (i.e., leukemia-selective response). Modified from Pemovska et al. Individualised systems medicine - next-generation precision cancer medicine and drug positioning.[177]

2.3. Prediction of kinase addictions (I)

The selective sDSS of kinase inhibitors were further used to predict sample-specific kinase addictions. We compared sample-specific sDSS responses with target profiles for 35 kinase inhibitors overlapping between our compound panel and the panel biochemically profiled against more than 400 kinases by Davies et al.[178] For each kinase target, we calculated a kinase inhibition sensitivity score by averaging the sDSS values among those compounds that selectively targeted the kinase. These putative selective kinases were compared to gene expression to exclude non-expressed targets and the remaining kinases defined a putative "kinaddictome" for each patient sample. For displaying purposes, the resulting kinases were depicted in a kinase inhibitor target similarity network.[179]

3. NEXT-GENERATION SEQUENCING (NGS)

3.1. Exome sequencing and somatic mutation analysis (I-III)

Genomic DNA was isolated using the DNeasy Blood & Tissue kit (Qiagen, Hilden, vGermany). Exome capture was performed using the Nimblegen SeqCap EZ v2 (Roche NimbleGen, Madison, WI, USA), Agilent SureSelect v5 Exome or Agilent SureSelect XT Clinical Research Exome (Agilent, Santa Clara, CA, USA) capture kits and the HiSeq 1500 or 2500 instruments (Illumina, San Diego, CA, USA). Exome sequence reads were processed and aligned to the GRCh37 human reference-genome primary assembly as previously described.[180] Somatic-mutation calling was done for the exome-capture target regions and the flanking 500 bps. High confidence somatic mutations were called for each tumor sample using the VarScan2 somatic algorithm[181] with the following parameters: strand-filter 1, min-coverage-normal 8, min-coverage-tumor 6, somatic-p-value 1, normal-purity 1, and min-var-freq 0.05. Mutations were annotated with SnpEff 4.0 (ref.[182]) using the Ensembl v68 annotation database. To filter out misclassified germline variants, common population variants included in dbSNP database version 130 (National Center for Biotechnology Information, Bethesda, MD, USA) were removed. The remaining mutations were visually validated using the Integrative Genomics Viewer (Broad Institute, Cambridge, MA, USA).

In study I in order to evaluate the frequencies of mutations identified in samples not meeting the criteria for high-confidence mutations, we retrieved the variant frequencies and read counts for each mutation from a set of unfiltered variant calls generated by VarScan2 using the following parameters: strand-filter 0, min-coverage-normal 8, min-coverage-tumor 1, somatic-p-value 1, normal-purity 1, min-var-freq 0. In addition, we used variant allele frequencies from the control–leukemia pairs to identify regions of heterozygosity loss.

3.2. Transcriptome sequencing and data analysis (I-III)

We used the total RNA isolated from patient MNCs was used for the depletion of ribosomal RNA (Ribo-Zero™ rRNA Removal Kit, Epicentre, Madison, WI, USA) and reverse transcribed to double-stranded cDNA (Ribo-Zero rRNA Removal Kit, Epicentre, Madison, WI, USA; or in study II: NEBNext® mRNA Library Prep Master Mix Set 1, New England BioLabs, Ipswich, MA, USA). RNA-seq libraries were prepared by Illumina-compatible Nextera Technology (Epicentre, Madison, WI, USA) and sequenced on the Illumina HiSeq 1500 or 2500 instruments. Sequenced reads were filtered and aligned to the GRCh37 human reference-genome using TopHat.

In study II, we reconstructed the *STAT5B* haplotype of the index patient's relapse sample regarding mutations N642H, T648S, and I704L extracting from Tophat alignment pairs (38 in total), properly aligned and overlapping with the affected exons. The pairs supporting each of the possible haplotypes were counted manually using the Integrated Genomics Viewer (Broad Institute). In total, we had 23 pairs supporting the triple mutation (N642H, T648S, I704L); 15 pairs supporting the triple WT, and no pairs supporting other combinations.

In study III, mapped reads were counted for each genomic feature (gene) with the FeatureCount read-summarization program from the Subread package (WEHI, Melbourne, Australia).[183] The Trimmed Mean of M-values (TMM) method from the edgeR package was applied to normalize raw read count and to find out differential gene expression signatures between sensitive and resistant samples from 53,893 (Ensembl 67) genes[184].

3.3. Amplicon sequencing (I and II)

We prepared samples according to an in-house targeted polymerase chain reaction (PCR) amplification protocol. Each amplicon was generated using locus specific PCR primers carrying Illumina compatible adapter sequences (primer sequences are presented in the supplementary material in original studies I and II). All oligonucleotides were synthesized using Sigma-Aldrich (St. Louis, MO, USA). The PCR reaction was performed in an extracted sample DNA, with Phusion High-Fidelity PCR Master Mix (Thermo Scientific Inc., Waltham, MA, USA), locus specific primers, and adapter primer carrying Illumina grafting P7 sequence, or P5 sequence (the sequences are presented in supplementary material of the original publications). Following PCR amplification, samples were purified using Performa® V3 96-Well Short Plate (EdgeBio, Gaithersburg, MD, USA) and QuickStep™2 SOPE™ Resin (EdgeBio, Gaithersburg, MD, USA) or with Agencourt® AMPure® XP beads (Beckman Coulter Inc., Beverly, MA, USA) and then pooled together without exact quantification. Purified sample pools were analyzed on Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA) to quantify the amplification performance and yield. Sequencing of PCR amplicons was performed using the

Illumina MiSeq instrument with MiSeq Control Software v2.2.29 or later versions (Illumina, Inc., San Diego, CA, USA). Samples were sequenced as 101 paired-end reads and one 7pb (study I) or 151 or 251 bp and two 8 bp index reads (study II). Data obtained from sequencing were processed using an in-house amplicon pipeline. *STAT5B* mutations were validated from the index patient with capillary sequencing.

4. RQ-PCR (II AND III)

Total RNA was prepared from BM or PB MNCs using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was prepared from total RNA using SuperScript III reverse transcriptase and random primers (Life Technologies, Carlsbad, CA, USA), including RiboLock RNase inhibitor (Thermo Scientific, Waltham, MA, USA). The reference genes were chosen based on a uniform expression in all samples. For study II POLR1B and GUSB were identified as the best reference candidates, whereas for study III *GAPDH* and *PGK1* were chosen based on uniform expression in all samples. For study II qPCR was performed for four target transcripts: *BCL2, BCL-XL, BCL-XS,* and *MCL-1*. The primer sequences are listed in the supplementary material of study II.

For study III RQ–PCR was performed for *B2M*, *BCL-2*, *BCL-XL*, *MCL-1*, *BIK*, *BAX*, *BAK1*, *BID*, *BCL2L12*, *BCL2L11* (*BIM*), *BCL2A1*, *BBC3* (*PUMA*), and *BAD* as well as for *HOXA1-A7, HOXA9, HOXA10-A13, HOXB1-B9,* and *HOXB13* mRNAs. The primer sequences are listed in the supplementary material of study III. BCL-XL and BCL-XS represent alternative transcripts of the BCL2L1 gene, such that the primers were transcript-specific. Other targets were not specific for transcript variants. qPCR reactions were performed using iQ SYBR Green Supermix (Bio Rad, Hercules, CA, USA), while the specificities of the amplification products were verified using a melting curve analysis. Gene expression was quantified using the Pfaffl method based on the calculated primer efficiencies and other genes, while the DDCq method was applied to the analysis of homeobox (HOX) genes.[185]

5. FUNCTIONAL STUDIES

5.1 *STAT5B* mutagenesis (II)

An expression plasmid pCMV6-XL6 containing the wild-type (WT) coding sequence of *STAT5B* (OriGene, Rockville, MD, USA) was modified to include the N642H, T648S and I704L mutations. After generating the single mutated constructs the double (N642H+T648S, N642H+I704L, T648S+I704L) and triple (N642H+T648S+I704L) mutated constructs were prepared. The mutant constructs were created using the

GENEART Site-Directed Mutagenesis System (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. The primer sequences used for the mutagenesis are listed in the supplemental table 3 in study II. The entire *STAT5B* cDNA sequence with mutations was confirmed using capillary sequencing (the primer sequences are presented in the supplemental material).

5.2. Cell culture, transfection and Western blot analysis (II)

HeLa cells were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Carlsbad, CA, USA) containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Transfection was carried out using STAT5B expression plasmids mixed with luciferase reporter plasmid pGL4.52 (Luc2P/STAT5RE/Hygro, Promega, Madison, WI, USA) using the Fugene HD transfection reagent (Promega) with a Fugene HD:DNA ratio of 3.5:1. A master mix was prepared for each mutant construct + luciferase plasmid and appropriate amounts were added to each well.

One day after transfection, the HeLa cells were lysed in a RIPA buffer. Lysates were then sonicated and after the addition of a Laemmli buffer, protein was loaded to 10% SDS-PAGE gels. The detection of phopho–STAT5 and total STAT5 was completed using two separate gels run in parallel with the same protein lysates. The proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), after which the membranes were blocked with a 5% bovine serum albumin (BSA). Primary antibodies for STAT5 were obtained from Cell Signaling Technology (Cell Signaling Technology, Danvers, MA, US): STAT5 (rabbit polyclonal antibody, cat. 9363) and phospho-STAT5 (Tyr694/699, rabbit mAb, cat. 9359). Anti-α-tubulin was obtained from Sigma Aldrich (T9026 mouse mAb). Membranes were incubated with diluted primary antibodies and, then, with diluted secondary infrared antibodies: goat anti-rabbit IRDye 800 CW and donkey anti-mouse IRDye 800 CW (LI-COR Biosciences, Lincoln, NE, USA). The proteins were visualized using the Odyssey imaging system (LI-COR Biosciences) and version 3.0 of the application software was used to quantify band intensity levels.

5.3. STAT5B reporter assay (II)

After 24 h incubation at 37 °C, transfected HeLa cells were lysed and luciferase activity was assessed using the One-Glo luciferase detection reagent (Promega, Madison, WI, USA). The assay was repeated three times and the mean fold change in transcriptional activity induced by the different mutant constructs compared to the activity of the WT STAT5B construct was calculated (reported as standard deviations [SDs]).

6. PROTEIN ANALYSIS

6.1. Proteomic analysis (I and II)

The phosphoproteomic analysis in study I for AML patient samples was performed using Proteome Profiler antibody arrays (R&D Systems) according to the manufacturer's instructions. Lysates containing 300 μg of protein were applied to the arrays and fluorescently labeled streptavidin (IRDye 800 CW streptavidin, LI-COR) and an Odyssey imaging system (LI-COR) were used for detection.

6.2. Protein structure analysis

The human STAT5B dimer homolog was produced as described previously.[137] The visualization of the protein structure was produced using the Swiss PDB viewer.[186]

5. STATISTICAL ANALYSIS (I-III)

Statistical analyses were performed using the Prism software versions 5 and 6 (GraphPad Software, San Diego, CA, USA). The datasets were subjected to normality testing using the Shapiro-Wilk normality test. Differences between responses modeled by a Gaussian (normal) distribution were analyzed using *t* tests. In all other cases, we used the Mann-Whitney *U* test or the Wilcoxon matched-pairs signed-rank test. Correspondingly, statistical dependence between two variables was assessed using the Pearson's correlation or Spearman's rank correlation coefficient. All tests were two-tailed and we considered $P < 0.05$ as statistically significant. The clustering of drug sensitivity profiles across patient and control samples was performed using unsupervised hierarchical complete-linkage clustering using Spearman and Euclidean distance measures for the drug and sample profiles, respectively. Euclidean distance measures were also used to cluster samples according to gene expressions.

RESULTS

1. INDIVIDUALIZED SYSTEMS MEDICINE (ISM) FOR TAILORING THERAPY IN AML

1.1. ISM platform

In study I, we developed an individualized systems medicine (ISM) platform, which combines the genomic profiling data with the drug sensitivity profile. Thus, ISM can be utilized (1) to rapidly identify personalized treatment options for patients with relapsed or refractory AML, (2) to gain an understanding of factors driving drug resistance (and sensitivity), (3) to gain an understanding of the disease biology in a specific patient, and (4) to offer the possibility of repurposing approved agents, also in a boarder context. (**Figure 1**)

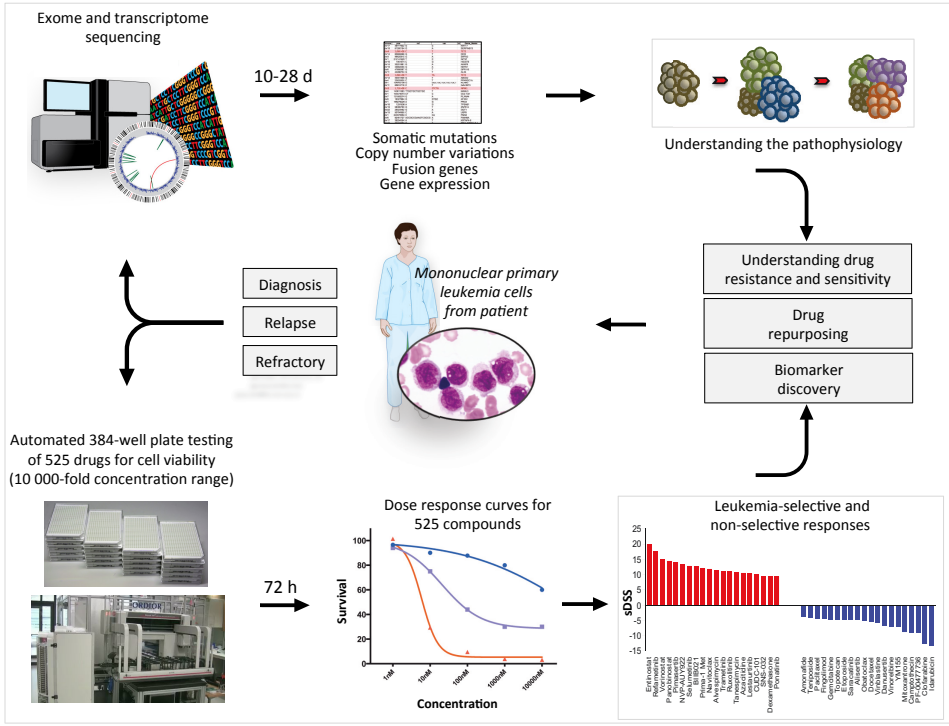


Figure 1. The individualized systems medicine (ISM) platform.
Currently, the platform involves DSRT against a comprehensive library of 525 anticancer compounds (both approved and experimental (pre-clinical and phase I–III trials)), next-generation genomic profiling (exome and transcriptome sequencing), and the integration of these to clinical follow-up data. This data may be used to improve understanding of the mechanisms of sensitivity, resistance, and individual disease biology, and introduce novel therapeutic options. Turn-around times for DSRT: 3 days; for genomic profiling: exome sequencing: 10–12 days; transcriptomic profiling: 28 days. Abbreviations: sDSS, selective drug sensitivity score.

The DRST methods are discussed in detail in the methods section. In brief, in study I DSRT was performed on fresh BM MNCs using a panel of all FDA- or EMA-approved small molecule compounds and conventional cytotoxic anticancer drugs, as well as investigational compounds (n = 202) in five concentrations over a 10,000-fold concentration range (e.g., 1–10,000 nM). A leukemia-specific drug sensitivity score (sDSS) derived from dose response AUC calculations was used as the efficacy variable by comparing leukemia cell results with those from normal BM cells. DSRT was performed on 28 samples, obtained from 18 patients with AML. In total, 18 samples were collected from relapsed patients whereas 10 were obtained upon initial diagnosis.

1.2. DSRT identifying subgroup-specific responses

DSRT identified a subset of AML samples exhibiting a selective response to targeted therapy agents. This response pattern differed from conventional chemotherapeutic agents, where the average sensitivity profiles were highly similar to those observed in healthy BM, thus reflecting the limited therapeutic window for these agents (**Figure 2a**). Importantly, DSRT identified several signal transduction inhibitors as potential AML-selective compounds. The primary patient samples exhibited leukemia-selective responses (sDSS > 5) for several TKIs, including the multi-kinase inhibitors dasatinib, sunitinib, and sorafenib. Moreover, leukemia-selective responses to more specific pathway inhibitors such as ruxolitinib (JAK1/2 inhibitor), trametinib (MAP–ERK kinase (MEK) inhibitor)) and quitzartinib (FLT3 inhibitor) were observed. Samples also demonstrated a sensitivity to rapalogs (temsirolimus) and the dual inhibitors PI3K/mTOR such as dactolisib and AKT-inhibitor MK-2206 (**Figure 2b**).

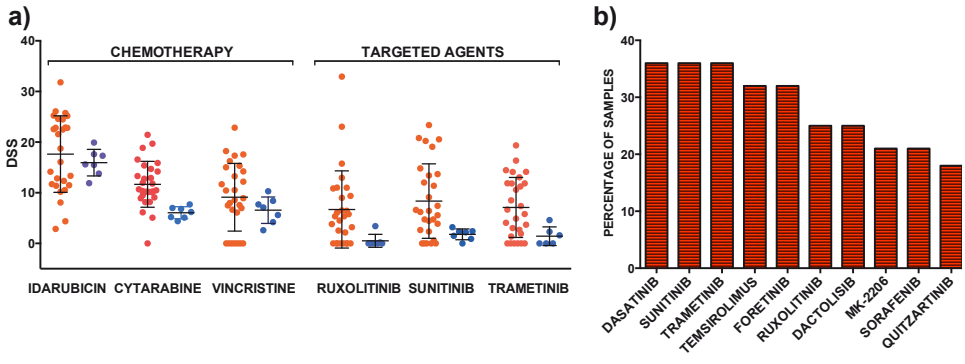


Figure 2. Responses in healthy donors and primary AML samples.

(a) Compared with healthy donors, targeted therapies caused selective responses in a subset of AML patient samples. **(b)** The percentage of primary AML samples exhibiting leukemia-selective responses to targeted agents. Color key for panel a: red, leukemia samples; blue, healthy controls.

Unsupervised hierarchical clustering analysis was used to visualize the various DSRT response patterns. Although the genomic profile for each sample is individual, the clustering divided the responses into five functional groups (groups I–V; **Figure 3**). Overall, 19 of 28 (68%) samples were sensitive to TKIs.

Specifically, group I displayed a selective response to navitoclax and appeared non-responsiveness to a majority of the other tested compounds. Group II showed a very limited response to receptor TKIs, but were quite sensitive to immunosuppressive drugs such as corticosteroids as well as JAK and MEK inhibitors. Groups III, IV, and V were all selectively sensitive to a broad range of TKIs, indicating a pathway dependence for tyrosine kinase signaling. Group III was further characterized by a similar sensitivity to inhibitors HSP90, HDAC, and PI3K/mTOR. Group IV exhibited leukemia-selective responses to MEK and PI3K/mTOR inhibitors, whereas group V showed selective responses to several receptor TKIs targeting ABL, the vascular endothelial growth factor receptor (VEGFR), the platelet-derived growth factor receptor (PDGFR), FLT3, and KIT proto-oncogene receptor tyrosine kinase (KIT). We also observed topoisomerase II sensitivity in this group.

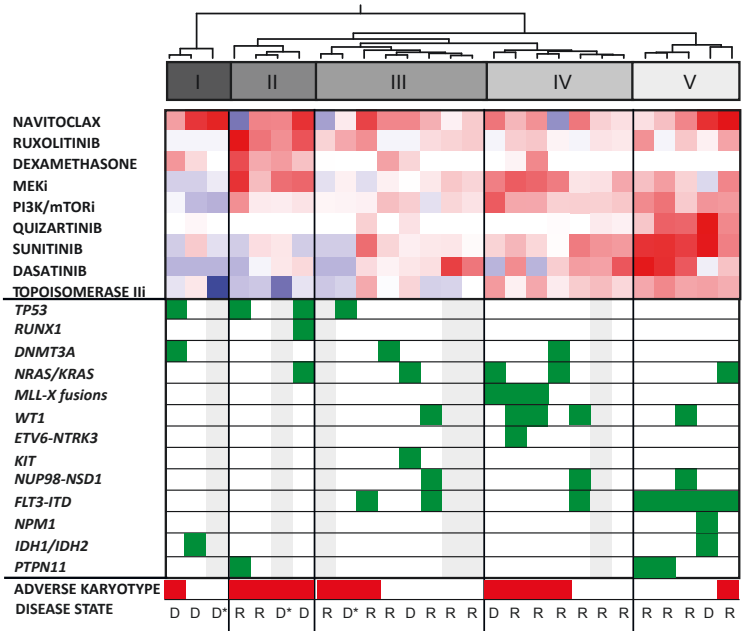


Figure 3. The clustering of DRST responses aligns AML patients into five distinct groups.
The recurrent AML mutations and gene fusions, disease stages, and adverse karyotype status of the patient samples are also presented. Abbreviations: D, diagnosis; D*, secondary AML diagnosis; R, relapsed and/or refractory. Color key: For drug sensitivity: red, sensitive; blue, resistant. For mutations: green, mutation detected; grey, data not available.

All samples in group V had *FLT3* mutation. As expected, several FLT3 inhibitors, such as sunitinib and quizartinib, exhibited the highest leukemia-selective responses. Notably, also those TKIs not inhibiting FLT3, such as dasatinib, showed highly selective responses in group V implying *FLT3* driven AML to depend on other tyrosine kinase signals as well. Mutations in *RAS* genes correlated significantly with leukemia-selective responses to MEK inhibitors.

1.3. Clinical implementation of ISM data

We considered DSRT results clinically implementable among patients with no treatment options available if (1) we observed a distinct leukemia-selective response pattern and (2) drugs with the most leukemic-selective responses were clinically available (as compassionate or on/off-label use) within a sufficient timeframe. Three of seven such patients responded to DSRT-guided therapy based on the ELN response criteria (**Table 1**). Patient 600 (dasatinib-sunitinib-temsirolimus) had CRi response, whereas patients 718 (sorafenib–clofarabine) and 800 (dasatinib–clofarabine–vinblastine) reached a morphological leukemia-free state. Additional three patients had responses not meeting ELN criteria. Patient 252 had an eight-week progression-free state during dasatinib monotherapy (BM blasts 65-40-70%). Patient 560 showed a rapid clearance of PB blasts after five days of treatment with dasatinib and sunitinib, after which gastrointestinal toxicity led to therapy cessation. When receiving ruxolitinib-dexamethasone therapy, patient 1145 showed an improvement in PB counts leading from a constant need for transfusions to normal blood counts.

In study I, two patient cases, 600 and 784, were examined in detail. Patient 784, a 37-year-old woman, was diagnosed with high-risk AML with the *MLL–ELL* fusion gene (t(11;19)(q23;p13.1)) and *FLT3-ITD*. Since the patient was chemotherapy resistant, we explored further treatment options using the ISM platform. Initial DSRT revealed leukemia-selective responses to several compounds, including TKIs such as dasatinib, and to rapalogs, and MEK inhibitors. The patient achieved a transient response with dasatinib-sunitinib-temsirolimus therapy; BM blasts decreased from 70 to 35%, but then the disease progressed rapidly. The repeated DSRT indicated a loss of sensitivity to both dasatinib and sunitinib, although the sensitivity to ATP-competitive mechanistic target of rapamycin (mTOR) inhibitors remained. Furthermore, the resistant sample gained new vulnerabilities, such as sensitivity to BMS-754807 (insulin-like growth factor-I receptor (IGF-IR) / Trk inhibitor), to several immunomodulatory and differentiating agents, as well to crizotinib (TKI) and tipifarnib (farnesyl-transferase inhibitor). Comparing the putative driver kinases in these two samples revealed a conversion in the kinase dependence along with gaining an addiction to ALK and Trk family receptor tyrosine kinases and a loss of an addiction to SRC family kinases, PI3K, and mitogen-activated protein kinase (MAPK). Importantly, the dasatinib-sunitinib-temsirolimus resistant sample showed

more than a 1000-fold enrichment of two fusion transcripts, *ETV6–NTRK3* and *STRN-ALK*, suggesting that resistance might have resulted from the selection of a resistant clone. The *ETV6–NTRK3* results in the TEL–TrkC fusion protein which, interestingly, appears to lead to IGF-IR activity and to the hyperactivation of mTORC1.[187-189]

Patient /sample	DSRT guided therapy	Therapy duration (days)	Disease state	Treatment response (ELN)	Additional information	Time to progression (weeks)	Treatment related toxicity
252	Dasatinib	59	Relapsed, resistant disease	Resistant disease	Bone marrow blasts: 65-40-75%	8	No
560_1	Dasatinib-temsirolimus	34	Relapsed, remission	Resistant disease	Induction with plerixafor-MAC	4	No
560_2	Dasatinib-sunitinib	5	Relapsed	Not evaluable	Blood blasts: 34-0% Monocytes: 7-49%	N/A	Neutropenic fever, diarrhea
600	Dasatinib-sunitinib-temsirolimus	44	Relapsed, resistant disease	CRi		6	Diarrhea (gr 3), neutropenic fever (gr 2)
718	Sorafenib-clofarabine	63	Relapsed, resistant disease	Morphologic leukemia-free state	Hypoplasia	Lost to follow-up	Bone marrow hypoplasia (gr 4)
784_1	Dasatinib-sunitinib-temsirolimus	13	Resistant disease	Resistant disease	Bone marrow blasts: 70-35-85%	Not evaluable	Neutropenic infection (gr 2)
784_2	Cytarabine-etoposide-sunitinib-vinblastine	30	Resistant disease	Resistant disease	Bone marrow blasts 85-30%	Bridged to HSCT	Neutropenic infection (gr3)
800	Clofarabine-dasatinib-vinblastine	6	Resistant disease	Morphologic leukemia-free state	Hypoplasia	Hypoplasia, no progression	Infection (gr 4), death in aplasia (gr 5)
1145	Ruxolitinib-dexametason	6	Relapsed, resistant disease	Resistant disease	Hematologic improvement	6	No

Table 1. Responses in patients treated with DSRT-guided therapies.

Patient 600 was a 54-year-old man at the time of diagnosis with cytogenetically normal, *FLT3-ITD*-positive AML M5. Upon diagnosis, a high PB blast cell count and signs of extramedullary leukemia (splenomegalia and gum hyperplasia) were observed. Induction therapy with cytarabine, idarubicin, and thioguanine was initiated and BM hypoplasia was achieved. A treatment delay due to an infection-related multi-organ failure resulted in leukemia relapse. Three re-induction regimens (cytarabine-idarubicine-thioguanine, clofarabine-cytarabine-etoposide, and plerixafor-clofarabine-idarubicin-lenalidomide) were administered without a response. **Figure 4a** provides a summary of the response to the targeted therapy. In DSRT, the blasts showed a selective sensitivity to the mTOR inhibitor temsirolimus

(sDSS 8,6) multikinase inhibitors dasatinib (sDSS 12,8), and sunitinib (sDSS 16,5) (**Figure 4b**). After initiating this combination, the BM blast cell count rapidly decreased and the PB neutrophil count normalized, although the platelet count remained low (CRi). At 30 days from response, relapse occurred. Repeat DSRT showed a loss of sensitivity reflecting a solid correlation between *ex vivo* and *in vivo* sensitivity and resistance (**Figure 4b** and **4c**).

Transcriptome (RNA) sequencing revealed a *NUP98-NSD1* fusion. The *NUP98-NSD1* fusion gene represents a recurrent aberration in pediatric AML, occurring in 17% of cytogenically normal AML patients and strongly associated with *FLT3-ITD* mutations. In adult AML, the fusion is detected in 2% to 3% of patients.[190] The transforming property of *NUP98–NSD1* involves a block in cellular differentiation and enforced leukemic progenitor self-renewal through the persistent overexpression of distinct *HOX* genes. The *NUP98–NSD1* fusion was also detected in the diagnostic sample and all later follow-up samples, verifying that this fusion represented the initiating event in the disease. Exome sequencing showed a diverse clonal architecture highlighted by *FLT3-ITD* and *WT1* mutations. After induction chemotherapy, the predominant *FLT3-ITD* subclone was no longer detectable, while subsequent therapies, including combining dasatinib, sunitinib, and temsirolimus combination, induced a redistribution of the subclones containing *WT1* mutations and a second *FLT3-ITD*. (**Figure 4d**)

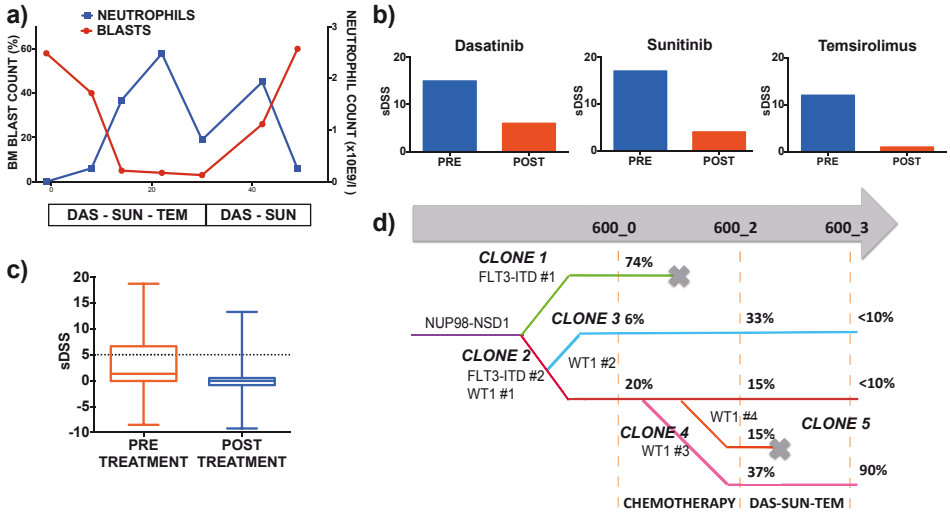


Figure 4. Clinical implementation of DSRT results and clonal evolution in a treatment-refractory AML patient. (a) The effect of DSRT-guided therapy (dasatinib-sunitinib-temsirolimus) on the BM blast count and PB neutrophil count. The combination therapy resulted in a rapid decrease in blasts and a normalization of neutrophils (CRi). **(b)** The blasts showed a selective sensitivity to the mTOR inhibitor temsirolimus and multikinase inhibitors dasatinib and sunitinib. At the time of relapse, repeated DSRT showed a loss of sensitivity. **(c)** The DSRT for all tested agents show a general loss of sensitivity in the post-treatment sample. **d)** Clonal evolution of AML from diagnosis to the last relapse in a patient. Abbreviations: Das, dasatinib; Sun, sunitinib: Tem, temsirolimus; Pre, pre-treatment; Post, post-treatment.

2. NOVEL ACTIVATING *STAT5B* MUTATIONS AS DRIVERS OF T-ALL

2.1 The functional effects of different *STAT5B* mutations

In study II, we performed exome sequencing on the blast cells of an 18-year-old woman, who previously received chemotherapy and HSCT for T-ALL and then relapsed 40 months after HSCT. At the time of relapse, exome sequencing identified three different *STAT5B* mutations in the blast cells. These included the N642H and T648S located in the SH2 domain, with the third mutation I704L located in the transactivation domain (**Figure 5**). Capillary sequencing further also validated these mutations. Other mutations detected in exome sequencing were *NOTCH1*, *KRAS*, *MED12, SUZ12*, and *KDM6A.*

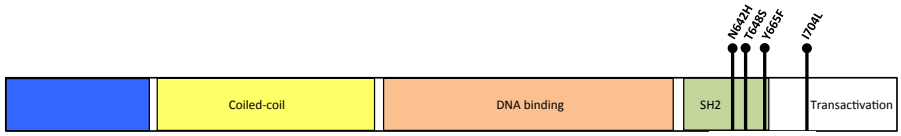


Figure 5. Representation of STAT5B protein domains and the localization of the identified mutations. The mutations N642H, T648S and Y665F are located in the SH2 domain, while the mutation I704L is located in the transactivation domain.

We first evaluated the functional effects of all three different *STAT5B* mutations detected in the index patient. To evaluate the effect of these mutations on STAT5B phosphorylation, we used STAT5B expression plasmids containing WT, single, double or triple mutations to transfect HeLa cells. Western blot analysis showed the mutation in N642H to induce strong constitutive phosphorylation of STAT5B, whereas the I704L mutation carried a smaller effect on phosphorylation (**Figure 6a**). By co-transfecting HeLa cells with luciferase transporter plasmids, we compared the effect of different mutations on the transcriptional activity. Compared with WT *STAT5B*, the N642H and I704L mutants led to a 26- and 17-fold increased transcriptional activity, respectively, while the mutation in T648S had no effect. The construct with both the N642H and I704L mutations led to a slightly enhanced transcriptional activity compared with the activity induced by either mutation alone, whereas a construct containing all three *STAT5B* mutations (i.e., N642H, T648S, and I704L) led to a 38-fold increase in transcriptional activity (**Figure 6b**).

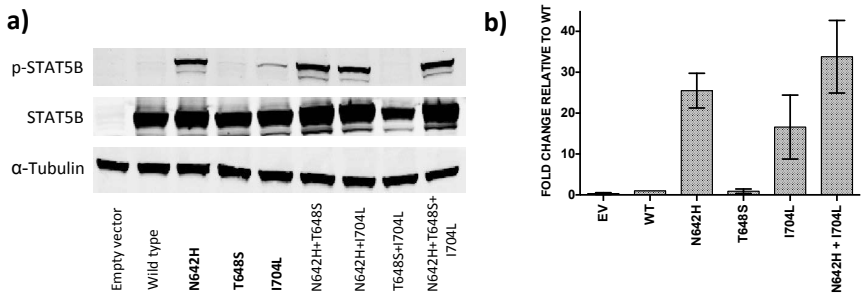


Figure 6. The functional effect of detected *STAT5B* mutations.
(a) STAT5B expression plasmids containing single, double, or triple mutations were used for the transfection of HeLa cells. One day after transfection, we performed a Western blot analysis. Parallel gels with identical loading were run from the same protein lysates to quantify the phosphorylated STAT5 (pSTAT5; Tyr699) and total STAT5 levels. Alpha-tubulin was used as an internal control. **(b)** The transcriptional activity of selected *STAT5B* mutations and co-mutations. HeLa cells were co-transfected with luciferase transporter plasmids and mutation constructs. The graph shows the X-fold change and SDs relative to WT from three repeated experiments. Abbreviations: EV, empty vector; WT, wild type.

2.2. *Ex vivo* drug sensitivity of *STAT5B* mutated blast cells

DSRT showed that the blasts exhibited sensitivity to the pan-BCL-2 navitoclax (EC_{50} 82 nM). The blasts did not display sensitivity (EC_{50} >1 µM) to PI3K inhibitors (idelalisib and XL147), RAC-alpha serine/threonine-protein kinase (AKT1) inhibitors (MK-2220), or rapalogs (temsirolimus and everolimus). The blasts were also non-responsive to dual inhibitors of PI3K and MTOR (PF-04691502 and dactolisib) as well as to the JAK inhibitors (ruxolitinib and tofacitinib) (**Figure 7**). In addition to the navitoclax sensitivity, the blasts also responded to corticosteroids and MEK inhibitors. The sensitivity to MEK inhibitors likely results from the *NRAS* mutation observed in the relapsed sample.

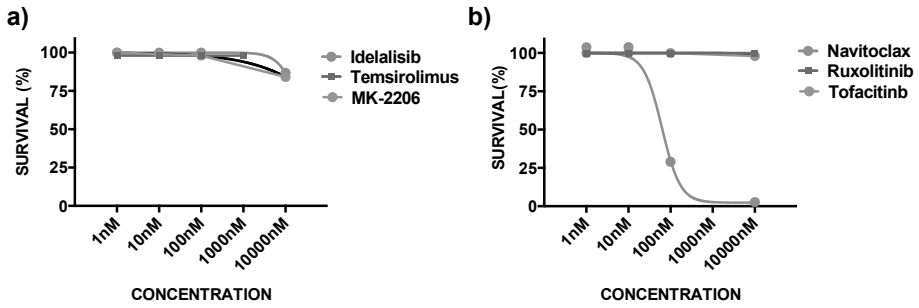


Figure 7. The ex vivo drug sensitivity of index patient blast cells. The ensitivity of inhibitors targeting the PI3K-AKT-MTOR pathway (**a**) and JAK-STAT pathway, including navitoclax (**b**).

To evaluate the expression of different anti-apoptotic *BCL-2* family members (e.g. *BCL-2, BCL-XL,* and *MCL-1*) in the index patient, we performed RQ-PCR on the blast cells. RNA extracted from the blast cells of two T-ALL patients with no *STAT5B* mutation and a CD3-positive lymphocyte fraction from a healthy donor PB served as controls. *STAT5B*-mutated blasts showed a similar expression on *BCL-2* and *MCL-1*, whereas, interestingly, *BCL-XL* expression in the *STAT5B*-mutated blasts were 12- and 4-fold in the diagnostic and relapse samples, respectively.

2.3. Prevalence of *STAT5B* mutations in T-ALL

Finally, to evaluate the prevalence of *STAT* mutations in T-ALL, we sequenced the SH2 dimerization and the transactivation domains of *STAT5B* (exons 14–19), exon 21 of *STAT3,* and also exon 17 of *STAT5A*, which encode the SH2 dimerization domains. The initial sample cohort included 17 patients from the Helsinki University Hospital. Since we found no *STAT3* or *STAT5* mutations in this cohort, we collected a larger validation cohort of 64 adult and 4 pediatric T-ALL patients. Sequencing revealed *STAT5B* mutations in five additional patients, all of which occurred in the SH2 domain (**Table 2**). One patient carried Y665F mutation, whereas four patients shared the same N642H mutation as the index patient. No mutations in *STAT3* or *STAT5A* were found.

Sex	Immunologic subtype	Age	Mutation	Variant allele frequency
Male	Cortical T	39	N642H	0.92
Male	Cortical T	44	N642H	0.40
Male	Mature T	45	Y665F	0.19
Male	Mature T	59	N642H	0.47
Female	Pre-T	18	N642H	0.46
			I704L	0.48
			T648S	0.49
Male	Pre-T	24	N642H	0.6

Table 2. *STAT5B* mutations detected in 6/68 patients. All patients, except one, had mutation in locus 642. Most mutations were likely heterogeneous. One patient with high (0.92) variant allele frequency had likely lost the wild type copy of the gene (i.e. loss-of-heterozygosity).

3. FACTORS PREDICTING SENSITIVITY TO BCL-2 INHIBITION IN AML

3.1. Correlation and grouping of responses to BCL-2 inhibition

In study III, we explored the potential biomarkers for BCL2-inhibitor sensitivity in AML. We first evaluated the venetoclax and navitoclax sensitivity in AML, CLL and in MNC fraction of healthy BM. MNC of healthy donors all showed a low responsiveness, whereas AML samples, by contrast, showed heterogeneous responses to BCL-2 inhibition (DSS values presented in **Figure 8**). All CLL samples tested exhibited a high sensitivity to BCL-2 inhibition: for venetoclax sDSS values were 31.0, 30.4 and 32.1; and for navitoclax respectively: 23.8, 21.0 and 29.1. The BCL-2-selective venetoclax had a slightly weaker effect in healthy control MNCs compared with the dual BCL-2/BCL-XL inhibitor navitoclax (median DSS 7.3 versus 10.2; paired *t*-test $P = 0.03$).

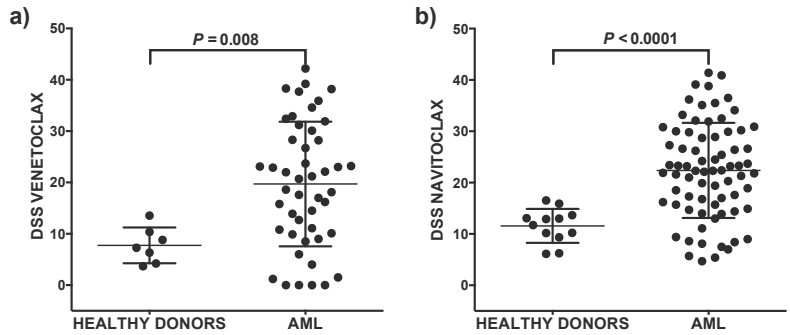


Figure 8. Responses of venetoclax and navitoclax in AML and healthy control BM MNC fractions. Both venetoclax (**a**) and navitoclax (**b**) exhibit AML-selective responses. Mean, SD and results of unpaired *t*-test are shown.

The responses for navitoclax and venetoclax were highly correlated (Pearson's r 0.88, $P < 0.0001$; **Figure 9a**). The diagnostic samples showed a slightly higher sensitivity to venetoclax, while the refractory samples exhibited a lower sensitivity to venetoclax compared to the dual-inhibitor navitoclax. These differences were minor, and thus unlikely to carry biological relevance. Importantly, in addition to the diagnostic samples, we observed an intermediate or high sensitivity in samples from relapsed or refractory samples (diagnostic: 13/19, 68%; relapsed or refractory: 19/28, 67%; **Figure 9b)**.

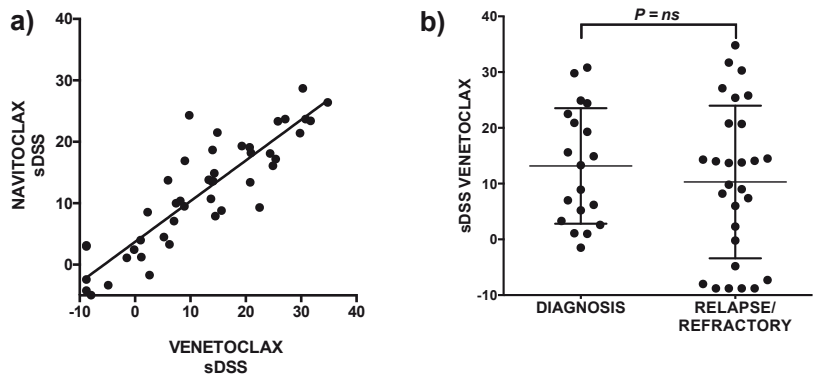


Figure 9: Correlation of navitoclax and ventoclax responses (a) and venetoclax response in different disease states (b).

To determine whether specific biomarkers correlate with molecular profiling analyses, we divided the venetoclax responses into three groups. First, the resistant group included samples exhibiting lower or comparable responses to those observed in healthy donors. This group was defined by the upper confidence level (95%) of the median DSS for venetoclax among healthy controls (sDSS < 5.8) and included 15 of 47 (32%) of all venetoclax tested AML patients. Second, the highly sensitive subgroup was defined by a BCL-2 inhibitor response similar to that observed among samples from CLL patients. This group included 7 of 47 (15%) of AML patients. Finally, the intermediate group included 25 of 47 (53%) of AML patients exhibiting a sensitivity in between the highly sensitive and resistant groups (translating to sDSS values 5.8–25.5). (**Figure 10**)

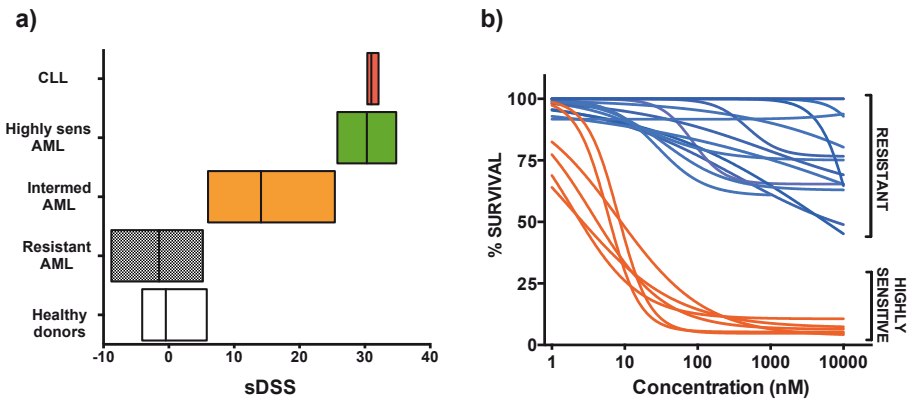


Figure 10. Venetoclax responses. (**a**) The responses were divided into three groups: resistant, intermediate, and high ("CLL-like") sensitivity. (**b**) Dose response curves in the highly sensitive and resistant samples. Abbreviations: intermed, intermediate sensitivity.

3.2. Gene expression studies

RNA-sequencing data from four venetoclax resistant and three highly sensitive samples were compared to identify possible biomarkers for sensitivity. Normalized read counts were used to produce differential gene expression between these groups. The analysis showed 322 differentially expressed genes between the sensitive and resistant groups with a false discovery rate of <0.05. Next, 41 overexpressed genes in the sensitive group were further analyzed for their biological function. Interestingly, we found that several *HOX* genes were overexpressed in sensitive samples. We next clustered all 16 samples with the available RNA sequencing data according to the expression of HOX genes. Importantly, we detected a general *HOXA* and *HOXB* overexpression profile in the sensitive samples and a low expression in the resistant samples (**Figure 11**). Samples exhibiting an intermediate sensitivity primarily clustered between these two groups. We validated these results using RQ-QPR by comparing the mRNA expression of resistant samples to the expression observed in highly sensitive samples. Venetoclax sensitivity correlated significantly with seven *HOX* genes (*HOXA2*, *HOXA3*, *HOXA5*, *HOXA6*, *HOXA7*, *HOXA9* and *HOXB2).*

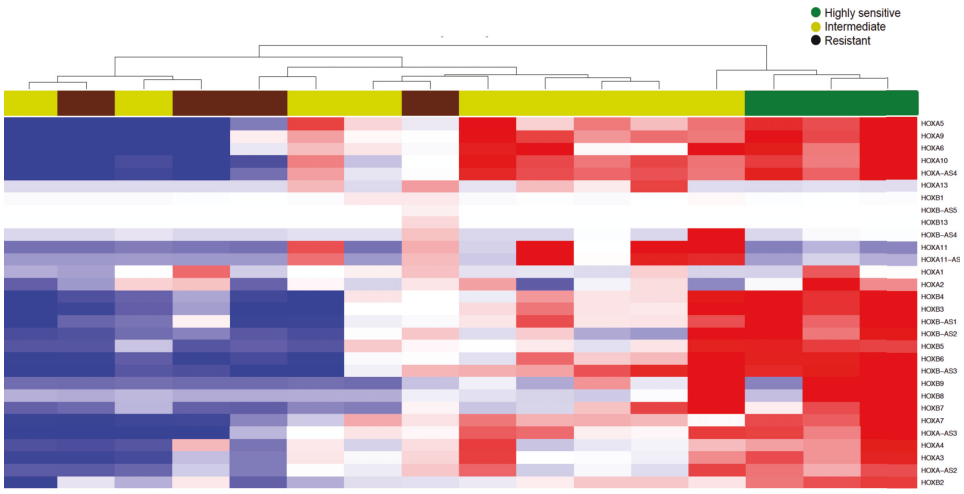


Figure 11. Expression of *HOXA* and *HOXB* family genes in 16 AML patient samples (4 resistant, 9 intermediate, and 3 highly sensitive samples) derived from RNA sequencing data. Samples were clustered using Euclidean distance measures of mean-centered log2 of the reads per kilobase of transcript per million mapped reads (RPKM) values. Color key for gene expression: red, overexpressed; blue, underexpressed.

We also explored the correlation of BCL-2 family gene mRNA expression and venetoclax sensitivity. We observed no correlation between the venetoclax response and the expression of anti-apoptotic *BCL-2* or *MCL1* genes. Although we observed a positive correlation between *BCL-XL* expression and resistance, we also detected

unexpected correlations (e.g., an inverse correlation for two pro-apoptotic mRNAs, *BIM* and *PUMA*). Thus, since several BCL-2 family members are modified during or after transcription and translation, it is likely that the mRNA level of BCL-2 family genes insufficiently reflects the status of the anti-apoptotic balance and are not potential biomarkers. Interestingly, we observed a high mRNA expression for *B2M* in venetoclax-resistant samples and a significant inverse correlation with sensitivity. B2M represents a well-established biomarker in lymphatic malignancies, but the precise effect of B2M to BCL-2 resistance still requires further investigation.

3.3. Exome sequencing studies

Next, we investigated data from 48 exome sequenced AML samples. As previously reported[114], all six samples with *IDH1* or *IDH2* mutations exhibited a sensitivity to BCL-2 inhibitors. Importantly, we found that all samples with mutations in histone-modifying genes (three samples with *NUP98-NSD1* translocation, three samples with *MLL* fusions and one sample with *ASXL1* mutation) displayed a sensitivity to BCL-2 inhibitors.

We also observed responses to the BCL-2 inhibition in samples with a monosomal karyotype. Indeed, four of six samples with a loss of function for *TP53* or *17p* deletion showed sensitivity to BCL-2 inhibition. One patient (samples 3443_3 and 3443_6) with an observed heterozygous *17p* deletion was resistant to BCL-2 inhibition. Subsequently, while we observed a trend towards a higher venetoclax sensitivity in samples with noncomplex karyotypes, the difference did not achieve statistical significance (median sDSS for complex karyotype samples 8.9; for noncomplex karyotype, 13.7. $P = 0.23$).

4. ESTABLISHMENT OF A NATIONAL BIOBANK

4.1. Background and methods

In study IV, we developed a national biobank allowing researchers to access high-quality samples, thus enabling the research community in general to develop treatment, diagnostics, and an understanding of the pathophysiology of hematological malignancies. The establishment of the Finnish Hematology Registry and Biobank (FHRB) represents a collaborative effort among three agencies, the Finnish Association of Hematology (FAH), the Finnish Red Cross Blood Service (FRCBS), and the Institute for Molecular Medicine Finland (FIMM). FHRB, a nation-wide disease-oriented and population-based biobank, was originally launched as a research project in November 2011 and received authorization as a licensed biobank in 2014.

Samples are collected based on broad informed consent as outlined by the Finnish Biobank Act. Samples were collected at three time points: diagnosis, potential remission, and relapse. Sample types include BM (diagnosis and relapse: 30 mL, remission 10 mL), blood (40 ml), and skin punch biopsy (diameter 4 mm) in order to obtain information on the germline genome. In addition, comprehensive clinical data is collected through a clinical web-based registry. All samples are shipped to FRCBS where they are coded, processed, aliquoted, and frozen. Sample collection occurs at all university hospitals (n = 5) as well as several central hospitals (n = 6). All participating hospitals are contractual partners of FHRB (**Figure 12**).

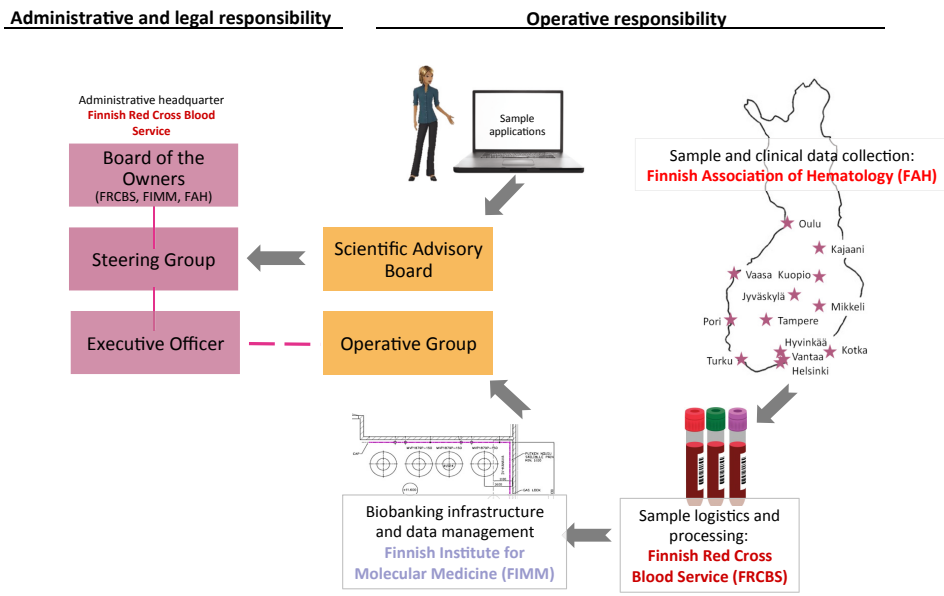


Figure 12. Organization of FHRB. Samples and data are collected nationally by participating hospitals. Sample processing, storage, and delivery are centralized at FRCBS and FIMM. The stars on the map represent the hospitals currently collecting samples and corresponding clinical data.

4.2. Sample collection, quality and access principles

As of February 2017, FHRB contained 68,000 sample aliquots collected from 1,270 patients diagnosed with a hematological disease. Most of the samples (70%) were collected at the time of diagnosis. **Figure 14** presents the proportion of diagnoses and the accumulation of samples. All processes in FHRB are based on standard operating procedures. Detailed directions describe how samples are collected, transported, processed, and stored. The quality of the frozen samples is evaluated on a regular basis. **Figure 15** provides the results of the quality control assessments.

FHRB accepts sample requests from both academic and industrial research groups worldwide. Requests are reviewed by an independent panel of experts, which evaluates the feasibility and scientific significance of the proposed study.

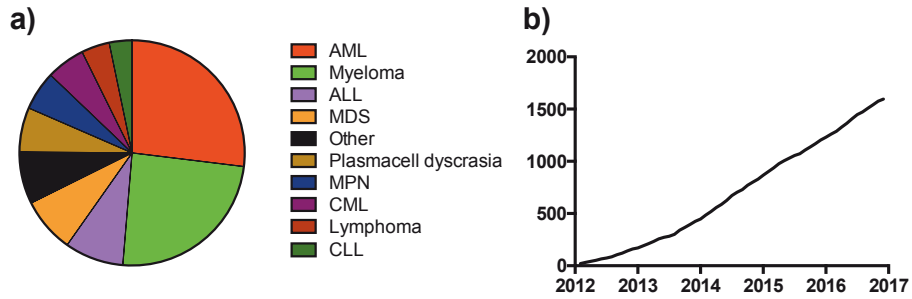


Figure 14. FHRB samples (as of February 2017). **(a)** The proportion of diagnoses in FHRB. **(b)** The accumulation of samples.

Based on the recommendation of the Scientific Advisory Board, the Steering Group makes the final decision on sample requests. Coded specimens, together with the corresponding annotated clinical data, are then provided to research groups. Results from the research projects are returned to the biobank to enhance future projects.

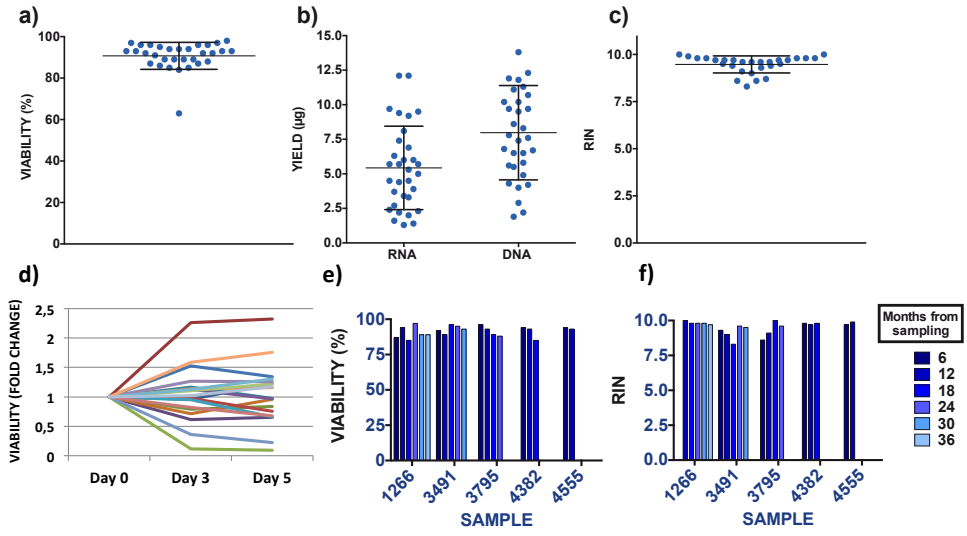


Figure 15. Quality control of vitally frozen biobanked AML samples. (a) Viability of vitally frozen cells after thawing**. (b)** DNA and RNA yield from thawed cells. **(c)** RNA integrity number (RIN) values of RNA extracted from thawed cells**. (d)** The viability of biobanked, thawed BM MNC in a culture at day 3 and day 5 in conditioning media (Riikka Karjalainen et al. submitted). **(e)** The viability of thawed BM MNC. The viability is measured biannually from sampling. **(f)** RIN values measured from the same samples. In panels a, b, and c, the mean and SD are shown.

DISCUSSION

The modern era of chemotherapy to treat acute leukemia was initially started with the folic acid antagonist aminopterin in late 1940s by Sidney Farber.[191] Because monotherapy failed to produce long-lasting remissions, the road to current therapy protocols—and to a potential cure—was paved by NCI-driven combination trials in the 1960s, led by Frei and Freireich.[192] Although the therapy has remained nearly unchanged since that era, the absence of progress has not resulted from a lack of effort. Decades later, our understanding of AML pathophysiology has significantly improved but, as yet, it has not translated to successful therapies in most AML patients. In part, this is due to our inability to characterize individual disease in the requisite detail and, consequently, the evolutionary potential of each individual cancer remains unidentified.[193]

In this study, we integrated genomic and molecular profiling data with systematic drug-testing results from a comprehensive library of anti-cancer agents in patients with acute leukemia. We showed that the individualized systems medicine (ISM) platform can produce comprehensive patient-to-patient data to facilitate understanding of an individual patient's disease biology and, in select cases, translate this information into successful guided therapies. We have demonstrated that the platform can be utilized as a tool to repurpose anti-cancer agents. In addition, comprehensive data facilitate the discovery of novel biomarkers and efficiently characterize new genomic subsets of acute leukemia with specific vulnerabilities to targeted therapies.

1. INDIVIDUALIZED SYSTEMS MEDICINE (ISM) PLATFORM

Despite abiding efforts, the *ex vivo* assessment of drug sensitivity to predict anticancer effects in patients remains a distant goal.[144] As such, our studies have several advantages over previous efforts. First, we concentrated on targeted therapy agents, whereas several previous studies focused on conventional chemotherapeutics.[145-151] The response assessment of conventional chemotherapeutics partly depends on *in vitro* proliferation and, thus, results from non-proliferative primary patient samples are both difficult to interpret and difficult to translate into clinical patient care. Second, we focused on leukemias, in which representative tumor samples are easily accessed—even for repeated sampling. Third, we developed a method to assess leukemia-specific responses, making it possible to identify compounds with less toxicity to healthy hematopoiesis. Finally, we also utilized genomic and transcriptomic data to evaluate the biology of individual disease and to complement the weaknesses of functional testing alone.

In study I, we found that several approved anticancer agents such as dasatinib, sunitinib, temsirolimus and ruxolitinib to exhibit leukemia selective responses in

AML patient samples.[194] We also identified several investigational compound classes such as inhibitors of MEK, JAK, BCL-2/BCL-XL and PI3K/AKT/mTOR pathways to demonstrate a leukemia-selective effect. Thus, the ISM platform can identify promising compounds, both investigational and approved, for further evaluation in clinical AML trials. We also demonstrated that the platform-produced data are useful for guiding therapies for relapsed and resistant AML patients. We observed an overall response rate of 33% (3/9), which is a favorable result in this patient population. Since all responses appeared short-lived, we could study distinct resistance mechanisms by combining data from different platforms. For those cases studied in detail, the resistance was mediated either by clonal shift (as exemplified in patient 784), or through the loss of pathway dependence with no acquired mutations (as exemplified in patient 600).

Despite the genomic heterogeneity of AML, an unsupervised hierarchical clustering analysis divided patient-specific drug responses into five categories. This finding is of particular interest, since other large genomic studies have shown AML to divide into limited number of subgroups. For example, in The Cancer Genome Atlas (TCGA) study, the 200 *de novo* AML patients clustered into seven separate groups according to the gene expression profile. This grouping also partially reflected the prognosis.[33] Interestingly, a recent study showed that patterns of co-mutation divide AML patients into 11 classes, each with distinct diagnostic features and clinical outcomes.[31] Thus, this drug response–driven grouping may identify specific leukemia subsets sensitive to specific targeted therapies.

Parallel to our work, a systematic approach using *ex vivo* drug sensitivity testing at Oregon University was published in 2013.[195] This study also illustrated the potential of *ex vivo* screening to identify functionally important kinase targets. The group assessed the sensitivity of 151 primary leukemia patient samples using a panel of 66 small-molecule kinase inhibitors. Comparable to our results, the leukemia samples exhibited a heterogeneous variety of drug sensitivities: overall, 70% of the samples exhibited a sensitivity to one or more of the kinase inhibitors tested. Investigators developed an algorithm to predict kinase pathway dependence, and as a proof-of-concept, they used this algorithm to tailor therapy for a relapsed, refractory patient with a rare FLT3 mutation. The therapy utilizing both sorafenib and sunitinib led to a three-month treatment response.[195] Later, Tyner adopted the term "functional genomics"[196] to refer to a similar approach in which a patient's cancer cell sample was both sequenced and tested using high-throughput drug sensitivity screening. In addition to individualized treatment decisions, such a platform allows data collection from a large number of patient samples, ultimately yielding sufficient data to create algorithms to produce informed recommendations for treatment choices based only on sequencing results. Such data are currently being collected in the Beat AML 1.0 project.[174,196]

In a broader context, these platforms provide tools to unravel specific vulnerabilities for certain disease subsets, potentially offering novel or repurposed therapies for patients. This potential was demonstrated in our study on *BCR-ABL*-driven diseases with T315I point mutations. These mutations lead to the impaired binding of TKIs to the ABL1 kinase domain and, thus, broad-range TKI resistance. Interestingly, we found patients with T315I-mutated *BCR-ABL1* to exhibit leukemia-selective responses to VEGFR inhibitor axitinib, thus presenting a novel treatment option for these patients.[197] Similarly, Tyner and colleagues applied integrated data from patients with atypical CML to unlock the pathogenesis and drug sensitivity of this disease. Oncogenic mutations within two distinct regions of *CSF3R* appear to activate downstream kinase signaling either through Src family-TNK2 or Janus family kinases (JAKs), which could be selectively targeted using dasatinib and ruxolitinib, respectively.[198]

These approaches share several limitations. In *ex vivo* high-throughput drug screening, antibodies, or other biological therapies cannot be routinely explored, since these compounds require a sufficient amount of other cell populations (including lymphocytes) to demonstrate their efficacy. Furthermore, the effect of BM and stoma-cell adhesion signaling remains inadequately captured. Co-culturing primary blasts with stroma cells or with media isolated from stromal cell cultures may mimic the protective effect of stroma,[199] but additional studies to correlate *ex vivo* and clinical responses are still needed. Additionally, existing platforms remain suboptimal for exploring the sensitivity of slow-acting and differentiating compounds such as hypomethylating agents (i.e., azacytidine and decitabine). We may be able to evaluate their effect with a longer incubation using readouts also capable of capturing *in vitro* differentiation. A drug-screening assay using the iQue PLUS high-throughput flow cytometry instrument (Intellicyt, Albuquerque, NM, USA) may allow for a more comprehensive understanding of the drug effects (including differentiation) on AML blasts.[200] This approach might also allow the use of unselected patient-derived material to evaluate biological compound efficacy. Finally, while the complexity of individual disease can be understood, a complete understanding of the evolutionary power leading to the selection of different clones or a switch in the pathway dependence remains an elusive goal. Currently, amplicon-based methods enable retrospective reconstruction of the clonal architecture from diagnosis to relapse.[47] As the sequencing turn-around time shortens, in future these applications might also be applied to real-time monitoring. Rather opportunely, the platform carries as yet unexplored capabilities. Interestingly, several multi-kinase inhibitors (such as midostaurin and sorafenib) providing clinical benefits in combination with conventional chemotherapy[70,71] also inhibit ABC proteins.[67,68] Thus, the effect of mutations or polymorphisms modifying ABC protein functioning as well as the potential effect of ABC protein inhibitors on drug sensitivity should be explored in a more comprehensive manner.

2. DISSECTING DISEASES TO SUBENTITIES - *STAT5B* MUTATIONS IN T-ALL

In study II, we found *STAT5B* N642H mutation to be a recurrent event in T-ALL, with a prevalence of 9% in an adult-biased population.[139] In addition, our work showed that in T-ALL, *STAT5B* mutations are activating, leading to the enhanced phosphorylation of STAT5B and to increased target-gene expression. Previous work on transgenic mouse models showed that the overexpression of STAT5A or STAT5B leads to thymic T-cell lymphoma.[201] Similarly, in T-LGL, the *STAT5B* N642H mutation had been associated with a very unusual aggressive and fatal phenotype.[137] Thus, we hypothesized that *STATB* mutations probably represent a novel activation mechanism to the IL7R-JAK-STAT pathway and may also alter the disease phenotype. Subsequently, other groups verified our findings that the N642H mutation represents a recurrent event in T-ALL. In pediatric patients, *STAT5B* N642H mutations have been detected in 6.3% of diagnostic and in 9% of relapsed cases.[141] Pediatric patients with the *STAT5B* mutation also appear to have a higher risk of relapse.[141] In another cohort including both pediatric and adult patients, *STAT5B* N642H mutation was detected in 6.5% of patients. An additional 2 of 31 patients had *STAT5B* mutations located at a different position (i.e., S434L and Y665F).[202] Furthermore, a subsequent study detected aberrations in the JAK-STAT pathway to associate with a higher risk of relapse, as well as inferior overall and event-free survival.[203] Contrary to these studies, Ma et al. failed to demonstrate that *STAT5B* represents a recurrent event. They found a N642H mutation only in 1 of 28 pediatric patients, and found it missing completely from a cohort of 65 adult T-ALL patients.[204] Taken together, we and others demonstrated *STAT5B* mutation to be recurrent event in T-ALL; importantly, later work found that *STAT5B* N642 mutation carries prognostic significance, thus strengthening the hypothesis of its biological significance.

Curiously, our index T-ALL patient had three different somatic *STAT5B* mutations, all located in the same allele. Mutation T648S alone had no effect on transcriptional activity, while N642H led to strong constitutive phosphorylation. This suggests that this SH2 domain mutation stabilizes the phosphorylated dimer, whereas the transactivation domain mutation I704L possibly enhances the transcriptional activity. Although the finding of three co-occurring *STA5B* mutations is fascinating, subsequent work has failed to demonstrate similar patient cases with several mutations. Importantly, we observed high *BCL-XL* expression in our index patient, while observing the expression of other *BCL-2* family genes at levels similar to those among controls. Together with the data on navitoclax sensitivity, this finding suggests that in *STAT5B*-mutated cells inhibiting BCL-XL can trigger apoptosis. Correspondingly, several preclinical cell line studies demonstrated that STAT5B induces *BCL-XL* expression by directly binding to the promoter of the *BCL-XL* gene.[205-207] On the contrary, however, Bandapalli and coworkers showed that the N642H mutation led to elevated BCL-2 expression, accompanied by strong

overexpression of both PIM and SOCS2.[141] In their study, they did not explore the expression of *BCL-XL*. Subsequent studies showed T-ALL cell lines and primary patient samples to be dependent on the BCL-2 family in a maturation state–related manner. As such, immature T-ALL samples, including ETP-ALL, depend on BCL-2 expression, whereas more mature T-ALL samples depend on BCL-XL expression and may, thus, be exploited by navitoclax.[208,209] Future studies are needed to establish the effect of IL7R-JAK-STAT activation and *STAT5B* mutation on BCL-2 family dependence and vulnerabilities.

3. DETECTION OF BIOMARKERS – BCL2 INHIBITOR SENSITIVITY AND *HOX* GENES IN AML

In study III, we evaluated possible biomarkers for BCL-2 sensitivity in AML.[210] Our most important observation included the detection of the broad-spectrum overexpression of both *HOXA* and *HOXB* genes in venetoclax-sensitive samples and a low or absent *HOX* gene expression in resistant samples. In healthy human hematopoiesis, *HOX* gene expression is largely restricted to hematopoietic stem cells and progenitor cells.[25,26] As in AML, *HOX* expression appears to be highly regulated and, intriguingly, recent work suggests that the similarity between normal and leukemic expression patterns indicates that most *HOX* expression in AML reflects a normal stem cell state captured in transformed cells.[27] Interestingly, previous work has demonstrated that hematopoietic stem cells depend on MCL-2, whereas leukemic stem cells depend on BCL-2.[99] Correspondingly, this was illustrated in a preclinical study where the apoptosis of progenitor cells of patients with high-risk MDS or secondary AML was triggered by BCL-2-only inhibition.[211] Thus, we can argue that previous findings on stem or progenitor sensitivity, taken alongside our findings on *HOX* gene expression, may characterize a stem/progenitor cell-like subgroup of AML sensitive to targeted BCL-2 inhibition using venetoclax. Thus far, no other studies exploring gene expression as a biomarker for venetoclax sensitivity have been published. These results require validation in upcoming clinical trials.

Our study shows that several mutations associate with a leukemia-selective response to BCL-2 inhibition. *IDH1/2* mutated and 6 of 7 *WT1* mutated samples exhibited a leukemia-selective response to BCL-2 inhibition. *IDH1/2* and *WT1* mutations appear to share similar biological effects: *WT1* mutant patients have decreased 5-hydroxymethylcytosine levels, which is consistent with the reduced TET2 function observed in *TET2/IDH1/IDH2* mutant AML.[212] However, because *IDH1/2* mutations induce venetoclax sensitivity by a 2-HG mediated mechanism, it is likely that *WT1*-mutated AML presents different mechanism of sensitivity than *IDH1/IDH2*-mutated AML. In addition, samples with *MLL* fusions or *NUP98-NSD1* translocations exhibited a leukemia-selective response to BCL-2 inhibition. Previously, *MLL* fusion harboring AML cell lines, as well as ALL xenograft models, demonstrated venetoclax sensitivity, suggesting that *MLL* fusions have a common

role in BCL-2 mediated anti-apoptotic effects.[213,214] The proliferation and immortalization of HOXA9-driven leukemia specifically depend on *BCL-2* expression.[215] Intriguingly, both *MLL* fusion and *NUP98-NSD1* translocation have a similar biological effect; both fusions prompt the up-regulation of specific *HOXA* genes. In the case of *NUP98-NSD1,* this upregulation also accompanies the overexpression of distinct *HOXB* genes and *MEIS1*.[190,216] *TP53* mutations are enriched in secondary AML and are typically associated with chemoresistance.[217] Leukemia-selective responses were observed in 4 of 6 *TP53* mutated or deleted samples, which was expected, based on recent CLL studies showing responses in 17p-deleted patients.[105] Because *TP53* mutated AML patients presently lack treatment options, BCL-2 inhibitors should be considered for clinical trials in this patient group. Concordantly, we also observed responses across all cytogenetic groups, including among complex and monosomal karyotypes.

In addition, we found that venetoclax sensitivity correlated highly with navitoclax responses observing similar response profiles for both agents across all points in the disease phase (i.e., diagnosis, relapse, and refractory). In a phase II trial, responses were observed in 19% of relapsed/refractory AML patients.[98] Although patients responding in this trial received venetoclax therapy for a median 117 days, very limited data exist on the mechanisms leading to the loss of sensitivity. Several studies, primarily preclinical, demonstrated that high initial BCL-XL or MCL-1 protein levels both predict and generate acquired venetoclax resistance.[98,112,218] Rather, aberrations of BH3-domain causing venetoclax resistance appered infrequently.[119] Thus, the overexpression of anti-apoptotic proteins not targeted by venetoclax remains the most likely resistance mechanism, although its regularity remains unknown. Interestingly, recent preclinical studies on CLL show that the multikinase inhibitor sunitinib can overcome venetoclax resistance by downregulating the expression of BCL-XL and MCL-1.[219] Perhaps surprisingly, in ALL xenografts, effective anti-leukemic activity requires the concurrent inhibition of both BCL-2 and BCL-XL. Venetoclax alone appears capable of delaying disease progression, although the dual inhibition of BCL-2 and BCL-XL is necessary for an appropriate response. Similar to AML, BCL-XL expression predicted a poor response to venetoclax.[213] Additional studies are needed to explore venetoclax resistance mechanisms in AML and whether they can be circumvented through the dual or sequential inhibition of BCL-2 and BCL-XL.

Future work should attempt to unravel the mechanistic explanation for the link between *HOX* gene expression and venetoclax sensitivity. In ALL, the fusion gene *MLL/AF4*, which is produced through translocation t(4;11), appears to directly control the activation of the *BCL-2* expression via DOT1L-mediated H3K79 methylation. This results in a high *BCL-2* expression and sensitivity to BCL2-only inhibition using venetoclax.[220] Importantly, H3K79 methylation induces a concomitant elevated HOX gene expression.[216] As discussed above, we did not detect

a correlation between *BCL-2* gene expression and venetoclax sensitivity. However, because *BCL-2* expression coincides with *HOX* gene expression, further functional experiments should attempt to clarify the role of this interaction in AML.

4. ESTABLISHING RESOURCES FOR FUTURE RESEARCH

Although new therapy options carry the potential to improve treatment outcomes, identifying factors that predict responses and allowing for the use of targeted therapies only among patients expected to benefit remains challenging.[5] In order to achieve this, we must accumulate adequate high-quality patient sample collections together with comprehensive annotated clinical data that enables an understanding of factors that advance resistance or sensitivity. Samples and data are also needed to improve our understanding of the molecular mechanisms leading to disease progression and to identify potential new vulnerabilities.[221,222] In study IV by developing biobank-collected samples with accompanying clinically annotated data, we demonstrated that a nationwide collection is feasible and, importantly, capable of producing high-quality sample material for ongoing and further research.

5. FUTURE PERSPECTIVES

Most clinical trials still focus on monotherapy or, in the add-on setting, where novel agents are combined with existing chemotherapy. In most cases, this leads to increased toxicity and diminishes the potential benefits of the agent investigated.[223,224] Moreover, targeting post-onset driver mutations (e.g., *FLT3-ITD* and *RAS*) has produced disappointing short-lived responses, perhaps because of their inability to target disease-initiating mutations.[225] Success may lie in identifying ways to combine modern therapies in a more effective manner. Similar to Frei and Freireich, perhaps we need to return to the lessons learned from tuberculosis treatment. With an adequate understanding of AML pathophysiology and our current anti-leukemic arsenal, we may finally design increasingly successful combination therapies. To achieve efficiency, these combinations will hit several independent targets. Such targets include the leukemia-initiating aberrations and activated signaling (if any), combined with therapies independent of these events, such as immunological therapies, agents targeting leukemic stem cells, and conventional chemotherapeutic agents among responsive patients. We need to monitor pathway dependence in real-time and modify therapy accordingly, as well as further our understanding of pre-leukemic clones. This may include distinguishing a subgroup of patients with high-risk characteristics benefitting from pre-emptive therapy using novel agents. In addition, we must examine the effect of these agents in a maintenance therapy setting. To meet this challenge, we must also obtain comprehensive high-quality sample datasets with extensive clinical information from very distinct disease subsets.

CONCLUSIONS

In this study, we developed and evaluated novel techniques for individualizing therapy for acute leukemia patients. Our primary findings are as follows:

1. The ISM platform appears feasible for repurposing therapies for both individual AML patients and distinct disease subgroups. As such, the ISM platform allows us to study each disease and possible sensitivity and resistance mechanisms in a comprehensive manner.
2. These methods were used to uncover the prevalence of *STAT5B* mutations in T-ALL. We determined that these mutations represent recurrent events and demonstrated their activating characteristics. We presented a hypothesis regarding the possible vulnerability of *STAT5B*-mutated blasts for BCL-XL inhibition.
3. An extensive dataset from different platforms enables the detection of biomarkers for individualizing therapy. We identified a specific *HOX* gene expression pattern serving as a robust biomarker for venetoclax sensitivity *ex vivo*. Our results may identify BCL-2 inhibitor-sensitive AML subgroups for validation in upcoming clinical trials.
4. We developed a nationwide biobank for the collection of high-quality sample material accompanied by clinically annotated data to facilitate further research.

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