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BIOLOGICAL CHARACTERIZATION OF PHILADELPHIA CHROMOSOME-POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA

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ACADEMIC DISSERTATION

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To my dear husband Henri

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LIST OF ABBREVIATIONS

ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
alloHSCT	allogeneic hematopoietic stem cell transplantation
ASO	allele-specific oligonucleotide
BCL2	B-cell lymphoma-2
BCR	breakpoint cluster region
BM	bone marrow
CAR T	chimeric antigen receptor T cell
CD	cluster of differentiation
CML	chronic myeloid leukemia
CNA	copy number alteration
CNS	central nervous system
CR	complete remission
CRS	cytokine release syndrome
CTLA4	cytotoxic T-lymphocyte-associated protein 4
CVAD	cyclophosphamide, vincristine, doxorubicine, dexamethasone
DC	dendritic cell
ddPCR	digital droplet polymerase chain reaction
DSRT	drug sensitivity and resistance testing
DSS	drug sensitivity score
FDA	the United States Food and Drug Administration
FFPE	formalin-fixed and paraffin-embedded
FHR	Finnish Hematology Registry
FHRB	Finnish Hematology Registry and Clinical Biobank
FLG	Finnish Leukemia Group
HDAC	histone deacetylase
HRP	horseradish peroxidase
IKZF1	IKAROS family zinc finger 1
KD	kinase domain
MEA	mitoxantrone, etoposide, cytarabine

mDC1	myeloid dendritic cell type 1
MDM2	mouse double minute 2 homolog
MDSC	myeloid-derived suppressor cell
mIHC	multiplex immunohistochemistry
MLPA	multiplex ligation-dependent probe amplification
MNC	mononuclear cell
MRD	measurable residual disease
NAMPT	nicotinamide phosphoribosyltransferase
NGS	next-generation sequencing
NK cell	natural killer cell
NOPHO	Nordic Society of Paediatric Haematology and Oncology
OS	overall survival
PCR	polymerase chain reaction
PD1	programmed cell death protein 1
PDGFR	platelet derived growth factor
Ph+	Philadelphia chromosome-positive
Ph⁻	Philadelphia chromosome-negative
Ph-like	Philadelphia chromosome-like
RFS	relapse-free survival
RNAseq	whole transcriptome sequencing
RT-PCR	reverse transcriptase polymerase chain reaction
TBS	Tris-buffered saline
TIM3	T cell immunoglobulin domain and mucin domain 3
TKI	tyrosine kinase inhibitor
TMA	tissue microarray
TSA	tyramide signal amplification
VAF	variant allele frequency
VEGFR	vascular endothelial growth factor
WBC	white blood cell

LIST OF ORIGINAL PUBLICATIONS

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

I. **Hohtari H***, Brück O*, Blom S, Turkki R, Sinisalo M, Kovanen PE, Kallioniemi O, Pellinen T, Porkka K, Mustjoki S. Immune cell constitution in bone marrow microenvironment predicts outcome in adult ALL. Leukemia (2019) 33(7):1570-1582.
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II. **Hohtari H**, Kankainen M*, Adnan-Awad S*, Yadav B, Potdar S, Ianevski A, Dufva O, Heckman C, Sexl V, Kytölä S, Mustjoki S*, Porkka K*. Targeting apoptosis pathways with BCL2 and MDM2 inhibitors in adult B-cell acute lymphoblastic leukemia. Hemasphere (2022) 6(3):e701. https://doi.org/10.1097/HS9.0000000000000701

III. **Hohtari H**, Pallisgaard N, Kankainen M, Ellonen P, Brück O, Siitonen T, Säily M, Sinisalo M, Pyörälä M, Itälä-Remes M, Koskenvesa P, Elonen E, Mustjoki S, Porkka K. Copy number alterations define outcome in Philadelphia chromosome-positive acute lymphoblastic leukemia. Haematologica (2022) 107(8):1971-1976.
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ABSTRACT

The prognosis of Philadelphia chromosome-positive (Ph+) acute lymphoblastic leukemia (ALL) has significantly improved with the introduction of tyrosine kinase inhibitors (TKIs). As the incidence of Ph-positivity increases with age, a substantial number of elderly Ph+ ALL patients are ineligible for intensive treatment modalities. Currently, a proportion of patients experience prolonged survival with TKI-based therapies only, and many succumb eventually to non leukemia-related causes.

The aim of this thesis was to identify potential predictive biomarkers for more personalized risk stratification in Ph+ ALL, including characterization of the immune microenvironment in ALL bone marrow (BM). We also wanted to assess the drug sensitivity of primary patient samples to identify potential novel or repurposed drugs, with especially non-fit patients in mind, and to study the prevalence of copy number alterations and other secondary mutations.

In study I, we collected archived formalin-fixed and paraffin-embedded BM biopsies from Ph+ (n = 31) and Philadelphia chromosome-negative (Ph^-; n = 21) ALL patients and non-leukemic controls (n = 14). The samples were constructed to tissue microarrays and analyzed with multiplex immunohistochemistry and automated image analysis. The immune contexture of Ph+ and Ph^- ALL BM did not differ significantly. Instead, ALL BM was characterized by an increased amount of immune cells associated with immunosuppression when compared to healthy controls. Further, the higher proportion of CD4+PD1+TIM3+ T cells, older age, and lower platelet count at diagnosis segregated a group with poor survival.

In study II, we analyzed the drug sensitivity of 18 primary B-ALL BM samples (Ph+ n=10, Ph^- n=8) to a selection of 64 drugs by using a well-established drug sensitivity and resistance testing assay. The results were combined with whole transcriptome sequencing and publicly available gene expression data. Apoptosis-modulating BCL2 inhibitors and MDM2 inhibitors were widely effective. BCL2-selective venetoclax was more effective in Ph^- samples, whereas BCL2, BCL-W, and BCL-XL targeting navitoclax showed uniform potency. *BCL2* expression was significantly higher in Ph^- ALL, whereas *BCL-W* and *BCL*-XL were overexpressed in Ph+ ALL, explaining the differential drug responses. In addition, the sequencing strategies recognized three previously undiagnosed Ph-like patients with a sensitivity to TKIs.

In study III, we investigated the frequency and significance of copy number alterations (CNAs) and other secondary mutations in Ph+ ALL by applying

targeted next-generation sequencing (NGS) gene panel and multiplex ligation-dependent probe amplification to diagnostic (n=40) and relapse-phase (n=11) BM samples. We also assessed the prevalence of subclonal T315I kinase domain mutations. The results were combined with clinical registry data. Deletions of *IKZF1* together with deletions in *CDKN2A/B* and/or *PAX5* were common, and they stratified a group with dismal outcome. Other secondary mutations at diagnosis were rare.

In conclusion, this thesis shows Ph+ ALL BM immune contexture did not differ from Ph⁻ ALL. Instead, ALL BM immune microenvironment differs from healthy controls, and immune profiling can serve as a tool in identifying novel prognostic biomarkers. Copy number alterations (CNA) defined a subset in Ph+ ALL with dismal outcome, and we recommend incorporating CNA analysis to routine diagnostic procedures. In addition, with *ex vivo* drug testing, we identified several potential compounds to be further tested in clinical trials.

TIIVISTELMÄ

Tyrosiinikinaasiestäjät (TKE) ovat parantaneet merkittävästi Philadelphia-kromosomipositiivisen (Ph+) akuutin lymfaattisen leukemian (ALL) ennustetta. Koska Ph+ ALL :n yleisyys kasvaa iän myötä, merkittävää osaa näistä iäkkäämmistä tai heikkokuntoisemmista potilaista ei voida kuitenkaan hoitaa tavanomaisilla intensiivisillä hoito-ohjelmilla hoitoon liittyvien haittojen vuoksi. Toisaalta osa potilaista saa hyvän vasteen pelkälle TKE-pohjaiselle kevennetylle hoidolle, ja monet menehtyvät lopulta leukemiaan liittymättömiin syihin.

Tämän väitöskirjatyön tavoitteena oli selvittää potentiaalisia biomarkkereita Ph+ ALL :n yksilöllisemmän riskinarvioinnin kehittämiseksi, sekä kuvata immuunijärjestelmän koostumusta ALL :n luuytimen mikroympäristössä. Analysoimme myös potilasnäytteiden herkkyyttä lupaaville lääkeaineille ajatellen erityisesti hauraampien potilaiden ilmeistä tarvetta tehokkaille ja samalla turvallisille lääkehoidoille. Arvioimme myös kopiolukumuutosten ja muiden sekundaaristen mutaatioiden esiintyvyyttä Ph+ ALL:ssa.

Ensimmäisessä osatyössä keräsimme vanhoja luuydinbiopsioita Ph+ (n=31) ja Philadelphia-kromosominegatiivista (Ph^-; n=21) ALL:ia sairastavilta potilailta sekä terveiltä kontrolleilta (n=14). Näytteistä koostetut kudosblokit värjättiin multipleksatulla immunohistokemialla ja analysoitiin käyttäen apuna automatisoitua kuva-analyysia. Ph+ ja Ph^- ALL -potilaiden luuytimen immunologinen mikroympäristö ei eronnut merkittävästi toisistaan. Sen sijaan ALL-potilailla immuunivasteen heikentämiseen liittyvien solutyyppien osuus oli korostunut verrattuna terveisiin kontrolleihin. Lisäksi CD4+PD1+TIM3+ T-solujen suurempi osuus, korkeampi ikä sekä matalampi verihiutaleiden määrä diagnoosihetkellä erottelivat monimuuttujamallissa ALL-potilaista huonoennusteisen ryhmän.

Toisessa osatyössä analysoimme 18 potilasnäytteen (Ph+ n=10, Ph^- n=8) herkkyyttä 64 eri lääkeaineelle käyttämällä vakiintunutta lääkeherkkyystestausmenetelmää. Näytteistä tehtiin myös RNA-sekvensointi, sekä tulokset yhdistettiin julkisista tietokannoista saatavilla olevaan geenien ilmentymistä kuvaavaan dataan. Ohjelmoitua solukuolemaa edistävät BCL2:n ja MDM2:n estäjät olivat tehokkaita valtaosassa näytteitä. Valikoivasti BCL2:een kohdistuva venetoklaksi oli tehokkaampi Ph^- näytteissä, kun taas laajemmin BCL2:een, BCL-W:een sekä BCL-XL:ään kohdistuva navitoklaksi oli tehokas lähes kaikissa näytteissä. *BCL2*-geenin ilmentyminen oli lisääntynyt Ph^- ALL-potilailla, kun taas *BCL-W*- ja *BCL-XL*-geenien ilmentymistasot olivat korkeampia Ph+ ALL:ssa tarjoten samalla mekanistisen selityksen eroille lääkevasteissa. Sekvensointi tunnisti lisäksi kolmen Ph^-

potilaan näytteessä geneettisiä muutoksia, jotka aiheuttivat herkkyyttä TKE-lääkkeille.

Kolmannessa osatyössä selvitimme kopiolukumuutosten ja muiden sekundaaristen geneettisen muutosten yleisyyttä ja merkitystä Ph+ ALL:ssa hyödyntämällä kohdennettua syväsekvensointia sekä MLPA-menetelmää (MLPA, *multiplex ligation-dependent probe amplification*) diagnoosi- (n=40) ja relapsivaiheen (n=11) luuydinnäytteissä. Arvioimme myös subklonaalisten T315I kinaasialueen mutaatioiden esiintyvyyttä. Tulokset analysoitiin yhdessä kliinisen rekisteridatan kanssa. *IKZF1*-geenin deleetiot yhdessä *CDKN2A/B* ja/tai *PAX5*-geenin deleetioiden kanssa olivat yleisiä ja erottelivat erityisen huonon ennusteen ryhmän. Muita sekundaarisia geneettisiä muutoksia esiintyi lähinnä relapsivaiheen näytteissä.

Tässä väitöskirjatyössä osoitimme, että Ph+ ALL:ia ja Ph$^-$ ALL:ia sairastavien potilaiden luuytimen immunologinen mikroympäristö ei eronnut merkittävästi toisistaan. Sen sijaan ALL:n luuytimen immunologinen mikroympäristö erosi terveistä kontrolleista, ja immuunijärjestelmän profilointia voidaan hyödyntää etsittäessä uusia ennusteeseen vaikuttavia biomarkkereita. Yhdistelmä epäsuotuisia kopiolukumuutoksia erotteli huonon ennusteen alaryhmän Ph+ ALL:ssa, ja suosittelemme kopiolukumuutosten rutiininomaista määrittämistä diagnoosivaiheessa. Lisäksi tunnistimme *ex vivo* -lääkeherkkyystestauksella useita ALL:n kliinisiin lääketutkimuksiin soveltuvia, lupaavia lääkeaineita.

1 INTRODUCTION

Philadelphia chromosome-positive (Ph+) acute lymphoblastic leukemia (ALL) forms the most common subgroup of ALL in adults.[1] Previously classified as a disease with very dismal prognosis, the advent of tyrosine kinase inhibitors (TKI) turned the course and has since improved survival significantly.[2] Despite this favorable development, a considerable proportion of Ph+ ALL patients are found unfit for intensive and more effective treatment modalities, especially as the prevalence of Ph-positivity increases with age.[3] These, typically elderly, patients are usually treated with TKI-based reduced regimens. Although a majority of these patients experience a relatively short relapse-free (RFS) and overall survival (OS), a proportion stays in remission for years, and many perish eventually to other causes than leukemia.

The most common reason for treatment failure in Ph+ALL is the emergence of *BCR::ABL1* kinase domain-mutated leukemic clones.[4] The factors governing clonal selection are mostly unknown and may be related to disease biology such as secondary mutation profile or *BCR::ABL1* transcript type, host factors such as immunological reconstitution, treatment response kinetics and level of measurable residual disease, or a combination of all these factors.

During the recent decade, also the treatment of relapsed and refractory ALL has experienced major breakthroughs, when immuno-oncological therapies such as bispecific and drug-conjugated antibodies and chimeric antigen receptor T cell therapy have proven their efficacy.[5] Despite the emerging immunotherapies and the essential role of the immune system in leukemia pathophysiology, the immune contexture in the ALL bone marrow (BM) microenvironment has not been thoroughly studied.

Although the treatment alternatives have rapidly expanded, most elderly Ph+ ALL patients nonetheless succumb to their disease, and the optimal use of TKIs in the treatment remains unresolved.[6] Recent research has suggested, that it may be possible to maintain long remissions with potent TKI and immunomodulatory therapies only, sparing patients from the short and long-term toxicities of allogeneic hematopoietic stem cell transplantation (alloHSCT).[7] Ideally, the selection of optimal therapies for individual patients would be based on robust predictive and prognostic biomarkers for treatment outcome and survival.

This thesis investigated potential biomarkers for more personalized risk stratification in Ph+ ALL and explores the efficacy of a selection of novel or repurposed drugs, with especially non-fit patients in mind. We also characterized the immunological landscape prevailing in ALL BM. By utilizing

a variety of different methods, such as next-generation sequencing, *ex vivo* drug assays, multiplex immunohistochemistry (mIHC), digital droplet PCR (ddPCR), and bioinformatics tools, we identified several potential markers and compounds to be tested in clinical trials. The ultimate aim in any research regarding Ph+ ALL is to transform the disease to one with an excellent prognosis, using treatments which are both effective and safe.

2 REVIEW OF THE LITERATURE

2.1 MODERN THERAPY OF ACUTE LYMPHOBLASTIC LEUKEMIA

ALL is an aggressive blood cancer, where rapidly dividing immature lymphoid blast cells infiltrate the bone marrow, causing anemia, neutropenia and thrombocytopenia, and consequently risk for bleeding and infections. The disease can be divided into T and B-ALL, depending on whether the lymphoblasts express T-cell or B-cell lineage immunophenotypic markers. ALL holds place as the most common type of childhood cancer, whereas in adults the disease is rare, with an annual incidence of 1/100,000.

Traditional ALL treatment consists of remission induction with combination chemotherapy followed by several rounds of consolidation chemotherapy. High-risk patients are directed to alloHSCT in first complete remission (CR). Incorporating intrathecal chemotherapy and corticosteroids to treatment is crucial to prevent central nervous system (CNS) relapse. If the patient does not proceed to alloHSCT, the treatment is continued with maintenance chemotherapy up to three years from diagnosis to avoid relapse.

In children, high-intensity multiagent chemotherapy with risk group-targeted alloHSCT has produced excellent long-term survival rates above 90%.[8] In adults, the outcome remains disappointedly inferior, with long-term survival rates around 40%.[9] However, the outcome of younger adults has improved with the adoption of pediatric protocols.[10] The difference in survival derives partly from the frequent necessity to reduce therapy intensities in adults, mostly due to patient age, comorbidities, and possible adverse effects. In addition, adult ALL more often displays high-risk features, such as unfavorable cytogenetics.[11]

The incorporation of pediatric ALL regimens to young adults and adolescents, the more precise recognition of potentially targetable genetic subtypes, and the emergence of potent immuno-oncological therapies is changing the treatment practices and outcome of adult ALL.

2.2 GENETICS OF B-ALL

The development and accessibility of contemporary sequencing techniques has led to increasing knowledge of ALL genetic subtypes and recognition of novel therapeutic targets. Most ALL patients have defining chromosomal or other genetic alterations, that are associated with distinctive prognosis.

Conventional cytogenetic analysis can provide information of the number of chromosomes and recognize also large structural changes that are visible with light microscopy. Hypodiploid karyotype is defined by the presence of less than 44 chromosomes, and it is associated with a poor prognosis. Hypodiploidy can be recognized in approximately 2-3% of ALL cases, and it can further be divided into low hypodiploidy (32-39 chromosomes) and near haploid (24-31 chromosomes) cases.[3] High hyperdiploidy (>50 chromosomes) is nearly exclusively observed in childhood ALL, and it is associated with favorable survival.[12] Depending on the source, complex karyotype is usually defined by the presence of more than three or five chromosomal aberrations, including at least one structural aberration. The prevalence increases in adults and is associated with poor outcome, at least in Philadelphia-negative disease.[13]

Chromosomal translocations are frequently observed in ALL, and they are typically an early event in the leukemogenesis. *BCR::ABL1* [t(9;22)(q34;q11)] positive, or more familiarly, Ph+ ALL, forms the most frequent genetic subgroup in adult B-ALL, with an incidence of 20-40%.[1] In children the occurrence of this subtype is low. Previously *BCR::ABL1* positivity was linked with inferior prognosis, but the incorporation of tyrosine kinase inhibitors (TKI) therapy has since improved survival.[2]

KMT2A (MLL) rearrangements, such as *KMT2A::AFF4* [t(4;11)(q21;q23)], and *ETV6::RUNX1* [t(12;21)(p13;q22)] are mainly encountered in children, with the previous associated with unfavorable outcome and the latter with an excellent one. *TCF3::PBX1* [t(1;19)(q23;p13)] is observed both in children and adults, and it is associated with good prognosis. Intrachromosomal amplification of chromosome 21 is another typical alteration in childhood ALL, associated with poor survival.[3]

The utilization of NGS techniques has led to the recognition of Philadelphia chromosome-like (Ph-like) ALL. This subtype of B-ALL is characterized by diverse genetic alterations, that result in similarly activated tyrosine kinase signaling and phenotype as in Ph+ ALL.[14,15] Most common alterations in Ph-like ALL include mutations and rearrangements in *CRLF2*, other *ABL* class gene fusions, *JAK2* and *EPOR* gene rearrangements, *JAK-STAT* and Ras pathway-activating mutations, and some less frequent kinase alterations such as *FLT3* mutations.[16] The incidence of this subtype peaks in young adults between 20-40 years, and decreases with advanced age.[17] Ph-like ALL has been associated with a poor prognosis, but incorporating relevant targeted therapy such as TKIs or JAK2 inhibitors into treatment regimens may improve survival.[17–19]

In addition to Ph-like ALL, modern genomic approaches such as whole transcriptome sequencing (RNAseq) have identified several other genetic

subtypes not detectable by conventional cytogenetics. These include alterations in B lymphoid transcription factor gene *PAX5,* and rearrangements involving genes such as *MEF2D*, *ZNF384*, and *DUX4*.[3,20] *MEF2D* rearranged B-ALL has been characterized by sensitivity to histone deacetylase (HDAC) inhibitors,[21] whereas *ZNF384*-rearrangements leads to upregulation of JAK-STAT signaling pathway. The latter alteration can occur also in mixed-phenotype acute leukemias. In B-ALL, *ZNF384*-rearrangered cases often express myeloid antigens, potentially hinting that the lesion has been acquired in common B/myeloid progenitor.[22]

ALL genomes evolve dynamically, typically acquiring secondary lesions during leukemia development and progression. These secondary alterations can involve DNA sequence mutations or larger structural changes, such as CNAs. The likelihood of gaining secondary mutations depends on the initiating lesion and leukemia subtype. For example, Ph+ ALL is characterized by deletions in lymphoid transcription factor *IKZF1*,[23] whereas the other Ikaros gene family members, *IKZF2* and *IKZF3*, are almost exclusively mutated in hypodiploid ALL.[24] CNAs in B-ALL typically affect genes that control apoptosis (such as *ETV6* and *ERG*), cell cycle regulation and tumor suppression (*CDKN2A/B*, *RB1*), or lymphoid transcription factors (members of Ikaros family, *PAX5*, *EBF1*). In addition, these secondary alterations may involve epigenetic changes.[25]

Some alterations may originate from rare leukemic subclones present already at diagnosis. *CREBBP* mutations that may impair sensitivity to corticosteroids can be found in approximately 20% of relapsed ALL patients, although they are rare at diagnosis.[26] *TP53* mutations, one of the most common alterations in solid cancers,[27] occur less frequently in ALL, but their incidence increases in more advanced phases of the disease.[28,29]

Although B-ALL is generally considered to originate from somatic mutations in early B-cell precursors, some leukemia-predisposing germline variants have been described, and they may play a role especially in childhood ALL.[30] In addition to contributing to leukemogenesis *per se*, germline variants may affect ALL treatment also indirectly, for example via genetic polymorphism of drug-metabolizing enzymes.[31]

2.3 PROGNOSTIC FACTORS

The terms "predictive" and "prognostic" biomarker are often used interchangeably, although their exact definition differs. A prognostic biomarker is used to identify likelihood of a certain clinical outcome, such as disease relapse in patients with a specific disease of interest. Instead, predictive biomarkers are used to identify patients who are more likely to

experience either a favorable or unfavorable response in relation to a particular treatment. However, prognostic and predictive biomarkers cannot often be distinguished when studying patients who have received only one kind of therapy. In addition, biomarkers can be both prognostic and predictive.

Prognostic factors can be roughly divided into patient-related, disease-related, and treatment-related entities. One of the most important defining factors is patient-related: the age. The long-term survival in childhood ALL exceeds 90%, whereas in adolescents and young adults the survival rates lie between 60-70%, and in adult ALL sinks to approximately 20-40%.[8,9,32] Age correlates strongly with patient fitness to tolerate more intensive treatment modalities, such as pediatric high-intensity chemotherapy regimens, and the ability to proceed to alloHSCT, when necessary.[33–36]

Other classical prognostic factors in ALL include initial leukocyte count, immunophenotype, and cytogenetics.[9] High white blood cell (WBC) count at diagnosis, typically defined as more than 30 x10^9/l for B-ALL and more than 100 x10^9/l for T-ALL, reflects the disease aggressiveness and higher leukemic burden, and is associated with poor prognosis.[37] The prognostic significance of ALL immunophenotype remains mainly undecided. The long-term survival rates of T-ALL and B-ALL are nowadays comparable, especially if the patient can be treated with intensive approaches.[38,39] However, certain immunophenotypes such as early T-cell precursor ALL, have a poor outcome.[40]

The most common cytogenetic abnormalities in B-ALL and their prognostic significance were covered in chapter 2.2. In addition to more established prognostic genetic alterations, there is increasing evidence how secondary lesions may affect outcome. Deletions in *IKZF1* constitute a hallmark in Ph+ ALL and Ph-like ALL, but occur also in other subtypes.[23,41,42] *IKZF1* gene constitutes of eight exons and codes for transcription factor Ikaros, which is required for the normal development of lymphoid lineage cells from hematopoietic precursors.[43] In cancer, wild-type Ikaros functions as a tumor suppressor by binding to DNA and regulating target gene expression.[44] Typical deletions involve exons 4 to 7 (Δ4-7) or exon 2. [23,45,46] The product of Δ4-7 deletion can dimerize with the wild-type Ikaros protein, thus exerting a dominant-negative effect.[47] Deletions that involve exon 2 lead to the loss of translation start site.[46] The inactivation of one functional allele leads to the loss of the normal tumor suppressive function of the gene and can mediate resistance to glucocorticoids.[44,48–50] The clinical relevance of *IKZF1* deletions has remained controversial.[51] Most studies, however, have defined their prognostic role as adverse.[42,52,53] There might also be differences related to the ALL subtype or the type of alteration, as some studies have reported inferior outcomes related especially to loss-of-function deletions.[54,55]

Response to treatment, such as achieving CR, and the persistence of measurable residual disease (MRD) constitute an inseparable part of modern ALL risk stratification.[56] Primary refractory and relapsed ALL have the most dismal prognosis, despite developments in the treatment.[57] MRD is usually defined by the presence of leukemic cells below the detection threshold of conventional morphological and cytogenetic methods.[56] An arbitrary sensitivity limit of 0.01% or 10^{-4} has been used in most studies. For the majority of ALL patients, a quantitative marker suitable for MRD follow-up can be detected by analysis of clonal rearrangements in immunoglobulin and T-cell receptor genes (allele-specific oligonucleotide [ASO]-PCR), multiparameter flow cytometry, or quantitative PCR-based detection of fusion gene transcripts and other aberrations.[58] Achieving MRD negativity during treatment is one of the strongest predictors of outcome in ALL.[59] The persistence of MRD despite intensive therapy signifies of a poor prognosis, and forms an indication for alloHSCT.[56,60] However, the clinical significance of MRD may vary depending on the disease subtype, designated therapy, the sensitivity of the used follow-up method, and the MRD level and kinetics.[61] In Ph+ ALL, discrepancies between ASO-PCR and *BCR::ABL1* MRD measurements have been described, possibly due to occasional expression of *BCR::ABL1* in non-lymphoid cells.[62,63]

Risk stratification of ALL is in constant transformation.[64] The increasing knowledge of genetic and other contributing variables has led to the recognition of novel disease subtypes and potential subgroup differences in terms of prognostic factors.[65] The implementation of immuno-oncological and other novel therapies may signify that all classical prognostic factors may not apply similarly as with traditional chemotherapy regimens.[66,67] With increasing sensitivity of MRD detection techniques,[68] the definition and clinical significance of "MRD negativity" may also require re-evaluation in context.

2.4 PHILADELPHIA CHROMOSOME-POSITIVE ALL

Philadelphia chromosome was first discovered in 1960 by Nowell and Hungerford when examining cytogenetic samples of chronic myeloid leukemia (CML) patients.[69] These patients had a small, changed chromosome 22, which later on turned out to be the result of a translocation between chromosomes 9 and 22 [t(9;22)(q34;q11)]. This translocation creates a pathogenic fusion gene *BCR::ABL1* producing constitutively active tyrosine kinase, which results in increased cell proliferation and survival.[70] *BCR::ABL1* is generally considered to act as a driver mutation in the leukemia-initiating process. Philadelphia chromosome forms the hallmark of CML, but occurs also in a subset of ALL, being the most frequent genetic abnormality in adult ALL.[1] In Ph+ ALL,

approximately 20-25% of patients harbor the longer *BCR::ABL1* transcript variant p210 (e13a2 or e14a2, previously named major), and approximately 75% a shorter variant p190 (e1a2, previously named minor), whereas in CML a majority (95%) of patients have the p210 variant.[71] The prognostic value of different transcript variants remains elusive.[72] Despite sharing the same chromosomal lesion, CML and Ph+ ALL differ profoundly related to disease biology, prognosis, and treatment.

Historically, Ph+ ALL represented the subset of adult ALL with the worst prognosis, with CR rates falling below 70 % and long-term survival below 20 %.[71,73] The golden standard of the treatment and the only potential cure rested on alloHSCT[74] - a treatment accessible only for the younger and fitter patients. As the incidence of Ph+ ALL increases with age, a significant fraction of Ph+ ALL patients were left without efficient treatment options.

2.4.1 TYROSINE KINASE INHIBITORS

ATP binding TKIs

In 2001, imatinib (imatinib mesylate, STI571), an oral BCR::ABL1-targeting TKI received the United States Food and Drug Administration (FDA) approval, revolutionizing not only the treatment of CML, but also of Ph+ ALL. After adding imatinib to standard treatment protocols, CR rates exceeded 90% and long-term survival rates increased up to 40%.[2,75] Imatinib binds to the ATP-binding pocket of ABL1 kinase, blocking its enzymatic activity. In addition to ABL1, it also targets other protein kinases such as c-kit and platelet derived growth factor (PDGFR). Treatment is generally well tolerated, and the most common side effects such as transaminitis, cytopenias, and gastrointestinal symptoms are often manageable with dose adjustments.[76]

After the release of the drug, it was soon noted, that although imatinib as a monotherapy could produce high initial response rates even in refractory and relapsed disease, it was uncapable of sustaining long-term remissions in Ph+ ALL, contrary to CML.[77] The most common reason for treatment failure is the emergence of P-loop mutations that alter the conformation of ABL1 and prevent imatinib from binding to the active site. This led to the development of more potent second generation (2G) TKIs, dasatinib, nilotinib, and bosutinib, which were able to target most imatinib-resistant mutations.

Dasatinib targets several kinases in addition to BCR::ABL1, such as SRC family kinases and ephrins, and possesses ABL1-independent immunomodulatory effects.[78,79] It crosses the blood-brain barrier and has shown efficacy also in CNS leukemia.[80] Peripheral blood large granular lymphocytosis and pleural effusions are common side effects of dasatinib treatment, which have been associated with improved survival.[81] Pulmonary arterial hypertension is a rare

adverse event related to dasatinib, but the condition is usually reversible after cessation of the therapy.[82] Other second-generation TKIs, nilotinib and bosutinib, are mainly used in CML, with only a few published studies of nilotinib and none of bosutinib in a first-line setting in Ph+ ALL. [83–85]

The T315I kinase domain mutation forms the leading cause for second generation TKI failure.[4] Third generation TKI ponatinib is a pan-BCR::ABL1-inhibitor with a 520-fold potency compared to imatinib. It targets several kinases besides BCR::ABL1, such as KIT, FLT3, vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor, PDGFR, and SRC kinases, and it can overcome resistance to T315I.[86,87] Initial studies of ponatinib in Ph+ ALL have proven very promising, with complete molecular remission rates of 79% and three-year OS rates reaching 79%.[88] Ponatinib harbors the highest risk for cardiovascular toxicity amongst BCR::ABL1 targeting TKIs, but the toxicity profile seems partly dose-dependent, and the drug has been successfully applied also to the treatment of older patients.[89,90]

A lack of randomized controlled studies regarding the choice of different TKIs in Ph+ ALL poses a challenge. In pediatric Ph+ ALL, dasatinib improved survival significantly compared to imatinib.[91] In adults, adding dasatinib to treatment protocols may lead to better outcome compared with imatinib, and ponatinib seems even more effective.[88] However, with evolving treatment modalities and improved supportive care resulting in overall better prognosis in ALL, a comparison without a randomized setting can hardly deliver any conclusive answers.

Other tyrosine kinase inhibitors

Asciminib, a novel allosteric inhibitor of BCR::ABL1, has received FDA-approval for CML, but the role in the treatment of Ph+ ALL remains to be seen. Asciminib does not bind directly to the ATP-binding pocket, therefore avoiding resistance to the common kinase domain mutations. Instead, the drug binds to the myristol-binding pocket causing conformational changes in the protein and locking the ATP-binding site to an inactive state.[92,93] Mutations leading to asciminib resistance have already been documented, but seem rare.[94] In preclinical data, combining ponatinib with asciminib restored efficacy against highly resistant BCR::ABL1 mutants,[95] and a combination therapy with ATP site and TKIs could become a potential treatment strategy.

Axitinib is a VEGFR targeting TKI approved for advanced renal cell carcinoma. It is inactive against native BCR::ABL1, but has been proven active against the T315I mutation.[96] No clinical data of Ph+ ALL exists. Crizotinib acts as a BCR::ABL1 targeting TKI with a dual mechanism, which combines both allosteric inhibition and ATP binding. In preclinical studies it has shown activity both against native and mutated BCR::ABL1 as well as compound

mutations.[97] Several other BCR::ABL1-targeting TKIs, such as flumatinib and olverembatinib, are currently in early clinical trials.[98]

2.4.2 KINASE DOMAIN MUTATIONS AND TREATMENT FAILURE

The most common reason for treatment failure in Ph+ ALL are point mutations in the ABL1 kinase domain (KD), which prevent the ATP-binding TKIs from binding to the site of action. Today, over 100 resistance-causing KD mutations have been described.[99] The development of 2G TKIs covered most of the imatinib-resistant mutations, but some mutations such as T315I and F317L evade also 2G drugs. Currently, third-generation (3G) ponatinib is the only FDA-approved TKI for Ph+ ALL that targets T315I.

Sanger sequencing has been the golden standard for detecting KD mutations, with a sensitivity of approximately 15-25%,[100] but PCR and NGS-based methods (sensitivity around 2%) are increasingly employed in the clinical laboratories,[101] and novel techniques, such as duplex sequencing might further lower the detection threshold.[102]

There are also *BCR::ABL1* independent mechanisms that may contribute to TKI resistance, such as decreased drug uptake or increased efflux from leukemic cells, activation of alternative survival pathways, dysregulation of epigenetic pathways, additional genetic aberrations, and decreased apoptosis.[99,103] Less investigated fields such as leukemia immune microenvironment most likely play a significant part in treatment resistance and disease recurrence,[104] but further studies are needed.

A sequential treatment with different TKIs may theoretically lead to increased risk for developing highly-resistant compound mutations.[105] As several reports have suggested that up to 25% of Ph+ ALL patients harbor subclonal resistance-causing KD mutations already at diagnosis,[106–108] screening for these pre-treatment mutations might have a clinical impact when choosing frontline therapy. Hypothetically, suboptimal TKI treatment might select a resistant subclone by offering it a growth advantage, in the end leading to treatment failure and relapse. This would support the idea of starting with the most potent TKI immediately at diagnosis, instead of enhancing the treatment only after insufficient response or failure. However, financial considerations and varying national reimbursement policies often impact real-life decisions, especially as there exists no definitive randomized data on the superiority of any specific TKI in long-term follow-up. Today, imatinib, and to lesser extent, dasatinib, are the most commonly used TKIs in frontline therapy of Ph+ ALL.[6]

2.4.3 TOWARDS MODERN THERAPY OF PH+ ALL

The introduction of TKIs has led to significant improvement in the long-term survival of Ph+ ALL, and the disease outcome currently compares favorably with Ph⁻ ALL. Questions regarding treatment intensity, the optimal use of TKIs, and the role of alloHSCT and immuno-oncological therapies have arisen concurrently with improving survival rates, but remain unanswered.

Myelosuppression, infections, and other treatment-related toxicity remain a common challenge when treating elderly ALL patients with conventional chemotherapy. Positive reports of elderly long-term survivors with TKI-based low-intensity or even chemotherapy-free regimens have raised interest in the possibility of de-escalating the intensity of chemotherapy or adapting completely chemotherapy-free regimens in Ph+ ALL.[109] Induction protocols with corticosteroids and TKIs have yielded excellent CR rates fully comparable or even better to more intensive approaches and with less treatment-related toxicity.[90,110,111] Low-intensity chemotherapy with TKIs have contributed to five-year OS around 45%, although majority of the published studies have reported only two and three-year OS rates.[106,112,113] Final conclusions cannot be drawn due to incomplete data on long-term survival, but the results thus far seem to advocate at least for less intensive induction treatment.

Promising reports of long-term survivors treated solely with TKI-based therapies imply that alloHSCT may be avoided in selected Ph+ ALL patients.[112,114,115] The dilemma lies in the absence of a proper definition and biomarkers for "low-risk" Ph+ ALL. Currently, MRD-based stratification for alloHSCT has been used, namely, achieving and retaining low levels of MRD with therapy.[116] An arbitrary ≥4 log reduction in MRD compared with baseline values has been used in most studies to define MRD "negativity".

However, MRD monitoring also has its pitfalls. Hovorkova et al reported discrepancies in a significant proportion of Ph+ ALL patients when monitoring MRD levels concomitantly with *BCR::ABL1* and Ig/TCR. They were able to recognize a novel CML-like disease subtype, where *BCR::ABL1* MRD positivity originates from clonal hematopoiesis from early progenitors or from mature cells of a different lineage (e.g. T-cells).[62] If a decision is made of not to proceed to alloHSCT, the patient will continue with TKIs indefinitely - an approach of whose long-term risks are yet to be established.

The use of TKIs after alloHSCT is often less well tolerated than before transplantation, and there is limited evidence supporting either prophylactic or preemptive use, at least in CR1.[117] However, the use of posttransplant TKIs unquestionably decreases the risk for relapse compared with pre-TKI era.[118] European Society for Blood and Marrow Transplantation recommends careful monitoring of MRD levels with rapid restart of posttransplant TKI in case of detectable MRD, or optional prophylactic administration in MRD-negative

patients. No one knows how long the TKIs should be administered. With patients undergoing alloHSCT in later remission than CR1, the TKIs are recommended to be continued indefinitely.[119]

Additional genetic lesions have prognostic value also in Ph+ ALL, and recent publications have demonstrated that some alterations, such as deletions of *IKZF1* combined with deletions in *CDKN2A/B* or *PAX5* ("*IKZF1* plus"), retain their adverse effect even in the context of alloHSCT.[113,120,121] An innovative trial of frontline chemotherapy-free regimen with dasatinib and blinatumomab reported two-year OS of 88% and disease-free survival of 80%, and confirmed the adverse prognostic role of additional genetic lesions.[122,123] However, the outcome of *IKZF1* plus patients was superior compared with reports from other trials, possibly indicating that blinatumomab may be able to partly reverse the negative prognostic impact. In addition, the transplantation-related mortality in patients who received an allograft was markedly low, possibly due to the lack of systemic chemotherapy.

Management of Ph+ ALL is in constant evolution. Increasing long-term data concerning the use and efficacy of modern immuno-oncological treatment modalities and more potent TKIs may further refine the field and even lead to paradigm shifts in the treatment.[7,124]

2.5 CANCER IMMUNOLOGY

Studying solely the properties of a solitary cancer cell can rarely decipher the intricate reality of a malignant disease. Instead, cancer is the product of a complex and dynamic network, where the intercourse of malignant and normal cells defines cancer emergence, progression, metastasis, and even response to treatment.[125] Although the emergence of cancer basically denotes failure of immune surveillance, the advent of immunotherapies has highlighted the role of the immune system in cancer.[126]

Hematologic malignancies such as ALL differ from solid malignancies, where tumors have clear center and margins. As the hematopoietic system is affected, normal hematopoiesis can be compromised, leading to potential defects in the normal immune function. While leukemias display one of the lowest mutation frequencies across cancer types, the number of potential immune activating neo-antigens is also theoretically low.[127] Although this would suggest for decreased efficacy of immune therapies in leukemias, one of the most studied immunological treatments has been particularly developed and applied for hematological diseases, namely alloHSCT.[128] Also many cutting-edge immuno-oncological approaches are currently successfully adapted for B-ALL.[5]

2.5.1 T CELLS IN CANCER

White blood cells, or leukocytes, form an integral part of the human immune system. They are produced in the BM from multipotent hematopoietic stem cells and differentiate first into myeloid and lymphoid lineage precursors and further stepwise into mature blood cells. Neutrophils, basophils, eosinophils, monocytes, erythrocytes, megakaryocytes and eventually platelets develop from the common myeloid progenitors. The common lymphoid progenitor evolves into T and B lymphocytes and natural killer (NK) cells (Figure 1).[129]

Based on two main strategies of action, the immune system can be divided into innate and adaptive immunity. Innate immunity forms the first line of defense against pathogens, and consists of physical barriers, biochemical complement system, and cellular defenses. The innate response is non-specific and rapid. Most of the leukocyte population, such as all myeloid cells and most NK cells, belong to the innate immune system. Adaptive immune response forms the second line of defense. It is long-lasting and highly specific to each encountered pathogen. B and T lymphocytes and adaptive NK cells form the adaptive immunity. B cells are responsible for humoral immunity and antibody production, while T cells account for cell-mediated response. Innate immune cells are essential for the normal function of adaptive immunity, and participate in actions such as antigen presentation and regulation of activated lymphocytes.[130]

Developing T cells migrate from BM to mature in the thymus. T cells can further be classified into subtypes according to their surface markers, such as T cell receptors and CD4 or CD8 surface proteins. CD8+ T cells destroy infected or dysfunctional cells by releasing cytotoxins such as perforin and granzymes, which leads to apoptosis of the target cell. CD4+ T cells activate cytotoxic T cells and memory B cells. Regulatory T cells form a distinct entity of T cells, that suppress immune responses and are critical in maintaining self-tolerance.[130]

T cells are regarded as the pivotal effector cell types in cancer immunology.[131] The density and location of T cells in the tumor has been shown to predict outcome in a variety of cancers.[132] Cancer can affect normal T cell function in several ways. Malignant cells secrete chemokines that tempt regulatory T cells, thus preventing the recognition and immune response against tumor cells.[133] The chronic inflammation in cancer can cause prolonged and excessive T cell stimulation, which further facilitates T cell exhaustion. Cell mediators released by the malignant cells upregulate the expression of inhibitory immune checkpoint molecules, such as PD1, TIM3, or CTLA4, which can lead to suboptimal T cell stimulation and T cell anergy. These exhausted or anergic T cells are then incapable of performing their normal effector functions.[134] Importantly, this T cell dysfunction can be reversed with cancer immunotherapy.[135,136]

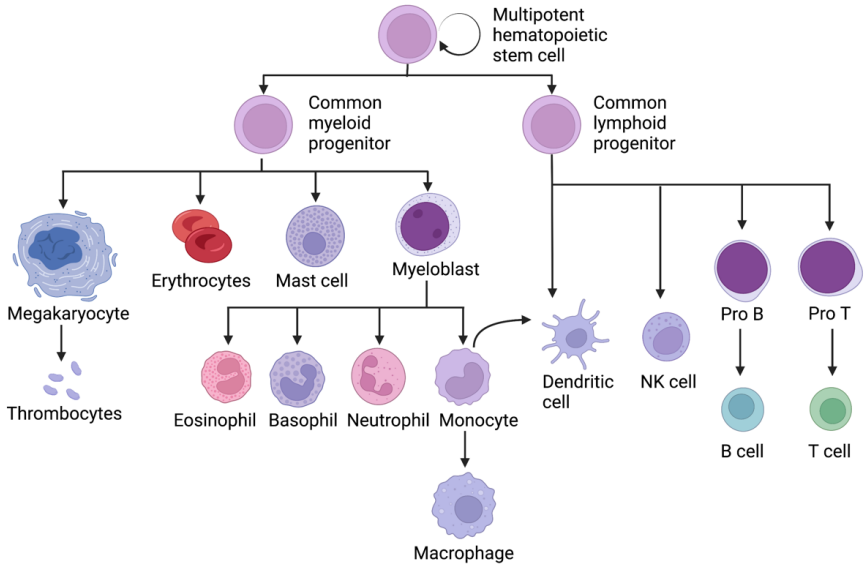

Figure 1 Simplified model of hematopoiesis (created with Biorender.com).

2.5.2 TUMOR MICROENVIRONMENT

Cancer can be depicted by accumulation of mutations that transform a normal cell into a malignant one. An altered cell forms a target for the immune system, and normal conditions in an organism represent a tumor-hostile environment. Tumors never consist only of cancer cells, but contain numerous other cell types, such as fibroblasts, endothelial cells, lymphocytes, and bone marrow-derived inflammatory cells. In addition to cells, the tumor microenvironment contains the extracellular matrix composed of collagen and proteoglycans. To avoid immune evasion and promote survival, cancer cells recruit a repertoire of seemingly normal cells, that contribute to angiogenesis, promotion of tumor growth, and immunosuppression by creating a favorable tumor microenvironment.[125] Despite that cancer tends to provoke at least some level of anticancer immune response, normal stromal cells in the tumor microenvironment can exclude T cells from the proximity of the malignant cells, thus preventing an efficient immune attack.[137] Tumors recruit proinflammatory cells, such as macrophages and mast cells, that paradoxically promote tumorigenesis by supplying proangiogenic molecules, growth factors, and enzymes capable of shaping the extracellular matrix.[125] Malignant cells present antigens and secrete mediators that directly promote immunosuppression, for example by attracting regulatory T cells or by activating negative regulatory pathways, also called immune checkpoints.[126,133]

The presence of immune cells in the tumor and its margins varies. The level of immunoactivation has been shown to affect prognosis in solid cancer and also response to immunologically targeted treatment, such as anti-PD1 or anti-CTLA4 therapies.[138] These immune checkpoint inhibitors work by blocking the immunological brakes on cancer-primed T-cells, thus releasing them to their full activation potential.[138] There are limited data of checkpoint inhibitor therapy in acute leukemias, but they have been successfully used to treat lymphomas with PD-L1 amplification.[139]

Immune-inflamed phenotype is characterized by the abundance of CD4+ and CD8+ T-cells, and often also myeloid cells. The immune cells are typically located in the close proximity of the tumor cells, and this phenotype usually responds to checkpoint inhibitor therapy. In immune-excluded phenotype the immune cells are located in the stroma surrounding the tumor instead of tumor parenchyma. There is usually no significant response to anti-PD1/PD-L1 therapy, as the tumor stroma limits T-cell migration. Immune-desert phenotype is characterized by the paucity of T-cells, reflecting the lack of pre-existing anti-tumor immune response and unresponsiveness to checkpoint inhibitor therapy.[126]

2.6 IMMUNO-ONCOLOGICAL THERAPIES IN B-ALL

Cancer immunotherapy aims at activating and harnessing the patient's own immune system to attack and eliminate the malignant cells.[140] In comparison, alloHSCT rests on the graft-versus-leukemia effect, where engrafting donor hematopoiesis clears the residual leukemia cells, often with the cost of graft-versus-host disease.[128] While previously adapted mainly as a salvage therapy, at present, immuno-oncological treatments continue to move towards the front-line regimens in B-ALL (Figure 2).[5]

2.6.1 MONOCLONAL ANTIBODIES

CD20 is expressed in approximately 40-50% of B-ALL cases, and it has been associated with inferior survival in adults.[141] Rituximab is a monoclonal antibody designed to target CD20. Incorporating rituximab into chemotherapy regimens may decrease the risk for relapse in younger adults with Ph⁻ ALL, but the effect on survival remained indefinite.[142–144] Ofatumumab is a second-generation CD20 antibody, that binds to a different epitope than rituximab and targets CD20 with greater affinity. It may prove more effective than rituximab.[145]

2.6.2 DRUG-CONJUGATED ANTIBODIES

CD22, a general B lymphocyte surface marker, is expressed in approximately 90% of B-ALL lymphoblasts.[146] Inotuzumab ozogamicin is an anti-CD22 monoclonal antibody conjugated to a cytotoxic agent, calicheamicin. Linkage to CD22 directs the cytotoxic drug into leukemic cells, thus both increasing the therapy potency and limiting the toxicity. In the INO-VATE study, adults with relapsed or refractory B-ALL were randomized to receive either inotuzumab or standard of care. In the inotuzumab arm, CR was achieved with significantly higher percentage (80.7% versus 29.4%), and more patients proceeded to alloHSCT. However, in Ph+ ALL, there was no difference in OS.[147] Currently, inotuzumab ozogamicin is indicated for the treatment of adults with CD22-positive relapsed or refractory B-ALL, and it is considered especially useful as a bridging therapy to alloHSCT. Increased risk for hepatotoxicity, including sinusoidal obstruction syndrome, has been associated with the use.[147]

2.6.3 BISPECIFIC ANTIBODIES

Blinatumomab is a bispecific T-cell engager antibody, that contains two binding sites: one for CD3 and one for CD19. CD19 is a common B-lineage surface antigen, that is expressed throughout the normal B-cell lymphopoiesis and in a majority of leukemic lymphoblasts.[146] Blinatumomab creates a synapse between CD3-positive T-cells and CD19-expressing B-lymphocytes, activating the cytotoxic T-cells and resulting in the lysis of the CD19+ cells.[148] The drug is administered as a continuous intravenous infusion, typically for 28 consecutive days per cycle. The relevant adverse events related to blinatumomab are fever, cytokine release syndrome (CRS), and neurological side effects, such as mental confusion and seizures, which typically are reversible with treatment interruption.[149] Treatment is usually well-tolerated also in elderly patients.[150] Blinatumomab has proven highly effective especially in eliminating low residual tumor burdens, and is currently used in second-line therapy or as a measurable residual disease eradicating therapy prior to alloHSCT.[149–154] Resistance to blinatumomab can develop through loss of CD19 expression in the blast cells, upregulation of PD1/PDL1 axis, or an increased number of regulatory T cells.[63,155–157]

2.6.4 CHIMERIC ANTIGEN RECEPTOR T CELL THERAPY

Chimeric antigen receptor T-cells (CAR T) are autologous T- cells, which have been genetically engineered to target specific cancer-associated antigens. Also allogeneic CAR T therapies are being developed, but there are not yet any approved products available. In case of B-ALL, the T-cells are first collected from the patient's bloodstream with apheresis, and then transduced with a CAR gene construct targeting CD19. The costimulatory domains in the CAR construct vary between different products. After genetic modification, the produced CAR T-cells are expanded and transferred back to the patient.[158] In

children and young adults with relapsed or refractory B-ALL, CAR T therapy has yielded excellent initial results with MRD negative CR rates between 80-90%.[159,160] In long-term follow-up, relapses remain nevertheless common.[161,162] Treatment failures have been associated with deficient CAR T cell expansion and persistence *in vivo*, loss or downregulation of CD19 expression, and potential T cell exhaustion.[163] Frequently encountered adverse effects related to CAR T therapy include fever, CRS, and neurological symptoms. The adverse effects may be severe, but are mainly transient and usually manageable with administration of interleukin-6 receptor antagonist tocilizumab.[160,164,165] Currently, tisagenlecleucel and brexucabtagene are the only FDA approved CAR T products with indication for the treatment of pediatric and young adults patients with relapsed or refractory B-ALL.[158]

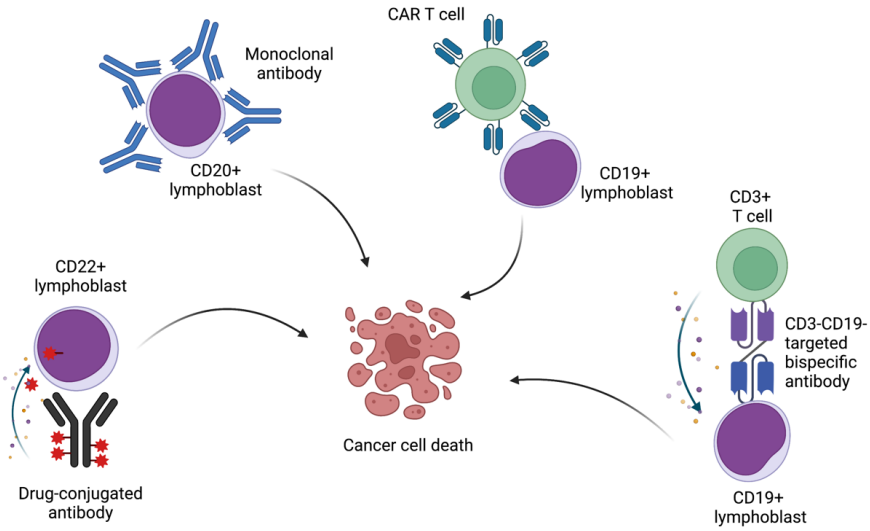

Figure 2 Common immuno-oncological therapies in B-ALL (created with Biorender.com).

2.7 NOVEL DRUGS

Although the treatment results of ALL have improved, the prognosis of relapsed or primary refractory disease and of elderly, non-fit adults remains especially poor.[32,57] There is an unmet need for effective yet less toxic treatment alternatives, and a variety of new or repurposed compounds for B-ALL are currently being tested in clinical trials (Table 1).

A significant number of ALL trials are investigating the efficacy of a selective BCL2-inhibitor venetoclax,[166] which has been approved for the treatment of chronic lymphocytic leukemia and AML, but has already shown some

promising preliminary results in ALL.[167] BCL2 inhibitors act by blocking the action of BCL2, an antiapoptotic protein located on the outer mitochondrial membrane. This inhibition activates the proapoptotic proteins, leading eventually to cancer cell apoptosis.[168] Several malignancies overexpress BCL2 and can become BCL2-dependent for their survival, offering a potential target for cancer therapy.[169]

Another BCL2 inhibitor, navitoclax, targets BCL2, BCL2L2 (BCL-W), and BCL2L1 (BCL-XL).[170] Platelets express BCL-XL, and dose-dependent thrombocytopenia decreased the interest to the drug in solid tumor trials.[171–173] Low-dose navitoclax has been tested in ALL in a phase I study in combination with venetoclax and chemotherapy. The combination was well tolerated with preliminarily promising results in a heavily pretreated patient population.[174]

Monotherapy with BCL2 inhibitors can activate alternative survival pathways in BCL2-dependent malignant cells, such as overexpression of antiapoptotic MCL1.[175,176] The activation of wild-type tumor suppressor TP53 promotes degradation of MCL1.[177] In malignant cells, the function of TP53 is often suppressed by an increased amount of regulatory protein MDM2.[178,179] Idasanutlin, an oral MDM2 inhibitor, acts by blocking the interaction between MDM2 and TP53, leading to restoration of normal TP53 function and enhanced apoptosis.[180] Mutations in *TP53* prevent the action of idasanutlin, but in ALL these mutations are less frequent than in solid tumours in general.[27,29] Thus, mechanistic rationale for a combination treatment with MDM2 and BCL2 inhibitors exists.[177] Currently, idasanutlin is being tested in combination with either chemotherapy or venetoclax in a phase1/2 clinical trial for relapsed or refractory ALL (NCT04029688, clinicaltrials.gov).

Table 1 *Novel or repurposed drugs that are currently being tested in clinical trials for B-ALL (clinicaltrials.gov, 9.9.2022). Cell therapy trials and dietary supplements have been excluded.*

Drug	Class	Number of studies
APG-2575	BCL2-inhibitor	1
Asciminib	BCR::ABL1 allosteric inhibitor	3
Azacitidine	Hypometylating agent	2
BMF-219	Menin inhibitor	1
Bortetzomib	Proteasome inhibitor	9
Carboplatin	Alkylkating agent	1
Carfilzomib	Proteasome inhibitor	1
Chidamide	Histone deacetylase inhibitor	1
Copanlisib	PI3K inhibitor	1
CPX-351	Liposomal daunorubicin/cytarabine	1
Daratumumab	Anti-CD38 antibody	3

Decitabine	Hypometylating agent	1
DS-1594b	Menin inhibitor	1
DSP-5336	Menin inhibitor	1
Epratuzumab	Anti-CD22 antibody	1
Everolimus	mTOR inhibitor	1
Flotetuzumab	CD3-CD123 bispecific antibody	1
Flumatinib	BCR::ABL1 inhibitor	3
Ibrutinib	BTK inhibitor	2
Idasanutlin	MDM2 inhibitor	1
IMGN632	CD123-targeted cytotoxin	1
Ipilimumab	Anti-CTLA4 antibody	1
Isatuximab	Anti-CD38 antibody	1
Ixazomib	Proteasome inhibitor	1
JNJ-75276617	Menin inhibitor	2
Lestaurtinib	JAK2, FLT3 and TrkA inhibitor	1
LP-118	BCL2 inhibitor	1
MRX-2843	MRTK/FLT3 inhibitor	1
Navitoclax	BCL2 inhibitor	1
Nilotinib	BCR::ABL1 inhibitor	1
Nivolumab	Anti-PD1 antibody	3
ONC201	Akt/ERK inhibitor	1
Ofatumumab	Anti-CD20 antibody	1
Olverembatinib	BCR::ABL1 inhibitor	2
Palbociclib	CDK4/6 inhibitor	3
Pembrolizumab	Anti-PD1 antibody	1
Pevonedistat	NEDD8 inhibitor	1
PIT565	Undefined	1
PLX3397	CSF1R inhibitor	1
Pyronaridine	Antimalarial drug	1
Ribociclib	CDK inhibitor	1
Ruxolitinib	JAK2 inhibitor	4
Sapanisertib	mTOR inhibitor	1
Selumetinib	MEK inhibitor	1
Selinexor	CRM1 inhibitor	1
SNDX-5613	Menin inhibitor	2
Sorafenib	Multikinase inhibitor	1
Tafasitamab	Anti-CD19 antibody	2
Tagraxofusp	CD123-targeted cytotoxin	2
Temozolomide	Alkylating agent	1
Topotecan	Topoisomerase inhibitor	1
Veliparib	PARP inhibitor	2
Venetoclax	BCL2 inhibitor	15
Zilovertamab	ROR1-targeted cytotoxin	1

2.8 PRECISION MEDICINE

The concept of precision medicine, also called personalized medicine, refers to an idea that individual biological characteristics of each patients' disease could be used for treatment tailoring, as opposed to treating all patients in a similar fashion.[181] By identifying different patient subgroups, a precision approach can avoid overtreatment and treatment-related toxicity, for example when a disease subtype is either known to respond equally well to less intensive regimens or to not respond to a certain therapy at all, or a known risk factor for adverse effects is recognized. On the other hand, a personalized approach can improve outcome in those subgroups, where more intensive approach is needed, or a potential actionable therapy target exists.[182]

Precision medicine leans greatly on modern NGS techniques, such as whole exome and transcriptome sequencing, but is not limited to them. Single genetic alterations can rarely decipher the complexity of cancer, and incorporating functional assays, such as *ex vivo* drug testing or immunological profiling, are essential for complementing the general picture.[183,184] With massive amounts of available genetic and other medical data, by applying efficient data mining algorithms on electronic health records and public data repositories, novel connections in rare diseases and subgroups could be established.[185] Ideally, each patient would be comprehensively profiled in terms of drug sensitivity and resistance, pharmacogenomics, genetic alterations in the tumor cells including potential molecular targets, and immunological status, which in combination with clinical data would guide the treatment decisions.

The growing knowledge of ALL genetic subgroups has increased the awareness of disease subtypes, such as Ph-like or *PAX5* altered ALL, and potential novel targets,[3] but generally the treatment decisions are based on classifying the patient as fit or non-fit (based on patient age, comorbidities, and performance score) and the disease as high-risk or non-high risk (according to simple disease traits such as certain chromosomal aberrations, WBC count, or response to given treatment).[9] A downsize of a personalized approach is, that with ever more smaller and smaller patient subgroups, such as the entire variety of genetic lesions in Ph-like ALL,[186] it is nearly impossible to test individualized therapies in reasonable-sized patient cohorts in traditional clinical drug trials.

Translational bench-to-bedside efforts have been made in situations, where all the common practices have failed. These attempts have yielded some positive results, although most treatment responses have been short lived in advanced

leukemia.[187] All in all, these reports give promise, that in the future treatment development towards an increasingly personalized direction is possible. Novel techniques, such as single cell sequencing and high-throughput flow cytometry or imaging-based drug sensitivity testing may enable a more sophisticated approach.[188,189] In addition, increasing knowledge of contributing epigenetic factors, leukemia microenvironment, and the role of immunology in leukemogenesis might offer breakthroughs in the near future.

2.8.1 *EX VIVO* DRUG SENSITIVITY AND RESISTANCE TESTING

Ex vivo drug sensitivity and resistance testing of primary tumor samples enables comprehensive functional profiling of potential cancer cell drug vulnerabilities, thus providing an efficient tool for precision medicine. In practice, viable leukemic cells are isolated from the bone marrow or peripheral blood and incubated with the drugs in desired concentrations. After the preset incubation time has elapsed, cell viability is measured, and raw counts transformed via bioinformatics pipeline into more descriptive sensitivity metrics (Figure 3).[190] Drug responses can be measured using the area under the dose response curve (AUC), the half maximal inhibitory concentration (IC50), or drug sensitivity score (DSS). DSS outperforms conventional IC_{50} measurements when assessing drug responses.[191] Sample cell viability and cell count, the proportion of cancer cells in the sample, and the cell medium in the drug assay can theoretically affect *ex vivo* drug responses, as *ex vivo* conditions can never mimic the original tumor microenvironment precisely.[192] By using only well-established and optimized assays, these potential confounding factors can be minimized, and with flow cytometry-based analysis, the leukemia-specific drug responses can further be segregated.[188,193] Importantly, *ex vivo* findings have been reported to correlate well with *in vivo* responses in several studies concerning acute leukemias.[187,194,195] Systematic high-throughput drug sensitivity screening, especially in combination with NGS-based molecular profiling, can be used to identify completely novel targets and sensitivities,[96,196] or it can be successfully incorporated into a clinical setting for a more personalized treatment approach.[197,198]

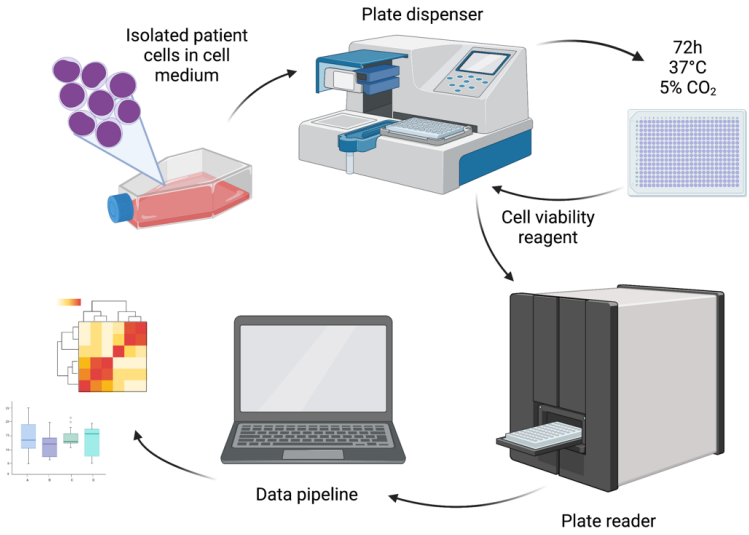

Figure 3 Schematic *ex vivo* drug sensitivity and resistance testing pipeline (created with Biorender.com).

3 AIMS OF THE STUDY

The aim of this thesis was to investigate potential biomarkers for more personalized disease stratification in Ph+ ALL, and to identify novel drug candidates for clinical trials, with particular focus on elderly, non-fit patients.

The more specific aims were:

To characterize the immunological microenvironment in pretreatment B-ALL BM, and to compare the BM immune contexture of Ph+ ALL patients to Ph$^-$ ALL patients. The prognostic value of BM immune profile at diagnosis was also evaluated. (I)

To explore the efficacy of 64 novel or repurposed drugs in an *ex vivo* assay to identify novel compounds for the treatment of B-ALL. In addition, by combining the drug data with gene expression data, to evaluate the link between molecular profile and *ex vivo* drug responses. (II)

To investigate potential clinical and genetic biomarkers for survival using targeted NGS sequencing and MLPA approaches combined with clinical registry data. In addition, to assess the frequency of subclonal pretreatment T315I mutations and their impact on treatment decisions. (III)

4 SUBJECTS AND METHODS

4.1 STUDY SUBJECTS

All clinical data was obtained from the Finnish Hematology Registry (FHR), a population-based centralized database, which covers all Finnish university hospitals. FHR includes data from Finnish Leukemia Group (FLG) ALL clinical trials dating from 1984, and stores information on clinical variables, treatments, treatment outcomes, laboratory values, and results from cytogenetic and molecular analyses. Bone marrow samples were retrieved from the clinical laboratories and from the Finnish Hematology Registry and Clinical Biobank (FHRB; https://www.fhrb.fi/) with appropriate ethics approval. All patients had signed a written informed consent for the studies and for collection of clinical data to FHR. The studies were approved by institutional ethics committee and conducted in accordance with the Declaration of Helsinki.

4.1.1 STUDY I

For mIHC analysis, we collected deposited, diagnostic-phase, formalin-fixed and paraffin-embedded (FFPE) BM biopsies of adult B-ALL patients (n = 52) from clinical laboratory pathology archives. The cohort included both Ph+ (n = 31) and Ph$^-$ (n = 21) patients. Five samples were obtained from Tampere University Hospital (Tampere, Finland) pathology department and the others from Helsinki University Hospital (Helsinki, Finland). Patients with a preceding malignancy were excluded.

We used BM biopsies from non-leukemic controls (n = 14) as a reference. Control patients were examined in the hematology or internal medicine outpatient clinics typically due to moderate changes in the blood counts, but no diagnosis of any hematologic malignancy, chronic infection, or autoimmune disease could be established in the examinations and in a 6-year follow-up (Table 2).

To validate the prognostic biomarkers found in the mIHC discovery cohort, we analyzed 31 vitally frozen, pretreatment adult B-ALL BM samples obtained from the FHRB. Both Ph+ (n = 13) and Ph$^-$ (n = 18) patients were represented. Altogether 76 ALL patients were studied, as seven patients had samples in both cohorts. All patient-related clinical data was attained from the FHR. Ph+ patients who were treated prior to the TKI era were excluded from the outcome analyses and were included only in the BM immunological characterization.

Patient characteristics of the subjects included in the survival analyses are listed in Table 3.

Table 2 *Characteristics of the control subjects (n=14) and indications for the bone marrow biopsy. Reproduced with permission from Leukemia (Springer Nature).*[199]

Gender	Age	Indication for bone marrow biopsy
Male	61	Persistent thrombocytosis
Male	56	Thrombocytosis
Female	57	Mild leukopenia and thrombocytopenia
Male	13	Mild neutropenia
Male	60	Fluctuating leukocytosis and mild thrombocytosis
Male	61	Mild thrombocytopenia
Male	44	Mild eosinophilia
Male	65	Unspecific, hypodense focal lesions in spleen
Female	40	Thrombocytosis and leukocytosis for several months
Female	25	Mild neutropenia
Female	44	Moderately elevated hemoglobin and mild thrombocytosis
Female	47	Iron-deficiency anemia with mild neutropenia
Male	54	Persistent erythrocytosis
Female	43	Mild anemia and lymphopenia

Table 3 *Patient characteristics of the subjects included in survival analyses. Of the diagnostic data, median value and range are reported.*

Variable	Discovery cohort (n=44)	Validation cohort (n=31)
Gender, male (%)	55	65
AlloHSCT (%)	55	52
Age (years)	47 (16–72)	43 (19–69)
Ph+ (%)	57	42
CD20+ (%)	36	58
Leukocytes (10E9/l)	15.6 (0.4–174)	18.4 (0.9–188.5)
Platelets (10E9/l)	47 (3–233)	45 (3–252)
BM blasts (%)	90 (50–100)	90 (50–100)
WHO[a] ≥1 (%)	66	71

alloHSCT allogeneic hematopoietic stem cell transplantation, *BM* bone marrow; [a]WHO-ECOG performance scale

4.1.2 STUDY II

For this study, we obtained cryopreserved, pretreatment bone marrow samples from the FHRB.

Initially, we received altogether 31 diagnostic-phase, adult B-ALL BM samples. Of these, 13 samples (Ph+ ALL, n = 3; Philadelphia chromosome negative [Ph⁻] ALL, n = 10) displayed low cell viability, and were discarded. Ultimately, 18 samples were subjected to further analysis (Ph+ ALL, n = 10; Ph⁻ ALL, n = 8).

The median age of Ph+ ALL subjects was 42 years (range 22-68) and of Ph⁻ ALL 43 years (range 24-68). There was no significant difference in the BM blast percentage (median 89.5%, range 54-98%).

4.1.3 STUDY III

FHR returned data of 141 Ph+ ALL adult patients (diagnosis years 1984-2020). A total of 82 Ph+ ALL patients were treated with TKI-based therapies in first line and were analyzed in more detail. Of these 82 patients, we retrieved 41 diagnosis-phase and 11 relapse-phase bone marrow samples from the clinical laboratories and from the FHRB. Patient characteristics of frontline TKI-treated patients are compared in Table 4.

Table 4 *Patient characteristics of the frontline TKI-treated patients (n=82). Continuous variables were independently compared with Wilcoxon signed-rank test. Fisher's exact test was used for comparing categorical covariates with two groups and chi-squared test for covariates with more than two groups.*

	All patients (n=82)	**AlloHSCT (n=43)**	**No alloHSCT (n=39)**	**P**
Males/females, n	41/41	24/19	17/22	ns
Year of diagnosis, median (range)	2012 (2001-2020)	2009 (2001-2017)	2014 (2002-2016)	**0.02**
Median age, y (range)	51 (19-79)	42 (19-69)	64 (28-79)	**<0.0001**
BM blast %, median (IQR)	90 (85-90)	90 (86-95)	90 (85-90)	ns
Median WBC, 109/l (IQR)	14.4 (4.9-44.9)	14.4 (5.4-38.6)	14.6 (3.5-62.7)	ns
WBC ≧30x109/l, n (%)	28 (34)	16 (37)	12 (31)	ns
Imatinib/dasatinib,	43/39	25/18	18/21	ns

n				
ACAs, n (yes/no/unknown)	27/27/28	18/13/12	16/6/1/16	ns
BCR subtype, n (m/M/both/unknown)	39/14/2/27	23/8/1/11	16/6/1/16	ns

alloHSCT allogeneic hematopoietic stem cell transplantation, *BM* bone marrow; *IQR* interquartile range, *WBC* white blood cell count, *ACAs* additional chromosomal abnormalities, *m* minor transcript, *M* major transcript, *ns* not significant

4.2 METHODS

4.2.1 TISSUE MICROARRAYS (I)

Tissue microarrays (TMA) were constructed from duplicate 1 mm diameter punches from selected areas of FFPE BM biopsies. The most representative areas with high blast cell infiltration were first marked by an experienced hematopathologist. Control punches from non-leukemic patients were taken from BM regions with high cellularity. The punches were cut into thin 4 um sections with microtome and set on slides. Compared with whole-section analysis, the TMA technique enables analyzing hundreds of samples simultaneously, providing benefits in terms of cost-effectiveness, time, data storage and analysis.[200]

4.2.2 MULTIPLEX IMMUNOHISTOCHEMISTRY (I)

We stained the TMA slides using a protocol originally published by Blom *et al.* and adapted by Brück *et al* (Figure 4).[201,202] The sections were stained both with 5-plex fluorescent and subsequent 3-plex chromogenic staining. The antibody panels were designed to detect immune cells, such as B and T lymphoid cells, natural killer (NK), and dendritic cells (DCs), macrophages, and myeloid-derived suppressor cells (MDSCs). In addition, the panels included antibodies for immune checkpoint receptors (PD1, LAG3, OX40, TIM3, CTLA4, HLA-ABC) and ligands (PD-L1, PD-L2, HLA-G), alongside with various activation markers (Table 5 and Table 6).

Table 5 *mIHC staining panel. Reproduced with permission from Leukemia (Springer Nature).*[199]

Panels	GFP	Cy3	Cy5	Cy7	LPR	VGR
T cell activity	GrB 1:100	CD57 1:400	CD8 1:25	CD4 1:25		CD3 1:250
Memory T-cells	CD27 1:500	CD25 1:25	CD8 1:25	CD4 1:25		CD3 1:250
Immune checkpoints 1	PD-1 1:1500	TIM-3 1:2500	CD8 1:25	CD4 1:25		CD3 1:250
Immune checkpoints 2	LAG-3 1:150	CTLA-4 1:150	CD8 1:25	CD4 1:25		CD3 1:250
Immune checkpoints 3	PD-1 1:1500	OX40 1:25	CD8 1:25	CD4 1:25	CD45RO 1:250	CD3 1:250
Cancer cell ligands	HLA G 1:25	PD-L1 1:50	TIM-3 1:100	HLAABC 1:100	PD-L2 1:250	CD34 1:100
B-cells, NK-cells and macrophages	CD56 1:300	pSTAT1 1:100	CD3 1:25	CD20 1:25	CMAF 1:150	CD68 1:250
Dendritic cells and MDSCs	CD11b 1:250	CD33 1:200	BDCA-3 1:25	HLA-DR 1:200	CD11c 1:250	BDCA-1 1:100

GFP green fluorescent protein, *Cy3* cyanine 3 (orange-fluorescent label), *Cy5* cyanine 5 (far-red-fluorescent label), *Cy7* cyanine 7 (near-infrared fluorescent label), *LPR* liquid permanent red, *VG* vina green, *GrB* granzyme B, *MDSCs* myeloid-derived suppressor cells.

Table 6 *Markers used in immune cell characterization. Reproduced with permission from Leukemia (Springer Nature).*[199]

Cell name	Marker combination
Cytotoxic T-cells	CD3+CD8+
Helper T-cells	CD3+CD4+
NK cells	CD3-CD56+
M1 macrophages	CD3-CD68+pSTAT1+cMAF
M2 macrophages	CD3-CD68+pSTAT1-cMAF+
MDSCs	CD11b+CD33+HLADR
mDC1	CD11c+BDCA1+

NK-cells natural killer cells, *MDSCs* myeloid derived suppressor cells, *mDC1* myeloid dendritic cells type 1.

Slides were first deparaffinized with xylene and then rehydrated in a series of ethanol and H_2O. Heat-induced epitope retrieval was achieved in 10 mM Tris-Hcl and 1 mM EDTA buffer, which was heated up to 99°C for 20 min (PT Module, Thermo Fisher Scientific, Waltham, MA). We used diluted H_2O_2 (0.9%) to block endogenous peroxidase activity (15 min). Normal goat serum (10%) in Tris-buffered saline (TBS) was used as a protein blocking reagent (15 min).

For fluorescent staining, a pair of primary antibodies was diluted in protein blocking solution, followed by secondary anti-mouse and anti-rabbit antibodies conjugated to horseradish peroxidase (HRP) and diluted 1:1 in washing buffer (0.1% Tween-20 and 10 mM TBS). The antibodies were applied for 1h45min and 45 min, respectively. Tyramide signal amplification (TSA) Alexa Fluor 488 (Thermo Fisher Scientific) in TBS was added to increase the signal. The first staining round was ended by repeating the heat-induced epitope retrieval, with the intention of quenching the enzymatic activity of the secondary antibodies and denaturating the primary antibodies.

The second staining round was started by repeating the peroxidase and protein blocks. This was followed by application of a different primary antibody, matching secondary antibody, and TSA Alexa Fluor 555 (Thermo Fisher Scientific). Heat-induced epitope retrieval, peroxidase block, and protein block were repeated. We incubated the slides overnight in +4°C with two additional primary antibodies that had been immunized in different species.

The following day, we applied secondary antibodies (45 min) diluted in 1:150 washing buffer and conjugated to AlexaFluor 647 and AlexaFluor 750 (Thermo Fisher Scientific). After washing, the nuclei were counterstained with DAPI diluted in TBS (15 min). Finally, we applied mountant (ProLong Gold, Thermo Fisher Scientific) and a coverslip on the slides. The slides were scanned the following day.

After fluorescence imaging, the coverslips were detached from the tissue slides by incubating them overnight (+4°C) in washing buffer. Heat-induced epitope retrieval, peroxidase block, and protein block were again repeated. Then, we added two primary antibodies immunized in separate species, and a 1:1 mixture of alkaline phosphatase and HRP-conjugated secondary antibodies. Antibody complexes were detected using red (Liquid Permanent Red, Dako) and green (VinaGreen, Biocare Medical, Concord, CA) chromogen dyes. After each chromogen reaction, the slides were washed in H_2O (30 s). Last, the slides were mounted with Pertex mounting media and coverslips.

We followed common antibody selection recommendations and selected antibody clones that were validated by the manufacturer and used in merited publications (Table 7).[203,204] The antibodies were tested by staining tissue sections of lymph node, appendicitis, healthy and leukemic BM, and brain.

Table 7 *List of the antibodies used in the staining panels.*

Marker	Manufacturer	Clone	Host species
CD3	Thermo Scientific	EP449E	Rabbit
CD4	Abcam	EPR6855	Rabbit
CD8	BioSB	C8/144B	Mouse
CD11b	BioSB	EP45	Rabbit
CD11c	Abcam	EP1347Y	Rabbit
CD20	BioSB	L26	Mouse
CD25	Abcam	EPR6452	Rabbit
CD27	Sigma	Polyclonal	Rabbit
CD33	LsBio	LS-C338084	Mouse
CD34	Dako	QBEnd 10	Mouse
CD45RO	Abcam	UCH-L1	Mouse
CD56	Cell Marque	MRQ-46	Rabbit
CD57	Sigma	VC1.1	Mouse
CD68	Abcam	KP1	Mouse
Granzyme B	Novocastra	11F1	Mouse
pSTAT1	Cell Signaling	D3B7	Rabbit
cMAF	Abcam	EPR16484	Rabbit
BDCA-1	Abcam	2F4	Mouse
BDCA-3	Abcam	EPR4051	Rabbit
HLA-ABC	MBL International Corporation	EMR8-5	Mouse
HLA-DR	Abcam	TAL 1B5	Mouse
HLA-G	Santa Cruz	4H84	Mouse
PD1	LsBio	PDCD1	Mouse
CTLA4	Santa Cruz	F-8	Mouse
TIM3	Cell Signaling	D5D5R	Rabbit
OX40	Biolegend	ACT35	Mouse
LAG3	Abcam	EPR4329	Rabbit
PD-L1	Cell Signaling	E1L3N	Rabbit
PD-L2	Sigma	Polyclonal	Rabbit

4.2.3 SLIDE DIGITIZATION AND IMAGE PREPROCESSING (I)

The slides were digitized with AxioImager.Z2 (Zeiss, Germany) microscope equipped with Zeiss PlanApochromat 20x objective (NA 0.8), CoolCube1 CCD camera (MetaSystems, Germany), and PhotoFluor LM-75 (89 North) metal-halide light source. For fluorescent signals, we used filters for DAPI, FITC, Cy3, Cy5, and Cy7. The exposure times of the fluorescent channels were

visually optimized. The digitized images were exported in JPEG2000 format (95% quality).

After slide digitization, the brightfield images were deconvolved to separate the individual chromogen staining signals.[205] We applied two-dimensional phase correlation method that used mean image of both fluorescent and brightfield channels for image registration.[206] Mean images were downsized by a factor of eight and image histograms were fitted to each other before registration. We used a numerical computing platform (MATLAB, MathWorks, Natick, MA, US) for image preprocessing.

The quality of the gray-scale images of each TMA spot was evaluated after registration. Small artefacts were manually curated before image analysis. Images with blurred focusing, typically due to air bubbles in mounting media or shattered tissue, and images that failed registration were disqualified. As we used duplicate spots, none of the leukemic subjects was excluded from the study.

4.2.4 CELL SEGMENTATION AND CLASSIFICATION (I)

The cell masks were segmented with parent immune cell markers, such as CD3 for T cells, by using Otsu's thresholding method. We used intracellular intensity patterns to separate the single cells from aggregates. Cell segmentation, marker intensity measurements, and cell classification were executed in CellProfiler 2.1.2, an open-source image analysis platform.[207–209] We calculated the cell count for each TMA spot from binary DAPI images with Fiji by dividing the thresholded area by the area of one cell. To avoid bias, we excluded TMA spots with less than 1000 cells from the analysis. We used FlowJo v10 to compute marker co-localization and cell classification with integrated intensity on a single-cell level.

Due to cell number variation between spots, different cell types were quantified either as a proportion of all cells in the TMA spot or as a proportion of a defined cell subtype, such as CD3+CD4+/PD1+TIM3+ T cells of all CD3+CD4+ T cells. We calculated the mean values of each class from the duplicate sample spots.

4.2.5 FLOW CYTOMETRY (I)

We used flow cytometry to validate the key findings in the mIHC cohort. Cryopreserved pretreatment B-ALL BM mononuclear cells (N = 31) were first thawed and then stained using CD3-PerCP-Cy5.5 (SK7, BD Pharmingen), CD8-FITC (SK1, BD Pharmingen), CD4-BV510 (SK3, BD Pharmingen), TIM3-PE-Cy7 (F38-2E2, Invitrogen), PD1-AlexaF647 (EH12.1, BD Pharmingen), CD45-BV421 (HI30, BD Horizon), CCR7-PE (150503, R&D Systems), and

CD45RA-AlexaF700 (HI100, BD Pharmingen) antibodies. Fluorescence was measured with FACSVerse (BD Pharmingen), and the cell proportions were defined from the non-debris cells with FlowJo v10 similarly as with the mIHC data. The proportion of CD3+CD4+/PD1+TIM3+ T cells was then used in the survival analysis correspondingly as in the discovery cohort.

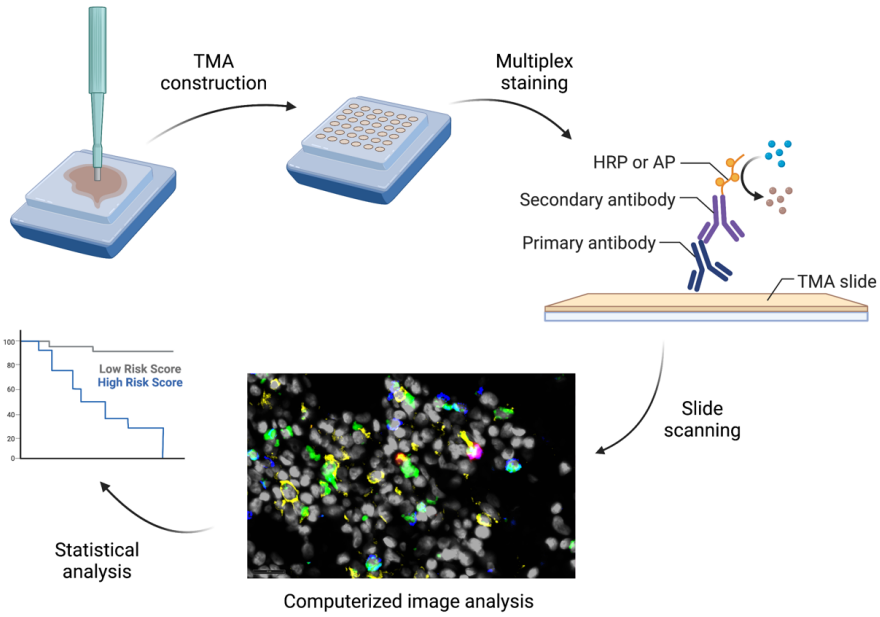

Figure 4 Tissue microarrays (TMA) were analyzed with multiplex immunohistochemistry and computerized image analysis (created with Biorender.com).

4.2.6 DRUG SENSITIVITY AND RESISTANCE TESTING (II)

First, we designed a custom drug plate with 64 clinically relevant drugs in 5 different concentrations, covering a 10,000-fold concentration range (Table 8). We mainly focused on potential actionable anticancer drugs, currently off-label for ALL, such as MDM2 antagonists, VEGFR, BCL2, BCL-XL, BET, MEK, JAK, Aurora kinase, PI3K, MTOR, IGF1R, ERK, STAT3, STAT5, HSP90, and nicotinamide phosphoribosyltransferase (NAMPT) protein inhibitors. The drug plate included also compounds from glucocorticoids and BCR::ABL1 inhibitors. Drug plates were constructed at Institute for Molecular Medicine Finland by a specialized High Throughput Biomedicine Unit.

We analyzed 18 primary adult B-ALL samples (Ph+ ALL, n = 10; Ph⁻ ALL, n = 8) following a previously published DSRT protocol.[190] Shortly, the cryopreserved BM mononuclear cells (MNC) were thawed and suspended in

mononuclear cell medium (PromoCell) supplemented with 50µg/ml gentamicin. Before counting, the cells were treated with DNase I (ThermoFisher Scientific). Then, 5,000 cells per well were plated by using automated microplate dispenser, and the cells were incubated with the drug plates for 72 hours in a humid environment at 37˚C and 5% CO_2. We counted cell viability using CellTiter-Glo (Promega) immediately after plating and after the three-day incubation. Cell viability was measured with PHERAstar FS (BMG Labtech) plate-reader, and the raw data were subsequently run through Breeze (https://breeze.fimm.fi) data analysis pipeline.[210]

The viability readouts were used to calculate drug sensitivity score (DSS), which measures the area under the dose response curve and takes into account both drug efficacy and potency.[191] DSS values >10 were defined as effective *ex vivo*, and DSS > 20 as highly effective. Low blast count (<10-20%) in the sample may confound DSS results, but in our study cohort the blast percentages were high.

Table 8 *Layout of the DSRT assay.*

Compound	Class	C1 (nM)	C2 (nM)	C3 (nM)	C4 (nM)	C5 (nM)
Alisertib	Aurora inhibitor	1	10	100	1000	10000
Axitinib	VEGFR inhibitor	1	10	100	1000	10000
Belinostat	HDAC inhibitor	1	10	100	1000	10000
BGB324	Axl inhibitor	1	10	100	1000	10000
BMS_754807	IGF1R inhibitor	1	10	100	1000	10000
Bosutinib	BCR::ABL inhibitor	1	10	100	1000	10000
CEP-32496	BRAF inhibitor	1	10	100	1000	10000
Cerdulatinib	JAK, SYK inhibitor	1	10	100	1000	10000
Cobimetinib	MEK1/2 inhibitor	0.1	1	10	100	1000
Danusertib	Aurora, Ret, TrkA, FGFR-1 inhibitor	1	10	100	1000	10000
Daporinad	NAMPT inhibitor	0.1	1	10	100	1000
Dasatinib	BCR::ABL inhibitor	0.1	1	10	100	1000
Dexamethasone	Glucocorticoid	1	10	100	1000	10000
Duvelisib	PI3K inhibitor	0.05	0.5	5	50	500
FRAX486	PAK inhibitor	0.5	5	50	500	500
Gedatolisib	PI3K/mTOR inhibitor	0.1	1	10	100	1000
Glasdegib	SMO inhibitor	0.1	1	10	100	1000
GSK_2334470	PDK1 inhibitor	1	10	100	1000	10000
GSK525762	BET inhibitor	1	10	100	1000	10000
Idasanutlin	MDM2 antagonist	1	10	100	1000	10000
Idelalisib	PI3K inhibitor	1	10	100	1000	10000
Imatinib	BCR::ABL inhibitor	1	10	100	1000	10000
JQ1	BET inhibitor	1	10	100	1000	10000
Lenalidomide	Immunomodulatory	10	100	1000	10000	100000
Lestaurtinib	FLT3, JAK2, TRK inhibitor	0.1	1	10	100	1000
LLL12	STAT3 inhibitor	1	10	100	1000	10000
Losmapimod	p38MAPK inhibitor	1	10	100	1000	10000

Luminespib	HSP90 inhibitor	0.1	1	10	100	1000
LY3009120	pan-RAF inhibitor	1	10	100	1000	10000
Mepacrine	anti-malaria	5	50	500	5000	50000
Methylprednisolone	Glucocorticoid	1	10	100	1000	10000
Milciclib	CDK2 inhibitor	1	10	100	1000	10000
MK_2206	Akt inhibitor	0.1	1	10	100	1000
MLN_0128	mTOR inhibitor	0.1	1	10	100	1000
Navitoclax	BCL2 and BCL-XL inhibitor	1	10	100	1000	10000
Nilotinib	BCR::ABL inhibitor	1	10	100	1000	10000
Nintedanib	VEGFR inhibitor	1	10	100	1000	10000
NVP_ABL001	allosteric inhibitor of BCR::ABL1	0.1	1	10	100	1000
NVP_AEW541	IGF1R inhibitor	1	10	100	1000	10000
NVP_LCL161	SMAC mimetic	1	10	100	1000	10000
Onalespib	HSP90 inhibitor	0.25	2.5	25	250	2500
Osimertinib	EGFR inhibitor	0.25	2.5	25	250	2500
Pexidartinib	KIT, CSF1R, FLT3 inhibitor	1	10	100	1000	10000
Pimozide	STAT5 inhibitor	0.01	0.1	1	10	100
Plicamycin	RNA synthesis inhibitor	1	10	100	1000	10000
Pomalidomide	Immunomodulatory	1	10	100	1000	10000
Ponatinib	BCR::ABL inhibitor	0.1	1	10	100	1000
PRI_724	CBP/beta-catenin inhibitor	1	10	100	1000	10000
Radotinib	BCR::ABL inhibitor	0.2	2	20	200	2000
Ralimetinib	p38MAPK inhibitor	1	10	100	1000	10000
Raloxifene	Selective estrogen receptor modulator	1	10	100	1000	10000
Ruxolitinib	JAK1&2 inhibitor	1	10	100	1000	10000
SAR405838	MDM2 antagonist	1	10	100	1000	10000
Saracatinib	Abl, Src inhibitor	1	10	100	1000	10000
SCH772984	ERK1&2 inhibitor	1	10	100	1000	10000
STAT5i	STAT5 inhibitor	0.05	0.5	5	50	500
Sunitinib	Broad TK inhibitor	0.1	1	10	100	1000
Temsirolimus	mTOR inhibitor	0.01	0.1	1	10	100
Tipifarnib	Farnesyltransferase inhibitor	1	10	100	1000	10000
Tivozanib	VEGFR inhibitor	1	10	100	1000	10000
Trametinib	MEK inhibitor	0.025	0.25	2.5	25	*250*
Ulixertinib	ERK inhibitor	1	10	100	1000	10000
Venetoclax	BCL2 inhibitor	0.1	1	10	100	1000
Vistusertib	mTOR inhibitor	1	10	100	1000	10000

4.2.7 TARGET ADDICTION SCORING (II)

Since drug responses are typically a complex result of multiple factors, we applied a targeted addiction score (TAS) approach to our DSRT data. TAS combines drug sensitivity profiles with drug-target interactions and takes into account also known off-target effects.[211] For TAS analysis, we collected altogether 463 targets for 64 drugs. The targets for 33 drugs were collected from kinase inhibitor bioactivity database (KiBA),[212] and the targets for the remaining 31 drugs were manually curated from literature. Last, TAS results

were compared, sample-wise, with gene expression read counts from the same targets.

4.2.8 WHOLE TRANSCRIPTOME SEQUENCING (II)

16 samples (9 Ph+ and 7 Ph$^-$) were subjected to whole transcriptome sequencing (RNAseq), as two of the subjects analyzed with the DSRT assay did not have sufficient material for RNA extraction. We constructed the RNAseq libraries and processed the RNAseq data as earlier described.[213]

First, RNA was extracted from BM MNC pellets using Qiagen miRNeasy kit (QIAGEN, Hilden, Germany). Ribo-depletion based RNAseq libraries were constructed by using 3 μg of good-quality RNA (RIN 8-10) per sample. The libraries were then sequenced using Illumina HiSeq and paired-end 100 bp reads. To avoid batch effect, all samples were sequenced in the same series. RNAseq data was processed by trimming low-quality reads and bases, mapping good quality reads to human reference genome build 38, producing expression estimates, and performing variant calling.

R v3.3.3 was used for analyzing transcriptomic data, with R package DESeq2 for differential expression (DE) analysis and maftools for visualization of variant data.[214–216] For DE analysis, we used DESeq2-normalized counts, alpha value 0.05, and a moderate threshold of 0.32 for log2 fold change. P-values were adjusted with Benjamini-Hochberg method.[217] Lowly expressed and non-protein-coding genes were pre-filtered. In DE analysis, we compared Ph+ ALL to Ph$^-$ ALL patients. Ph-like samples were kept in the DE model, but they were not included in the Ph$^-$ group.

To study sample purity, we applied Cibersort to our RNAseq data.[218] Similarly as in the DE analysis, we first filtered lowly expressed genes and kept only the protein-coding genes. Naive B-cells turned out the most prevalent cell type, as expected.

4.2.9 FUSION GENE PANEL (II)

Seven samples were analyzed with commercial, RNA-based Archer FusionPlex Pan-Heme Kit (ArcherDX, Boulder, CO), that targets >199 genes related to hematological malignancies. Besides point mutations and known fusions, the assay can identify also deletions in *IKZF1* and novel fusions in fusion panel target genes. First, RNA was extracted with RNeasy Mini Kit (QIAGEN, Hilden, Germany), and second, cDNA was synthesized from 250 ng of RNA using random primers to generate random start sites. Both ends of the end-repaired cDNA molecules were ligated to sample-specific indexes and specific molecular barcode adapters. Ligation Cleanup Beads (ArcherDX, Boulder, Colorado) were used to remove excess adapters. We used multiplex gene-

specific primers (GSP1) during the first round of PCR, and after cleaning, nested gene-specific primers (GSP2) during the second round of PCR to increase specificity. The final libraries were quantified with Ion Library TaqMan Quantification kit (Thermo Fisher Scientific, MA, USA) and the products were sequenced using the Ion Torrent sequencing platform (Ion Proton or Ion GeneStudio S5 system, Carlsbad, CA, USA). We obtained approximately 2.5 million reads per sample. Suite Analysis 6.0.4 Software (ArcherDX, Boulder, Colorado) was used for data analysis.

4.2.10 MICROARRAY DATA FROM PUBLIC DATABASES (II)

In addition to sequencing our own samples, we analyzed publicly available expression data in order to deepen our understanding of potential gene expression biomarkers for *ex vivo* drug responses. We used E-MTAB-5035 data set from the European Bioinformatics Institute's ArrayExpress database (http://www.ebi.ac.uk/arrayexpress).[219] The analyzed set included microarray expression data of 137 adult B-ALL patients (96 Ph⁻ and 41 Ph+) measured with Affymetrix Human Genome U133 Plus 2.0 Microarray. Furthermore, we obtained transcriptomic data from St Jude PeCan Data Portal (https://pecan.stjude.cloud) from a study published by Gu *et al.*[20] The St Jude cohort contained 670 adult and adolescent B-ALL patients (83 Ph+), with a median age of 40 years (range 16–79). Only genetic subgroups with more than 20 patients were included in the analyses. We also used data from HEMAP (hemap.uta.fi) to assess *MDM2* gene expression across different hematological diseases.[220] The HEMAP cohort included 1300 patients with B-ALL, the vast majority being pediatric cases. We analyzed the public expression data with R (v3.3.3), using R packages ArrayExpress, affy, and limma.[221–223]

4.2.11 CELL LINES AND DRUG COMBINATION TESTING (II)

To find possible synergy between the most interesting compounds, we tested five different two-drug combinations in human B-ALL cell lines, that represented Ph+ ALL (NALM-21), Ph⁻ ALL (Kasumi-2), and Ph-like ALL (MHH-CALL-4). The combinations in the assay were dasatinib+venetoclax, dasatinib+navitoclax, dasatinib+idasanutlin, venetoclax+idasanutlin, and navitoclax+idasanutlin. The drugs were pre-plated in five concentrations, covering a 10,000-fold concentration range (Table 9).

The cell lines were acquired from DSMZ (German Collection of Microorganisms and Cell Cultures) and cultivated according to the provider's recommendations in RPMI-1640 (Lonza) supplemented with 10%-20% fetal bovine serum, 2 mM L-glutamine (Lonza), 100 U/mL penicillin, and 100

μg/mL streptomycin (Gibco). The number of the passages was below 15 for every cell line at the time of plating.

After plating, the cells were incubated with the combination drug plates for 72 hours in a humid environment at 37˚C and 5% CO_2. After the three-day incubation, cell viability was measured using CellTiter-Glo (Promega) and PHERAstar FS (BMG Labtech) plate-reader. We used SynergyFinder to analyze the drug combination synergy data with ZIP model.[224]

Table 9 *Drug concentrations in the combination testing assay.*

Compound	C1 (nM)	C2 (nM)	C3 (nM)	C4 (nM)	C5 (nM)
Dasatinib	0.1	1	10	100	1000
Venetoclax	0.1	1	10	100	1000
Navitoclax	0.1	1	10	100	1000
Idasanutlin	0.1	1	10	100	1000

4.2.12 MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION (III)

Multiplex ligation-dependent probe amplification (MLPA) is a PCR-based method, that enables the amplification of multiple targets in the same reaction. When detecting CNAs, the probe signal intensities are compared to reference sample DNA known to harbor normal two copies of the gene in interest. We used the commercial SALSA MLPA Probemix P335 ALL-IKZF1 kit (MRC Holland, Amsterdam, the Netherlands) to discriminate CNAs in *IKZF1*, *CDKN2A/B*, *PAX5*, *EBF1*, *ETV6*, *BTG1,* and *RB1* genes. Genomic DNA was extracted from the BM samples with DNeasy Blood & Tissue Kit (Qiagen). We applied 50-100 ng of genomic DNA per reaction and performed the assay according to the manufacturer's protocol. The data was analyzed with Coffalyser.Net analysis software (MRC Holland, Amsterdam, the Netherlands). Intensity ratios between 0.7 and 1.3 were considered to represent a normal copy number, and ratios <0.7 were classified as deletions.

4.2.13 TARGETED NEXT-GENERATION SEQUENCING GENE PANEL (III)

First, genomic DNA was extracted from the BM samples with DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol. We processed 150ng of gDNA according to SeqCap EZ HyperCap Workflow User's Guide, v2.1 Enzymatic Fragmentation (Kapa Biosystems, Inc., Wilmington, MA, USA) using Unique Dual Index adapters (Integrated DNA Technologies, Coralville, IA, USA). LabChip GX Touch HT High Sensitivity assay (PerkinElmer, USA)

was used for library quality check. We used seven cycles for precapture amplification and performed SeqCap custom captures (170621_HG38_ALL-75G_EZ_HX3) in 6-7 samples multiplexed DNA Sample Library Pools (600 µg of each library). We used 10 cycles for post capture amplification and quantified the captured library pools with KAPA Library Quantification Kit (KAPA Biosystems, Wilmington, MA, USA) and 2100 Bioanalyzer High sensitivity kit. We sequenced the samples in three batches. Illumina HiSeq2500 system in HiSeq high output mode (v4 kits, Illumina, San Diego, CA, USA) was used for the first batch. The other batches were sequenced with Illumina NovaSeq system using S4 flow cell with lane divider (Illumina, San Diego, CA, USA) and v1.0 chemistry. In all batches, read length for the paired-end run was 2x101 bp.

For variant calling, the analysis of DNA read data was mainly performed as previously described.[225] First, the sequence data was first pre-processed for low quality, adapter sequences, and short read length using the Trimmomatic software.[226] Paired-end reads passing filters were then aligned to human reference genome build 38 (EnsEMBL v82) using BWA-MEM,[227] alignments were sorted by coordinate using the SortSAM, and PCR duplicates were marked with the MarkDuplicate module of the Picard toolkit (Broad Institute). We used default parameters.

We employed Genome Analysis Toolkit (GATK) toolset. Variant calling was based on the GATK somatic short variant best practice (version 3.5), supplemented with the estimation of the cross-sample contamination level and filtering of the 8-oxoguanine and deamination artefacts with GATK4 CalculateContamination, CollectSequencingArtifactMetrics, and FilterByOrientationBias tools.[228] GATK resources were converted from GRCh37 to GRCh38 using CrossMap, and chain files were downloaded from EnsEMBL.[229]

When separating variants with a low variant allele frequency from technical or biological artefacts, datasets were filtered after variant calling for vector contamination, RNA or pseudogene associated reads. In this process, reads from the final GATK alignment files were re-mapped to human reference genome build 38 (EnsEMBL v94) using STAR2 with the guidance of EnsEMBL v94 gene models. Alignments were sorted by coordinate using the SortSAM, PCR duplicates were marked with the MarkDuplicate, indels were left-aligned using the GATK toolkit, and duplicate pairs, unmapped pairs, and secondary alignment were removed. Read pairs with an internal gap ≥ 10 bp and insert size less than 50 kb or with an insert size of between 1 and 50 kb were classified as discordant. The fraction of discordant read pairs relative to undiscordant spanning exon-intron boundaries were then assessed per gene and exon.

Variants with a variant allele frequency ≤5% or with a contamination fraction + 2% were removed with the exception of variants supported by approximately same fractions of discordant and undiscordant reads (i.e. variant allele frequency in discordant read pairs × 0.8 ≤ variant allele frequency in undiscordant read pairs ≤ variant allele frequency in discordant read pairs × 1.2) at gene and exon level and variants residing in genes and exons without any discordant read pair.

Variants were annotated and filtered using the Annovar tool against the RefGene database.[230] At first, all variant calls were normalised using bcftools.[231] Variants other than those passing all MuTect2 filters with a TLOD ≥ 6.3 or a TLOD ≥ 5.0, and supported by five or more independent COSMIC samples were filtered.[232] Variant data were then filtered for false-positives by removing variants in 20 intronic and intergenic regions, with a total coverage ≤ 10, and not supported by at least one read in both directions as well as variants with variant quality value ≤ 40, variant allele frequency ≤ 5%, strand odd ratio for SNVs ≥ 3.00, and strand odd ratio for indels ≥ 11.00, minor allele frequency ≥ 1% in the 1KG database, minor allele frequency ≥ 3% in the EPS database, minor allele frequency ≥ 1% in general, African, Finnish, Latino, East Asian, and Non-European ExAC, gnomAD exome, or gnomAD genome databases, PHRED-like CADD score ≤ 3.00, and likelihood ratios score ≤ 2.00. Variants with a variant allele frequency ≥ 30% were accepted, if supported by five or more COSMIC samples. Finally, cancer associated mutations were picked by removing those without COSMIC identifier. Variants were manually curated, missed known cancer variants checked and rescued, and variants inspected using Integrative Genomics Viewer 2.3.66 (Broad Institute).

Table 10 *List of the gene panel targets.*

ABL1	*IL7R*	*RUNX1*
ABL2	*IRF4*	*SH2B3*
AICDA	*IRF8*	*SOX11*
BCL11B	*JAK1*	*STAT3*
BCL2	*JAK2*	*STAT5B*
BCL6	*JAK3*	*TCF3*
BCR	*KDM6A*	*TYK2*
BLNK	*KLF2*	*WT1*
BRAF	*KMT2A*	*ZCCHC7*
CD274	*KRAS*	*TP53*
CSF1R	*MPL*	*CDKN2A*
CRLF2	*MYC*	*CDKN2B*
CTLA4	*NF1*	*RB1*

DNM2	*NRAS*	*ERG*
DNTT	*NTRK3*	*P2RY8*
EBF1	*PAX5*	*ZNF384*
EPOR	*PBX1*	*MEF2D*
ETV6	*PDCD1*	*CHD1*
EZH2	*PDCD1LG2*	*NOTCH1*
FGFR1	*PDGFRA*	*CREBBP*
FLT3	*PDGFRB*	*FBXW7*
IDH1	*PTPN11*	*PAG1*
IKZF1	*PTK2B*	*NT5C2*
IKZF2	*RAG1*	*SEMA6A*
IKZF3	*RAG2*	*SETD2*

4.2.14 DIGITAL DROPLET PCR FOR DETECTING T315I (III)

To study subclonal T315I mutations, we designed a sensitive digital droplet PCR (ddPCR) assay with a variant allele frequency (VAF) detection limit of 0.04%. First, RNA was extracted with QIAamp RNA Blood Mini kit (QIAGEN, Hilden, Germany) on a QIAcube purification robot (QIAGEN) according to the manufacturer's protocol. RNA (2 µg) was converted to cDNA using SuperScript VILO cDNA Synthesis Kit (ThermoFisher, Waltham, MA) according to the manufacturer's protocol.

To select and amplify the translocated *BCR::ABL1*, we performed a 40 cycle PCR amplification using a forward primer located in *BCR* exon 1 and reverse primer in *ABL1* exon 10, using Q5 High Fidelity DNA polymerase (New England Biolabs, Ipswich, MA).[233] In a laminar flow cabinet in a separate laboratory, the PCR product was diluted 10^5 to 10^8 in nuclease free water. We ran ddPCR on the dilutions using ddPCR Supermix for Probes on a QX200 ddPCR system (Bio-Rad, Hercules, CA) with forward primer: GGTCTGCACCCGGGAG, reverse primer: AGGTAGTCCAGGAGGTTC, wild type probe: HEX-CCGTTCTATATCATCACTGAGTTCATGACC*TAGAACG*-BHQ1 and T315I probe: FAM-CCGTTCTATATCATCAtTGAGTTCATGACC*TAGAACG*-BHQ1.

We employed a novel probe design by adding seven base pairs to the 3'-end (shown in italic) that were reverse complimentary to the 5'-end of the probe (underlined). When temperatures sink below 65°C, the 5'-end and 3'-end of the probes form a hairpin structure, thereby decreasing non-specific binding to the mutant or wildtype DNA and improving the assay specificity.[234] Cycling conditions were 95°C (10 min), followed by 40 cycles in 94°C (30 sec) and 60°C (60 sec).

4.2.15 STATISTICAL ANALYSES (I-III)

For continuous variables with non-normal distributions, group differences were independently compared with a non-parametric Mann-Whitney U test (study I-II) or a Wilcoxon signed-rank test (study III), or in case of normally distributed variables, t-test. Normality of continuous variables was tested using the Shapiro-Wilk test and equality of variances was tested with the Levene's test. We used Fisher's exact test when comparing categorical covariates with two groups and chi-squared test for covariates with more than two groups. For multiple test correction, we applied Benjamini–Hochberg's method.[217]

Survival functions were estimated with Kaplan-Meier method and log-rank test. When analyzing relapse-free survival, relapse and death were treated as competing events, and the primary refractory cases were documented as an event occurring immediately after diagnosis. For univariate analysis, we applied Cox proportional hazards model. Gray's test was used for analyzing competing risks.[235]

In study I, all variables with $P < 0.20$ (log-rank test) in univariate Cox proportional hazards analysis were incorporated in a L1-penalized elastic net regression analysis.[236] The regression model was chosen for its capability of performing both the model shrinkage and variable selection without overfitting the model in a relatively small data set. The shrinkage parameter lambda (λ) was defined by minimum mean cross-validated error. The proportional hazards assumption was tested using scaled Schoenfeld residuals.

The model prediction was assessed by comparing the area under the receiver operating characteristic (AUROC) curves (bootstrap method, number of iterations: 4000), time-dependent ROC curves (inverse probability of censoring weighting approach), and C-statistic values.

We used R v3.3.3 (studies I-II) and R v3.6.3 (study III) for statistical analyses and data visualization.[214] In study I, we used R packages glmnet, corrplot, survminer, survival, ggplot2, cmprsk, pROC, plotROC, timeROC, and gplots. In study II, we used R packages ggplot2 and pheatmap, and in study III R packages survminer, survival, cmprsk, cmprsk2, swimplot, ComplexHeatmaps, and ggplot2.[237] Correlation plots were drawn with Prism (v7 in study I and v8 in study II; GraphPad Software Inc) and tested with non-parametric Spearman correlation.

5 RESULTS

5.1 IMMUNE PROFILING IN ALL BONE MARROW

5.1.1 CHARACTERIZATION OF THE BM IMMUNE MICROENVIRONMENT

The cancer microenvironment plays a significant role in carcinogenesis, treatment responses, disease recurrence, and metastasis.[125] Despite its fundamental contribution, ALL BM immune microenvironment has remained poorly investigated. We characterized the BM immune profiles of 52 adult B-ALL patients (31 Ph+ and 21 Ph^-) and 14 controls using TMA technique and mIHC. The mIHC panels covered 29 immune markers, including known immune checkpoint molecules and cancer ligands. We applied automated image analysis to recognize cells from digitized images and classified them into subtypes according to co-localized markers.

In hierarchical clustering, the control and ALL BMs clustered distinctly from each other, but there was no significant difference in the immune contexture of Ph+ ALL and Ph^- ALL BM (Figure 5). In general, immune cell phenotypes linked with cytolytic activity were decreased and markers associated with immune regulation were increased in ALL BM (Figure 6).

The proportion of antitumor-associated M1-polarized macrophages and the proportion of NK cells was decreased in ALL BM compared to the controls. Similarly, the proportion of activated T cells, deciphered by cytolytic CD8+granzyme B+CD57+ T cells as well as CD27+ T cells, was decreased in ALL BM.

Instead, cell subtypes associated with immune suppression, such as M2-polarized macrophages and myeloid derived suppressor cells (MDSCs) were enriched in ALL BM in comparison to controls. Also the expression of immunoregulatory PD1 and CTLA4 molecules on T cells was increased in ALL BM. However, the expression of inhibitory LAG3 and TIM3 molecules were decreased on ALL T cells compared to the control BM. The expression of immune-stimulatory OX40 was increased in CD4+ T cells but no difference could be observed in CD8+ T cells (Figure 7).

The proportion of antigen-presenting myeloid dendritic cell type 1 (mDC1) and the proportion of CD4+CD45RO+ memory T cells was elevated in ALL BM, but the percentage of CD8+CD45RO+ memory T cells did not reach statistical significance.

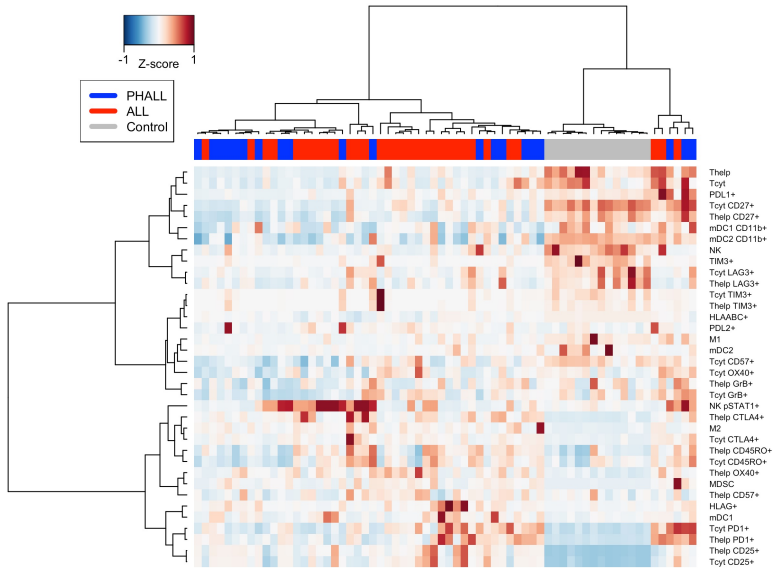

Figure 5 Visualization of immune cells quantified as a proportion of all cells and their immunophenotypes quantified as a proportion of the parent immune cell. Hierarchical clustering with Spearman correlation distance and Ward linkage method. Philadelphia chromosome-positive patients are annotated in blue and Philadelphia chromosome-negative patients in red. Reproduced with permission from Leukemia (Springer Nature)[199]

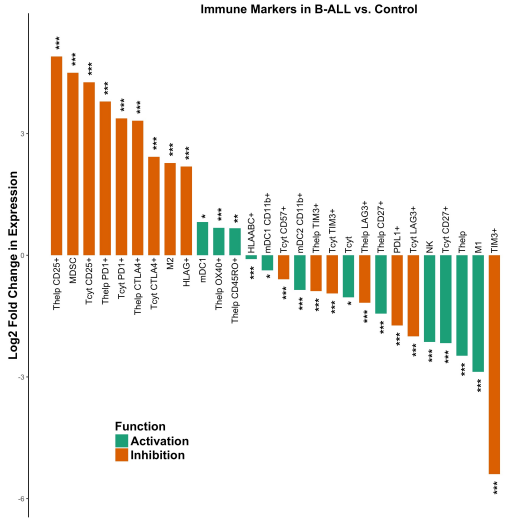

Figure 6 Two-fold logarithmic transformation of ALL-to-control ratios (median values). Only significant differences are included (***, adjusted P-value <0.05). The markers associated mainly with anticancer immunity are shown in green, and the markers associated with immunosuppression are shown in orange. Reproduced with permission from Leukemia (Springer Nature)[199]

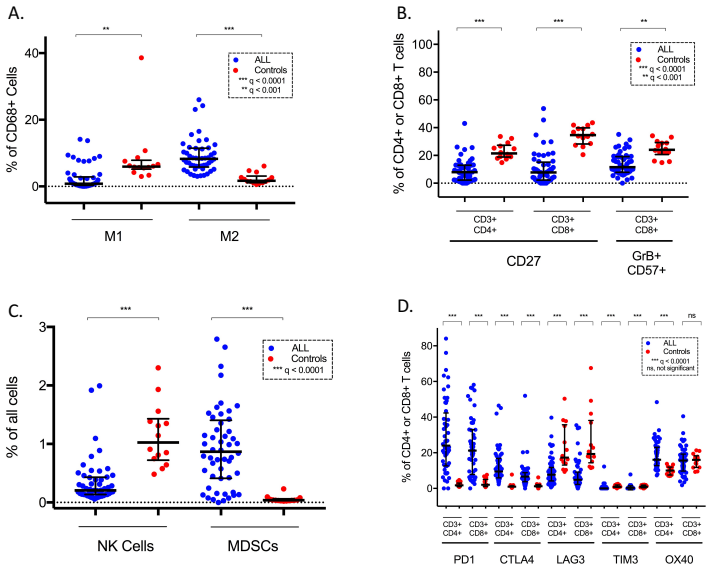

Figure 7 Proportion of A) M1-like and M2-like macrophages; B) CD27+ and granzymeB+CD57+ T cells; C) Natural killer (NK) cells and myeloid-derived suppressor cells (MDSCs); D) PD1, TIM3, CTLA4, LAG3, and OX40-expressing T cells. Comparison between ALL patients and controls (Mann–Whitney U test, p values adjusted (p.adj) with Benjamini–Hochberg method; **p.adj<0.001, ***p.adj <0.0001). Reproduced with permission from Leukemia (Springer Nature)[199]

5.1.2 SURVIVAL PREDICTION MODELS

Based on the immune profiles characterized with mIHC and clinical parameters, we created a risk model using L1-penalized Cox regression analysis. Preselected covariates with $P < 0.20$ in univariate Cox regression were inserted to the multivariate analysis. The resulting risk model was used to stratify patients into equally sized high-risk and low-risk groups.

High expression of CD4+PD1+TIM3+ T cells, older age, and low platelet count at diagnosis segregated a poor survival group. In the high-risk group, the hazard ratio (HR) for overall survival (OS) was 4.9 (95% confidence interval (CI) 1.8–13.3; $P = 0.0007$, log-rank test) and for RFS 3.7 (95% CI 1.4–9.6; $P = 0.004$, log-rank-test, Figure 8A-D). In the competing risk analysis (Gray's test), both deaths and relapses were more prevalent and censoring less prevalent in high-risk patients. Also in the univariate analysis, the higher expression of PD1+TIM3+ CD4+ T cells trended toward poor survival, though not reaching statistical significance.

When comparing other baseline patient characteristics, the proportion of BM blasts and the expression of CD20 at diagnosis was higher in the high-risk

group, but otherwise these two groups did not differ significantly. PD1+TIM3+ T cells were heterogeneously expressed in ALL BM, whereas in control BM this immunophenotype was practically absent (Figure 8E).

Next, we explored whether the PD1+TIM3+ expression on CD4+ T cells correlated with clinical or other immune parameters. There was no correlation between CD4+PD1+TIM3+ expression and baseline blood counts or age. However, the plentitude of T cells, and especially the granzyme B or TIM3 expressing CD4+ and CD8+ T cells correlated positively with CD4+PD1+TIM3+ T cells (Spearman correlation). In addition, there was a positive correlation with MDSCs.

The results of the generated risk model were validated with flow cytometry in a separate cohort (n=31). Similarly, as in the discovery cohort, higher proportion of CD4+PD1+TIM3+ T cells, older age (above cohort median), and low platelet count predicted poor OS (HR 4.7, 95% CI 0.98–22.5; P = 0.03, log-rank test) and RFS (HR 4.9, 95% CI 1.3–19.0; P = 0.01, log-rank test). The proportion of PD1+TIM3+ T cells varied greatly between the samples (mean 0.88%, range 0.00–10.11%, SD 1.88), correlating with the mIHC results. In the competing risk analysis, relapses were more frequent in the high-risk group (P = 0.014, Gray's test). The baseline patient characteristics of the high and low risk groups did not differ significantly.

Last, we compared our prediction model to risk stratification by MRD status at four months from the diagnosis. MRD negativity was defined as $<10^{-4}$ leukemic blasts per healthy cells, and all different MRD follow-up methods with sufficient detection sensitivity, such as ASO-PCR or multiparameter flow cytometry, were accepted. Our model predicted survival with higher confidence than the simple MRD measurement. However, when we combined the MRD status at 4 months with our risk model, the combined model prediction was improved, and each variable remained independent (P < 0.05; Figure 9A). We also compared our prediction model to the risk stratification defined by the treatment protocols NOPHO-ALL2008 or Finnish Leukemia Group ALL2000,[238] as the majority of the patients in the discovery cohort were treated with these regimens. There was no similar model improvement when our risk model was supplemented with NOPHO-ALL2008 and ALL2000 risk classification (Figure 9B).

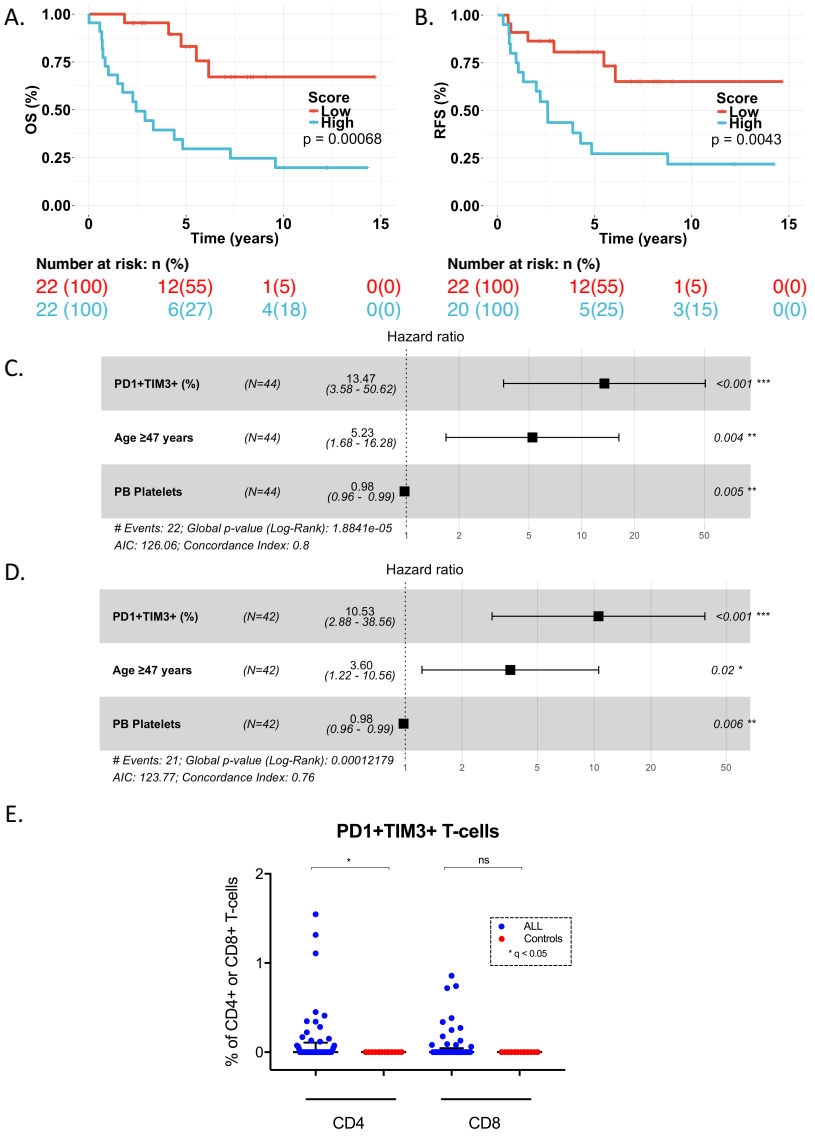

Figure 8 Survival analysis (Cox regression, log-rank test) in the immunohistochemistry cohort. Comparison between the dichotomous risk groups generated in the multivariate analysis, A) overall survival (OS), B) relapse-free survival (RFS). Forest plot of the risk stratification model for C) OS and D) RFS. E) Levels of PD1+TIM3+ T cells in ALL and control bone marrow (Mann-Whitney U test, adjusted p value). Reproduced with permission from Leukemia (Springer Nature)[199]

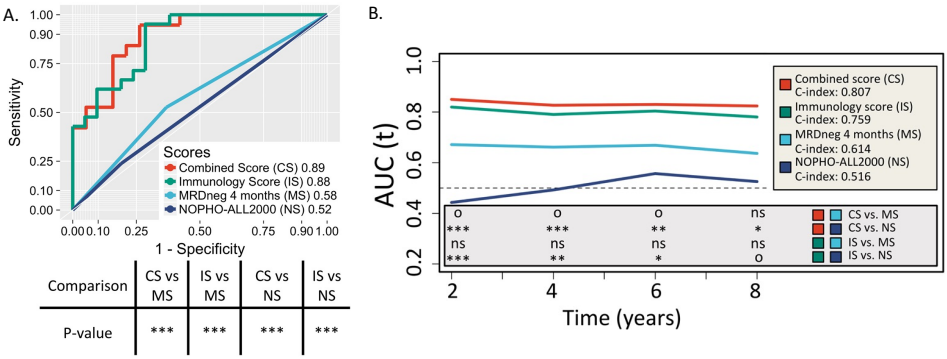

Figure 9 A) The area under the receiver operating characteristic curves (AUROC) of our immunology score (green line) was compared to prediction of relapse-free survival (RFS) by measurable residual disease (MRD) status (4 months from the diagnosis; light blue line) using the bootstrap method (number of iterations: 4000). A combination of these two models (red line) was compared to prediction by MRD status at 4 months and original treatment protocol (ALL2000 or NOPHO-ALL2008) risk classifications (dark blue line). *P < 0.05, **P < 0.01, ***P < 0.001. B) The time-dependent receiver operating characteristic (ROC) curves and C-statistic values for RFS at different time points. Our immunology score (green line) was compared to prediction by MRD status (light blue line), and the combined model (red line) was compared to stratification by MRD status and the treatment protocol classifications (dark blue line). oP < 0.10, *P < 0.05, **P < 0.01, ***P < 0.001. Reproduced with permission from Leukemia (Springer Nature)[199]

5.2 EX-VIVO DRUG SENSITIVITY TESTING

In the DSRT assay (Ph+ ALL, n = 10; Ph⁻ ALL, n = 8), especially glucocorticoids, BCL2 family inhibitors venetoclax and navitoclax, HDAC inhibitor belinostat, NAMPT inhibitor daporinad, MDM2 inhibitor idasanutlin, BET inhibitor JQ1, HSP90 inhibitor luminespib, and antineoplastic antibiotic plicamycin showed pan-ALL efficacy. Sensitivity to the other drugs varied greatly between individual patients, reflecting the heterogenous molecular background of ALL (Figure 10).

5.2.1 TKI SENSITIVITY DRIVES THE CLUSTERING OF EXPRESSION DATA

In the heatmap analysis, DSRT data clustered patients into two main groups. This clustering was mainly driven by sensitivity to BCR::ABL1 targeting tyrosine kinase inhibitors. By visual inspection, patient age, survival, or other somatic mutations did not clearly affect the clustering. Three Ph⁻ patients showed sensitivity to tyrosine kinase inhibitors. Pt_6 and Pt_4 were sensitive

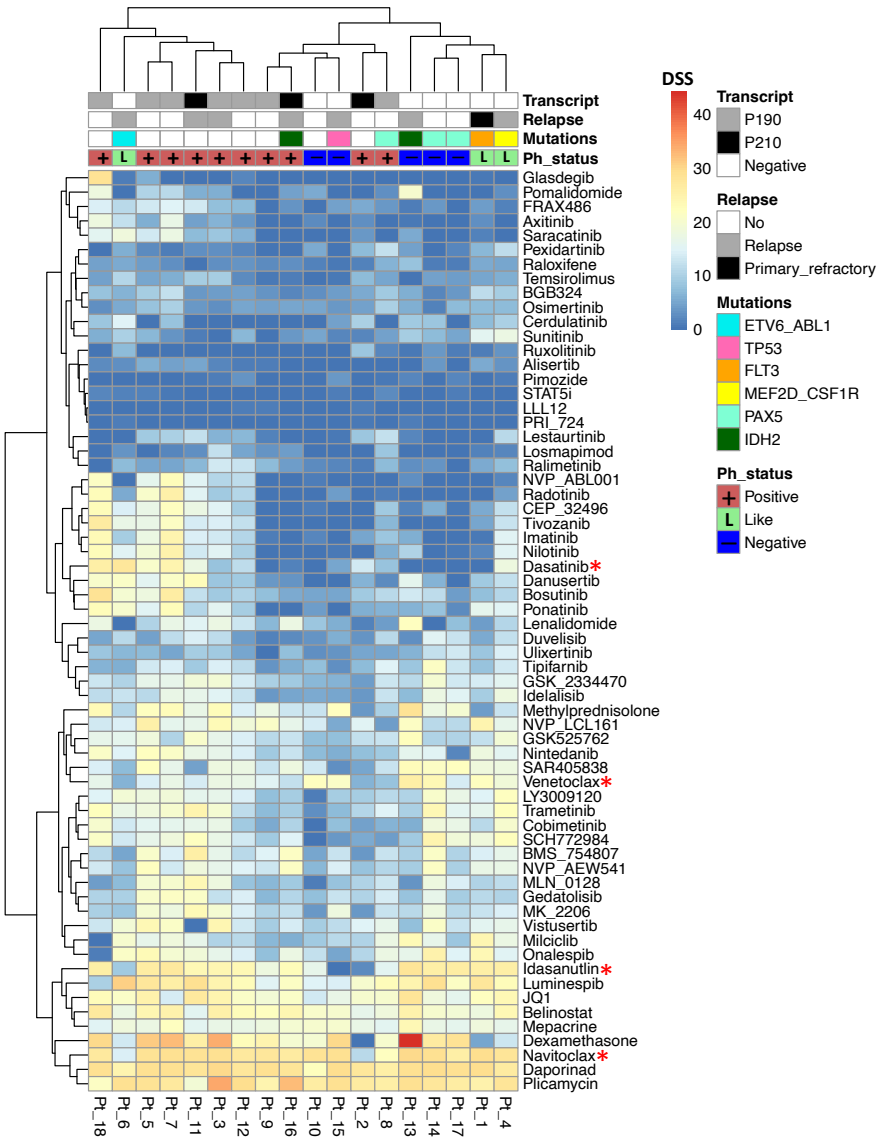

Figure 10 Heatmap visualization of the drug plate results, clustering by Euclidean distance measurement and complete clustering method. Drug efficacy is color-coded according to drug sensitivity score (DSS). DSS >10 denotes efficacy and DSS >20 excellent efficacy. Philadelphia chromosome-positive patients (n = 10) are marked in red, Philadelphia chromosome-negative patients in blue (n = 5), and Philadelphia-like patients in light green (n = 3). The type of *BCR::ABL1* transcript, later occurrence of a relapse, and mutations defined from the sequencing data are annotated in additional tracks. The compounds marked with red asterisk were included in combination assays. Reproduced with permission from Hemasphere (Wolters Kluwer)[239]

to several BCR::ABL1 targeting TKIs, and Pt_1 showed sensitivity to broad-spectrum kinase inhibitor sunitinib and ponatinib.

When analyzing transcriptomic data (9 Ph+ and 7 Ph⁻) with principal component analysis, the first variance component clustered Ph⁻ samples Pt_1 and Pt_4 together with the Ph+ patients. Further analysis of the RNAseq data revealed a tyrosine kinase-activating *MEF2D::CSF1R* fusion gene in Pt_4 and a *FLT3* kinase point mutation Y842H in Pt_1. Pt_6 did not have material for RNAseq, but in FusionPlex Pan-Heme fusion screen, the patient was revealed to harbor an *ETV6::ABL1* fusion gene, classifying all these three patients to the Ph-like subgroup, and further explaining the sensitivity to TKIs in the DSRT.
Unexpectedly, four Ph+ samples showed varying resistance to several BCR::ABL1-targeting TKIs. One sample measured borderline cell viability after the three-day DSRT experiment, and another one possessed lower blast count than on average, which may explain the lower TKI responses in these two samples. However, we did not detect any technical aberrances with the remaining two patient samples, and these abnormal TKI responses did not correlate with clinical outcome.

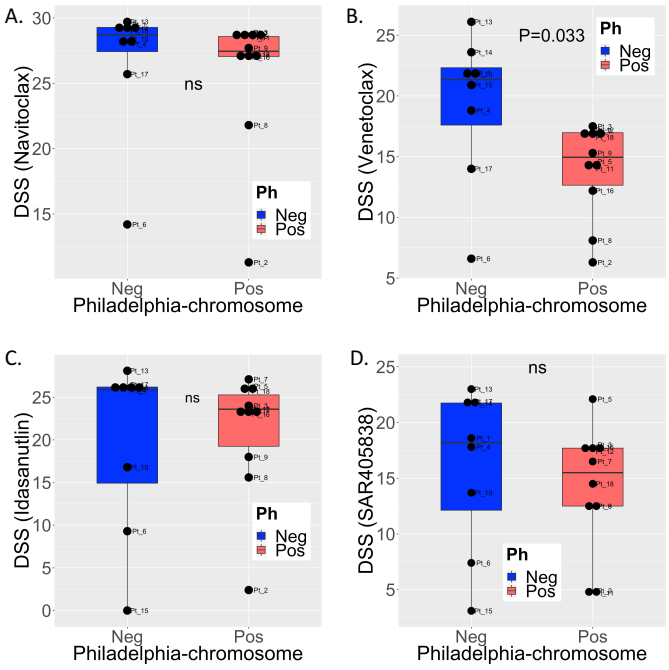

Figure 11 The efficacy of A) navitoclax, B) venetoclax, C) idasanutlin, and D) SAR405838 in Philadelphia chromosome-positive (n = 10) and negative (n = 8) patient samples (Mann-Whitney U test). DSS = drug sensitivity score; ns, not significant. Reproduced with permission from Hemasphere (Wolters Kluwer)[239]

5.2.2 BCL2 AND MDM2 INHIBITORS ARE EFFECTIVE AND SHOW SYNERGISM

Several apoptosis-promoting compounds showed promising efficacy in the drug screen. Navitoclax, a BCL2, BCL-XL, and BCL-W inhibitor, was highly effective (DSS >20) in 89% of the samples. 83% of all samples showed at least moderate sensitivity (DSS >10) to selective BCL2-inhibitor venetoclax, although Ph+ samples were significantly less sensitive than Ph⁻ samples. MDM2 inhibitor idasanutlin was highly effective in 67% of the samples, with no apparent difference regarding the Ph status. A second MDM2 inhibitor SAR405838 was less effective than idasanutlin, although showed responses in 78% of the samples (Figure 11). A sample completely resistant to MDM2 inhibitors turned out to harbor a TP53 mutation in the RNAseq data.

To dissect the potential molecular background behind the different venetoclax and navitoclax responses between Ph⁻ and Ph+ ALL patients, we analyzed gene expression data from two publicly available datasets, E-MTAB-5035 and B-ALL1988.[20,219] *BCL2* expression was significantly downregulated in Ph+ ALL patients compared with Ph⁻ patients in both cohorts, whereas *BCL-W* was upregulated in Ph+ samples. Also the expression of *BCL-XL* trended towards upregulation in Ph+ patients in both cohorts (Figure 12). In addition, we analyzed *MDM2* expression from HEMAP database.[220] In B-ALL, *MDM2* was clearly overexpressed compared with other hematological diseases such as AML, multiple myeloma, or chronic lymphocytic leukemia, corresponding with the strong idasanutlin responses in our drug panel.

In the differential gene expression analysis, altogether 242 protein-coding genes were differentially expressed (adjusted P value < 0.05) between Ph+ and Ph– patients. The drug testing data was complemented with gene expression based TAS analysis, with the intention of obtaining additional information of the targets behind the observed drug responses. In the analysis histone deacetylase, *NAMPT*, *BCL-XL*, *MDM2*, and *HSP90* class gene targets were highly addicted, and these target genes were highly expressed throughout the samples.

We further tested five different two-drug combinations in three human B-ALL cell lines: NALM21 (Ph+ ALL), Kasumi2 (Ph⁻ ALL), and MHH-CALL4 (Ph-like ALL). The selected drugs in the combination screen were venetoclax, navitoclax, dasatinib, and idasanutlin. BCL2 inhibitor venetoclax and MDM2 inhibitor idasanutlin showed synergy in all cell lines, and all dasatinib-combinations were synergistic in Ph+ ALL cell line NALM21. In addition, a combination of navitoclax and idasanutlin was synergistic in Kasumi2.

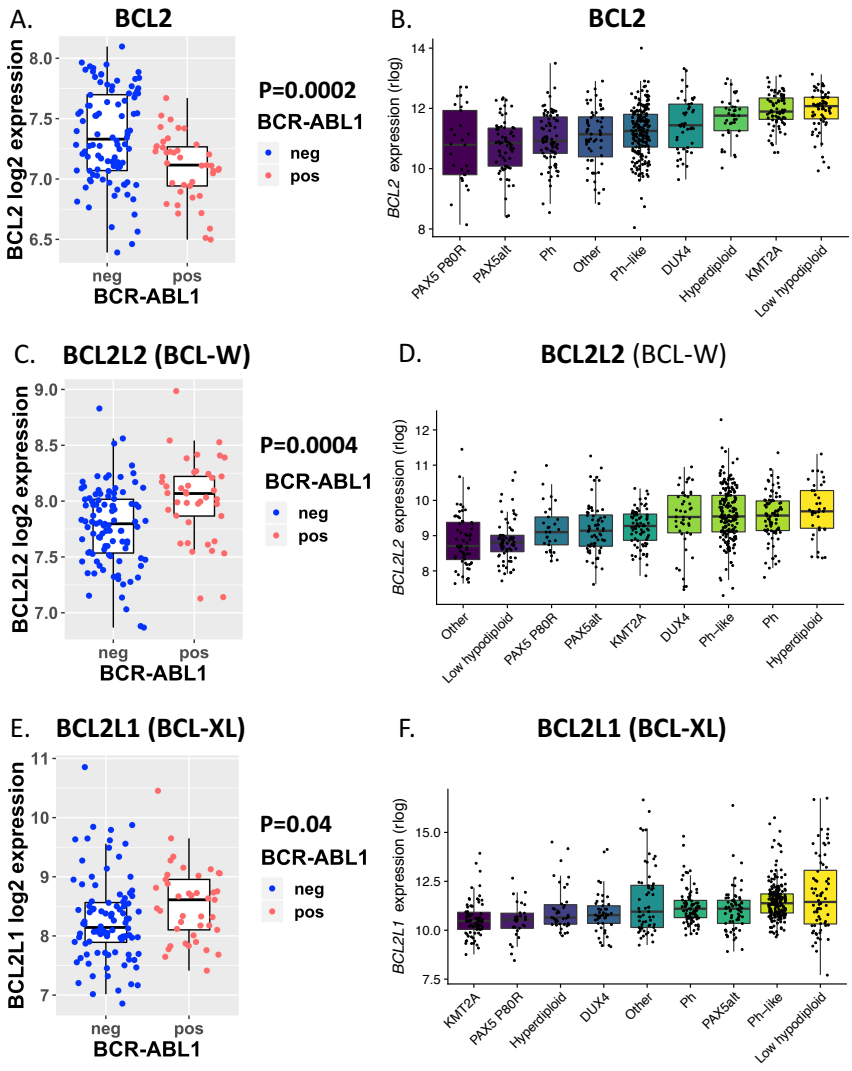

Figure 12 The expression of *BCL2*, *BCL-W*, and *BCL-XL* genes encoding venetoclax and navitoclax targets in Philadelphia chromosome-positive and negative samples. Comparison of the expression of *BCL2* in (A) E-MTAB-5035 and (B) B-ALL 1988 cohorts, the expression of *BCL-W* in (C) E-MTAB-5035 and (D) B-ALL 1988 cohorts, and the expression of *BCL-XL* in (E) E-MTAB-5035 and (F) B-ALL 1988 cohorts (Mann-Whitney U test). In the B-ALL 1988 dataset, the genetic subgroups that included at least 20 patients were visualized and annotated as in the original data. Reproduced with permission from Hemasphere (Wolters Kluwer)[239]

5.3 DETECTING COPY NUMBER ALTERATIONS

5.3.1 STEM CELL TRANSPLANTATION IS NOT ASSOCIATED WITH IMPROVED OUTCOME IN A DASATINIB-TREATED POPULATION

We analyzed nationwide registry data of altogether 141 adult Ph+ ALL patients (year of diagnosis 1984-2020). Median OS of the patients who were treated prior the TKI era (n= 59) reached 19.5 months (95% confidence interval (CI) 15.0-25.8 months), with all long-term survivors associated with alloHSCT.

A total of 82 patients had received frontline TKI-based regimens and were analyzed in more detail. Majority of the patients were treated according to NOPHO ALL2008 or FLG ALL2000 treatment protocols.[238,240] The most common induction regimens in the TKI era were cyclophosphamide+vincristine+doxorubicine+dexamethasone (CVAD; n=36), CVAD+pegasparaginase (n=9), mitoxantrone+etoposide+cytarabine (MEA, n=18) and NOPHO ALL-2008 non-high risk induction (n=13). Six patients received customized induction treatments, such as a combination of TKI and steroids (n=3). Two of these TKI and corticosteroid-treated patients deceased rapidly, but one patient has remained in remission for over 6 years from the diagnosis.

There was no significant difference regarding the outcome of imatinib (n=43) and dasatinib-treated (n=39) patients. The three-year and five-year OS estimates in the imatinib cohort were 67% and 58%, respectively, and in the dasatinib-treated patients 64% and 51%. However, contrary to the imatinib-treated cohort, allogeneic hematopoietic stem cell transplantation (alloHSCT) did not improve the outcome of the dasatinib-treated patients, although the non-allotransplanted patients were significantly older (Figure 13).

Altogether 52% (n=43) of the TKI-treated patients received alloHSCT, which was associated with better outcome. As expected, the non-allotransplanted patients were older (median age 64 years vs. 42 years, P<0.0001, Wilcoxon signed-rank test), but the baseline characteristics of these two groups did not otherwise differ significantly. However, after excluding over 65-year-old patients, who are often considered ineligible for alloHSCT, from the outcome analysis, the survival advantage disappeared, even though the non-allotransplanted patients were further significantly older than the transplanted ones (median 54 vs. 42 years, P=0.003, Wilcoxon signed-rank test; Figure 14).

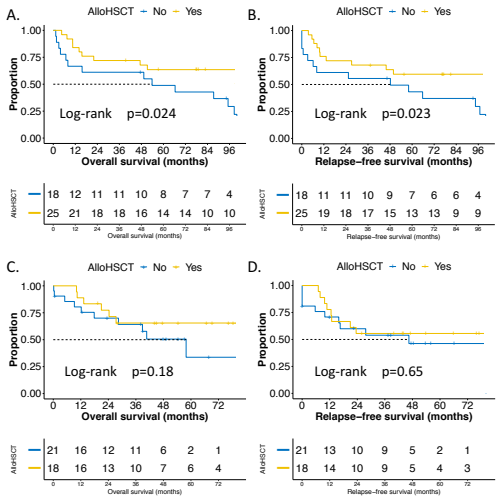

Figure 13 A) Overall survival (OS) and B) relapse-free survival (RFS) of allotransplanted and non-allotransplanted patients who received frontline imatinib. C) OS and D) RFS of allotransplanted and non-allotransplanted patients who received frontline dasatinib. For visualization purposes, events after 100 and 80 months are not shown (Kaplan-Meier estimate, log rank test). Reproduced with permission from Haematologica (Ferrata Storti Foundation).[241]

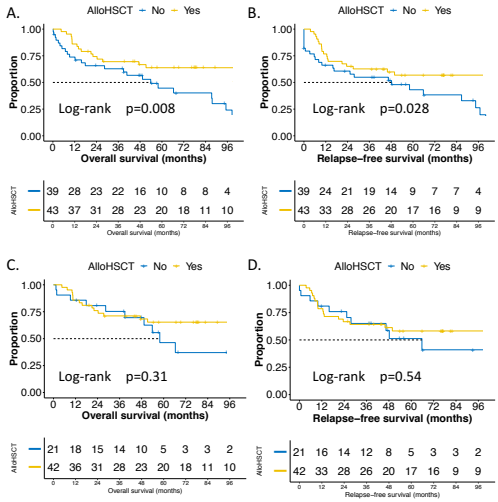

Figure 14 A) Overall survival (OS) and B) relapse-free survival (RFS) of allotransplanted and non-allotransplanted patients who were treated with frontline tyrosine kinase inhibitors (TKI). C) OS and B) RFS of allotransplanted and non-allotransplanted patients (over 65-year-olds were excluded from the analysis) who were treated with frontline TKI. For visualization purposes, events after 100 months are not shown (Kaplan-Meier estimate, log rank test). Reproduced with permission from Haematologica (Ferrata Storti Foundation).[241]

When analyzing the causes of death in the non-allotransplanted patients, 27% (7/26) succumbed to non-leukemia-related reasons (such as heart failure or solid malignancies), 50% (13/26) of the deaths were leukemia-related, and 23% (6/26) treatment-related, typically induction deaths. In the allotransplanted patients, transplantation-related causes explained 65% (11/17) of the deaths, and relapses 29% (5/17). One death was caused by other reasons. When analyzing competing risks, both leukemia-related and other than leukemia or treatment-related causes of death were more frequent in the non-allotransplanted patients.

5.3.2 *IKZF1* PLUS GENOTYPE PREDICTS OUTCOME

Other mutations besides *BCR::ABL1* were rare in the targeted NGS panel, as it detected only a single missense *PAX5* mutation (p.V26G) in the diagnostic samples (n=41). Even though alterations in *IKZF1* and *CDKN2A/B* are frequent in Ph+ ALL, the changes are typically larger deletions, and our targeted panel did not detect any point mutations in these genes. In the relapse-phase samples (n=11), the panel detected one *TP53* mutation (p.C176F), and the other detected alterations were *ABL1* kinase domain mutations known to cause resistance to TKIs (p.T315I, n=5; p.E355A, n=1; p.F317I, n=1; p.T315I+p.Y253F, n=1; p.E255K+p.Y253F, n=1; Figure 15A).[233]

Several publications have reported that a significant fraction of Ph+ ALL patients harbor subclonal *ABL1* mutations already at diagnosis.[106–108] Considering that the T315I mutations contribute to a notable proportion of treatment failures,[4] we designed a ddPCR assay for detecting T315I with a sensitivity of 0.04% (VAF). In total, we analyzed 32 samples (26 pretreatment, 6 relapse-phase) from 25 patients. We found only one baseline sample positive for T315I with a VAF of 0.10%. The patient was treated with frontline imatinib but relapsed in less than a year with an E255K mutation. The TKI was switched to dasatinib, only to be followed by a quick relapse with T315I. The ddPCR assay detected T315I mutations with a high VAF >25% in all matching relapse-phase samples (5/6) as the clinical *ABL1* mutation analysis and the targeted NGS panel.

For analyzing CNAs in the genes of interest, we employed a commercial MLPA ALL kit. MLPA recognized *IKZF1* deletions in 75%, *CDKN2A/B* deletions in 42% and *PAX5* deletions in 28% of the analyzed pretreatment samples (n=40). Other alterations turned out infrequent. 38% of the cases fulfilled the criteria for a *IKZF1* plus genotype, where *IKZF1* deletion was accompanied by a deletion in *CDKN2A/B* and/or *PAX5* genes (Figure 15B).

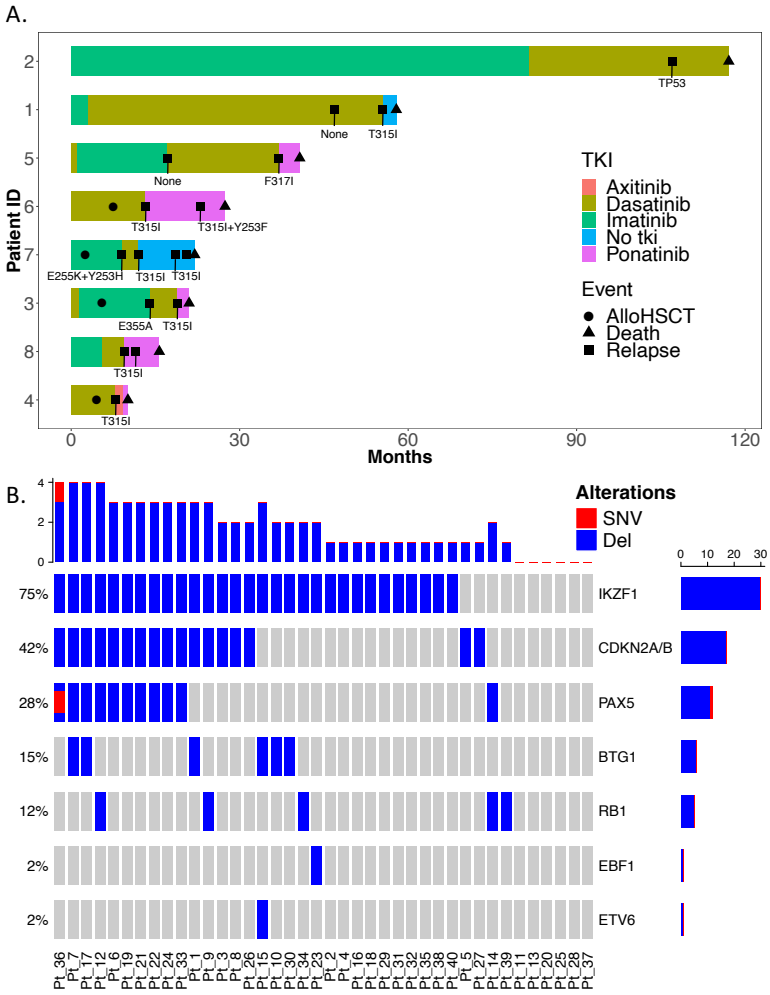

Figure 15 The detected mutations A) in the relapse-phase samples and their relation to the given tyrosine kinase inhibitor treatment, and B) in the diagnostic samples. Reproduced with permission from Haematologica (Ferrata Storti Foundation).[241]

IKZF1 deletions had no clear effect on survival, whereas the outcome of *IKZF1* plus patients turned out inferior (Figure 16). *IKZF1 plus* patients had higher WBC count at diagnosis, but the median age or other baseline characteristics did not differ significantly between IKZF1 plus and non-plus patients. In both cohorts 60 % of the patients were allotransplanted, with no difference in the number of given reduced-intensity conditioning regimens. Although the cohort size was limited, alloHSCT did not improve survival within the *IKZF1* plus group. At 12 months after alloHSCT, the cumulative incidence for relapse

was 56% in *IKZF1* plus patients compared to 7% in non-plus patients (non-relapse mortality as a competing event; Figure 17A). Non-relapse mortality did not differ significantly between these two cohorts (relapse as a competing event; Figure 17B). In Cox regression univariate analyses age, alloHSCT, and IKZF1 plus genotype were the only significant predictors for survival in frontline TKI-treated patients (Table 11).

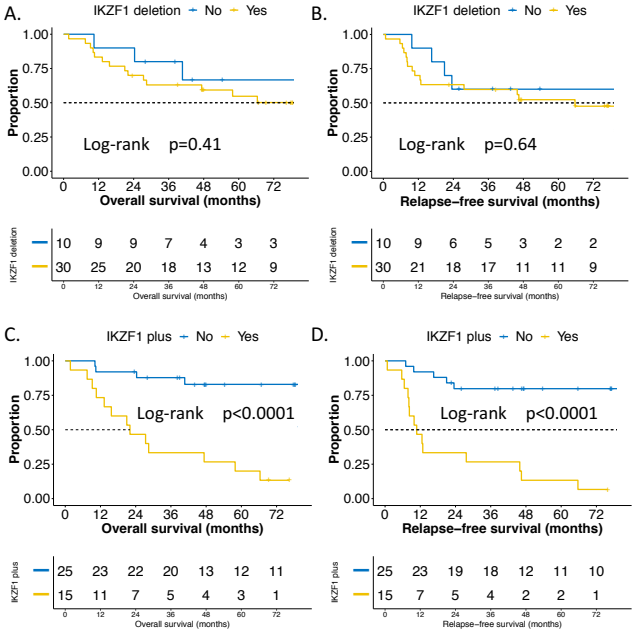

Figure 16 A) Overall survival (OS) and B) relapse-free survival (RFS) according to the presence of a *IKZF1* deletion. C) OS and D) RFS according to the presence of *IKZF1* plus genotype. For visualization purposes, events after 80 months are not shown (Kaplan-Meier estimate, log rank test). Reproduced with permission from Haematologica (Ferrata Storti Foundation).[241]

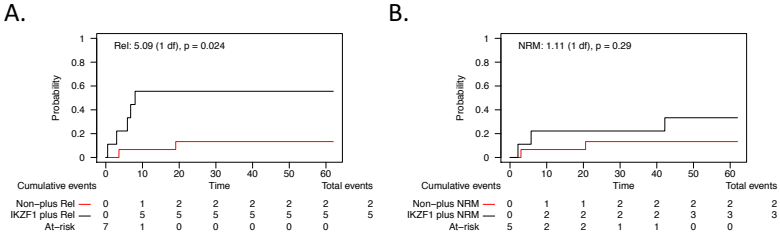

Figure 17 A) Cumulative incidence of relapse and B) cumulative incidence of non-relapse mortality after allogeneic hematopoietic stem cell transplantation in *IKZF1* plus and non-plus patients (Gray's test). Reproduced with permission from Haematologica (Ferrata Storti Foundation).[241]

Table 11 *Cox regression univariate analysis of overall (OS) and relapse-free survival (RFS) in patients treated with frontline tyrosine kinase inhibitor-based regimens.*

	OS HR (95% CI)	P	RFS HR (95% CI)	P	N of obs
Age, cont.	1.04 (1.02-1.06)	**0.0004**	1.04 (1.01-1.06)	**0.001**	82
Age, cat. (>65 vs ≦ 65 y)	3.32 (1.75-6.26)	**<0.0001**	3.04 (1.63-5.67)	**0.0002**	82
AlloHSCT	0.45 (0.24-0.82)	**0.008**	0.51 (0.28-0.93)	**0.03**	82
Dasatinib vs. imatinib	1.16 (0.60-2.24)	0.7	1.21 (0.64-2.28)	0.6	82
WBC	1.003 (0.999-1.007)	0.2	1.003 (0.998-1.007)	0.2	79
WBC≧30x109/l	1.81 (0.98-3.37)	0.06	1.76 (0.96-3.23)	0.06	79
WBC≧50x109/l	1.77 (0.89-3.50)	0.1	1.64 (0.83-3.23)	0.1	79
Hemoglobin	1.002 (0.990-1.014)	0.7	1.003 (0.99-1.02)	0.6	78
Platelets	0.998 (0.993-1.002)	0.3	0.998 (0.993-1.002)	0.3	78
LDH	1.00 (0.9997-1.001)	0.5	1.00 (0.9998-1.001)	0.5	74
BM blast%	1.02 (0.99-1.06)	0.2	1.03 (0.99-1.06)	0.1	53
P210 vs P190	0.50 (0.17-1.46)	0.2	0.61 (0.23-1.64)	0.3	53
ACA (karyotype)	1.05 (0.48-2.27)	0.9	0.92 (0.43-1.93)	0.8	54
MRDneg in 3 months (no vs yes)	0.65 (0.32-1.33)	0.2	0.61 (0.31-1.21)	0.2	68
***IKZF1* deletion**	1.59 (0.52-4.84)	0.4	1.27 (0.46-3.46)	0.6	40
***IKZF1* plus**	8.37 (2.71-25.83)	**<0.0001**	8.56 (3.04-24.07)	**<0.0001**	40

HR, hazard ratio; CI, confidence interval; N of obs, number of observations; cont., continuous; cat., categorical; y, years; AlloHSCT, allogeneic hematopoietic stem cell transplantation; WBC, white blood cell count; LDH, lactate dehydrogenase; BM, bone marrow; ACA, additional chromosomal abnormalities; MRD, measurable residual disease.

6 DISCUSSION

6.1 DISTINCT IMMUNOPROFILE IN ALL BM

In study I, we constructed TMAs from archived FFPE ALL BM samples and analyzed them using mIHC. FFPE samples provide ideal source material for retrospective studies, as the preservation methods has less effect on cell number and phenotype compared with cryopreservation.[242] The TMA format enables simultaneous analysis of hundreds of samples and tens of different marker combinations,[200] whereas mIHC supplemented with automated image-analysis allows objective and fast, in-depth assessment of distinct immune cell subtypes in their native BM microenvironment.

The immune system in cancer is a constantly evolving, complex, and dynamic interplay of diverse cell populations.[126] Our antibody staining panels covered a comprehensive selection of well-established immune cell populations and phenotype markers with previously described clinical significance.[126,243] As the T cells play a crucial role in anticancer immunity,[131] we especially focused on characterizing different T cell subpopulations. Even though the mIHC cannot replace functional assays, our results strongly imply that ALL BM is inclined towards immunosuppression compared to healthy controls. We did not detect any significant differences in the BM immune cell constitution between Ph+ and Ph⁻ ALL patients, although specific genetic alterations may lead to identifiable changes in the tumor immune microenvironment, such as *TP53* mutations in MDS and AML.[244]

M2-polarized macrophages and MDSCs can suppress Th1-type adaptive immunity and promote tumor growth.[245,246] In contrast, M1-like macrophages produce inflammatory cytokines and other effector molecules, which mediate antitumor responses.[245] When compared to healthy BM, the proportion of M1-skewed macrophages was decreased, and the proportion of M2-polarized macrophages and MDSCs was increased, consistent with previous studies.[247,248] Similarly, cell subtypes associated with anticancer-immunity such as NK cells, activated CD27+ T cells, and cytotoxic CD8+granzyme B+CD57+ T cells were decreased in ALL BM.[249]

Myeloid dendritic cells present cancer-related antigens and are essential in T cell priming. mDC1s can also produce high amounts of interleukin-12, thereby inducing a Th1-fashioned immune response.[250] In ALL BM, the proportion of mDC1s and CD4+ memory T cells was increased, which may refer to enhanced antigen-presentation capability.

Immune checkpoints PD1 and CTLA4, markers associated with immune regulation,[243] were increased in ALL compared to the controls. Then, the expression of immune suppressive checkpoint molecules LAG3 and TIM3 was decreased and the expression of immune stimulatory OX40 increased,[243] although only on CD4+ T cells. The explanation for these seemingly contradictory expression patterns may be caused by different regulatory signaling pathways. It is also fair to note, that the same immune checkpoints may represent distinct functional activation states in separate contexts, for example in different stages of T cell differentiation.[251]

As the immune contexture in solid tumors has been shown to predict survival, we wanted to assess if the immune cell constitution in ALL BM is associated with outcome, as well. In solid malignancies, especially the infiltration rate of T cells in the tumor core and margin are able to predict treatment responses to both immune checkpoint therapy and traditional chemotherapy.[138,184] Leukemias and other blood cancers cannot be spatially divided into central tumor and invasive margin, thus making similar stratification inapplicable. However, considering the success of CAR T cell therapy and T-cell engaging antibodies in B-ALL,[5] and also the well-known leukemia-eradicating capability of alloHSCT, which is based on the immunological activity of donor T cells,[128] the immune system unquestionably contributes to the disease course of acute leukemias, as well. Immune checkpoint inhibitor therapy has become routine in several solid cancers,[252] but the clinical trials in ALL have been sparse, and with lack of demonstrable efficacy.[253] Some preliminary data indicate that combining checkpoint inhibitors with other agents such as blinatumomab or TKIs may increase the efficacy of both therapies in ALL.[254,255]

In our study, higher proportion of CD4+PD1+TIM3+ T cells, older age, and lower platelet count at diagnosis identified a group with poor survival. This result was validated in a separate cohort using flow cytometry, which is a routinely used method in clinical diagnostics. In the study cohort, our risk model outranked simple MRD-based risk-classification and the original study protocol risk stratifications when predicting long-term survival. When our model was complemented with MRD prediction, the accuracy increased even further. The high-risk group showed a trend towards higher BM blast proportion and initial WBC count, both markers of prominent tumor burden. With retrospective study design in a heterogenous and relatively small study population, the limitations of this risk score are obvious, and the true significance lies in the concept of adapting immunological parameters for more precise clinical risk stratification.[256]

Although the purpose of our study was not to functionally test different immune cell subtypes, PD1+TIM3+CD4+ T cells might present an immune-exhausted phenotype arising from prolonged immune response against

malignant cells or chronic inflammation related to the expansion of leukemic BM.[257,258] Similarly, the decreased effector and increased immunoregulatory cell subtypes that prevail in ALL BM may indicate a switch from activated to immunosuppressive state, although further functional studies are needed for confirmation. It should also be noted, that as aging is known to cause biological changes in the immune system,[259] our results might not apply to pediatric ALL.

6.2 APOPTOSIS-PROMOTING DRUGS ARE EFFECTIVE IN ALL

In study II, we tested the *ex vivo* drug sensitivity of primary B-ALL patient samples to 64 novel or repurposed compounds, and molecularly profiled the same specimens with whole-transcriptome sequencing. We complemented the analysis by assessing publicly available expression data in parallel.

Apoptosis-promoting BCL2 inhibitors venetoclax and navitoclax were widely effective across the samples, although BCL2-selective venetoclax[166] was significantly more effective in Ph$^-$ patient samples compared to Ph+ ALL. There was no significant difference in the efficacy of BCL-2, BCL-W, and BCL-XL targeting navitoclax[170] between Ph+ and Ph$^-$ patients, as the drug showed high potency in nearly all samples. When analyzing gene expression data of BCL family genes, *BCL2* was downregulated and *BCL-W* upregulated in Ph+ ALL, thus offering a mechanistical explanation to the observed drug responses.

Currently, venetoclax has been approved for the treatment of chronic lymphocytic leukemia and AML, but in preclinical and early clinical trials, BCL2 inhibitors have shown efficacy in a variety of hematological cancers and other malignant conditions.[168,260] Venetoclax in different drug combinations is currently under keen investigation for relapsed or refractory ALL (clinicaltrials.gov). In preclinical ALL models, venetoclax has shown potency as a monotherapy,[261–263] but especially combination strategies, such as with inotuzumab ozogamicin and dexamethasone, have raised expectations.[167,264]

The early clinical trials of navitoclax in solid tumors raised limited clinical interest due to grade III-IV thrombocytopenia in higher drug doses and the limited efficacy of low-dose navitoclax monotherapy.[172,173] BCL-XL is a prosurvival protein expressed normally on platelets, and the side effect is inevitably linked to the drug mechanism.[171] However, navitoclax-related thrombocytopenia is both reversible and dose-dependent, and can often be managed with stepwise dose escalations and adjustments.[172] Navitoclax potentiates the efficacy of other anticancer drugs, and it might be especially useful in combinations with other agents.[265] The combination strategy would

also allow treatment with lower navitoclax doses, thus ameliorating the adverse effects of the drug. We hypothesize, that navitoclax is a potent compound to be tested in ALL in combinations, especially considering that the management of transient thrombocytopenia forms an inevitable part of any current leukemia treatment. Our results showed that Ph+ ALL samples were more sensitive to navitoclax than to venetoclax and combining navitoclax with TKIs could prove a successful strategy. In a limited patient population with relapsed or refractory Ph+ ALL, where a combination of venetoclax and ponatinib was tested, none of the patients responded to the standard 400 mg dose of venetoclax, but required higher doses.[266] Today, there is only limited clinical data of navitoclax in ALL. In a phase I dose-escalation study, patients with relapsed or refractory ALL were treated with low-dose navitoclax in combination with venetoclax and chemotherapy with promising efficacy and manageable side effects. Although the study cohort was heavily pretreated and included patients who had failed therapies such as alloHSCT, blinatumomab, CAR T, and inotuzumab ozogamicin, 60% achieved CR and third of the patients were able to proceed to alloHSCT.[174]

Tumor suppressor gene TP53 is commonly mutated in solid malignancies, but only in 15% of new ALL cases.[27,29] In cancer, also the function of wild-type TP53 is often compromised by an increased amount of MDM2, which binds to TP53 and regulates its proapoptotic features.[178,179] MDM2 inhibitor idasanutlin acts by blocking the MDM2-TP53 interaction. This restores the normal tumor suppressor functions of TP53, thereby leading to increased cancer cell apoptosis.[180]

Malignant cells can become dependent on BCL2, but overcoming this dependency is not that straightforward. BCL2 inhibition can lead to activation of alternative survival routes, such as overexpression of antiapoptotic MCL1,[267] and monotherapy with BCL2 inhibitors unlikely offers any permanent solutions. Activation of wild-type TP53 can reverse the upregulation of MCL1, providing a mechanism how a combination of MDM2 and BCL2 inhibitor can overcome resistance to apoptosis.[177] A combination of BCL2 inhibitor venetoclax and MDM2 inhibitor idasanutlin was synergistic in all three human B-ALL cell lines we tested. The cell lines represented Ph+, Ph$^-$, and Ph-like ALL. A combination of MDM2 and BCL2 inhibition was effective in resistant AML xenografts,[177] and idasanutlin is currently in a phase 1/2 clinical trial in combination with venetoclax or chemotherapy for relapsed or refractory ALL (NCT04029688, clinicaltrials.gov).

By combining molecular profiling with *ex vivo* drug testing, we identified targetable lesions, which were dismissed at the diagnosis by the targeted fusion screen and other routinely used clinical methods. All the detected lesions were classified into Ph-like subgroup, and the samples showed corresponding sensitivity to selected TKIs in the drug screen. A patient with

ETV6-ABL1 fusion gene was refractory to first induction, remained MRD positive after achieving CR, and relapsed and died rapidly after allotransplantation. A second Ph-like patient with *FLT3* point mutation Y842H succumbed to primary refractory disease, and the third patient with *MEF2D-CSF1R* fusion experienced relapse in less than a year from the diagnosis, with fatal consequences. All these patients could have been potential candidates for targeted therapy with TKIs or FLT3 inhibitors,[16,19] but, the opportunity was missed because Ph-like status was not recognized.

Ex vivo drug testing is a useful tool for evaluating drug candidates for clinical trials,[96] and is also currently tested in a personalized medicine setting to design customized therapies for heavily pretreated relapsed or refractory patients.[187] However, the method has its limitations, such as its lesser capacity to predict responses to drugs that require persistent exposure for several days or week, such as differentiation-inducing drugs. The capability of *ex vivo* assays to predict *in vivo* responses has been and will remain an important question,[268] but by using systematic and validated approaches, the confounding effects can be minimized.[193] For example, the *ex vivo* and *in vivo* responses of venetoclax have been reported to correlate well.[194] In a very heterogenous cell population, the conventional drug testing method might not recognize resistant subpopulations. Developments in the field regarding more sensitive readouts, such as flow cytometry, image analysis, and single-cell transcriptomics based drug testing assays can further refine this approach.[188,269] In conclusion, combining molecular profiling with *ex vivo* drug testing can assist in identifying actionable targets and therapies for ALL patients.

6.3 COPY NUMBER ALTERATIONS DEFINE OUTCOME

An increasing number of Ph+ ALL patients experience prolonged survival with TKI-based therapies only,[106,111,270] and many succumb to non-leukemia-related causes, such as 27 % of patients in our cohort. TKIs modulate the immune system, and part of their effect might stem from overcoming the immunosuppressive state that prevails in the leukemic bone marrow.[78,79] Despite their distinct features, very few randomized studies have investigated the efficacy between different TKIs. Compared to imatinib, dasatinib penetrates the blood-brain barrier, encompasses more potent and broader kinase inhibition, and has possible anti-leukemic immunomodulatory effects.[78–80,271] In a non-randomized setting, the benefit of alloHSCT seems no longer evident in a dasatinib-treated population.[113,270,272] In pediatric Ph+ ALL, a prospective randomized study showed that dasatinib had significant survival benefit compared to imatinib.[91]

In our observational study, the outcome of imatinib and dasatinib-treated patients did not differ significantly. However, in contrast to the imatinib-treated patients, in the dasatinib-treated patients alloHSCT was not associated with superior survival, despite that the non-transplanted patients were significantly older. Presently Finnish Leukemia Group recommends dasatinib as the TKI-of-choice for frontline adult Ph+ ALL treatment, and the first-line dasatinib-treated patients in our study reflect a more modern treatment era with improved patient care and monitoring, which may partly explain the difference in survival.

It is also noteworthy, that when analyzing all TKI-treated patients, the survival advantage of alloHSCT disappeared after we excluded over 65-year-old-patients from the analysis. This was done to diminish the apparent difference between these two cohorts and to allow more realistic comparison, as 65 is often considered the upper age limit for alloHSCT, although this theoretical border is steadily increasing. Especially in an observational, real-world data, direct comparison between allotransplanted and non-transplanted patients poses challenges, as these patient cohorts typically represent somewhat differing entities. The non-transplanted patients tend to be often older and they possess more frequently comorbidities that reduce the availability of intensive treatment modalities. Randomized studies regarding alloHSCT in Ph+ ALL would prove elemental, but due to the rarity of the disease, this kind of study design will unlikely be established.

Up to 25 % of Ph+ ALL patients have been reported to harbor minor TKI-resistant clones already at diagnosis.[106–108] As T315I mutations cause resistance to all other FDA-approved TKIs except for ponatinib,[87] we designed a sensitive ddPCR assay capable of detecting T315I mutations down to 0.04% VAF. Contrary to previous reports, we detected only a single pre-treatment T315I mutation. Commonly used RT-PCR-based methods may involve a greater risk for producing artificial mutations in the absence of standard Taq-polymerase proofreading activity when detecting low-level targets.[273,274] The proofreading of the Q5 polymerase used in our study was tested and found to be very high.[275] Combined with the novel probe design this decreases the risk of artificial mutations. In addition, the use of ddPCR decreases the number of iterative PCR cycles, reducing the error rate.[276] Concordantly with our results, Short et al reported only a single baseline T315I mutation in 63 pre-treatment Ph+ ALL samples by using highly accurate duplex-sequencing.[277] Importantly, the existence of minor TKI-resistant mutations at baseline did not correlate with later treatment failure. In view of these data, screening for T315I mutations should be directed to situations such as inadequate treatment response, imminent relapse, or treatment failure.

A majority of Ph+ ALL patients are known to harbor *IKZF1* deletions, which have been associated with worse survival,[23,55,278] although their role in the risk

stratification has not been established.[54,120,279] Recently, a combination of an *IKZF1* deletion with *CDKN2A/B* and/or *PAX5* deletion, so called *IKZF1* plus, has been associated with an especially dismal outcome.[113,120,121,280] In our study, we used an established MLPA assay to study CNAs in *IKZF1*, *CDKN2A/B*, and *PAX5*. Altogether 75 % of the patients harbored *IKZF1* deletions and 38 % of the patients represented *IKZF1* plus. The survival of the *IKZF1* plus group turned out especially disappointing; more than half of these patients had deceased or relapsed in 12 months from the diagnosis.

No consensus exists of the optimal treatment strategy for *IKZF1* plus patients. Unfortunately, alloHSCT does not seem to improve the survival in this group.[113,120,121] Limited data indicate that a combination of dasatinib and blinatumomab might prove more effective in this group than traditional treatment approaches.[122] In younger patients, a modern MRD-driven, intensive chemotherapy regimen might also prove useful.[281] *IKZF1* plus genotype may cause primary resistance to TKI-based therapies at the progenitor cell level, and more detailed mechanistic studies may open new treatment strategies.[50] Currently, potential compounds such as crizotinib, retinoids, immunomodulatory drugs, and a combination of ponatinib and asciminib are being investigated, and they might provide an alternative also for the elderly and more fragile patients.[95,97,282,283]

7 SUMMARY AND FUTURE DIRECTIONS

The key results and conclusions drawn from the studies I-III can be summarized as follows:

ALL BM microenvironment was characterized by immunosuppression compared to healthy controls, and the BM immune cell composition was associated with clinical response to therapy.

Apoptosis-targeting BCL2 and MDM2 inhibitors showed promising efficacy in B-ALL *ex vivo*, also in combination. Molecular profiling and *ex vivo* drug testing provided a powerful instrument for identifying novel therapies and actionable targets in ALL.

AlloHSCT did not appear to improve survival in Ph+ ALL patients who were treated with dasatinib-based regimens. It is likely that some patients do not benefit from allogeneic transplantation in first CR.

IKZF1 plus genotype distinguished a subgroup in Ph+ ALL with an especially poor prognosis and in desperate need of novel treatment alternatives. *IKZF1* plus patients may benefit from immuno-oncological or intensified treatment approaches, and they should be guided to appropriate clinical trials, when possible.

CNA testing should be incorporated to routine clinical diagnostics of Ph+ ALL. Instead, screening for baseline T315I KD mutations seems unwarranted, and the mutation testing should be reserved for situations such as persistent or rising MRD, or overt relapse.

Nationwide disease registries and biobanks are indispensable for research regarding rare diseases such as Ph+ ALL, and their efficient use should be enabled and encouraged by research institutes and legislation.

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