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INTEGRATIVE GENOMIC AND DRUG SENSITIVITY PROFILING OF HIGH-RISK CHRONIC MYELOID LEUKEMIA PATIENTS IN THE CONTEXT OF PERSONALIZED MEDICINE

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To my Uncle Hatem,

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Original publications

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

I. **Adnan Awad S**,* Kankainen M,* Ojala T, Koskenvesa P, Eldfors S, Ghimire B, Kumar A, Kytölä S, Kamel MM, Heckman CA, Porkka K, Mustjoki S. Mutation Accumulation in Cancer Genes Relates to Nonoptimal Outcome in Chronic Myeloid Leukemia. *Blood Adv*. 2020;4(3):546–559.

II. **Adnan Awad S**, Dufva O, Ianevski A, Ghimire B, Koski J, Maliniemi P, Schreiber A, Heckman CA, Koskenvesa P, Korhonen M, Porkka K, Branford S, Aittokallio T, Kankainen M,* Mustjoki S.* RUNX1 Mutations in Blast Phase Chronic Myeloid Leukemia Associate with Distinct Phenotypes, Transcriptional Profiles and Drug Responses. *Leukemia*. 2021; 35(4):1087-1099.

III. **Adnan Awad S**, Kim D, Hohtari H, Javarappa KK, Brandstoetter T, Potdar S, Heckman CA, Kytölä S, Porkka K, Doma E, Sexl V, Kankainen M, Mustjoki S. Integrative Characterization of p190-BCR-ABL in Chronic Myeloid Leukemia (CML) Reveals Specific Signaling Pathways and Therapeutic Targets. *Leukemia*. 2020; https://doi.org/10.1038/s41375-020-01082-4.

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Abbreviations

2G/3G	Second/Third generation
ABL1	Abelson murine leukemia viral oncogene homolog 1 gene
ACA	Acquired chromosomal abnormalities
AID	Activation-induced cytidine deaminase
AKT	Protein kinase B
ALL	Acute lymphoblastic leukemia
Allo-SCT	Allogeneic stem cell transplant
ALOX5	Arachidonate 5-Lipoxygenase
AML	Acute myeloid leukemia
AP	Acceleration phase
APOBEC	Apolipoprotein B MRNA Editing Enzyme Catalytic subunits
ARCH	Age-related clonal hematopoiesis
ASXL1	ASXL transcriptional regulator 1
AUC	Area under curve
BCL	B-cell lymphoma
BCOR	BCL6 corepressor
BCORL1	BCL6 corepressor like 1
BCR	Breakpoint cluster region
BM	Bone marrow
BMA	Bone marrow aspirate
BP	Blast phase
BRCA2	Breast cancer 2
BRD3	Bromodomain containing protein 3
C/EBPα	CCAAT Enhancer Binding Protein Alpha
CAR	Chimeric antigen receptor
Cas9	CRISPR associated protein 9
CCyR	Complete cytogenetic response
CD	Cluster of differentiation
CDK	Cyclin dependent kinases
CFA	Colony forming assay
CHIP	Clonal hematopoiesis of intermediate potential
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
CMR	Complete molecular response
CNV	Copy number variations
CP	Chronic phase
CPM	Counts per million mapped reads
CREBBP	CREB Binding Protein
CRISPR	Clustered regularly interspaced short palindromic repeats
CRKL	Crk-like protein
CVAD	Cyclophosphamide, Vincristine, Doxorubicin (Adriamycin) and Dexamethasone
CyR	Cytogenetic response
DE	Differentially Expressed
DH	Dbl homology
DMR	Deep molecular response
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-DSBR	DNA-double-strand break repair
DNA-MMR	DNA mismatch repair
DNMT3A	DNA methyltransferase3 alpha
DSRT	Drug sensitivity and resistance testing
DSS	Drug sensitivity score
E:T	Effector:Target
EDTA	Ethylenediaminetetraacetic
ELN	European Leukemia Network
ELTS	EUTOS long-term survival
EMA	European medicines agency
ERK	Extracellular signal-regulator kinase
EUTOS	European Treatment and Outcome Study
FBS	Fetal bovine serum
FDA	Food and drug administration
FISH	Fluorescence in situ hybridization
FLT3	Fms-like receptor tyrosine kinase
FPD	Familial platelet disorder
FU	Follow up
GFP	Green fluorescence protein
GUS	β-glucuronidase
HDAC	Histone deacetylase
HLA	Human leukocyte antigen
HSC	hematopoietic stem cell
IAPs	Inhibitors of apoptosis
IC50	Inhibitory concentration killing 50% of cells
IDH	Isocitrate dehydrogenase
IFN	Interferon

IL-1RAP	Interleukin 1 receptor accessory protein
IRF8	Interferon response factor 8
IS	International Scale
JAK	Janus associated kinase
KD	kinase domain
KMT2D	Lysine Methyltransferase 2D
LOH	Loss of heterozygosity
LSC	Leukemic stem cell
MAPK	Mitogen activated protein kinase
MEME	Multiple EM for Motif Elicitation
miRNA	microRNA
MLL	Mixed-lineage leukemia
MMR	Major molecular response
MNC	Mononuclear cells
MR	Molecular response
MSH6	MutS homolog 6
mTOR	Mammalian target of rapamycin
mut	Mutated
MYC	V-Myc Avian Myelocytomatosis Viral Oncogene Homolog
NA	not applicable
NGS	Next-generation sequencing
NR3C1	Nuclear Receptor Subfamily 3 Group C Member 1
PAK1	P21 (RAC1) Activated Kinase 1
PB	Peripheral blood
PD-1	Programmed cell death protein 1
pDC	Plasmacytoid dendritic cells
PDGFR	Platelet derived growth factor receptor
Ph	Philadelphia
PH	Pleckstrin homology
PHF6	PHD finger protein 6
PI3K	phosphatidylinositol 3 kinase
PML	Promyelocytic leukemia
PP2A	Protein phosphatase 2A
PRC1/2	Polycomb repressor complex 1/2
PRKDC	Protein Kinase, DNA-Activated, Catalytic Subunit
PTPR	Protein tyrosine phosphatase receptor
RAG	Recombination activating gene
RARA	Retinoic acid receptor alpha
RAS	Rat sarcoma
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPKM	Reads Per Kilobase of transcript per Million mapped reads
RSS	Recombination signal sequences
RT-PCR	Reverse transcriptase-polymerase chain reaction
RUNX1	Runt-related transcription factor 1
scRNA	single cell RNA
sDSS	selective drug sensitivity score
SETD1B/2	SET Domain Containing 1B/2
SH	Src homology
SHM	Somatic hypermutation
SIRT1	Sirtuin 1
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variation
Src	V-Src Avian Sarcoma Viral Oncogene Homolog
STAT	Signal transducer and activator of transcription
SV	Structural variant
TF	Transcription factor
TFR	Treatment free remission
TKI	Tyrosine kinase inhibitor
TLOD	Tumor log odds
TLR	Toll like receptor
TNF	Tumor necrosis factor
UBE2A	Ubiquitin Conjugating Enzyme E2 A
UV	Ultraviolet
VAF	Variant Allele Frequency
VEGFR	Vascular endothelial growth factor receptor
WES	Whole exome sequencing
WGS	Whole genome sequencing
WHO	World health organization
wt	Wild type
WT1	Wilms Tumor 1

Abstract

Chronic myeloid leukemia (CML) is an adulthood leukemia that accounts for 15% of all leukemia patients. The hallmark of the disease is the *BCR-ABL1* fusion gene. The development of tyrosine kinase inhibitors (TKI) targeted therapy, which inhibits BCR-ABL1 kinase activity, has led to dramatic improvement in CML management with the life expectancy of CML patients approaching normal rates. Nevertheless, certain challenges in CML management remain unmet. CML progression from the chronic phase (CP) to the blast phase (BP) occurs in 3–5% of CML patients and is associated with dismal survival. Additionally, a minority of CML patients express p190 as the sole BCR-ABL1 isoform at diagnosis, which is associated with inferior response to TKI treatment and increased risk of progression. The knowledge about genomic mechanisms underlying the pathogenesis of high-risk CML patients is still in its infancy. Furthermore, there is unmet need for optimized therapeutic approaches in high-risk patients.

In this project, we aimed to characterize the role of somatic mutations at different phases of CML and to identify genetic biomarkers that allow better risk assessment and CML management optimization. We also aimed to investigate the molecular and signaling mechanisms underlying the pathogenesis of high-risk CML. Furthermore, we integrated genetic data with ex-vivo drug testing to identify potentially relevant drugs in a personalized medicine approach.

In study I, we applied exome sequencing, targeted sequencing, and RNA sequencing to a cohort of 59 CML patients, which included 19 BP and 40 CP patients. BP patients demonstrated higher mutational load in comparison to CP patients and were associated with recurrent somatic mutations involving the *ABL1, ASXL1, RUNX1,* and *BCOR* genes. The mutational landscape of CP patients revealed an abundance of epigenetic modifier gene mutations, representing about half of the mutations at diagnosis. Furthermore, non-silent mutations were more frequently encountered in CP patients with poor treatment responses compared to patients achieving suboptimal and optimal responses. Genetic analysis of matched diagnosis-follow up samples from 30 patients enabled the study of clonal dynamics in CML patients under TKI treatment. Using RNA sequencing, we identified fusion genes in most BP patients, in contrast to CP patients, suggesting their potential role in CML progression. We demonstrated upregulation of pathways, including TNF, JAK/STAT signaling and inflammation-related pathways in BP patients. Finally, we presented two BP patients who were managed using a personalized approach and achieved good responses to drug sensitivity and resistance testing (DSRT)-based therapy, highlighting the potential power of this strategy in BP management.

RUNX1 was selected as an example driver somatic mutation in CML progression. In study II, we performed genetic profiling for eight BP patients using exome sequencing. After integrating genetic data of additional 12 BP patients from a previously published study, we demonstrated *RUNX1* mutations in 35% of patients. *RUNX1*-mutated samples showed frequent association with *PHF6, BCORL1* mutations and *IKZF1* deletion. Mutational signature and motif discovery analyses revealed AID/RAG axis off-target activity specific to *RUNX1*-mutated patients. In addition to upregulation of TNF- and IFN-signaling pathways, transcriptional profiling demonstrated the overexpression of lymphoid markers and transcription factors that was translated into an aberrant expression of lymphoid antigens (CD19/CD7) in *RUNX1*-mutated myeloid BP patients. Ex-vivo drug screening revealed enhanced sensitivity of *RUNX1*-mutated blasts to mTOR, BCL2, and VEGFR inhibitors. We further functionally validated transcriptional and drug sensitivity findings in cell lines using CRISPR/Cas9 gene editing to model *RUNX1* mutations. Finally, we demonstrated the efficiency of CD19 CAR T-cell immunotherapy in targeting *RUNX1*-mutated blasts of lymphoid-BP and myeloid-BP patients expressing aberrant lymphoid markers.

In study III, we investigated the characteristics of p190-BCR-ABL1 isoform in CML. To elucidate the molecular pathogenesis in p190-CML patients, we performed exome sequencing, RNA sequencing as well as phosphorylation profiling for four p190-CML patients. We demonstrated novel evidence for the p190-specific activation of interferon IFN/JAK1/STAT1 pathway in CML. Other important signaling pathways included Src and PAK1 signaling. In concord, our transcriptional and signaling findings were confirmed in two hematopoietic progenitor cell lines (Ba/f3 and HPC-LSK). Furthermore, we screened the drug sensitivity profiles of p190 and p210 isoforms in both models and identified novel p190-specific vulnerabilities, including Inhibitors of apoptosis (IAPs), P21-activated kinase (PAK), and Src inhibitors in addition to glucocorticoids. Finally, we validated JAK/STAT signaling findings, as well as in vitro sensitivity to selected drugs, in a cohort of 10 Ph+ ALL patients.

In conclusion, our study provides novel insights into the molecular pathogenesis of high-risk CML patient groups, including BP-CML and p190-CML patients. We identified potential biomarkers, including mutational load, somatic mutations and BCR-ABL1 isoform, which can be involved in the risk-assessment of CML patients. Integration of genetic and drug screening data enabled the identification of promising drug candidates for high-risk CML patients that potentially allow for further improvement of the current therapeutic approaches. Taken together, we highlighted the potential value of the personalized medicine approach, integrating genetic and functional data for optimizing the management of high-risk CML patients.

Introduction

Chronic Myeloid Leukemia (CML) is a clonal hematopoietic stem cell (HSC) myeloproliferative disorder that accounts for 15% of leukemia cases in adults.[1] CML is a model of cancer evolution, where a founder oncogene, *BCR-ABL1*, is capable of inducing full-blown CML phenotype. The *BCR-ABL1* fusion gene is encoded by the Philadelphia (Ph) chromosome, which is formed upon reciprocal translocation between chromosomes 9 and 22.[2] Typically, the natural course of CML has three phases: indolent chronic phase (CP), acceleration phase (AP) and aggressive blast phase (BP).[3] CML is considered to be a success story of targeted therapy in cancer. The first tyrosine kinase inhibitor (TKI), imatinib, targeting *BCR-ABL1*, was developed and implemented in CML management, followed by more potent second-generation TKIs (2G-TKIs). TKIs have led to a dramatic improvement in CML management, with CML survival rates approaching the rates of age-matched healthy individuals.[4]

Despite efficient treatment, certain CML patients show inferior response to TKI treatment and represent clinical challenges. The most serious challenge is the progression of CML into BP, which occurs in 2–3% of the patients and is associated with very dismal survival rates.[5] In addition, about 5% of CML patients are already diagnosed in BP, with inferior outcome.[6] Another challenging CML entity includes patients who express the p190-BCR-ABL1 isoform with reported inferior response to TKI treatment and high risk of progression.[7] Studies have linked progression of CML into BP with the accumulation of somatic mutations. Moreover, somatic mutations in cancer genes are linked to suboptimal responses and TKI resistance in CP patients.[8] To date, little is known about the molecular pathogenesis underlying high-risk CML and the prognostic value of genomic data.

Personalized medicine typically aims to provide the right treatment to the right patients at the right time. Integration of multi-omics genetic data with functional analysis and drug screening allows for the discovery of common druggable targets of cancer as well as for the identification of specific individualized responses. Furthermore, the identification of biomarkers that potentially predict targeted therapy response is a current research interest that enables better tailoring of treatment protocols.

This study focused on the characterization of high-risk CML patient groups and on the identification of the underlying pathogenic mechanisms linking genetic lesions to phenotypic and clinical characteristics. Through integration of drug screening, the study aimed to identify potential biomarkers and efficient targeted therapies that can, hopefully, be translated into optimized management protocols for those high-risk patients.

Review of the literature

1. *Chronic myeloid leukemia (CML)*

1.1. Historical review

CML is a clonal hematopoietic disorder arising from the multipotent HSC compartment and is mainly characterized by a proliferative expansion of myeloid lineage, although it also affects other hematopoietic lineages.[3] Historically, CML was the first type of leukemia to be described in 1845 by Bennett, Craigie, and Virchow[9] An important step occurred almost 100 years later, when Nowell and Hungerford discovered a minute Y-like chromosome in a cohort of CML patients, which was named the Philadelphia chromosome (Ph) later in that same year.[10] The next step occurred in 1973, when Rowley identified Ph as a truncated derivative of chromosome 22 that results from a reciprocal translocation between chromosomes 9 and 22.[11] The molecular pathology of CML was uncovered 10 years later when the human homologue of the murine Abelson gene (*ABL1*), normally located on chromosome 9, was reported to be translocated to the Ph chromosome in CML.[12] The partner gene on chromosome 22 was, two years later, located and identified as the "breakpoint cluster region" or the *BCR* gene.[13] In subsequent years, the ABL1 protein was shown to have abnormal size and enhanced kinase activity in CML patients with later identification of the *BCR-ABL1* fusion gene.[14] The oncogenic ability of *BCR-ABL1* to induce CML was finally confirmed in 1990 when it was shown that retroviral infection of HSCs with *BCR-ABL1* can efficiently induce CML-like disease in mice.[15] Being one of the most comprehensively studied human neoplasms, this remarkable history established CML as a model of cancer evolution and a success story of molecular biology research.[16]

1.2. Epidemiology

CML is among the most common myeloproliferative neoplasms and accounts for about 15% of the newly-diagnosed leukemia cases in adults.[17] The annual incidence of CML is 1–2 cases/100,000 individuals and it has been relatively even worldwide.[18,19] Recently, a trend of increasing CML incidence has been reported in the USA.[20] CML incidence increases with age, with the median age of CML diagnosis being 57–60 years, and it also shows a slight male dominance.[18,19] Contribution of other ethnic and geographic variables to CML risk is not clear.[18,19] The prevalence of CML is progressively increasing due to the improved life expectancy of patients, led by TKI treatment.[21]

1.3. Clinical picture

CML is a chronic disease with a slowly progressive course that can be classified into three phases: a chronic phase (CP), an accelerated phase (AP), and an eventual blast phase (BP).[3] Most CML patients (90–95%) present in CP-CML. Patients with CP-CML can present with non-specific symptoms (fatigue, malaise, weight loss, and

abdominal discomfort), splenomegaly (the most consistent sign in about half of patients), hepatomegaly (10% of patients) and, rarely, with serious manifestations that include bleeding, thrombosis, priapism, and leukostasis syndrome. CP-CML can, however, be asymptomatic and discovered accidentally during routine blood investigation as an unexplained elevation of granulocyte counts and, occasionally, thrombocytosis. The diagnosis is then confirmed by detection of the *BCR-ABL1* fusion gene. Bone marrow (BM) and peripheral blood (PB) show hypercellularity due to the expansion of the myeloid lineage with accumulation of immature granulocytes, basophils, and eosinophils, as well as low blast counts (< 10% of BM or PB counts).[2,22]

If left untreated, CP-CML naturally evolves into the terminal BP-CML within 4–5 years, often passing through a transient AP in most patients. BP has an aggressive course that is associated with manifestations of BM failure, including anemia, recurrent infections, and coagulopathies. In BP, accumulated blasts can be of the myeloid phenotype (50%) and, less commonly, of the lymphoid (25%) or ambiguous phenotype (25%). Despite the development of efficient treatment, BP has an invariably dismal prognosis with less than one year median survival rates.[3] Two diagnostic criteria, by the World Health Organization (WHO)[23] and the European Leukemia Net (ELN),[24] are commonly used to diagnose AP and BP primarily on the basis of the proportion of blasts in BM and/or PB. The criteria incorporated in the definitions of AP and BP are summarized in Table 1.

1.4. CML pathogenesis

1.4.1 Structure and signaling activity of BCR-ABL1

The hallmark of CML is the *BCR-ABL1* fusion gene, encoded by the Ph chromosome that results from the reciprocal t(9;22) (q34;q11) translocation. After juxtaposition, the 5` end of *BCR-ABL1* fusion gene is made by the *BCR* gene, while the proto-oncogene *ABL1* makes the 3` end.[25] This fusion yields a series of structural and functional changes that are essential for leukemogenesis. The *ABL1* gene encodes a constitutively expressed tyrosine kinase that is predominantly localized in the nucleus but transiently traffics between the nucleus and cytoplasm. On the other hand, the *BCR* gene encodes for a kinase protein whose function remains unclear. The N-terminal part of BCR-ABL1 is made by BCR domains that include, importantly, a serine/threonine kinase domain containing tyrosine residue 177 (Y177), which is critical for BCR-ABL1 tetramerization and activation, and CML transformation.[26] Most ABL1 domains are kept in BCR-ABL1, including a proline-rich domain, DNA and actin-binding domains, and Src homology domains (SH2/SH3). BCR-ABL1, however, lacks the N-terminus region of ABL1 that contains a myristoylation site, which is critical for autoinhibition of SH2 kinase activity of the ABL1 protein.[27] Autophosphorylation and loss of myristoylation moiety renders BCR-ABL1 into a pathogenic constitutive active kinase status. Additionally, the coiled-coil domain of BCR favors dimerization and tetramerization of BCR-ABL1 with subsequent autophosphorylation and activation.[28]

In contrast with ABL1 residing in the nucleus, the BCR-ABL1 kinase localize in the cytoplasm, enabling further interaction and activation of various signaling pathways.[29]

Table 1. Criteria for the definition of AP and BP, as recommended by the WHO[23] and ELN[24] guidelines.

	Acceleration phase (AP)		**Blast phase (BP)**	
	WHO	**ELN**	**WHO**	**ELN**
Hematological Criteria	- Blasts in BM/PB **10%-19%***	- Blasts in BM/PB **15%-29%, or blasts plus promyelocytes >30% with blasts <30%**	- Blasts in BM/PB **≥20%***	- Blasts in BM/PB **≥30%**
	- Basophils >20%	- Basophils >20%	- Extramedullary blast proliferation, apart from spleen	- Extramedullary blast proliferation, apart from spleen
	- Persistent thrombocytopenia <100 × 10^9/L, unrelated to therapy	- Persistent thrombocytopenia <100 × 10^9/L, unrelated to therapy	- Large foci or clusters of blasts in the bone marrow biopsy	
	-Thrombocytosis >1000 × 10^9/L, unresponsive to therapy			
	- Persistent or increasing spleen size unresponsive to therapy			
	- Persistent or increasing white blood cell count (>10×10^9/L) unresponsive to therapy			
Cytogenetic Criteria	- At diagnosis, additional chromosomal abnormalities (ACAs) in Ph+ clones, including "major route" ACAs (second Ph, +8, i17q, +19), complex karyotype, or abnormalities of 3q26.2	- Acquired ACAs during treatment		
	- Acquired ACAs during treatment.			

*****The finding of bona fide lymphoblasts in BM/PB, even if < 10%, should be considered for lymphoblastic BP transformation, and further clinical and genetic investigations are warranted.

The enhanced kinase activity of BCR-ABL1, in addition to its sequestration in the cytoplasm, enables the interaction, phosphorylation, and recruitment of various adaptor and substrate proteins with subsequent activation of several key signaling pathways. Among the most characterized BCR-ABL1-activated key signaling pathways in CML are JAK/STAT, PI3K/AKT, RAS/MEK/ERK, CRKL, and Src family kinases pathways as well as the deregulation of autophagy and apoptosis pathways.[30,31] Many of these pathways are clinically relevant and represent potential targets for targeted therapy. JAK2/STAT5, MYC, and PI3K/AKT pathways have been demonstrated to play a critical role in the development and maintenance of the CML phenotype as well as in CML progression.[32–34] Furthermore, BCR-ABL1 inhibits

apoptosis by inducing the expression of anti-apoptotic proteins, BCL2 and BCL-XL, through STAT5 and PI3K pathways.[35] The excessive activation of the above-mentioned pathways, in addition to loss of apoptosis, results in uncontrolled expansion of the myeloid lineage and the characteristic CML phenotype. BCR-ABL1 also activates DNA damage and reactive oxygen species (ROS) pathways and inhibits DNA repair machineries, contributing to genomic instability status that has been suggested to be a driving force of CML progression.[36,37]

1.4.2 BCR-ABL1 isoforms

The genomic breakpoints exhibit high variability in both *BCR* and *ABL1* partners, resulting in formation of different BCR-ABL1 isoforms (Figure 1). Regardless of the breakpoint in *ABL1*, the first two alternative exons (1a and 1b) are mostly spliced out while the remaining exons (2–11) are involved in the *BCR-ABL1* formation. On the other hand, the *BCR* gene has three common breakpoints—major (M-BCR), minor (m-BCR), and micro (μ-BCR)—which result in the formation of the three main BCR-ABL1 isoforms, designated based on their apparent molecular weight as p210, p190 (e1a2), and p230 (e19a2), respectively.[1] Based on the fusion of intron 13 or 14 of the BCR gene, p210 can be further subdivided into e13a2 or e14a2 transcripts (referred to as b2a2 and b3a2, respectively). Other rare transcripts include fusion genes lacking *ABL1* exon2 (e13/e14a3), e6a2, and exonic breakpoints.[30] The mechanisms underlying the BCR-ABL1 translocation are largely unknown; however, some mechanisms—for example, RAG rearrangements—have been suggested to contribute to the formation of different isoforms.[38]

Different BCR-ABL1 isoforms show clear association with distinct leukemia phenotypes. p210 accounts for ~98% of CML and about one fourth of Ph-positive acute lymphoblastic leukemia (Ph+ALL) cases. In contrast, p190 accounts for ~75% of Ph+ALL patients and is present in a minority of CML patients (1–2%). The p230 isoform has been associated with the rare CML phenotype, characterized by prominent mature neutrophilic expansion and/or thrombocytosis.[39,40] In p210-CML patients, differences between b2a2 and b3a2 isoforms have been related to platelet counts and subsequent response to treatment.[41] The mechanisms underlying the phenotypic preferences of different isoforms are still unclear. Experimentally, the three isoforms demonstrated similar potentials for developing CML-like phenotype in mouse models,[42] however, p190 was occasionally reported to induce a B-ALL-like phenotype with short latency.[43] One theory is that different isoforms are restricted to different hematopoietic cells of origin. While p210 of CML is suggested to originate from an early multipotent HSC, p190 has tendency to occur in a committed lymphoid B-cell progenitor.[44] This is supported by the ability of the CML blast to give rise to either a lymphoid or myeloid phenotype at the terminal BP. Additionally, the intrinsic kinase activity and structure of different isoforms have been suggested to contribute to phenotypic differences.

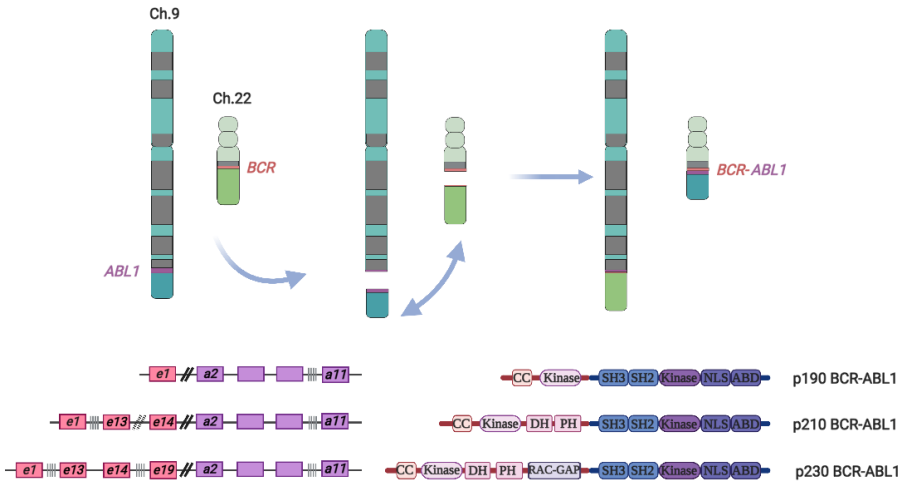


***Figure 1**. Schematic representation of the formation and different transcripts and isoforms of the BCR-ABL1 fusion.*

In contrast to the p210 isoform, The p190 isoform has a higher kinase activity and lack the Dbl homology (DH) and Pleckstrin homology (PH) domains.[45] The differences in the interactome and signaling pathways between BCR-ABLl isoforms have been systematically investigated in cell line models in few studies.[46,47] Differences included key signaling pathways—namely, the JAK/STAT pathway, where p190 demonstrates enhanced interaction and activation of STAT6 and STAT1, in contrast with STAT5 and STAT3 preferences of p210. Furthermore, p190 exhibited preferential interactions with cytoskeletal and adaptor proteins in comparison with plasma membrane protein dominant interactome of p210.[47,48] The molecular pathology underlying the phenotypic differences of BCR-ABLl isoforms remains to be explored, especially in CML primary cells.

1.5. Diagnosis of CML

The cornerstone of CML diagnosis is the detection of the Ph chromosome by conventional cytogenetics (Figure 2) or the detection of *BCR-ABL1* fusion by fluorescence in situ hybridization (FISH) or by reverse transcriptase-polymerase chain reaction (RT-PCR) applied on BM or PB samples. Other hematological investigations are providing important information in relation to disease staging and prognosis. BM aspirate (BMA) morphology indicates the proportions of blasts, eosinophils, and basophils that are among the criteria for defining the disease stage and calculating risk scores. BM biopsy has a potential prognostic value by assessing marrow fibrosis and ability to identify blast nests, which can be missed in BMA.[49]

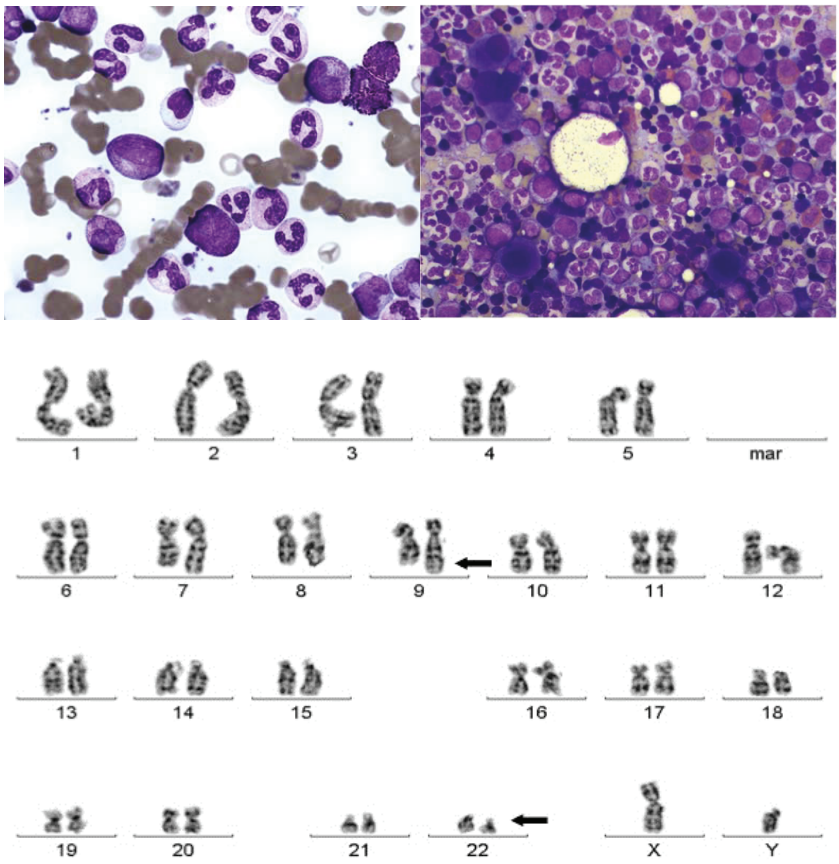


Figure 2. *Morphology of peripheral blood (upper left panel) and bone marrow (upper right panel) in CML at diagnosis. Courtesy of Satu Mustjoki. G-banding Karyotype (lower panel) of a CML patient showing the translocation between chromosomes 9 and 22 (indicated with arrows), with formation of the characteristic Philadelphia chromosome which is a derivative chromosome 22. Courtesy of Kirsi Autio.*

Conventional cytogenetics remains the gold standard for detecting Ph chromosome as well as for disease monitoring. Furthermore, it provides information about additional cytogenetic abnormalities (ACAs), an important criterion for disease staging, as well as clonal evolution in Ph-positive/negative cells during treatment.[50] Common ACAs include trisomy 8, isochromosome 17, double Philadelphia, trisomy 19, and others. However, conventional G-banding has low sensitivity (detection limit is 1–5% Ph-positive cells in the sample) and requires BM dividing cells for metaphase analysis. Cryptic and complex Ph-rearrangements can also be challenging to detect using conventional cytogenetics.[51] FISH is an assay that is commonly used to complement conventional G-banding for diagnosis and treatment monitoring. It uses genomic probes specific for *BCR* and *ABL1* to identify the colocalization of the genes. It is a rapid and specific assay that can be applied on interphase cells and does not necessitate

metaphases.[52] It is of specific importance in cryptic and complex Ph-rearrangements in addition to cases with positive RT-PCR and negative karyotyping. Despite better specificity and sensitivity in comparison to conventional G-banding, FISH has a lower sensitivity when compared to molecular methods and cannot be used to detect ACAs.[53,54]

RT-PCR is a highly sensitive method that has been widely used for CML diagnosis and monitoring of treatment. The technique has been developed to detect the *BCR-ABL1* fusion though amplification of the regions flanking the breakpoints in *BCR* and *ABL1* genes. RT-PCR can be performed as either a qualitative or a quantitative test.[55] Recent ELN recommendations indicated that a qualitative RT-PCR test is mandatory at diagnosis for detecting and identifying the type of *BCR-ABL1* that can be followed during monitoring of treatment responses.[56] As RT-PCR primers have been optimized for the common major (M-bcr) and minor (m-bcr) breakpoints, other rare breakpoints and *BCR-ABL1* transcripts may yield false negative results. When rare transcripts are suspected, multiplexed PCR protocols can be used.[57] Quantitative RT-PCR has become the main method for monitoring treatment response, especially when *BCR-ABL1* level falls below the sensitivity levels of FISH and karyotyping.[53,54] Additionally, achieving molecular responses (i.e., reduction of the *BCR-ABL1* transcript level compared to its quantified values at initial diagnosis) demonstrates a prognostic significance and has been incorporated in the risk-stratification of CML patients.[58]

1.6. Risk stratification and CML prognosis

1.6.1. Baseline criteria and risk stratification

At diagnosis, four scoring systems have been developed to stratify CP-CML patients regarding the predicted prognosis and survival rates. These systems are Sokal,[59] Hasford,[60] EUTOS,[61] and ELTS[62] scores, which are arithmetically-derived scoring systems that integrate simple clinical and hematological data, such as age, spleen size, platelet count and blast count in peripheral blood. Sokal and Hasford scores were developed in the pre-TKI era, while EUTOS and ELTS are TKI era-based scores. The Sokal score has been widely used in CML studies, however, recently the ELN has recommended the use of ELTS scoring, developed to predict CML-related deaths in the TKI era, as it shows enhanced predictive efficiency.[56,63] Sokal, Hasford, and EUTOS scores can predict treatment outcomes in imatinib-treated patients with the EUTOS system being superior,[64] but their efficiencies remains unclear in frontline 2G-TKI treatment. Furthermore, low diagnostic risk scores are among the suggested requirements for TKI discontinuation in clinical practice.[65]

Additional risk factors, not included in the scoring systems, include BM fibrosis, ACAs, and expression of p190-BCR-ABL1 at diagnosis. Increased BM fibrosis, that can be assessed by histochemical staining in BM biopsy, has an inferior impact on

treatment outcomes.[66] ACAs can be classified into high-risk and low-risk groups, where high-risk ACAs include +8, +ph, i(17q), +17, +19, +21, 3q26.2, 11q23, -7/-7q and complex karyotype, while the low-risk group includes other ACAs.[67] High-risk ACAs confer a poor prognostic impact and an increased risk of progression.[67,68] Patients with high-risk ACAs are recommended to be treated as high-risk patients regardless of the clinical score system prediction.[56,65]

1.6.2. Treatment milestones

Monitoring the levels of molecular response at the recommended milestones (3,6, and 12 months) has a strong prognostic value. Treatment responses can be classified into optimal, warning (suboptimal), and failure groups according to the molecular responses at different milestones (Table 2). These definitions apply to first and second lines of TKI treatment.[56,65] This risk-stratification determines whether a patient should continue or switch the current TKI treatment and the pace of molecular monitoring. Early molecular response (BCR-ABL1 IS < 10% at 3 months) was demonstrated to have a significant prognostic impact.[69] The 6-month mark is another important milestone for a TKI switch decision. Patients achieving BCR-ABL1 IS < 1% at 6 months have been associated with better survival rates.[69,70] Achievement of complete cytogenetic response (CCyR)/MR^2 at the 12-month milestone is associated with almost normal survival rates.[71] Patients achieving deeper molecular responses—major molecular response (MMR) and complete molecular response (CMR)—have demonstrated improved event-free survival rates but modest survival benefits.[72] With adoption of TFR as a treatment goal, the achievement of sustained deep molecular responses (MMR/CMR) is among the eligibility criteria for TKI discontinuation. The duration of deep response prior to TKI stop shows the strongest correlation with TFR rates.[56,73]

Table 2. Molecular definitions for the patient response groups at the recommended milestones, as expressed by BCR-ABL1 levels on the IS scale.

	Optimal	Warning (suboptimal)	Failure
3 months	≤10%	>10%	>10%*
6 months	≤1%	1-10%	>10%
12 months	≤0.1% (MMR)	0.1-1%	>1%
> 12 months	≤0.1%	0.1-1%	>1%

BCR-ABL1 level is expressed on the IS scale as a ratio of the baseline/diagnostic level.
*Failure is considered if *BCR-ABL1* level is confirmed as > 10% within 1–3 months after the 3-months milestone.

1.7. Treatment of CML

CML represents a paradigm for the power of targeted therapy in cancer management. Over a century, the treatment of CML has evolved from arsenical treatment and splenic radiotherapy through the use of chemotherapeutic agents (busulfan and hydroxyurea), interferon alpha (IFNα), and allogenic stem cell transplantation (Allo-SCT), to finally reach TKI-targeted therapy.[74] The development of molecular-based TKI treatment ushered in the era of precision medicine and the power of genomic information in successful cancer management.[75] With remarkably improved outcomes in the TKI era, the optimal goal of CML treatment is the eradication of all Ph-positive clone cells, which would enable "CML cure."[74]

1.7.1 Tyrosine kinase inhibitors (TKIs)

The introduction of TKIs has had a dramatic effect on improving treatment outcomes and on changing the natural history of CML from a deadly disease with median overall survival (OS) rates of only 5 years into a controllable disease with nearly normal life expectancy.[76] In a population-based study from Sweden including 2662 CML patients, the life expectancy of CML patients were approaching that of general population, with estimated loss of less than three life-years as a result of CML[4]. The tremendous progress in understanding the molecular pathogenesis of CML made in the second half of the twentieth century and the unraveling the role of the *BCR-ABL1* oncogene, has led to the remarkable story of the development of the first TKI, imatinib, followed by 2G- and 3G-TKIs. TKIs are small molecules designed to inhibit BCR-ABL1 kinase activity by occupying the ATP-binding pocket of the oncoprotein, which blocks BCR-ABLl downstream signaling. Furthermore, the binding of TKI to BCR-ABL1 holds its activation loop in an inactive conformation and blocks further activation.[77]

The first generation TKI, imatinib (imatinib mesylate, STI571), was developed by Brian Druker and scientists at Ciba-Geigy in Basel (now Novartis) in 1994.[78] The International Randomized Study of Interferon and STI571 (IRIS) demonstrated the efficiency of imatinib in reverting the CML phenotype, inducing CCyR in the majority of patients, and significantly reducing progression rates.[79] The revolutionary results from the IRIS study, confirmed by other academic trials, have led to the approval of imatinib as a frontline treatment for CML in 2003.[80] Ten-years follow-up studies have demonstrated the ability of imatinib to induce MMR in the majority of patients, associated with marked improvement of overall survival rates.[81] Long-term imatinib treatment is generally safe and serious adverse effects, including gastrointestinal disturbances, musculoskeletal pains, and cytopenias, are tolerable.[82] It is noteworthy that, despite the excellent profile, a significant proportion of patients discontinues imatinib treatment due to unsatisfactory treatment responses or intolerance.[83] Generic imatinib became available in 2016 and was reported to have comparable safety and efficacy profile to branded imatinib—however, longer follow-up is recommended.[84]

The remarkable success of imatinib has encouraged the development of more potent 2G-TKIs, including nilotinib, dasatinib, and bosutinib. Nilotinib was developed as a modification of imatinib with better selectivity and higher potency.[85] As a frontline TKI, nilotinib achieves faster and deeper molecular responses and lessens need to switch treatment in comparison to imatinib, and with similar survival rates. Regarding adverse effects, nilotinib exhibits higher incidence of cardiovascular events in comparison to imatinib (20% vs. 5% of patients, respectively) in addition to acute pancreatitis (5%) and cytopenia.[86] Dasatinib is another 2G-TKI that is structurally unrelated to imatinib and binds to BCR-ABL1 with higher affinity regardless of the activation loop conformation. As a kinase inhibitor, dasatinib is 300-fold more potent than imatinib and inhibits several kinases in addition to BCR-ABLl, including Src and c-KIT kinases.[87] Deeper and faster molecular responses were achieved with dasatinib in comparison to imatinib, with similar survival and treatment switch rates.[88] Dasatinib is known to exert immunomodulatory effects, involving lymphocytes and natural killer cells, that can potentially modulate the treatment outcome.[89] Adverse effects of dasatinib treatment include pleural and pericardial effusions and, rarely, pulmonary hypertension.[90] Bosutinib, a third 2G-TKI, also shows higher rates of one-year MMR in comparison to imatinib. It has a generally safe profile, with few side effects that include transient diarrhea (30% of patients) and transient elevation of liver transaminases.[91] Ponatinib is a highly potent broad spectrum 3G-TKI that has been approved for refractory CML patients who show resistance to ≥ 2 TKIs and for those carrying T315I resistance mutation. Despite the fact that such patients are usually heavily treated, they could achieve durable CCyR (50%) and/or MMR (40%) with ponatinib treatment at the 5-year follow up. The main adverse effect of ponatinib is cardiovascular toxicity in 30% of patients.[92]

1.7.2 Considerations of TKI treatment

Currently, all above-mentioned TKIs— with the exception of ponatinib—have been approved for use as front-line treatments in newly diagnosed CML patients. 2G-TKI were approved as front-line treatments because they showed higher rates of CCyR and MMR in comparison to imatinib—however, they did not show an OS advantage over imatinib. Other factors, including the safer toxicity profile of imatinib and introduction of generic imatinib, make imatinib the most commonly used front-line treatment for CP-CML, which is also the current recommendation by ELN for CML treatment.[56] Use of 2G-TKIs in front-line management has gained interest in high-risk CML patients and with respect to the trials of treatment discontinuation.[93,94] Generic dasatinib will soon be released and could significantly reduce the treatment costs, thus enabling further front-line use of 2G-TKIs.[95]

A significant fraction of the CML patients receiving frontline imatinib treatment will have to switch to 2G-TKIs within 5 years. Reasons for imatinib treatment failure are resistance and intolerance.[83] Furthermore, lack of an early molecular response to

imatinib treatment is recently defined as "warning" sign in clinical practice[56] and such patients are recommended to be switched to 2G-TKI.[96] 2G-TKIs—dasatinib, nilotinib, and bosutinib—have comparable response rates in clinical settings, though head-to-head comparisons are not available. The choice of specific 2G-TKI should incorporate data on the *ABL1* mutational status, patient comorbidities, and drug toxicity profile. More than 100 mutations located within the ABL1 kinase domain (ABL1-KD) have been identified, conferring resistance to TKI treatment.[97] Investigation of ABL1-KD mutations is mandatory when resistance to TKI treatment has been developed. Next-generation sequencing (NGS) is more sensitive than Sanger sequencing in the detection of ABL1-KD mutations and is recommended for patients with TKI resistance.[98] Except for ABL1-T315I, 2G-TKIs are active against several imatinib-resistant ABL1-KD mutations; however, each drug has its own unique resistance mutation list. As mentioned above, the toxicity profiles and contraindicating comorbidities vary between 2G-TKIs and affect treatment choice.[56,99] About one third of CML patients in clinical trials have to switch to another TKI, regardless of initial use of imatinib or 2G-TKI.[100]

Despite efficient initial response to 2G-TKIs as a second line treatment, a significant proportion of patients eventually shows resistance or drug-related toxicities that lead to treatment discontinuation. Third line treatment includes switching to another 2G-TKI or to ponatinib. The decision depends on the mutational status of *ABL1*, the presence of T315I mutation, and the comorbidities of the patient. Patients with unsatisfactory responses or intolerance to at least two TKIs are considered for Allo-SCT and investigational treatments.[56]

1.7.3 Monitoring of response to TKI treatment

Monitoring of the residual disease in patients on TKI treatment is a critical part of CML management. It provides information for risk stratification of patients and assessment of the need to switch treatment. With adoption of the achievement of treatment-free remission (TFR) as new goal of CML treatment, the monitoring of the residual disease has become increasingly important in order to identify the potential candidates for discontinuation and further follow up of patients.[101] In the TKI era, treatment responses can be assessed using hematological, cytogenetic, and molecular criteria[102] (Table 3). Total and differential blood counts should be assessed every two weeks until complete hematological response is achieved. Cytogenetic responses can be assessed by examining BM cells for Ph-positive metaphases or using FISH. FISH can be conveniently carried out on PB samples. Despite being less sensitive in comparison to molecular monitoring, cytogenetic monitoring is of particular value for patients with atypical *BCR-ABL1* transcripts as well as for assessing ACAs as a sign of progression.[103]

Molecular monitoring is currently the gold standard for assessing TKI treatment response. Quantification of *BCR-ABL1* transcripts using RT-PCR is recommended to be carried out at 3,6, and 12 months during the first year, and at 3 to 6 months intervals in the later follow up. RT-PCR results are expressed as a ratio of the *BCR-ABL1* transcript copy number to the copy number of a control gene, including *ABL1* or β-glucuronidase (*GUS*). For result standardization, values are recommended to be expressed using the International Scale (IS) conversion approach. The decline of *BCR-ABL1* levels can be expressed as a log10 reduction from the initial untreated value.[58] BMA sampling is no longer necessitated for molecular monitoring because RT-PCR can be efficiently done from PB. Other PCR techniques, including nested PCR, multiplex PCR, digital PCR, and DNA-based PCR, may have improved sensitivities or abilities to detect atypical variants—however, they are not as widely used as RT-PCR. Additional value of RT-PCR in CML monitoring lies in its ability to detect and monitor resistance-associated mutant *BCR-ABL1*.[101,104]

Table 3. Definitions of hematological, cytogenetic, and molecular responses as recommended by the European Leukemia Net (ELN) and the U.S. National Comprehensive Cancer Network (NCCN) guidelines.[102,105]

Type of response		Definitions
Hematological	Complete (CHR)	• White blood cells <10x10^9/L • normal differential (no immature cells) • Platelets <450x10^9/L • No signs or symptoms of CML
	Partial	• White blood cells <10x10^9/L • Persistence of immature cells • Platelets <50% of pre-treatment count, but >450x10^9/L • Persistent splenomegaly (<50% reduction of size)
Cytogenetic	Complete (CCyR)	0% Ph+ metaphases
	Partial (PCyR)	1-34% Ph+ metaphases
	Major (MCyR)	0-34% Ph+ metaphases (=complete and partial)
	Minor (mCyR)	35-65% Ph+ metaphases
	Minimal (minCyR)	66-95% PH+ metaphases
Molecular	Complete (CMR)*	BCR-ABL1 mRNA is undetected (≥ 5 log reduction)
	Deep (DMR)**	≥ 4 log-reduction of BCR-ABL1 (i.e. ≤0.01% IS)
	Major (MMR)	≥ 3 log-reduction of BCR-ABL1 (i.e. ≤0.1% IS)

IS: International Scale converted values.
* The recent ELN2020 recommends avoiding the term CMR, replacing it with the term "molecularly undetectable leukemia."[56] ** DMR refers to deep molecular responses (ex: MR,[4] MR[4.5]), including down to undetectable transcripts (i.e., CMR).[104]

1.7.4. TKI cessation and treatment free remission

Adherence of CML patients to life-long TKI treatment is recommended due to the inability of different TKIs to completely eradicate the CML leukemic stem cells (LSCs) and achieve "biological cure." However, a significant number of patients with sustained deep molecular response (DMR) can successfully discontinue TKI treatment and achieves TFR. The goal of CML management has gradually been shifted from improving survival rates to achieving successful TFR on TKI discontinuation. Definition of safe selection criteria and identification of biomarkers that can predict successful TFR are critical for clinical translation.[74,106] Given the increasing prevalence of CML in the TKI era, the treatment costs and cost-effectiveness of TKI have become important clinical and social factors in CML management.

Since the first TKI discontinuation trial, Stop Imatinib 1(STIM1), several trials have been conducted with slightly different entry and TKI-resuming criteria. A meta-analysis of imatinib stop trials, including 15 studies, demonstrated a mean molecular relapse rate of 51%.[107] Similar results were demonstrated by meta-analysis involving 2G-TKI stop trials.[108] Inclusion criteria of TKI treatment for at least three years and sustained DMR for $\geq$ 1 year were consistent for most of the trials. In the largest stop trial, EURO-SKI study, the duration of TKI treatment, duration of DMR, and prior IFNα treatment were important prognostic factors predicting TFR.[73] The immune system has been suggested to play role in maintaining TFR and keeping CML-LSCs in control.[109]

Most of the relapses, which is defined as MMR loss (i.e., elevation of *BCR-ABL1* transcript above MR^3 level), occur within the first year of TKI cessation. Thus, close monitoring is critical in the early period after stopping. Relapsed patients usually restart the same TKI treatment. Some patients showed fluctuating values below the MMR level and require close monitoring. The phenomenon of maintaining remission with or without detectable *BCR-ABL1* levels in either on- or off-treatment patients is called "operational cure." Relapse after the first year is uncommon. TKI stop is generally safe and events like resistance mutation or BP progression are rarely encountered. The most common side effect associated with TKI discontinuation is polymyalgia and joint pains in 20–30% of patients, known as the "TKI withdrawal syndrome."[110] The mechanisms underlying TKI withdrawal syndrome remain largely unaddressed. Potential mechanisms of TKI withdrawal syndrome include prolonged inhibition of BCR-ABL1-independent pathways such as c-KIT and platelet derived growth factor receptor (PDGFR),[111,112] as well as imatinib induced changes in bone remodeling and metabolism.[113] ELN recommendations for TKI discontinuation are summarized in Table 4.

Table 4. TKI discontinuation requirements as recommended by the ELN.[56]

Criteria	Minimal (stop allowed)	Optimal (stop recommended)
Duration of TKI therapy	>5 years (1st line TKI) >4 years (2nd line TKI)	> 5 years
Duration of DMR	>2 years	>3 years (MR^4) >2 years ($MR^{4.5}$)
Transcript type	Typical p210 transcripts (e13a2 or e14a2)	
Treatment	No prior resistance or failure*	
Mandatory criteria	• CP-CML • Access to high-quality quantitative PCR using IS • Motivated patient with structured communication • Patient's agreement of more frequent monitoring after TKI stop (monthly: 1st 6 months, every 2 months: 6-12 months and every 3 months thereafter)	

*Patients on second line TKI can be considered if the reason for switching the treatment was intolerance.

1.7.5 Other treatments in CML

hydroxyuria (hydroxycarbamide) is a well-tolerated drug that was historically introduced in CML treatment in the 1970s but has minimal effect on the disease course. Currently, a short course of hydroxyurea may be used as a cytoreductive agent before initiating TKI treatment.[114] In the last two decades of the twentieth century, Allo-SCT was the first line treatment for eligible CML patients and is still the only proven curative treatment. In the current TKI era, Allo-SCT became seldomly used for CML.[115] Indications for Allo-SCT include CP-CML patients with resistance or intolerance to multiple TKIs as well as for advanced-phase BP-CML patients.[56,115,116]

IFNα treatment demonstrated improved responses in comparison to hydroxyurea and became the treatment of choice for CML in the 1990s,[117] before being replaced with imatinib after the IRIS study in 2001.[79] IFNα monotherapy can induce complete cytogenetic responses in about 20% of patients. The combination of IFNα with cytarabine was associated with improved responses but also with increased toxicities.[118] Immunomodulatory effects of IFNα are suggested to mediate the response in CML patients[119], but the precise mechanism of action is still unclear. In the TKI era, several trials have combined IFNα with imatinib or 2G-TKIs in different schedules.[120] The combination of TKIs and IFNα was associated with an earlier onset and higher rates of DMR but also with a higher incidence of side effects.[121] Combination of 2G-TKIs with IFNα showed promising responses in high-risk CML patients with ABL1-KD mutations.[122] Additionally, the potential benefits of the TKI-IFNα combination on the outcomes of TKI discontinuation have been investigated

with promising results.[123] Optimization of the TKI-IFNα combination dosage can reduce the incidence of side effects and enable further clinical translation.

In addition to the TKIs in current clinical use, other TKIs have been developed but are not yet widely approved for clinical practice in CML, including radotinib and asciminib. Radotinib is a 2G-TKI that has thus far been approved as a first line CML treatment only in South Korea, and it shares structural and resistance profile similarities with nilotinib.[124] Asciminib is a novel allosteric TKI that inhibits BCR-ABL1 by targeting the myristoyl pocket rather than the ATP binding pocket targeted by other TKIs.[125] Promising results of phase III clinical trials using asciminib in CP-CML patients previously treated with ≥ 2 TKIs have recently been reported.[126] Asciminib is not influenced by ABL1-KD mutations, including the T315I mutation—however, mutations in the myristoyl domain can cause asciminib resistance. Dual targeting of BCR-ABL1 using asciminib and nilotinib has potentially enabled the eradication of the leukemic clone.[127] Newer 3G-TKIs with improved selectivity and limited off-target activity, including HQP1351[128] and K0706,[129] are still in the early phase I/II studies.

1.8. Clinical challenges and high-risk CML patients

1.8.1. BP-CML

Progression of CML remains the main clinical challenge in CML management.[130] Despite the major improvement of survival rates and quality of life in CP-CML patients achieved in the TKI era, the prognosis of BP-CML remains dismal. TKIs have efficiently reduced progression rates to 1–3% per year in comparison to 20% in the pre-TKI era, and the cumulative incidence has dropped from 70% prior to TKIs to 5% in imatinib-treated patients after 8 years of treatment.[5] In contrast, even 2G- and 3G-TKIs could only achieve a modest improvement of the prognosis of BP-CML patients, with median survival rates of 8–12 months in comparison to 3–4 months in patients treated with conventional chemotherapy.[130] Older age, prior TKI treatment, myeloid phenotype, and low platelet counts have been reported to be associated with reduced survival rates in BP-CML patients.[131] Provided that a suitable donor is available, treatment with a TKI-chemotherapy combination followed by Allo-SCT is the best treatment option for BP-CML patients, with 5-year OS rates of 40–49%.[131,132]

The knowledge of the mechanisms underlying CML progression is still in its infancy. Several studies have linked progression to the BCR-ABL1-induced genetic instability and acquisition of additional genetic aberrations. Uncontrolled BCR-ABL1 activity induces a state of genetic instability by enhancing DNA damage via reactive oxygen species (ROS) production and impairing DNA repair mechanisms.[133,134] This results in the acquisition of a wide spectrum of genomic aberrations, including somatic mutations, copy number variations (CNV), and fusion genes as well as ACAs.[37] The acquired lesions further increase genetic instability and activate various signaling

pathways that underlie the characteristics of the BP-CML phenotype, including enhanced self-renewal, differentiation arrest, and *BCR-ABL1*-independent growth via activation of other survival pathways.[135,136] In addition to activation of the above-mentioned *BCR-ABL1* signaling network, transcriptional reprogramming in BP-CML includes dysregulation of key signaling molecules, including MYC, P53, IRF8, C/EBPα, and SIRT1 as well as autophagy and apoptotic dysregulation.[136]

Given the dismal outcome and limited therapeutic options, prevention and regular monitoring are critical in BP-CML management. Achieving early and deep reduction of *BCR-ABL1* and properly switching TKIs in high-risk CML patients can efficiently reduce progression rates. Risk stratification and regular monitoring of CP-CML patients for response milestones and signs of acceleration, enable the timely switch of high-risk patients to intensive treatment. Further understanding of BP-CML molecular biology will potentially allow for the targeting of the underlying active pathways and for the tailoring of better management approaches.[137] Several targeted therapeutic approaches are under investigation, including TKI combinations with BCL-, JAK-, HDAC- and other inhibitors.[130,137] Given the genetic heterogeneity of BP-CML, patients are expected to show variable responses to targeted therapies. Hence, a personalized approach may hold great promise for these patients.

1.8.2. p190-BCR-ABL1 isoform in CML

At diagnosis, the p190 isoform is expressed as the sole transcript in about 1–2% of CML patients, while it is co-expressed with p210 in 5–7% of patients.[7,138,139] Sole expression of the p190 isoform in CML patients is associated with distinct hematological features, including monocytosis, absence of basophilia, frequent incidence of ACAs at diagnosis, and increased risk of progression to BP, especially with the lymphoid phenotype,[140,141] Additionally, p190-CML patients demonstrate an inferior short-lived response to TKI treatment with reduced survival rates.[141,142] The p190 isoform has been identified as an independent predictor of progression and poor survival using multivariate analysis[143] and the inclusion of p190 as a high-risk criterion in CML management has been suggested.[65] The genomic profile and pathogenic mechanisms of p190-CML remains largely obscure.

1.8.3. CML leukemic stem cells

CML-LSCs are the main culprits causing treatment resistance and CML relapse upon TKI discontinuation. LSCs that were first identified in AML as CD34+/CD38- cells capable of engraftment and of initiating leukemia in immunodeficient mice were then subsequently identified in CML as well.[144–146] LSCs share many characteristics with HSCs, including self-renewal, proliferative capacity, and ability to switch between cycling and quiescence. Additionally, CML-LSCs have unique transcriptional, metabolic, and signaling pathways that provide survival adventages.[144,147] Genomic

reprogramming in LSCs includes epigenetic dysregulation through interactions of polycomb family (PRC1/PRC2), BMI1 and histone deacetylase (HDAC) family proteins, as well as miRNA and telomere hemostasis.[148–150] Metabolic reprogramming includes dysregulation of apoptosis and autophagy pathways in addition to upregulation of drug transporter proteins.[151–153] Other active pathways are β-Catenin, hedgehog/Smo, PP2A, PI3K/AKT, and leukotriene/ALOX5.[154–156] The cross-talk between CML-LSCs and BM microenvironments plays an important role in CML leukemogenesis.[157] Furthermore, the immune evasion mechanisms of CML-LSCs and their interactions with the immune system have recently been highlighted.[158,159] In the context of TKI discontinuation trials, the immune system has been suggested to keep residual CML-LSCs under control and to induce an "operational cure" in CML patients, achieving TFR. Characterization of an active anti-CML-LSCs immune response is another important goal in CML research.[109,160] Phenotypically, only few hits can serve as CML-LSCs specific markers in contrast with HSCs, including CD26, CD25, and IL-1RAP, with a potential value in disease monitoring as well as immunotherapy.[161–163] CML-LSCs' specific pathways and targeting approaches are summarized in Figure 3.

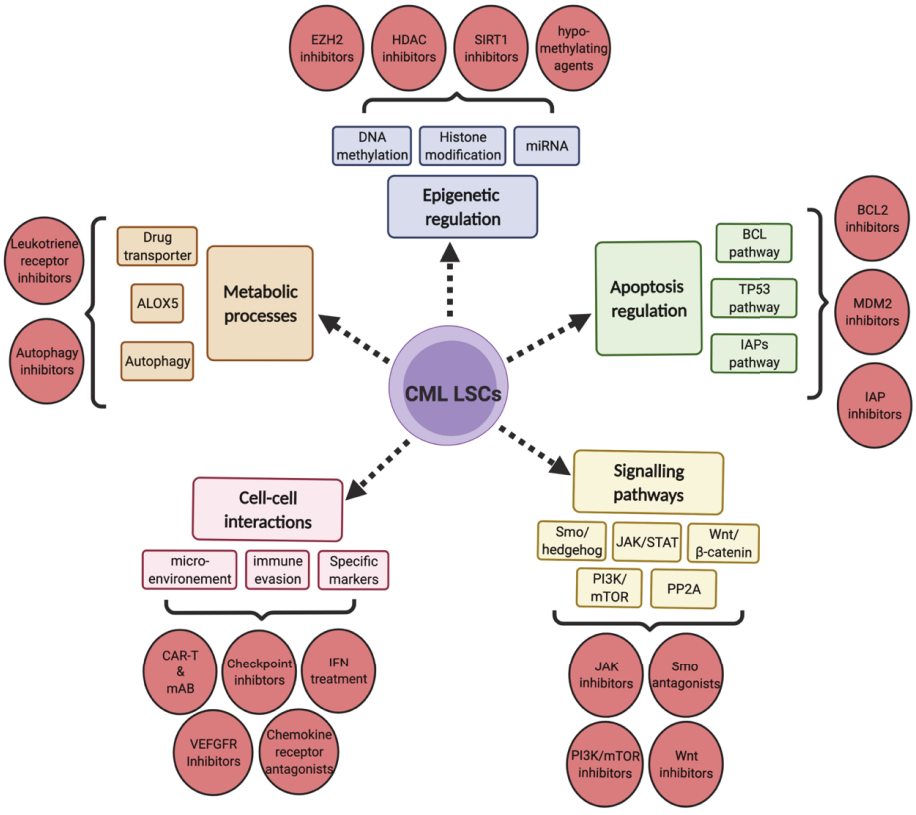


***Figure 3**. Summary of specific active pathways in CML LSCs and potentially targeting therapies.*

TKIs, including more potent 2G-TKIs, fail to eradicate CML-LSCs even in patients achieving deep responses.[164,165] Furthermore, TKI treatment has been reported to enhance the stemness of CML-LSCs,[166] which was recently been confirmed using single cell analysis that revealed enrichment of the quiescence gene expression signature in CML-LSCs under TKI treatment.[167] Resistant quiescent CML-LSCs have low *BCR-ABL1* expression levels and activated stem cell-inherited survival pathways. Targeting the BCR-ABL1-independent survival pathways is critical for enabling CML-LSCs eradication.[168] Targeting CML-LSCs gains further clinical importance with growing interest in TFR as a new goal in CML management.[169]

2. *Genetic background of high-risk CML*

Somatic mutations are well-known oncogenic drivers of tumorigenesis and leukemogenesis. A wide range of genetic aberrations, including point mutations, fusion genes, copy number, and structural variations, contributes to the molecular pathogenesis of leukemia.[170] In AML and ALL, somatic mutations and translocations affecting a core set of genes have been recurrently identified and established as prognostic biomarkers of treatment outcome.[171,172] Genetic data, mainly somatic mutations, have successfully been integrated in risk-stratification and management of acute leukemia patients.[173] Additionally, several targeted therapies have successfully been developed to target mutated oncogenes (e.g., FLT3 and IDH inhibitors).[174,175] Similar effort is also undergoing for other types of leukemia.[176] Characterizations of the mutational profile of CML and the potential value of genetic data in CML management are still in their infancy.

Several mutagenic mechanisms are implicated in the acquisition of somatic mutations and contribute to the mutational profile in variable proportions, according to cancer and tissue type.[177,178] Analysis of patterns of single nucleotide alterations and small indels among different cancers has led to the identification of distinct constellations of mutation types termed as "mutational signatures."[177,179] These mutational signatures have been partly attributed to specific mutagenic processes, including defects in DNA repair machineries and age- and smoking-related signatures. Furthermore, mutational signatures related to aberrant activity of specific DNA-modifying enzymes, including AID/APOBEC and polymerase-η/ AID/RAG axes, were also identified.[180,181] Study of the mutational signature provides insights into the molecular pathogenesis of different cancers and potential ways to tackle cancer development. Recent studies have characterized mutational signature profiles of different leukemias,[182] However, the mutational signature profile of CML remains largely uncharacterized.

In addition to the established connection between somatic mutations and cancer development, growing evidence suggests accumulation of somatic mutations in different tissues as a part of the aging process.[183] Most of these mutations may have a

silent effect, including synonymous mutations and mutations in non-coding DNA. When mutations occur in certain genes, they can confer proliferative advantages to the affected cells that lead to clonal expansion.[184] In blood, this pattern in which HSCs accumulate somatic mutations with age—and some of these mutations are associated with fitness advantage and expansion—is known as age-related clonal hematopoiesis (ARCH). Prevalence of ARCH increases with age, from 10–20% in individuals 60–70 years of age up to 40% in individuals over 90 years of age.[185,186] Studies applying highly sensitive NGS that can detect very low variant allele frequency (VAF) mutations have reported higher prevalence rates of ARCH.[187] Many of the genes recurrently mutated in ARCH are well-known leukemia-associated genes, including *DNMT3A, TET2, ASXL1,* and *TP53*.[188,189] Several studies have reported increased risk of hematological malignancies, cardiovascular diseases, and death in individuals with ARCH.[190,191] This has led to the development of the term "clonal hematopoiesis of intermediate potential" or CHIP, which describes leukemia-associated gene mutations that are detected with low VAF (≥ 2%) in individuals with no overt leukemia.[192] The role of CHIP in acute leukemia has actively been investigated. Given the elderly onset of CML, the expected potential role of CHIP in CML pathogenesis remains to be addressed.

2.1. Somatic mutations in CML

2.1.1. Somatic mutations in CP-CML

Historically, CP-CML has been considered to be a genetically uniform disease, where *BCR-ABL1* fusion is the principal event and is capable of establishing the disease phenotype.[15,16] Nevertheless, the clinical outcomes of TKI treatment targeting BCR-ABL1 are heterogeneous, suggesting that other independent mechanisms are implicated in CML pathogenesis.[97,193] Early genetic studies have employed Sanger sequencing to screen samples of individual cases or small cohorts of CP-CML patients for mutations affecting selected genes with known leukemia association. Two of the earliest studies have screened *RUNX1* gene in CP-CML patients with trisomy 21 and have reported *RUNX1* mutations in two patients associated with poor outcomes.[194,195] Other Sanger sequencing studies revealed high frequency of *ASXL1* mutations in CP-CML patients in contrast to rare incidences of other AML-associated mutations (e.g., *IDH1/2* mutations).[196–198] Recent NGS studies, including targeted sequencing and whole exome sequencing (WES), have highlighted the genetic heterogeneity of CP-CML, with about one third of the patients carrying non-silent mutations in cancer-associated genes.[199,200] In concord with earlier reports, *ASXL1* mutations were the most common mutations in about 10% of CP-CML patients. Other recurrent mutations involved *RUNX1, TET2, DNMT3A, KMT2D, CREBBP,* and *TP53* genes.

Recent studies have suggested a potential role of somatic mutations as predictors of treatment outcome in CP-CML patients (Figure 4). Mutations occurring in epigenetic

modifier genes were recurrently identified in CP-CML patients and associated with inferior outcomes to frontline imatinib treatment.[199,201,202] Additionally, a study of the dynamics of somatic mutations in longitudinal samples under TKI treatment has revealed distinct patterns of mutation clearance, acquisition, or persistence that was associated with different outcomes.[199] Furthermore, somatic mutations—defined as preleukemic (detected in both Ph-positive and Ph-negative cells) as well as Ph-negative specific mutations—have recently been identified in TKI-treated CP-CML patient samples.[203,204] A considerable variation is seen between genetic studies of CP-CML patients regarding patient selection, source of the samples, and sequencing method. Further analyses of larger patient cohorts that apply standardized methods are warranted to provide a systematic overview of CP-CML genetics.[8]

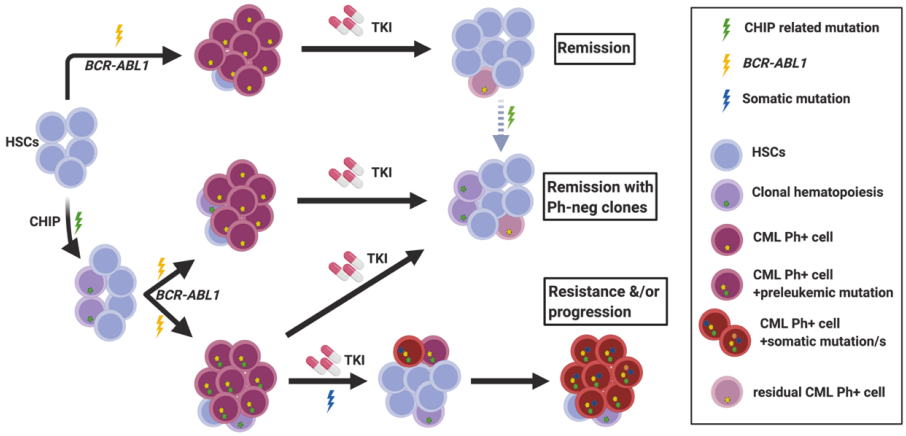


***Figure 4**. Proposal of clonal evolution of CML and dynamics of somatic mutations under TKI treatment. Somatic mutations may precede the incidence of BCR-ABL1 (such as age-related CHIP mutations), or can be acquired in Ph-positive cells during TKI treatment where it is potentially associated with resistance of progression to BP.*

2.1.2. Somatic mutations in BP-CML

Given the suggested association of CML progression with acquisition of somatic mutations, genetic studies of CML have initially focused on characterizing the mutational profile of BP-CML patients. Data accumulated from early Sanger studies and recent NGS studies revealed the ubiquitous presence of cancer-relevant mutations in almost all BP-CML patients.[205–210] In the TKI era, the ABL-KD mutation is the most recurrent mutation identified in almost half of the patients. The most common non-*ABL1* progression-associated mutations are *RUNX1, ASXL1* mutations and *IKZF1* deletions. Other recurrent mutations involved *BCOR/BCORL1, TP53, SETD1B, SETD2, UBE2A, IDH1/2, GATA2, NRAS,* and *WT1* mutations. Some mutations showed specific association with the BP phenotype, such as *IKZF1* deletions in lymphoid BP and *ASXL1* mutations in myeloid BP.[205,211,212]

Similar to CP-CML, genetic studies of BP-CML have demonstrated considerable variation regarding sequencing methods and metrics. Additionally, many of these studies have sequenced individual cases or specific genes of interest, which precludes definitive conclusions. Some known cancer genes, such as *TP53* and *WT1*, were reported at high frequency in pre-TKI studies in comparison to the recent NGS studies.[207,213] Additionally, recent WES-studies[205,210] are reporting a higher prevalence of *IKZF1* deletions than earlier SNP array-based studies.[211] Furthermore, emerging mutations that involve certain genes, including *UBE2A*[208] and *BRCA2*,[210] were uniquely reported at high frequencies in individual studies.

Only a few studies have analyzed matched samples from diagnosis and progression timepoints of BP-CML patients, which would enable the identification of progression-associated mutations as well as persisting CP mutations.[205,208,210] ABL-KD mutations were the most recurrent progression-specific mutations, followed by *RUNX1* mutations and *IKZF1* deletions. In contrast, *ASXL1* mutations demonstrated variable patterns of acquisition, persistence, and loss during CML progression. Further genetic studies of BP-CML patients are still required to improve our understanding of the molecular pathogenesis of CML progression as well as to investigate the potential role of genetic data in BP-CML management.

2.2. Fusion genes in CML

Chromosomal translocations that result in the formation of fusion genes are among the earliest and the most frequently identified events in leukemia.[214] Fusion genes, of which one of the partners is a leukemia-associated gene, have been described in acute leukemia with an established leukemogenic role and a distinct phenotypic and transcriptional criteria.[215] Additionally, fusion genes have demonstrated a prognostic value in acute leukemia patients, and have been incorporated in the diagnostic workup and risk stratification of the patients.[23,215] Interestingly, targeted therapies have been successfully used for managing leukemia patients with specific fusion genes, including *PML-RARA* and *BCR-ABL1*.[216,217] Fusion genes previously described in leukemia (e.g., *CBFB-MYH11*, *MLL* rearrangements) or involving leukemia-associated genes (e.g., *RUNX1, EVI1*) have been described in CML as progression-associated events.[218–221] Identification of known and novel fusion genes in leukemia using RNA sequencing has been described.[222] In a recent RNA sequencing study of 33 BP-CML patients, fusion genes were detected in 42% of patients, including *CBFB-MYH11* and *MLL* rearrangements. Additionally, novel fusion events associated with Ph-chromosome formation were identified in both CP and BP samples.[205] Further RNA sequencing studies are required for characterizing the landscape of fusion genes in BP-CML and their potential role in BP-CML pathogenesis and management.

2.3. Transcriptional and epigenetic regulation in CML

In addition to somatic mutations, other genomic data, including gene expression and epigenetic programming, provide important information about the potential mechanisms underlying treatment failure and disease progression in CML. Early transcriptional studies have compared gene expression profiles between different CML phases and identified progression-specific transcriptional signatures.[223,224] Pathways that include apoptosis regulations, inflammation, TNF, JAK/STAT, and other signaling pathways, as well as several transcription factors and markers, were dysregulated in association with CML progression. Recently, single cell RNA (scRNA) sequencing has been used to explore LSC-specific, therapy-related and progression-associated molecular signatures, providing insights into the molecular pathogenesis of high-risk CML.[167,225]

Epigenetic dysregulation is an established mechanism that contributes to tumor development and has recently been highlighted in CML.[136] Given the biology of CML as a stem cell disease, different epigenetic processes-including DNA methylation, histone modification, and non-coding RNAs—are expected to play an important role in CML pathogenesis and CML-LSCs specific reprogramming.[226] Additionally, mutations that affect epigenetic modifiers are frequently encountered in different CML phases. Aberrant DNA methylation has been described in CML, where it has been linked to treatment failure and disease progression.[227] Progression-specific DNA methylation changes were associated with transcriptional reprogramming of several survival pathways.[228] Furthermore, *BCR-ABL1* was found to induce dysregulation of many histone modifiers—namely, the polycomb complex (PRC1/PRC2) reported to be dysregulated in association with TKI resistance and CML progression.[210,229] Regulation of PRC1/PRC2 complex components, especially *EZH2*, is also crucial for the maintenance of CML-LSCs.[230] In addition, microRNA (miRNA) have been demonstrated to be involved in regulating self-renewal, TKI resistance, and CML progression, in addition to posttranscriptional regulation of several key transcription factors, e,g., *MYC*.[231,232]

2.4. Genetic aberrations and CML management

Genetic data have become an integral part of classification, risk-stratification, and management of acute leukemia.[233] In contrast, risk stratification and selection of frontline treatment for CML patients is mainly decided based on clinical scoring systems.[234] Nevertheless, several reports have suggested a predictive role for genetic data in CML. For example, *BIM* and *ASXL1* germline mutations were associated with poor treatment responses in CP-CML patients,[235,236] where integration of mutational data added to the predictive power of Sokal score.[237] In CP-CML, mutations in epigenetic modifiers have been reported to be associated with inferior responses to TKI treatment—namely, imatinib.[199,201] In addition, some of the recurrently mutated genes in BP-CML represent potential therapeutic targets, including *RUNX1* and *TP53*.[238,239] A personalized approach that integrates genetic and drug sensitivity data

is a promising approach for AML[240] and could potentially improve the outcomes of BP-CML patients.

3. *Personalized medicine in leukemia*

Goals of CML treatment include normal survival, improved quality of life and achievement of sustained treatment-free remission[56]. The major improvement of survival outcomes of CP-CML patients in the TKI era has shifted the focus of CML management toward achieving good quality of life without life-long treatment[241]. Patient-centered management in CML include risk stratification, tailored frontline treatment according to personal comorbidities, and assessment of the need for TKI switch based on the drug tolerability and monitoring of the response[242]. Investigations of the mutational profile of CML patients can help improving risk stratification and treatment choice. Furthermore, identification of biomarkers predicting sustained deep molecular response and successful TFR will potentially enable expansion of the eligibility criteria for TKI discontinuation. On the other hand, improvement of survival outcomes remains as the main treatment goal in BP-CML patients[56,241]. Integration of genetic and drug sensitivity testing data hold potential promise in improving treatment outcomes of these dismal prognosis patients.

The term of personalized medicine refers to the strategy of tailoring treatment according to the individual characteristics of each patient, aiming at giving the right drug in the right schedule to the right patient.[243] An integral part of personalized medicine is the characterization and stratification of patients using integrated genetic and molecular data with clinical criteria of the patients.[243,244] Personalized medicine, precision medicine or genomic medicine, are all emphasizing the value of molecular data in designing treatment strategies for individual patients and patient groups.[245,246] This approach enables better understanding of the pathogenic mechanisms of cancer development, identification of potential biomarkers, and discovery of novel druggable targets, all of which can translate into efficient treatment of cancer patients.[243–245] Implementation of the precision medicine approach, where targeted therapies are used to target discovered genetic drivers, has led to improved management of various cancer types.[247]

Tools for personalized medicine include next-generation sequencing (NGS) in addition to drug sensitivity screening. The comprehensive molecular characterization of hematological neoplasms has led to the incorporation of genetic data into the classification and diagnosis of leukemia, with established prognostic superiority over the traditional histology-based systems.[23,248] Additionally, genetic profiling has enabled the identification of driver events in leukemogenesis, which represent attractive targets for targeted therapy. In addition to the paradigm development of imatinib, targeting BCR-ABL1 in CML, several targeted therapies have been approved in AML, including FLT3 and IDH1/2 inhibitors.[174,249] In addition to the

genetic heterogeneity of leukemia, other processes including epigenetic, proteomic, and metabolic regulations are contributing to treatment responses. Hence, the sole use of sequencing approaches is not enough for exploring all potentially effective therapies. Functional analysis—namely, ex-vivo drug sensitivity profiling—represent an appealing approach that can further guide the clinical translation of genetic data.[250,251] Several studies have systematically investigated the drug sensitivity profiles of AML patients.[251,252] Nevertheless, implementation of a personalized approach into CML management, especially BP-CML, remains to be investigated.

3.1. Next-generation sequencing (NGS)

Advances in sequencing technologies from Sanger sequencing to NGS techniques have been associated with the production of large amounts of genomic data, which have guided the design of treatment strategies and the selection of targeted and immunotherapies.[253,254] NGS techniques include DNA and RNA sequencing. Different DNA sequencing methods are available, including whole genome sequencing (WGS), WES, and targeted amplicon-based sequencing. WES analyzes the protein-coding regions, "exons," which represent about 3% of the whole genome.[255] This approach prioritizes genetic aberrations that are predicted to have a high impact on protein function and it also provides information about CNVs and structural variations in a resolution comparable to the more extensive WGS. Restriction of sequencing to exonic regions is time-saving and improves the ability to identify causative mutations by screening more individuals.[256] Recent advances in sequencing technology have enabled the implementation of WES in clinical care.[257] On the other hand, targeted sequencing of a pre-selected panel of genes is more popular in clinical practice.[258] Besides being less laborious than WES, targeted sequencing allows improved depth of sequencing to be achieved, enabling discovery of rare and low variant allele frequency mutations as well as small clones. The flexible design to adapt disease-based gene panels and the ability to pool and screen large cohort sizes make the use of targeted sequencing favorable in clinical practice. Furthermore, targeted sequencing is of particular benefit in samples with limiting DNA amount or quality.[259] However, dissecting structural variations and copy number changes using targeted sequencing can be challenging.[260] Additionally, novel mutations that involve genes that are not included in the screening panel cannot be recapitulated.

RNA sequencing provides information about gene expression levels and disease-specific transcriptional programing that complements the genetic data in personalized medicine.[261] These data enable the identification of potential biomarkers and active transcriptional pathways that can be targeted using targeted therapy. A prototype of the value of RNA sequencing in identifying distinct transcriptional signature is the identification of 'Ph-like ALL' entity, whose transcriptional profile mimics the *BCR-ABL1*-specific signature of Ph+ALL. This was later clinically translated into the successful use of TKIs to treat Ph-like ALL patients.[262] Similar to DNA sequencing,

RNA sequencing can provide mutational data (e.g., SNVs). Additionally, RNA sequencing is a powerful tool for identifying fusion genes, which are known drivers in leukemogenesis.[263]

Application of recently developed single-cell sequencing technologies in leukemia has enabled the delineation of genetically heterogenous LSCs, the identification of distinct LSCs subpopulations, as well as the characterization of LSCs subpopulation dynamics and response to treatment.[264] In AML, single-cell DNA sequencing has provided novel insights into clonal architecture and clonal evolution patterns in addition to characterizing leukemia-initiating mutations and frequently co-occurring mutations with subsequent phenotypic sequelae.[265] Moreover, scRNA sequencing has enabled the characterization of distinct molecular signatures in LSCs subpopulations with potential contribution to leukemia pathogenesis and treatment responses.[266] In CML, scRNA data has provided evidence for LSCs heterogeneity and clonal evolution in BP-CML patients.[167] Analysis of scRNA data from CML patients has revealed subpopulation-specific transcriptional changes in CML LSCs in response to TKI treatment and has highlighted a fraction CML LSCs with a primitive quiescent molecular signature as a potentially TKI-resistant subpopulation.[225]

3.2. Ex-vivo drug sensitivity and resistance testing

Drug sensitivity profiling is an emerging approach of personalized medicine.[267] Systematic drug sensitivity screening is a powerful tool for linking the genomic data with the identification of active targeted drugs.[240] Ex-vivo drug testing of cancer cells can potentially reflect drug vulnerabilities of the tumor. This includes isolation of leukemic cells, incubation with drugs, and assessment of drug-induced inhibition or killing of cancer cells.[268] The data are used to construct dose response curves from which different metrics, including IC_{50}, area under the curve (AUC), or recently drug sensitivity score (DSS), are used to express drug sensitivity. DSS scores are more robust and superior to IC_{50} in reflecting drug responses.[269] High throughput drug testing has been recently suggested as an effective tool for identifying novel drugs or for repurposing approved drugs for use in acute leukemia. In a study from University of Helsinki, axitinib was identified and successfully implemented to treat BP-CML patients harboring the ABL-KD (T315I) mutation.[270] More recently, the Beat-AML study has integrated mutational and transcriptional data together with ex-vivo drug testing data for a personalized approach in AML.[271]

Aims of the study

The aim of this PhD project was to investigate the molecular pathogenesis of high-risk CML patients, such as BP-CML and p190 CML, and to elucidate the role of somatic mutations in driving CML progression, taking *RUNX1* mutations as an example driver oncogene. Furthermore, we applied functional analysis—including ex-vivo drug testing, cell line modeling, and CRISPR/Cas9 gene editing—to discover new therapeutic options for these patients. Finally, we demonstrated the potential benefits of using a personalized medicine approach in BP-CML management.

The project consisted of three parts:

- Characterizing the mutational profile of different CML phases and exploring the molecular mechanisms underlying CML progression.

- Investigating the phenotypic, genomic, and drug sensitivity criteria of BP-CML patients harboring *RUNX1* mutations.

- Characterizing the molecular mechanisms, phosphorylation, and drug sensitivity profiles specific to the p190-BCR-ABL1 isoform in CML.

Patients and methods

5. *Patients, materials and cell lines*

5.1 Patients

A total of 70 CML patients were included in studies I–III (Table 5). Definition of CML diagnosis and progression were performed based on the World Health Organization (WHO) 2016 criteria. Demographic criteria of patients included in this project are shown in Table 6. Most of the samples were collected from Finnish patients and healthy donors at Helsinki University Central Hospital, University of Helsinki, Finland. Additional samples were collected from other centers in Sweden, Norway, and Egypt. The studies were conducted in accordance with the Declaration of Helsinki and were approved by the Helsinki University Central Hospital Ethics Committee. Written informed consent was given by all patients and healthy controls.

In study I, the mutational profiles of CML patients in different phases were characterized by sequencing the samples from BP-CML (n = 19) and CP-CML (n = 40) patients. Phase-specific transcriptional regulations were investigated via RNA sequencing of samples from seven BP-CML and five CP-CML patients. For CP-CML patients, DNA and RNA were extracted from CD34+ cells. Germline DNA was extracted from skin biopsies of BP- and CP-CML patients and analyzed with WES. For CP-CML patients analyzed using target sequencing, DNA extracted from matched T-cell fraction or remission PMNCs were used as control DNA. DNA extracted from PMNCs of five healthy donors and RNA extracted from BMNC CD34+ of four healthy donors were used as controls for DNA and RNA sequencing, respectively. Additionally, matched samples (samples from individual patient at different time points) were available for 30 CML patients. Matched diagnosis follow-up samples were available from 28 CP-CML patients, two of which had a follow-up sample that was in progression to BP. Matched diagnosis relapse samples were also available for two CML patients diagnosed with BP. Finally, BMNCs from the diagnosis and relapse timepoints of two patients were analyzed using drug sensitivity testing (DSRT).

Study II patient cohort consisted of eight BP-CML patients. Comprehensive workflow integrating flowcytometry, WES, RNA sequencing, and DSRT were performed for the entire cohort to identify patients with *RUNX1* mutations and to characterize the specific criteria of *RUNX1*^{mut} BP-CML patients. Additionally, previously published WES and transcriptional data from 25 BP-CML patients were reanalyzed and integrated as a validation cohort.

In study III, exome sequencing was performed for four p190-CML patients from PMNCs samples at diagnosis. Additionally, RNA was extracted from three p190 and three p210-CML PMNCs samples for RNA sequencing. DSRT was performed for 10 Ph+ALL patients to identify isoform-specific drug sensitivity profile.

Table 5. Patient materials used in different studies and performed analyses.

Pt	Frontline treatment	Prognosis	St	Samples			Analysis
				Diagnosis	Follow up	Control	
1	imatinib	poor	**I,II**	CP	AP and BP	skin	WES, RNA seq, DSRT
2	imatinib	poor	**I,II**	CP-CD34	BP	skin	WES, RNA seq, DSRT
3	imatinib+ IFN	poor	**I,II**	CP-CD34	BP	na	targeted, RNA seq, DSRT
4	imatinib	na	**I,II**	BP	relapse	skin	WES, RNA seq, DSRT
5	dasatinib	na	**I,II**	BP	relapse	skin	WES, RNA seq, DSRT
6	nilotinib	na	**I,II**	CP-CD34	AP	skin	WES, RNA seq, DSRT
7	dasatinib+ chemo	na	**I,II**	BP	na	skin	WES, RNA seq, DSRT
8	Chemo + alloHSCT	na	**I,II**	BP	na	skin	WES, RNA seq, DSRT
9	dasatinib+ chemo	na	**II**	BP	na	skin	WES, RNA seq, DSRT
10	imatinib	na	**I**	BP	na	na	targeted
11	chemo+ alloHSCT	na	**I**	BP	na	na	targeted
12	imatinib	na	**I**	AP-CD34	na	na	targeted
13	imatinib	na	**I**	BP	na	na	targeted
14	imatinib	na	**I**	BP	na	na	targeted
15	imatinib	na	**I**	AP-CD34	na	na	targeted
16	chemo+ alloHSCT	na	**I**	BP	na	na	targeted
17	imatinib	na	**I**	BP	na	na	targeted
18	imatinib	na	**I**	BP	na	na	targeted
19	imatinib	na	**I**	BP	na	na	targeted
20	imatinib	na	**I**	BP-CD34	na	skin	targeted
21	dasatinib	suboptimal	**I**	CP	FU-12	skin	WES
22	dasatinib	poor	**I**	CP	FU-3	skin	WES
23	nilotinib	optimal	**I**	CP	na	skin	WES
24	imatinib	poor	**I**	CP	na	skin	WES
25	imatinib	optimal	**I**	CP	na	skin	WES, RNA seq
26	imatinib	suboptimal	**I**	CP	FU-3 CD34	skin	WES, targeted
27	dasatinib	optimal	**I**	CP	FU-6 CD34	skin	WES
28	imatinib	suboptimal	**I**	CP	FU-6 CD34	skin	WES
29	dasatinib	optimal	**I**	CP	na	skin	WES
30	imatinib	na	**I**	CP	na	skin	WES
31	imatinib	optimal	**I**	CP-CD34	FU-3 CD34	MMR PMNC	targeted
32	dasatinib	suboptimal	**I**	CP-CD34	FU-6 CD34	CMR PMNC	targeted
33	imatinib	optimal	**I**	CP-CD34	FU-9 CD34	T-cells	targeted
34	imatinib	suboptimal	**I**	CP-CD34	FU-12 CD34	MMR PMNC	targeted
35	imatinib	optimal	**I**	CP-CD34	FU-19 CD34	MMR PMNC	targeted

36	imatinib	optimal	**I**	CP-CD34	na	MMR PMNC	targeted
37	imatinib	suboptimal	**I**	CP-CD34	FU-30 CD34	MMR PMNC	targeted
38	imatinib	optimal	**I**	CP-CD34	FU-6 CD34	CMR PMNC	targeted
39	imatinib	suboptimal	**I**	CP-CD34	FU-6 CD34	MMR PMNC	targeted
40	imatinib	suboptimal	**I**	CP-CD34	FU-6 CD34	T-cells	targeted
41	dasatinib	optimal	**I**	CP-CD34	FU-6 CD34	T-cells	targeted
42	imatinib	suboptimal	**I**	CP-CD34	FU-6 CD34	T-cells	targeted
43	dasatinib	suboptimal	**I**	CP-CD34	FU-6 CD34	CMR PMNC	targeted
44	imatinib	optimal	**I**	CP-CD34	FU-3 CD34	T-cells	targeted
45	dasatinib	optimal	**I**	CP-CD34	FU-6 CD34	T-cells	targeted
46	nilotinib	optimal	**I**	CP-CD34	FU-3 CD34	T-cells	targeted
47	nilotinib	optimal	**I**	CP-CD34	FU-3 CD34	T-cells	targeted
48	dasatinib	suboptimal	**I**	CP-CD34	FU-12 CD34	T-cells	targeted
49	dasatinib	poor	**I**	CP-CD34	FU-3 CD34	T-cells	targeted
50	dasatinib	optimal	**I**	CP-CD34	FU-3 CD34	MMR PMNC	targeted
51	dasatinib	optimal	**I**	CP-CD34	FU-3 CD34	T-cells	targeted
52	imatinib	suboptimal	**I**	CP-CD34	na	T-cells	targeted
53	na	na	**I**	CP-CD34	na	T-cells	targeted
54	imatinib	optimal	**I**	CP-CD34	na	T-cells	targeted, RNA seq
55	dasatinib	suboptimal	**I**	CP-CD34	na	T-cells	targeted
56	imatinib	optimal	**I**	CP-CD34	na	T-cells	targeted
57	dasatinib	optimal	**I**	CP-CD34	na	T-cells	targeted
58	nilotinib	na	**I**	CP-CD34	na	T-cells	targeted
59	imatinib	optimal	**I**	CP-CD34	na	T-cells	targeted
60	imatinib	poor	**I**	CP-CD34	na	T-cells	targeted
61	na	na	**I**	CP-CD34	na	na	RNA seq
62	na	na	**I**	CP-CD34	na	na	RNA seq
63	na	na	**I**	CP-CD34	na	na	RNA seq
64	imatinib	na	**III**	CP	na	na	WES, RNA seq, WB
65	imatinib	poor	**III**	CP	na	na	WES, RNA seq, WB
66	imatinib	poor	**III**	CP	na	na	WES, RNA seq, WB
67	imatinib	poor	**III**	CP	na	na	WES
68	imatinib	poor	**III**	CP	na	na	RNA seq, WB
69	imatinib	poor	**III**	CP	na	na	RNA seq, WB
70	imatinib	suboptimal	**III**	CP	na	na	RNA seq, WB
	healthy controls		**I**	BM CD34 (4 samples)			RNA seq
	healthy controls		**I**	PMNC (5 samples)			targeted
	AML samples		**I**	Re-analysed data of 11 patients[240]			WES
	BP-CML samples		**II**	Re-analysed data of 25 patients[205]			WES, RNA seq
	Ph+ALL samples		**III**	Diagnosis samples of 10 patients			DSRT, p-flow

Pt: patient, St: study, FU: follow up on-TKI sample, FU-n: n indicates the time of sampling (months after TKI start), chemo: chemotherapy, p-flow: phospho-flowcytometry. Samples are MNC unless it has been stated as "CD34" which indicate sorted CD34+ samples.

Table 6. Demographics and clinical characteristics of patients.

	CML	AP/BP	CP					
			CP-all	Progressive	Poor	Responsive		
						All	Suboptimal	Optimal
Patients, n	70	17	53	3	9	34	14	20
Age, median (range) y	51.5 (24-80)	50 (24-74)	52 (27-80)	41 (35-46)	54 (27-80)	50.5 (29-78)	48 (29-67)	59 (29-78)
Sex, male:female, n (%)	37:26 (59:41)	10:7 (59:41)	27:19 (59:41)	1:2 (33:67)	6:3 (67:33)	18:14 (56:44)	8:6 (57:43)	10:8 (56:44)
Disease stage at diagnosis, CP/AP/BP, n (%)	61/3/6 (87:4:9)	8/3/6 (47/18/35)	53/0/0 (100/0/0)	3/0/0 (100/0/0)	9/0/0 (100/0/0)	34/0/0 (100/0/0)	14/0/0 (100/0/0)	20/0/0 (100/0/0)
Hb at diagnosis, median (range), g/L	118 (78-157)	118.5 (78-157)	118 (78-156)	130 (85-144)	111 (93-147)	122 (78-156)	114.5 (78-144)	122 (91-156)
WBC counts at diagnosis, median (range), $X10^9$/L	51 (3.78-365)	55.5 (0.9-317)	48 (6.4-365)	21.6 (16.9-174.1)	230 (6.4-345)	44 (14-365)	58.5 (15.3-327)	44 (14-365)
Platelet count at diagnosis, median (range), $X10^9$/L	445 (19-2674)	113 (19-1048)	491 (120-2674)	860 (156-2570)	565 (393-584)	480 (120-2674)	325 (120-620)	655 (127-2674)
PB blast at diagnosis,n (%)	1 (0-70)	10 (4-70)	0 (0-6)	0 (0-6)	2 (2-3)	0 (0-6)	0 (0-6)	0 (0-4)
BM blast at diagnosis,n (%)	3 (0-90)	32 (6-90)	3 (0-8)	2 (2-3)	3 (2-4)	3 (0-8)	2 (0-6)	3 (0-8)
Spleen size at diagnosis, median (range), cm	13 (0-30)	15 (0-30)	13 (0-30)	12 (0-15)	25 (13-30)	12 (0-23)	13.75 (0-23)	9 (0-21)
Sokal score median (range) low/intermediate/high/NA	0.89 (0.51-3.45) 12/10/11/37	NA	0.89 (0.51-3.45) 12/10/11/20	1.18 (0.56-7.4) 1/1/1/0	1.02 (0.73-1.89) 1/1/1/6	0.89 (0.51-3.45) 10/8/9/7	0.82 (0.52-1.76) 5/3/2/4	0.9 (0.51-3.45) 5/5/7/3
Hasford score median(range) low/intermediate/high/NA	890.5 (100-2384) 11/13/8/38	NA	890.5 (100-2384) 11/13/8/21	720 (328-1299) 1/2/0/0	908 (199-2176) 1/1/1/6	873 (100-2384) 9/11/5/8	866 (124-1306) 4/5/0/5	1106 (100-2384) 5/6/5/5
Additional cytogenetic abnormalities at diagnosis, n/N (%)	15/64 (23.4)	11/17 (64.7)	4/47 (8.5)	0/3 (0)	2/9 (22)	2/34 (6)	0/14 (0)	2/20 (10)
Frontline treatment, Imatinib/2G TKI/Chemotherapy/NA, n	41/22/3/4	10/4/3/0	31/18/0/4	3/0/0/0	7/2/0/0	19/15/0/0	9/5/0/0	10/10/0/0
Clinical responses, Poor/suboptimal/optimal/NA, n	12/14/20/24	NA	12/14/20/7	3/0/0/0	9/0/0/0	0/14/20/0	0/14/0/0	0/0/20/0
Interval between diagnosis and Follow up samples, median (range), months	NA	NA	6 (3-30)	NA	3 (3)	6 (3-30)	6 (3-30)	4.5 (3-19)
Response at Follow up sampling time minCyR/PCyR/CCyR/MMR, n	NA	NA	3/2/15/5	NA	2/0/0/0	1/2/15/5	1/1/9/0	0/1/6/5
Months to achieve MMR, median (range)	NA	NA	12 (3-70)	NA	NA	12 (3-70)	32 (15-70)	6 (3-12)
MMR at 12mon/MMR after 12mon/never in MMR/NA, n			18/14/9/12		0/0/6/3	18/14/0/2	0/14/0/0	18/0/0/2

Progressive refers to cases progressed to AP/BP, Poor: patients who failed to achieve MMR at any time point, Responsive: patients who achieved MMR either by 12 months (optimal) or after 12 months (suboptimal).
Hb: Hemoglobin, WBC: white blood cells, PB: peripheral blood, BM: bone marrow, 2G: second generation, MMR: major molecular response, CCyR: complete cytogenetic response, PCyR: partial cytogenetic response, minCyR: minimal cytogenetic response. NA: no available data. Clinical data were missing for 7 CP patients.

5.2. Sample processing and preparation

Mononuclear cells were separated from the peripheral blood (PB) and bone marrow (BM) samples of CML patients using Ficoll-Paque™ PLUS (GE Healthcare). The cells were either processed immediately, for DNA and RNA extraction and DSRT, or they were viably frozen for subsequent experiments. For freezing purposes, cells were resuspended in fetal bovine serum (FBS) containing 10% dimethyl sulfoxide (DMSO) at a concentration of 5–10 million cells/ml and stored at a temperature of -70 °C for 24 h before being transferred into liquid nitrogen, or -150 °C, for long term storage. In study I, the cells from CP-CML patients were further sorted into CD34+ (CML progenitors) and CD3+ (T-cells, control) populations using magnetic bead sorting (Miltenyi Biotech). The purity of the selected populations was confirmed by flowcytometry.

5.3. Cell lines

In study II, the human CML K562 cell line was used to model the effect of *RUNX1* mutations on the CML phenotype and drug sensitivity profile. Additionally, the murine Ba/f3 cell line transduced with the p210-BCR-ABL1 (b3a2) transcript was used as a CP-CML model for CRISPR gene editing of *RUNX1* and subsequent functional studies. Both cell lines were cultured in RPMI-1640 (Lonza) supplemented with 10% FBS, 2 mM L-glutamine (Lonza), and 100 U/mL penicillin as well as 100 µg/mL streptomycin (Gibco). In study III, the Ba/f3 cell line was retrovirally transfected with BCR-ABL1-GFP encoding either p190/p210 to investigate isoform-specific signaling and drug sensitivity profile. In addition, the murine hematopoietic progenitor cell line, HPC-LSK, was similarly retrovirally transfected with either GFP-marked transcript and used for validation of results. HPC-LSK cells were cultured in IMDM media (Sigma) and supplemented with 5% FBS, $0.75 \times 10{-}4$ M 1-Thioglycerol (Sigma), 12.5 ng/ml recombinant human IL6 (Peprotech), 2 mM L-glutamine (Lonza), and 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco) (for both parental and transduced lines), in addition to the inhouse-prepared stem cell factor (SCF) (only for parental line). K562 was obtained from DSMZ (German Collection of Microorganisms and Cell Cultures). The Ba/f3 cell lines were a kind gift from Prof. Nikolas von Bubnoff (Universitätsklinikum Freiburg, Germany) and the HPC-LSK cells were provided by Prof. Veronika Sexl's lab (University of Veterinary Medicine of Vienna, Austria). All cell lines tested negative for mycoplasma.

5.4. Immunophenotyping of leukemia cells and cell lines

In study II, clinical immunophenotyping data were collected for diagnostic samples of patient cohorts. Additionally, viably frozen bone marrow mononuclear cells (BMNCs) were thawed and stained for 15 min with surface markers CD45-V500, CD34-FITC, CD3-Percp-Cy5.5, CD303/BDCA-APC, CD115-PE, CD123-BV421, CD19-APC-Cy7, and HLA-DR-PE-Cy7 (BD Biosciences), according to recommended manufacturer protocols for surface antibody staining. For CRISPR-edited Ba/f3 cell lines in the same study, a panel of rat anti-mouse CD45-PE, CD19-PE, and CD11b-

BV510 antibodies (BD Biosciences) was applied. A minimum of 1 x 10^6 cells were acquired using the FACSVerse and analyzed with the FlowJo (Version 10.0.8r1, TreeStar) software.

In study III, phenotyping of the HPC-LSK cells was done after growing for 7 days in the presence or absence of IFNα using the following panel of antibodies: CD19-PE (eBioscience, #12-0193-82), CD3-APC (#100236), CD45-APC-Cy7 (# 557659) from Biolegend, CD11b-PerCP-Cy™5.5 (#561114), CD45R/B220-BV421 (#562922), Ly-6G-PerCP-Cy™5.5 (#560602), Ly-6C-APC-Cy7 (#560596), and Ly-6A/E(sca-1)-Alexa Fluor®647 (#565355) (BD Bioscience).

6. Next-generation sequencing experiments

6.1. DNA and RNA extraction

DNA from whole MNC, CD34+, or CD3+ cells was extracted using the genomic Nucleospin® Tissue Kit (Macherey-Nagel) in accordance with the manufacturer's instructions. Total RNA was isolated from patient cells and cell line samples using the miRNeasy Mini kit (Qiagen), including DNAse I digestion. DNA and RNA concentrations were measured using the Qubit® 2.0 Fluorometer (Life Technologies). The quality was assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies).

6.2. Whole exome and targeted sequencing

DNA sequencing libraries were prepared from genomic DNA after fragmentation using the Episonic Multi-functional Bioprocessor 1100 (Epigenetik Group Inc) to a mean fragment size of 200bp. Exome capture was performed using SureSelect XT Clinical Research Exome kit (Agilent). For targeted sequencing, the ThruPLEX kit (Rubicon Genomics) was used for library preparation and target capture was performed using the SeqCap EZ Comprehensive Cancer Design panel (Roche NimbleGen) that comprises 578 cancer-associated genes. Sequencing libraries were sequenced using 2x100-bp paired-end sequencing protocol on HiSeq2000 instruments (Illumina). Average coverage depth for WES and targeted sequencing samples was 90x and 187x, respectively.

6.3. RNA sequencing

RNA sequencing libraries were prepared from 1.5 µg of total RNA using the ribo-depletion-based approach. RNA sequencing libraries were prepared using the ScriptSeq v2™ Complete kit for human/mouse/rat (Illumina), the Illumina compatible Nextera™ Technology (Epicentre), and the Truseq standard total RNA preparation kit (Illumina). RNA sequencing libraries were purified using SPRI beads (Agencourt AMPure XP, Beckman Coulter). The quality of the RNA libraries was evaluated using High Sensitivity chips by Agilent Bioanalyzer (Agilent). For all libraries, paired-end 100-bp read sequencing was performed on the Illumina HiSeq2000 instrument (Illumina).

6.4. Analysis of sequencing data

6.4.1. Variant analysis

In studies I–III, analysis of the DNA sequencing data was mainly performed using MuTect2 tools. Briefly, reads were pre-processed using Trimmomatic[272] to filter low quality reads. Paired-end reads were then aligned to the human reference genome build 38 (EnsEMBL v82) using BWA-MEM.[273] PCR duplicates were marked and removed using the MarkDuplicate module of the Picard toolkit (Broad Institute). For variant calling, the Genome Analysis Tool Kit (GATK) toolset (version3.5) were used,[274] and supplemented by CalculateContamination, CollectSequencingArtifactMetrics, and FilterByOrientationBias GATK4 tools to correct for cross-sample contamination. Variants were called from WES data using tumor-normal strategy. In addition to germline control, variants were filtered against a panel of normal samples, from 24 healthy unrelated Finnish individuals. For targeted sequencing (study I), tumor-only variant calling strategy was used. Variants were then filtered against the above-mentioned normal panel in addition to a panel of five unrelated age-matched healthy individuals and patient-matched control T-cell and remission samples, which had been sequenced using the same sequencing protocol. The CrossMap[275] tool was used to convert GATK resources from GRCh37 to GRCh38. The final GATK alignment files were then re-mapped to human reference genome build 38 (EnsEMBL v94) using STAR[276] with further filtration of low-quality and RNA or pseudogene associated reads.

Variant annotation was performed using the Annovar tool[277] and the RefGene database. Variant calls were first normalized using bcftools.[278] Filters were applied to variants, including MuTect2 filters with a TLOD $\geq$ 6.3 or a TLOD $\geq$ 5.0, and supported by five or more independent COSMIC[279] samples with failed variants filtered. Variant lists were further refined by removing variants in intronic and intergenic regions, variants with a total coverage $\leq$ 10, and those not supported by at least one read in both directions, as well as variants with variant quality value $\leq$ 40, variant allele frequency $\leq$ 2%, strand odd ratio for SNVs $\geq$ 3.00, and for indels $\geq$ 11.00, minor allele frequency $\geq$ 1% in the 1KG database, minor allele frequency $\geq$ 3% in the EPS database, minor allele frequency $\geq$ 1% in general, African, Finnish, Latino, East Asian, and Non-European ExAC, gnomAD exome, or gnomAD genome databases, with a PHRED-like CADD score $\leq$ 3.00 and a likelihood ratios score $\leq$ 2.00. Variants with a variant allele frequency $\geq$ 35% were only accepted if supported by 5 or more COSMIC[279] samples. Variants were manually curated, while the filtered known cancer variants were checked using the Integrative Genomics Viewer (IGV) 2.3.66 (Broad Institute) and rescued when applicable.

In study I, non-silent mutations were defined from the variant call set by removing synonymous mutations and non-frameshift variants. Furthermore, cancer-associated mutations were defined by selecting variants in genes that were mutated in two or

more patients in addition to variants with COSMIC identifiers. For mutational signature analysis in studies I and II, the deconstructSigs[280] software, with default parameters, was applied using the cancer profiles downloaded from the COSMIC web site in September 2017. Structural rearrangements were called with Manta[281] from the same alignment files that were subjected to somatic short variant calling. In study III, only variants with COSMIC incidence in hematopoietic neoplasms and those reported in ≥ 10 independent COSMIC samples were recovered.

6.4.2. Pathogen discovery analysis

In study I, DNA sequencing reads were classified into microbial taxa to identify potential pathogens. The Burrows-Wheeler Aligner (BWA),[282] with default settings, was used to map the Trimmomatic[272] pre-processed read-pairs against the rRNA sequences obtained from RFAM v12.3,[283] and then those read-pairs matching the rRNAs were filtered using samtools.[284] Passed reads were classified into different taxa using Centrifuge,[285] by applying an index made of 27,127 complete pathogen genome assemblies and 10,615 technical artefact sequences, in addition to the human genome, which were extracted from the RefSeq[286] database in February 2018. In data visualizations and statistical analyses, read counts were scaled by the total number of reads in the root times one million to obtain counts per million mapped reads (CPM).

6.4.3. MEME analysis and motif search

In study II, the Multiple EM for Motif Elicitation (MEME) algorithm[287] was used to identify the motifs enriched in the sequences spanning the breakpoints of structural variants (SV) resolved using Manta. The start and end coordinates of each of the resolved deletions, inversions, duplications, and insertions—in addition to the start coordinates of each translocation—were recorded, and Bedtools[288] were used to extract the surrounding up- and downstream sequence regions of the breakpoints by applying different bp length cutoffs. The MEME tool, with the command-line parameters -dna, -revcomp, -mod zoops, -nmotifs 3, -markov_order 3 and -objfun classic, was then applied to search for motifs that were enriched in the sequence that was set in comparison to the third order Markov chain constructed from the GRCh38 reference sequence.

6.4.4. RNA sequencing data analysis

The preprocessing of RNA sequencing data was performed similarly to that of WES data using Trimmomatic[272] to filter low quality reads. The STAR[276] tool, guided by the EnsEMBL v82 gene model to the respective human or mouse reference genome build 38, was used to align filtered paired-end reads using default settings, except for setting the overhang of the splice junctions to 99. Aligned reads were then sorted using the SortSAM and PCR duplicates were filtered using the MarkDuplicate module of

the Picard toolkit (Broad Institute). Feature counts were generated using SubRead[289] and expression estimates of feature counts were converted using Trimmed Mean of M-values (TMM) normalization.[290] Calculations of the CPM and RPKM values, as well as differential expression testing, were performed using the edgeR[291] software. Statistical comparisons between subject groups involved possible confounding factors, including sequencing kit and sample sorting status when applicable. The P-values were adjusted with the Storey's Q-value for multiple comparisons[292] and a cutoff Q-value of ≤ 0.05 was used to determine the differentially expressed genomic features. For the subsequent analyses and data visualization, we used batch-corrected CPM data that was calculated using the removeBatchEffect function of the limma package.[293] The clustering of gene expression profiles was performed with both genes and samples using the Euclidean distance and Ward linkage method. The pathway enrichment analysis of gene expression data was performed using the Gene set enrichment analysis (GSEA)[294] software (Broad Institute) and the Enrichr[295,296] tool.

6.4.5. Calling of fusion genes

In study I, fusion genes were called from un-processed RNA sequencing data using the FusionCatcher[297] with default parameters. The called fusions were filtered by excluding the fusion calls located in introns, called from healthy control samples, and the calls supported by ≤ 3 spanning unique reads. Fusion calls were rescued if they had clinical relevance in cancer or were supported by evidence from spanning pairs. Selected fusion genes were validated using single-step RT-PCR. The cDNA was synthesized from the total RNA using the QuantiNova Reverse Transcription kit (QIAGEN) and was then amplified with Phusion® High-Fidelity DNA Polymerase (New England Biolabs, NEB) by applying fusion-specific primers. PCR products (10ul) were stained with Gel Loading Dye, Purple (NEB), then run on a 2% agarose gel and UV-visualized. 5ul of PCR product were then cleaned up using ExoSAP-IT (ThermoFisher) and used for Sanger sequencing validation. Clinical FISH and karyotyping data were available for *BCR-ABL1* and *CBFB-MYH11* fusion genes.

7. Functional studies

7.1. Drug sensitivity and resistance testing (DSRT)

DSRT was performed on fresh or viably frozen patient samples as well as on cell lines in studies I–III. In studies I–II, the drug library, consisting of 125 FDA/EMA anti-cancer approved drugs and 127 investigational and preclinical compounds, was used to screen patient samples. For Ba/f3 and K562 cell lines in studies II–III, a comprehensive library of 528 oncology drugs (156 approved drugs and 372 preclinical and investigational compounds) was used. HPC-LSK was screened using a custom library of 65 selected oncology compounds. In all libraries used, each compound was tested in five different concentrations, covering a 10,000-fold range, pre-printed on 384-microwell plates (Corning) with an acoustic liquid handling device (Echo 550,

Labcyte Inc.). All compounds were purchased from commercial chemical vendors and dissolved in either 100% dimethyl sulfoxide (DMSO) or water.

In brief, the experimental workflow included dispensing 5 µl of culture medium per well and shaking the plates for 10 min to dissolve compounds. Viably-frozen primary cells were thawed and resuspended as a single-cell suspension in the Mononuclear Cell Medium (MCM; PromoCell), supplemented with 0.5 µg ml−1 gentamicin and 2.5 µg ml−1 amphotericin B, while cell lines were resuspended in the above-mentioned recommended media. Multi-Drop Combi peristaltic dispenser (Thermo Scientific) was used to seed cells at a concentration of 10,000 cells in 20 µl per well for primary cells and at a lower density of 2,500 cells in 20 µl per well for cell lines. Plates were then incubated for 72 h at 37 °C and 5% CO2. CellTiter-Glo 2.0 reagent (Promega) was used to measure cell viability, according to the manufacturer's instructions, using a Pherastar FS plate reader. Luminescence measurements of cell viability were normalized to 100 mM benzethonium chloride-containing wells (positive control) and DMSO-only wells (negative control). Drug responses for the tested concentration range were quantified as the drug sensitivity score (DSS)[269] using an inhouse-developed interface—Breeze.[298] Furthermore, selective drug sensitivity scores (sDSS) were calculated by comparing the DSS score from patient samples to the median values of healthy donors, which represent leukemia-specific drug responses. Drugs were considered to be selective or highly selective when having sDSS values above 5 and above 10, respectively.

7.2. Drug combination testing

In study II, selected drugs (everolimus, dexamethasone, cobimetinib, axitinib, venetoclax) that showed potent activity against *RUNX1*-mutated blasts were further tested in combination with imatinib. Viably frozen leukemia cells were thawed and suspended in StemSpan™ SFEM media (Stemcell technologies). Cells were seeded in a concentration of 5,000 cell/well concentration on 384-well plates, with dose-response matrices comprising 7 different concentrations for each drug as well as DMSO controls. Plates were further processed as described in the DSRT protocol above. The quantification of drug combination synergy scores was performed using the SynergyFinder[299] web application based on the model of the highest single agent (hsa) synergy score. A combinatorial activity was reported as synergistic if the hsa synergy score was above 3 and antagonistic if less than -3.

7.3. CAR T-cell coculture assay

In study II, CD19- and mock (non-targeting)-CAR T-cells were provided by the Finnish Red Cross Blood Service. The use of human material was approved by the Institutional Review Board of the Finnish Red Cross Blood Service and the CAR T-cells were manufactured mainly as previously described.[300] Viably-frozen BMNCs were thawed, treated with DNase I (ThermoFisher Scientific) to remove clumps, and

then resuspended to an appropriate volume in StemSpan™ SFEM media (Stemcell technologies). Cells were then plated on a 96 flat-bottom well plate in a concentration of $5x10^4$ cell/well in the presence of CD19- or mock-CAR T-cells at the indicated effector:target (E:T) ratio and/or the indicated imatinib concentration (1–10,000 nM) in a 100ul final volume. The experiments were conducted in triplicates per each condition. The plate was incubated for 24 h at 37 °C and 5% CO_2. Cell suspensions were then moved into a V-bottom 96-well plate, where it was washed using a staining buffer (PBS + 2 mM EDTA + 0.5% BSA) and then centrifuged at 300 g for 5 min. Cells were stained using an antibodies cocktail, involving CD34-FITC, CD4-PE-Cy7, CD3-APC, CD8-APC-H7 (all from BD Biosciences), CD19-Pacific Blue (clone SJ25-C1, Invitrogen), and CD69-BV510 (clone FN50, Sony Biotechnology), in a 25 µl staining buffer at room temperature and covered from light for 15 min. Subsequently, cells were washed and resuspended to a 25 µl Annexin V binding buffer containing AnnexinV-PE and 7-AAD (both from BD Biosciences). Data were acquired using an iQue Screener Plus flow cytometer and analyzed using the ForeCyt software (edition 6.2, Intellicyt). Counts of viable CD34+ cells per well in DMSO-only wells were used as a negative control.

7.4. CRISPR/Cas9 *RUNX1* gene editing

In Study II, the CRISPR/Cas9 gene editing technology was used to model *RUNX1* mutations in the murine CML model. Ba/f3-*BCR-ABL1* cells (GFP-positive) were transfected with pU6-(BbsI) CBh-Cas9-T2A-mCherry (Addgene plasmid #64324), expressing CRISPR-Cas9 and sgRNA targeting exon 4 of mouse *Runx1* gene. Transfection was performed using the Fugene HD reagent (Promega) to induce transient expression of Cas9-sgRNA in Ba/f3 cells. After 72 h, cells were sorted for GFP/mCherry double positive population using the Sony SH800 cell sorter (Sony Biotechnology) into a single cell/well in a round-bottom 96-well plate. Clones were expanded and screened by Sanger sequencing to detect gene editing efficiency, including indel size and frequencies. The predicted sizes of introduced indels and its effect on protein structure were assessed using the TIDE tool.[301] Successfully edited clones were selected for further expansion and analysis. The *RUNX1*$^{-/-}$ cell line had an out of frame (-1) deletion that affected both alleles (85% efficiency), while the *RUNX1*$^{-/mut}$ cell line had an in-frame deletion (-3) in one allele (45%), predicted to have a deleterious effect on protein function, in addition to an out of frame (-1) deletion (48%). The *RUNX1*$^{wt/wt}$ cell line had both alleles as a wild type *RUNX1* gene despite being similarly transfected using above-mentioned protocol. *RUNX1*$^{wt/wt}$ was used as a control, in addition to the parental cell line (non-transduced). The predicted effects were validated by western blotting (Figure 5). Similarly, the *RUNX1* gene was edited in the K562 human cell line using the sgRNA targeting exon 2 of human *RUNX1* in lentiCRISPRv2 plasmid (Addgene #52961). For re-expression of *RUNX1* in CRISPR-edited cell lines, the cDNA of the *RUNX1* gene was transferred from the pCMV5-AML1B plasmid (Addgene plasmid #12426) and cloned into the LeGO-iC2 lentivirus expression vector (Addgene plasmid #27345) using the ECORI-HF

restriction enzyme (NEB). Then, it was lentivirally introduced into the CRISPR-edited cell lines for re-expression of the wild type gene.

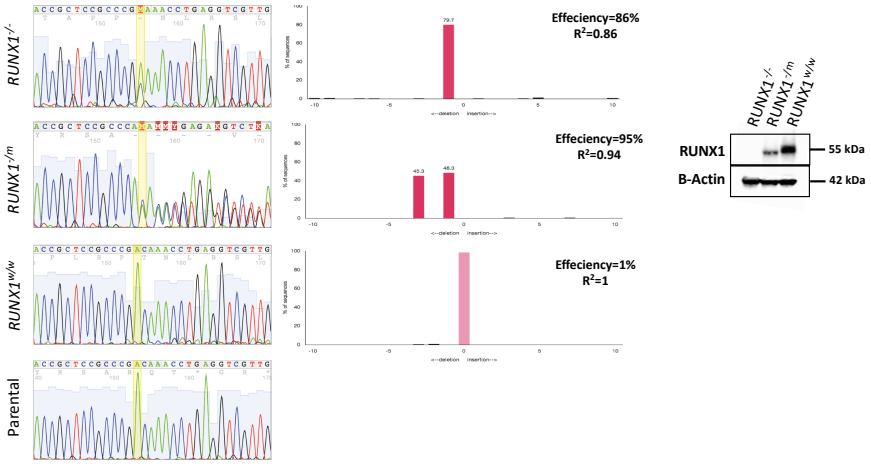


***Figure 5**. CRISPR-Cas9 editing of Runx1 in Ba/f3 cells.*

7.5. Mutagenesis assay

To explore the effect of missense *RUNX1* mutations, a *RUNX1*^{R162K} mutation that was identified in one BP-CML patient in our cohort was generated by applying the mutagenesis kit (GeneArt Site-Directed Mutagenesis System, Invitrogen) to the RUNX1-LeGO-iC2 plasmid in accordance with the manufacturer's protocol. The mutagenesis efficiency was validated by Sanger sequencing. *RUNX1*^{R162K} was lentivirally transduced in the Baf3 and K562 cell lines.

7.6. Phosphorylation profiling

7.6.1. Western blot analysis

To investigate the phosphorylation profiles of p190 and p210 CML patients, diagnostic samples from three p190 and three p210 CML patients were analyzed using Western blot for selected phospho-proteins. After being washed twice with cold PBS, the cells were lysed in an ice-cold RIPA buffer supplemented by a 1× protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). Samples were then centrifuged at 12,000 × g for 10 min at 4 °C. The Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) was used to measure the total protein concentration and the lysates were further processed using the Laemmli buffer (Bio-Rad Laboratories). Protein lysates were then separated using polyacrylamide gel electrophoresis on 7.5% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad). Proteins were transferred into a nitrocellulose membrane (0.2 µm pore-size nitrocellulose, Bio-Rad) using Trans-Blot® Turbo™ Transfer System (Bio-Rad). Primary antibodies against Src (Clone: 32G6, #2123T), phospho-Src Family (Tyr416) (Clone: D49G4, #6943T), Stat1 (#: 9172T), phospho-Stat1 (Tyr701) (Clone: D4A7, #7649T), Stat2 (Clone:

D9J7L, #72604S), Stat3 (Clone: 79D7, #4904), phospho-Stat3 (Tyr705) (Clone: D3A7, #9145S), Stat5 (#9363), phospho-Stat5 (Tyr694) (Clone: C71E5, #9314), Jak1 (Clone: 6G4, #3344T), phospho-Jak1(Tyr1034/1035) (Clone: D7N4Z, #74129S), c-Abl (#2862S), and β-Actin (Clone: 8H10D10, #3700S) were obtained from Cell Signaling Technology and phospho-STAT2 (Tyr690) (#SAB4503836) was purchased from Merck. Primary antibodies (1:1,000 dilution) were incubated overnight at 4 °C in the Odyssey blocking buffer (LI-COR Biosciences) containing 0.2% Tween-20. After wash, secondary antibodies (1:15,000 dilution) were incubated for 2 h at room temperature. Protein bands were visualized using the Odyssey Imaging Systems (LI-COR Biosciences).

7.6.2. Phosphorylation array

To perform extensive profiling of phosphorylation activities of the p190 and p210 isoforms in cell line models, we used a Tyrosine Phosphorylation Proarray (Full Moon Biosystems), featuring 228 phospho-tyrosine sites (6 triplicates for each site). Sample processing and preparation of array slides were performed according to the manufacturer's instructions. Array scanning, image analysis and feature extraction were performed by Full Moon Bioscience. During analysis, data were normalized to the median antibody signaling value.

7.6.3. Phospho-flow cytometry

In study III, the phosphorylation levels of selected proteins were investigated through flowcytometry analysis of samples from 10 Ph+ALL patients. First, viably-frozen samples were thawed, washed, and resuspended in appropriate volume of MCM media (PromoCell). Afterward, cells were pelleted and then fixed with 1.5% formaldehyde (BD Biosciences) for 15 min at room temperature. Samples were then permeabilized using 100% ice-cold methanol (BD Biosciences) and placed, meanwhile, on ice for 30 min. After washing, cells were stained using the following phosphoantibodies panel: Src (pY418)- Alexa Fluor® 488 (#560095), p38 MAPK (pT180/pY182)- Pacific Blue™ (Clone: 36/p38 (pT180/pY182) (RUO), # 560313), Stat1(pY701)- Pacific Blue™ (Clone:14/P-STAT1 (RUO), #560310), Stat3 (pY705)-PerCP-Cy™5.5 (Clone: 4/P-STAT3 (RUO), #560114), Stat4 (pY693)- Alexa Fluor® 647 (Clone: 38/p-Stat4, #558137), Stat5 (pY694)- Alexa Fluor® 647 (Clone 47/Stat5(pY694) (RUO), #612599), Stat6 (pY641)- PerCP-Cy™5.5 (Clone 18/P-Stat6 (RUO), #561195) (all purchased from BD Biosciences), in addition to STAT2(pY689)-Alexa Fluor® 488 (Clone 1021D, # IC8627G-025) from R&D Systems. Cells were acquired using the iQue Screener Plus flow cytometer (Intellicyt) and flow data were analyzed with the FlowJo software v10 (Treestar).

7.7. Colony forming assay (CFA)

To elucidate the differences of clonogenic potentials between the p190 and p210 isoforms, CFA was performed using HPC-LSK cell lines. In brief, 2.5 x 10^3 cells were suspended in 0.4 ml IMDM medium (Sigma) supplemented by 2% FBS (Gibco). Then the samples were each mixed with 4 ml of methylcellulose (MethoCult™ M3231, STEMCELL Technologies) without adding cytokines. Drugs (imatinib, dasatinib, LCL161, FRAX486, dexamethasone, idasanutlin) or recombinant mouse IFN-alpha (eBioscience, #14-8312-80) were then added into the indicated concentrations. The experiment was performed in triplicate wells (1.1 ml/well), on 35 mm dishes, according to the manufacturer's instructions. Colonies were counted using an inverted microscope after 14 days. For colony phenotyping, the colonies were harvested at day 14 and cells were stained with a panel of anti-mouse antibodies, including CD19-PE (eBioscience, #12-0193-82), CD11b-PerCP-Cy™5.5 (#561114), and CD45R/B220-BV421 (#562922) from BD Bioscience as well as CD3-APC (#100236) and CD45-APC-Cy7 (# 557659) from Biolegend. Cells were acquired using FACSVerse (BD Biosciences).

8. Statistical testing

In all studies, the two-tailed student t-test, Mann-Whitney U-test, Fisher's exact test, Spearman's correlation, and Pearson' correlation tests—along with a Fisher's exact test with simulated p-value on 1e + 07 replicates—were computed using the GraphPad Prism 7 or R 3.5.0 software. In study I, the statistical significance of the difference in microbial CPM was examined using the two-tailed student's t-test with unequal variance in Excel.

Results

9. Characterization of the mutational landscape of CML in different phases (Studies I–III)

To investigate the mutational profile of CML patients in different phases, we analyzed the samples obtained from 67 CML patients using WES (22 patients), RNA sequencing (3 patients), or targeted sequencing (42 patients). These included 47 CP-CML patients, 17 patients in AP or BP (BP-CML), and 3 patients with matched CP/BP samples. For CP-CML samples that were analyzed using targeted sequencing, the samples were enriched for CD34+ cells in order to enrich for variants of low abundance. Additionally, one or more follow-up samples from 30 patients were analyzed using WES or target sequencing, according to the method used to analyze the respective diagnostic sample.

9.1. Mutational landscape of CP-CML patients (Studies I, III)

We identified 25 potentially relevant mutations in 15 cancer-associated genes across the 50 diagnostic samples of CP-CML patients (mean = 0.5 mutation/sample). Only 38% of the CP-CML patients carried at least one cancer-associated mutation at diagnosis (Figure 6). Few genes were recurrently mutated in CP-CML patients. The *ASXL1* gene was the most recurrently mutated gene in 8/50 patients (16%). Other recurrently mutated genes included *DNMT3A, KIAA1549,* and *PRKDC* genes (each in 2 patients). *RUNX1* and *ABL1* mutations were rarely encountered in CP-CML at diagnosis (2%), and were identified in individual patients who later progressed to BP. Mutations identified in the p190-CML patients were comparable to those identified in the p210-CML patients, such as *ASXL1* and *DNMT3A* mutations. ACAs were rarely observed at diagnosis and were defined in only 4 out of 39 patients with available karyotyping data. Interestingly, karyotype data from one p190 patient revealed presence of ACA, in the form of +22, at diagnosis.

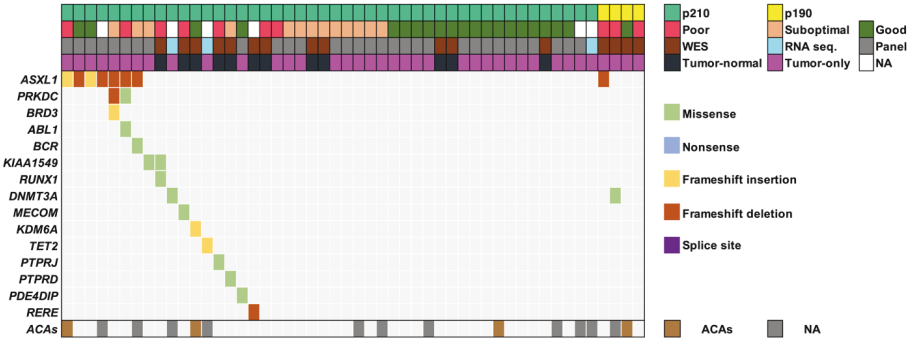


***Figure 6**. Mutational landscape of CP-CML patients (n = 50).*

9.2. Mutational burden and mutations in epigenetic modifier genes are potential biomarkers in CP-CML (Studies I, III)

By restricting the analysis of WES data to the target areas covered by targeted sequencing, we were able to pool all somatic SNV variants (both silent and non-silent ones) from both sequencing techniques and to calculate the mutational burden across the CP samples. Interestingly, CP patients that did not achieve MMR had a higher SNV mutational burden at diagnosis compared to CP patients that achieved MMR at 1 year or thereafter (mean SNV burdens of non-MMR patients and MMR patients were 3.7 vs. 1.3 SNV/mbp, $p = 0.046$) (Figure 7A).

At diagnosis, mutations involving epigenetic modifier genes showed potential value in predicting treatment responses of the CP-CML patients. Mutations in epigenetic modifier genes were identified in 13/19 CP-CML patient samples, harboring cancer-associated mutations. Mutations in epigenetic modifier genes were more frequently encountered in patients who later failed to achieve MMR at any timepoint (5/10, 50%) in comparison to those who achieved MMR (7/34, 20.6%), albeit with borderline statistical significance ($p = 0.066$) (Figure 7B).

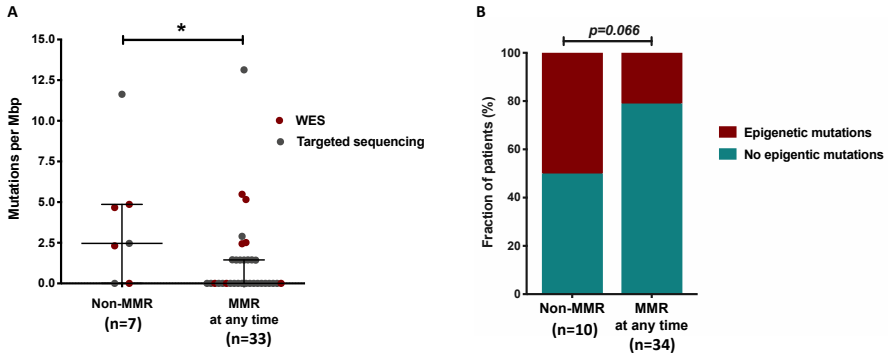


***Figure** 7. Comparisons of SNV Mutational burdens (A) and epigenetic modifier gene mutations (B) between CP patients grouped regarding achievement of MMR.*

9.3. Mutational landscape of BP-CML patients (Studies I, II)

Analysis of samples from 20 BP-CML patients revealed that 85% had at least one cancer-associated mutation. We identified 39 mutations in 18 cancer-associated genes across the BP-CML samples (mean = 1.95 mutation/sample). The most recurrent mutations were the ABL1-KD mutations (n = 7). Other recurrently mutated genes included *ASXL1* (n = 5), *RUNX1* (n = 4), *BCOR* (n = 4), *JAK3* (n = 2), and *BRD3* (n = 2) genes. Other known leukemia-associated genes—including *NRAS, SETD2,* and *BCORL1* as well as mutations affecting the genes that encode DNA repair components (*MSH6, PAPD7*) and protein tyrosine phosphatase receptor (PTPR) genes (*PTPRD, PTPRJ*)—were also identified in individual samples (Figure 8).

Another class of somatic events exclusively identified in the BP-CML patients was that of the fusion genes (other than the CML-hallmark *BCR-ABL1*). Fusion genes were

identified in 7/9 BP patients but in none of the five CP patients with available RNA sequencing data. The fusion genes included several known leukemia-associated genes, such as *TMEM236-MRC1* (in three patients) and *CBFB-MYH11, RUNX1-DYRK1A,* and *TPM4-KLF2* (each in one patient), in addition to other putative fusion genes. The identified fusion genes in study I were validated in relevant samples using either FISH or RT-PCR.



Figure 8. *Mutational landscape of BP-CML patients in study I (n = 20).*

9.4. Mutational burden and signature in BP-CML patients (Study I)

BP-CML samples had a significantly higher SNV mutational burden in comparison to CP-CML (mean SNV burdens of BP-CML and CP-CML were 5.5 vs. 1.8 SNV/mbp, $p < 0.0001$) (Figure 9a). The difference was still significant in separate WES analyses (0.16 vs. 0.12 SNV/mbp, $p = 0.045$) and targeted sequencing data (0.78 vs. 0.17 SNV/mbp, $p < 0.0001$, respectively). To elucidate the mutagenic mechanisms underlying the accumulation of mutations in BP samples, we performed mutational signature analysis of the variants pooled over BP and CP groups. Previously published[240] WES data of 11 AML patients were similarly processed for comparison. We observed a remarkable amount of age-related, DNA mismatch repair (DNA-MMR) and double-strand break repair (DNA-DSBR) signature type variants in CML samples (Figure 9b). Age-related and DNA-DSBR signatures were enriched in BP-CML samples in contrast with the enrichment of the DNA-MMR signature in CP samples. The mutational signature profile of BP-CML showed greater similarity with the AML profile ($r = 0.9$) rather than with the CP-CML profile ($r = 0.24$). Interestingly, we demonstrated the contribution of ultraviolet (UV)-related signature in CP samples, as well as somatic hypermutation (SHM) signature in samples from BP and poor-responder CP patients, which represent novel mutagenic mechanisms yet to be described in myeloid leukemia.

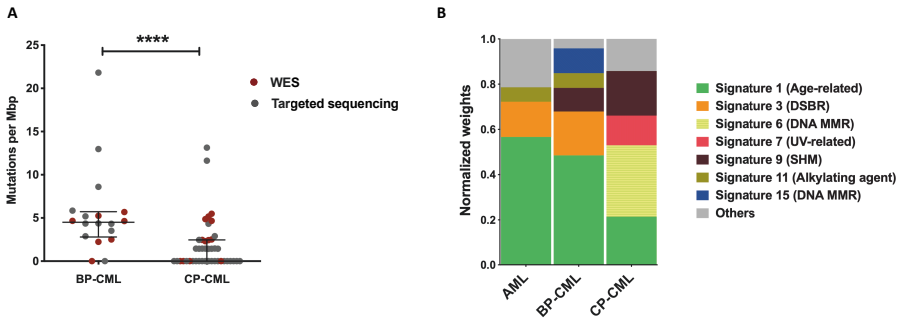


Figure 9. *Comparisons of SNV Mutational burden (A) and mutation signature profile (B) between CP and BP. Processed AML data are used as a comparison group in mutational signature analysis.*

9.5. *RUNX1* mutations as example driver events in BP-CML (Study II)

9.5.1. Mutational profile of RUNX1-mutated BP-CML patients

RUNX1 mutations are among the most frequently encountered genetic events in leukemia.[302] In BP-CML, the *RUNX1* mutations have recurrently been reported. For characterization of the *RUNX1*-mutated BP-CML patients, we combined WES data of BP-CML patients from our cohort (n = 8) with the reanalyzed WES data from a previously published study[205] (n = 12). In the combined data, *RUNX1* was the most frequently mutated gene identified in 7/20 patients. These 7 patients harbored 8 mutations (5 missense, 1 nonsense, 1 frameshift, and 1 splice site donor), all of which were located in the RUNT domain of the RUNX1 protein (Figure 10a). Frequently coexisting mutations in the *RUNX1*-mutated samples involved the *BCORL1* and *PHF6* genes in addition to *IKZF1* deletions (Figure 10b).

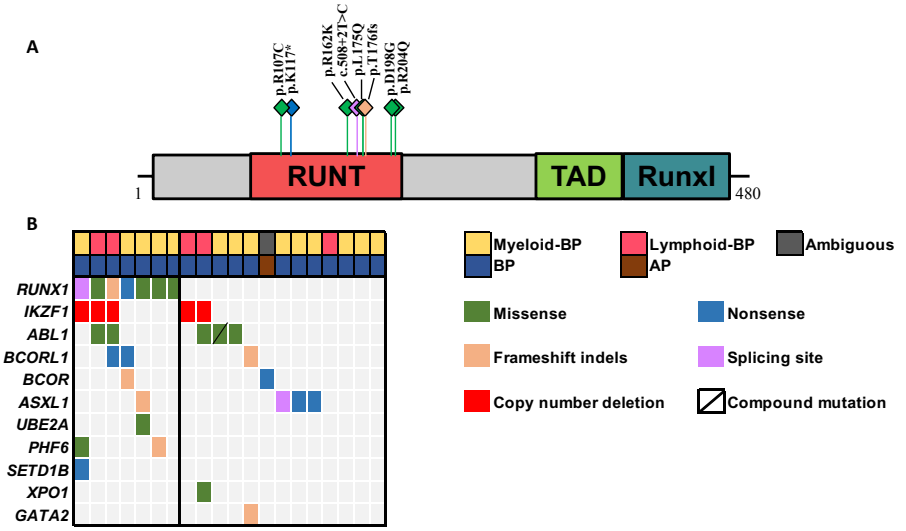


Figure 10. *RUNX1 mutations in BP-CML patients. A) Schematic diagram of the protein structure of RUNX1 protein and distribution of the identified mutations. B) Mutational landscape of BP-patients in study II (n=20)*

9.5.2. RUNX1 mutations are associated with enhanced AID/RAG activity and distinct mutational signature profile

To elucidate the active mutational mechanisms in *RUNX1*-mutated BP-CML, we analyzed the mutational signatures of the variants identified in BP-CML samples. As described above, the mutational signature profile of the BP-CML patients revealed a notable contribution of age-related, DNA-DSBR and DNA-MMR signature type of variants. Interestingly, the *RUNX1*-mutated samples showed specific enrichment of signature-9, related to polymerase η-induced SHM and AID/RAG activity (Figure 11). To investigate the contribution of RAG-mediated activity to the acquisition of SV in BP-CML, we performed an unsupervised motif search analysis to identify enriched motifs in the sequences spanning the SV breakpoints. Interestingly, a perfect heptamer sequence of the RAG enzyme was significantly enriched in the 20-bp spanning SV breakpoints in *RUNX*1-mutated patients (23/32, 79%) in comparison to the wild type group (16/39, 41%). Moreover, the RAG canonical RSS motif (conserved heptamer and nonamer sequences separated by a 12-bp spacer) was exclusively enriched around the SV breakpoints in the *RUNX1*-mutated samples, when the size of the output motif was increased (Table 7).

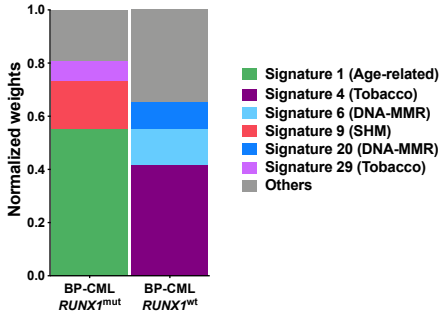
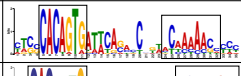
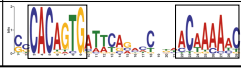
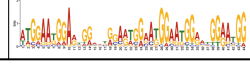


Figure 11*. Mutational signature profiles of RUNX1 mutated and wild type BP-CML patients.*

Table 7. Motif search using the MRME algorithm in BP-CML patients.

		***RUNX1*-mutated BP-CML breakpoints (n=32)**			***RUNX1*-wild type BP-CML breakpoints (n=39)**		
Pad length	Fragment size	Motif logo	E-value	Sites	Motif logo	E-value	Sites
6	12		1.2e-006	21		1.7e-009	22
10	21		1.7e-014	31		1.4e-014	20
20	41		8.8e-031	25		1.8e-031	15
50	100		8.0e-046	16		2.3e-036	38
100	200		6.8e-036	16		2.7e-084	19

9.6. Mutational dynamics in CML patients (Study I)

To investigate the spectrum of mutational dynamics in CML, we analyzed serial samples from 30 patients, including matched diagnosis follow-up samples from 5 poor, 11 suboptimal, and 12 optimal responder CP-CML patients, with a median follow-up sampling time of 6 months, in addition to 2 BP-CML patients with matched diagnosis-relapse samples.

In CP-CML patients who were under TKI treatment, we identified different patterns of loss, persistence, or acquisition of non-silent variants among treatment response groups (Figure 12a). Similar patterns were also identified by restricting the analysis to cancer-related mutations (Figure 12b). Interestingly, the patterns of persistence of truncal mutations or acquisition of cancer-related mutations were enriched in poor responders with TKI-resistance or progression in contrast to optimal and suboptimal patients. Moreover, we identified preleukemic mutations (variants with stable VAF at diagnosis and in follow-up samples despite decline of *BCR-ABL1*) and Ph-negative clones (acquired variants with low VAF coupled with *BCR-ABL1* decline), which involved mainly epigenetic modifier genes (*DNMT3A, TET2*) and were associated with mixed outcomes (Figure 12c). Analysis of matched samples from BP-CML patients revealed clonal evolution patterns, such as linear evolution and clonal drifting associated with progression of CP patients into BP as well as in relapsed BP patients.

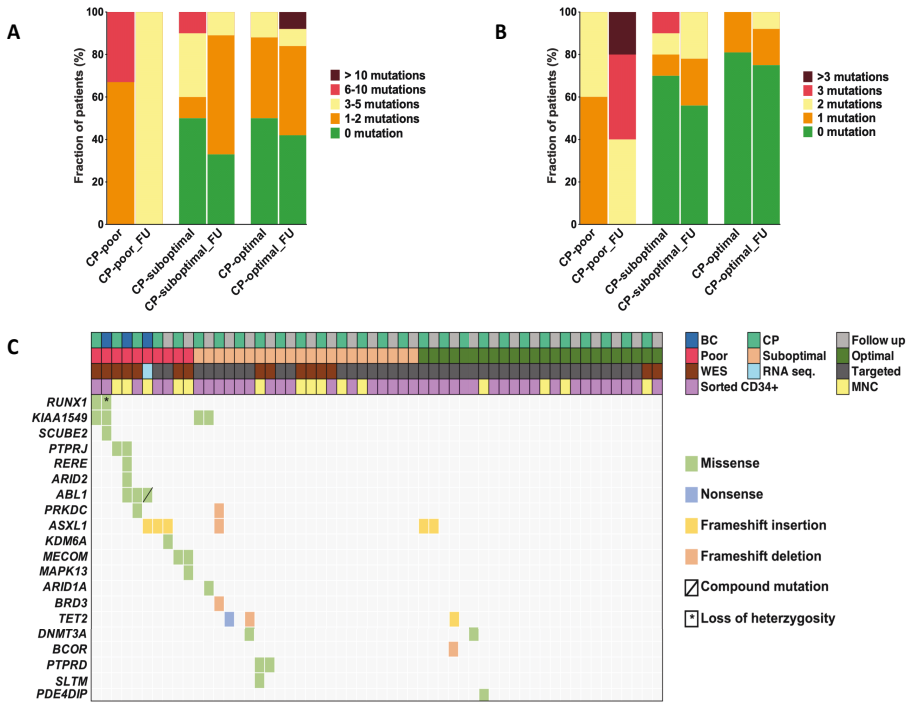


Figure 12. *Mutational dynamics in CP-CML patients. Fraction of cases with non-silent (A) and cancer-associated variants (B) at diagnosis and follow-up (FU) (n=28). P values of the comparisons between the three response groups using Fisher's exact test: A) diagnostic CP (p=0.08) and follow-up CP (p=0.44), B) diagnostic CP (p=0.003) and follow-up CP (p=.02). C) Paired diagnosis-follow up samples from each patient are presented in adjacent columns.*

10. Transcriptional and signaling regulation in high-risk CML patients (Studies I–III)

10.1. Transcriptional regulation in CML at different phases (Studies I, II)

To elucidate the transcriptional programming in CML, as well as progression-specific dysregulated pathways, we performed RNA sequencing of 9 BP-CML samples in addition to sorted CD34+ cells from 5 CP-CML patients and 4 healthy individuals. CML samples from both phases were clustered together in contrast to healthy CD34+ cells (Figure 13a). Expression analysis revealed 1,297 differentially expressed (DE) genes between CML and healthy samples. Individual comparisons between either BP or CP-CD34+ and healthy CD34+ identified 599 and 811 DE genes, respectively (Figure 13b).

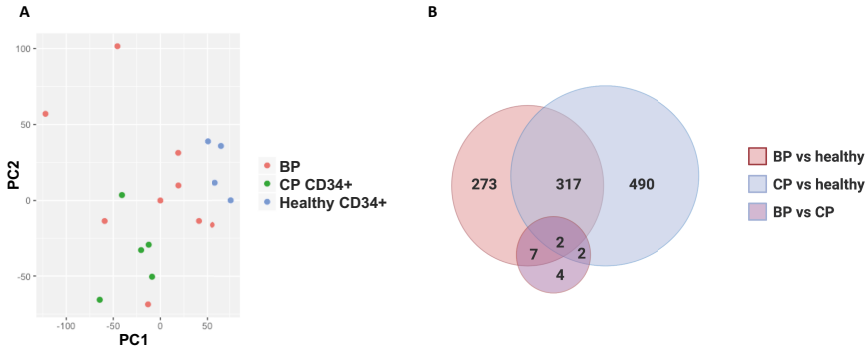


Figure 13. *Gene expression analysis of CML patients. A) PCA analysis of transcriptional data from CML and healthy control samples. B) Venn diagram showing the number of DE genes between CML phases and healthy samples.*

Among the most upregulated DE genes in CML in both phases, there were many CML-markers, including *RXFP1, CD26/DPP4, PRAME,* and *PIEZO2*, as well as cancer-related genes, such as *ST18, IL2RA/CD25, NT5E/CD73*, *GLI2,* and *MLLT4*. On the other hand, genes related to granulocytic maturation and function were among the most downregulated genes, such as *ELANE, AZU1,* and *MPO*. The pathways enrichment analysis revealed the enrichment of CML-signature, as well as activation of Hedgehog, IL12, and CTLA4 pathways in CML samples. In contrast, pathways related to TNF- and P53-signaling, inflammation, UV-response, and neutrophil degranulation were downregulated in CML samples in comparison to healthy CD34+ samples (Figure 14). Moreover, the analyses highlighted phase-specific alterations, such as deregulation of PD1 and coagulation pathways in BP-CML. Comparison between CML phases identified only a handful of DE genes between BP and CP, including *MRC1, HGF, SMAD1,* and *CHN1* as well as several cytokine receptors *IL21R, IL2RA,* and *IL10RA*. Nevertheless, the pathway enrichment analysis revealed a remarkable deregulation of IL2/STAT5, IL6/JAK/STAT3, HDAC, ERK/MAPK, CTCF, KRAS, and the cell cycle pathways in BP samples compared to CP samples (Figure 15).

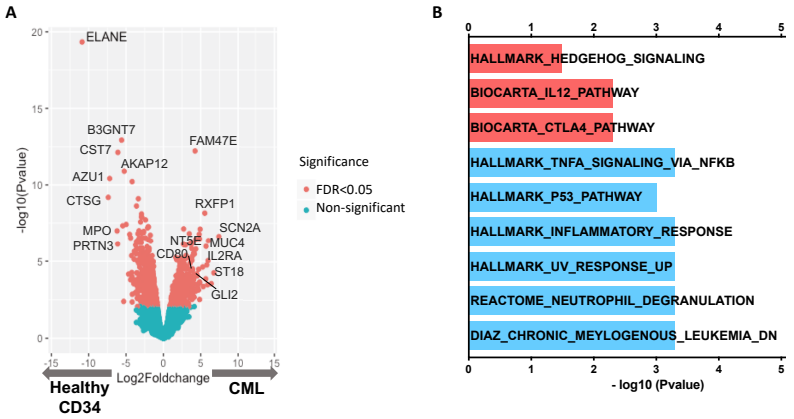


Figure 14. *Comparison of gene expressions between CML (n = 14) and healthy CD34+ (n=4) samples. A) Volcano plot of DE genes, B) Top deregulated pathways. The red color indicates the upregulated pathways while the blue color indicates the downregulated pathways in CML compared to healthy cells.*

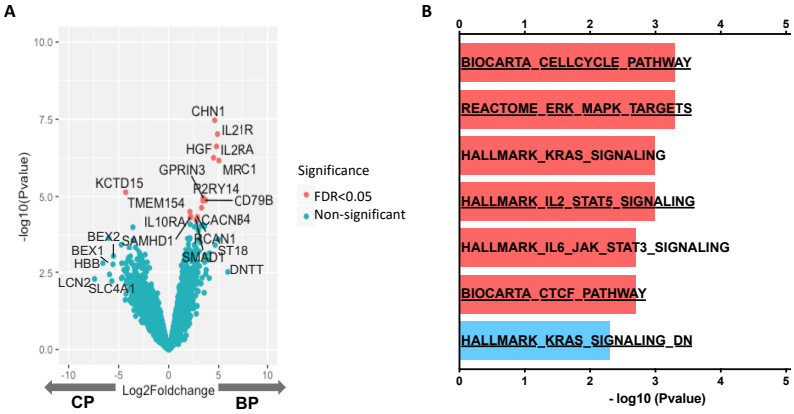


Figure 15. *Comparison of gene expressions between BP (n = 9) and CP-CD34+ (n=5) samples. A) Volcano plot of DE genes, B) Top deregulated pathways. The red color indicates the upregulated pathways while the blue color indicates the downregulated pathways in BP compared to CP.*

10.2. *RUNX1* mutations are associated with characteristic transcriptional alterations in BP-CML (Study II)

To investigate the transcriptional changes derived by *RUNX1* mutations, the BP-CML patient samples were divided into *RUNX1*-mutated (n = 4) and *RUNX1* wild-type (n = 5) groups. The *RUNX1*-mutated group included 3 myeloid and 1 lymphoid BP patients, while patients in the *RUNX1* wild-type group included 3 myeloid, 1 lymphoid, and 1 ambiguous phenotype patients. Expression analysis identified 396

DE genes between the 2 groups. BP samples revealed relative clustering according to the *RUNX1* mutation status (Figure 16). Notably, two *RUNX1* wild-type samples clustered with the *RUNX1*-mutated samples and they were, interestingly, of BP patients with lymphoid and ambiguous phenotypes.

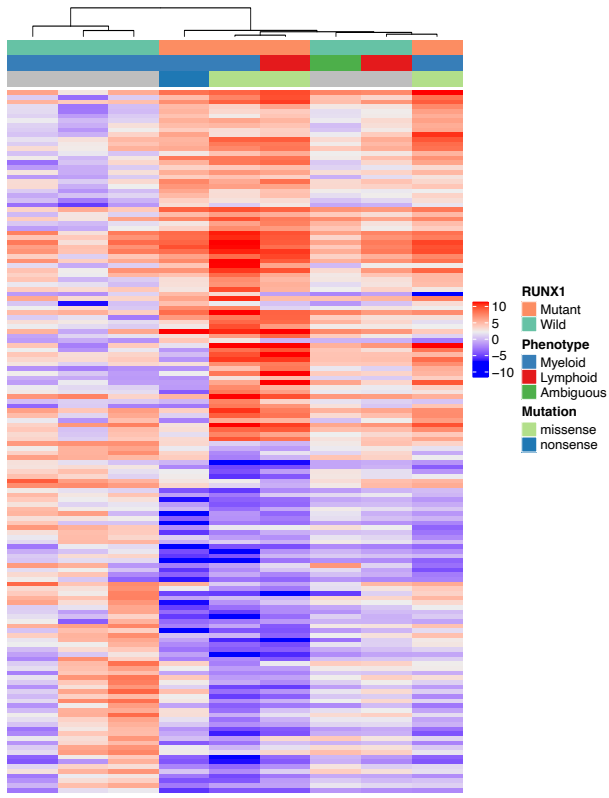


Figure 16. *Heatmap of top 150 DE genes between the RUNX1-mutated (n = 4) and RUNX1 wild-type (n = 5) BP-CML samples (Q < 0.05, logFC > 3.3).*

Genes implicated in HSCs-, lymphoid-, and plasmacytoid dendritic cells (pDCs)-specific pathways were enriched in *RUNX1*-mutated patients. This transcriptional profile was, interestingly, associated with the phenotypic expression of aberrant lymphoid antigens (CD19/CD7) in myeloid *RUNX1*-mutated BP patients. Moreover, genes related to the AID/RAG activity were overexpressed in *RUNX1*-mutated BP patients. Additionally, several immune regulatory molecules were dysregulated in *RUNX1*-mutated patients, such as *CIITA* and *CD74* as well as several *HLA-DR* and *TLR* molecules and cytokine receptors (Figure 17). Furthermore, antigen processing and presentation, interferon alpha and gamma signaling were the most upregulated pathways in the *RUNX1*-mutated samples—in addition to the TNF- and MAPK-signaling pathways. On the other hand, transcription factors and markers of megakaryocytic, erythropoietic, and granulocytic lineages, such as *ITG3B/CD61,*

PF4, and *ABO* genes, were downregulated in the *RUNX1*-mutated samples—in agreement with the downregulation of coagulation and complement pathways.

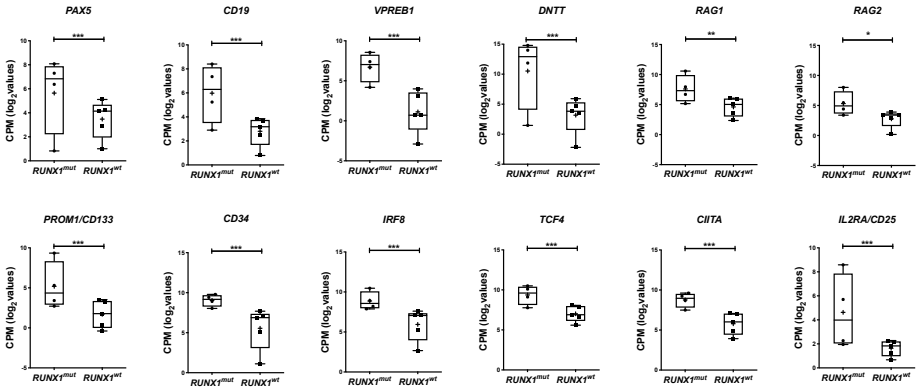


Figure 17. *Expression profiles of the RUNX1-mutated (n=4) and RUNX1 wild-type (n=5) patients for selected genes.*

To validate the specificity of the identified transcriptional changes to *RUNX1* mutations, we performed RNA sequencing of *RUNX1* CRISPR-edited cell lines. The cell line samples clustered according to the *RUNX1* mutations status. Enrichment analysis of the top 300 DE genes revealed remarkable downregulation of *RUNX1*-target genes in $RUNX1^{-/-}$ line in comparison to the $RUNX1^{wt/wt}$ line. $RUNX1^{-/mut}$ showed upregulation of IFN-signaling, antigen presentation, and inflammatory pathways, as well as downregulation of neutrophil differentiation and degranulation pathways, in concordance with expression profiles of *RUNX1*-mutated patients.

10.3. Active transcriptional and signaling pathways in p190-CP-CML patients (Study III)

10.3.1. Transcriptional profile of CP-CML patients expressing p190-BCR-ABL1 at diagnosis

To investigate the impact of the BCR-ABL1 isoform type on the transcriptional profile of CML cells, we performed RNA sequencing of diagnostic PMNC samples from three p190 and three p210 CP-CML patients. Transcriptional data showed notable clustering of samples according to the expressed BCR-ABL1 isoform. Given the small number of samples, only few DE genes could reach statistical significance ($Q < 0.05$) and were all upregulated in the p190-CML samples. The DE genes included several cytokine-signaling (*CCL20, CXCL8 (IL8), IL1R2)* as well as IFN-signaling related genes (*IFIT2, IFIT3*). In concordance, enrichment analysis highlighted the upregulation of IFN-, IL1R-, and TNF-signaling in addition to TP53-related pathways in p190-CML in comparison to p210-CML patients (Figure 18).

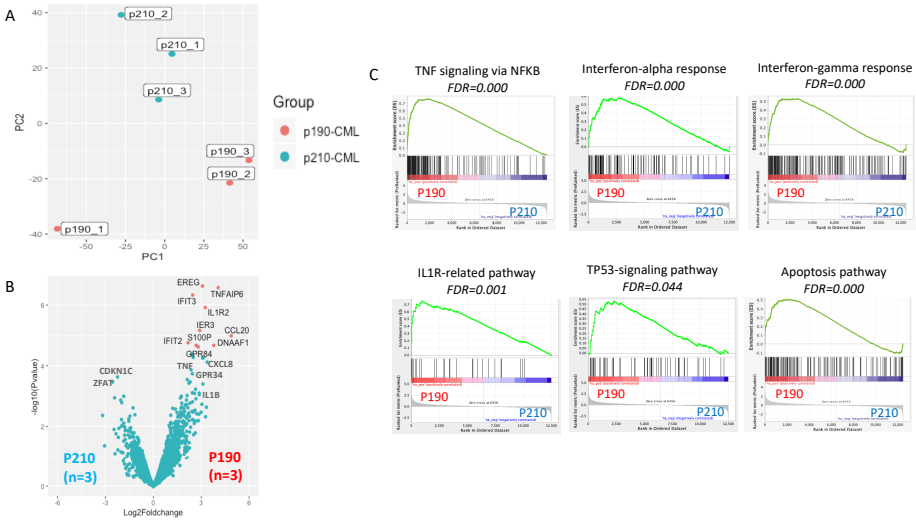


Figure 18. *Transcriptional profile of p190-CML patients. A) PCA of p190 and p210 CML samples. B) Volcano plot of DE genes. C) Top enriched pathways. FDR: false discovery rate, FDR=0.000 indicates that FDR is <0.001(highly significant).*

10.3.2. p190-BCR-ABL1-induced transcriptional reprogramming in cell line models

Given the scarcity of samples from p190-CML patients, we used two *BCR-ABL1*-transduced hematopoietic progenitor cell line models to gain deeper insights into isoform-specific transcriptional and signaling regulations. Ba/f3 is a mouse pro-B cell line that has extensively been used in BCR-ABL1 signaling studies.[46,47] In addition, we used a recently described mouse hematopoietic progenitor cell line, HPC-LSK,[303] to characterize isoform downstream signaling in more progenitor-like settings.

Transcriptional profiling of cell lines revealed a remarkable common *BCR-ABL1* signature, which is evident in *BCR-ABL1*-transduced cells in comparison to parental cell lines regardless of the isoform type. With respect to isoform-specific alterations, cells from both models showed notable clustering according to the isoform type (Figure 19a). Variable numbers of DE genes were identified in Ba/f3 and HPC-LSK cells between different isoforms (2,316 and 650 genes, respectively). To define the p190-speicifc signature, we combined data from both models and identified 111 significantly DE genes ($Q < 0.05$) between both isoforms, most of which were upregulated in p190 cells. Consistent with p190-CML patient profiles, many genes related to IFN-signaling, cytokine regulations, lymphoid kinases, and markers were upregulated in p190 cell lines. Moreover, the pathway enrichment analysis revealed the upregulation of IFN I and II signaling, STAT1-, chemokines-, and apoptosis-related pathways in p190 cells, while the *HIF-1a* pathway was enriched in p210 cells (Figure 19b).

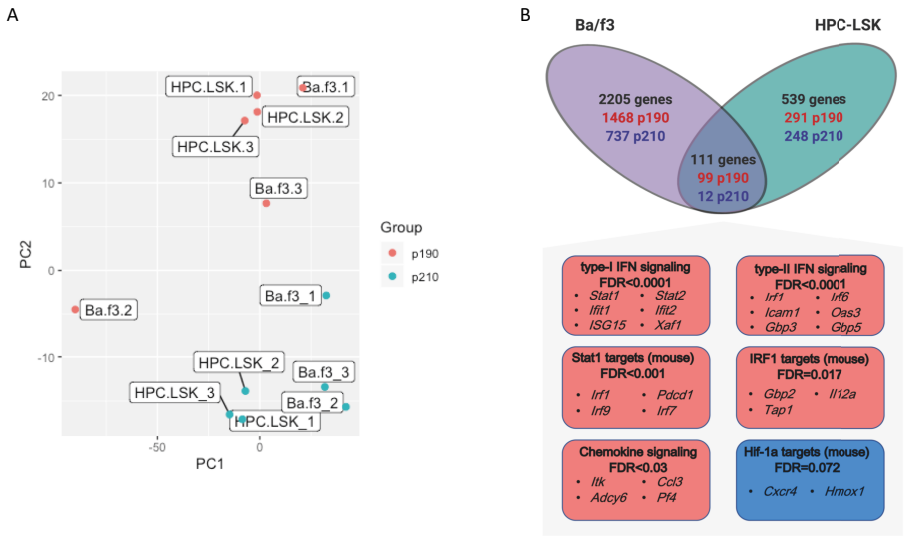


Figure 19. *Isoform-specific transcriptional programs in CML cell lines. A) PCA of p190 and p210 cell line samples showing phenotype-based clustering of samples. B) Venn diagram of the DE genes between p190 and p210 in Ba/f3 and HPC-LSK cell lines, and the top enriched pathways. The color indicates the pathway upregulation in either p190 (red) or p210 (blue).*

10.3.3. Differential phosphorylation profiles of p190- and p210-BCR-ABL1 in CML cells and cell lines

Given the identified isoform-specific transcriptional changes, we next aimed to investigate the differential signaling network and phosphorylation activities of p190- and p210-BCR-ABL1. We first screened the Ba/f3 and HPC-LSK models using an array that included 228 tyrosine residue sites. In both models, the top hit was Src kinase (pTyr418) with increased phosphorylation in p190 cells. Other p190-hyperphosphorylated residues involved sites in Dok1, Src, and Abl1 kinases, in addition to several JAK/STAT molecules (JAK1, STAT6, STAT4, STAT1) (Figure 20).

Given the evidence of transcriptional activation of IFN/STAT signaling, we then focused on the characterization of differential phosphorylation of the JAK/STAT pathway, as well as other key signaling molecules, using Western blot from patient samples and cell lines. In CML patient samples, p190 showed increased phosphorylation levels of STAT1 and, to a lesser extent, JAK1, STAT3, and STAT5 molecules. Moreover, key signaling molecules, such as Src and Pak1, showed enhanced phosphorylation in p190-CML patient samples (Figure 21a). In concordance, STAT1, STAT2, Src, and Pak1 molecules showed increased phosphorylation levels in p190 cell lines. STAT3 and STAT5 showed variable regulation in different models with enhanced phosphorylation in p190 cells in

comparison to p210 cells in the Ba/f3 model, while the HPC-LSK cells showed lower phosphorylation levels with no isoform-specific differences (Figure 21b).

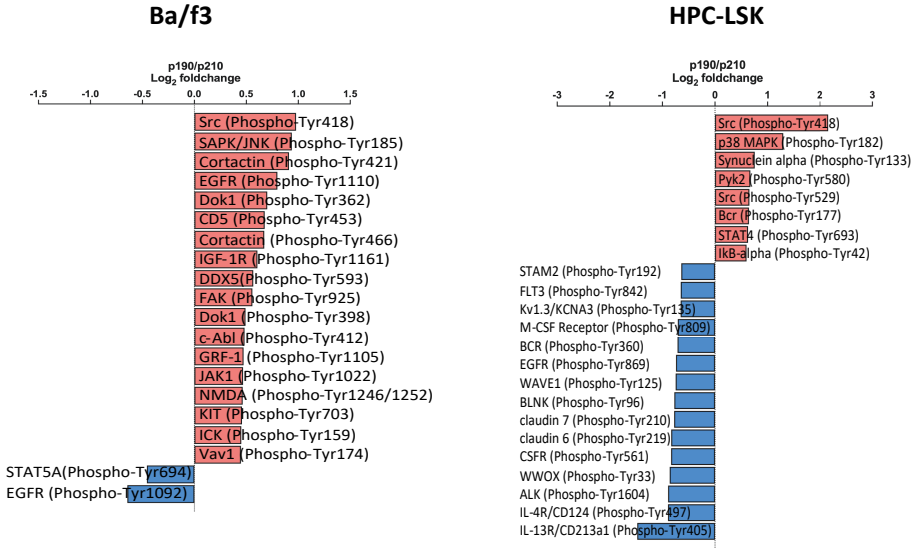


Figure 20. *Phosphorylation array results in CML cell lines. the cutoff value was set to ±33% in Ba/f3 cells and ±50% in HPC-LSK cells. the color indicates the direction of increased (red) or decreased (blue) phosphorylation in p190/p210 comparison.*

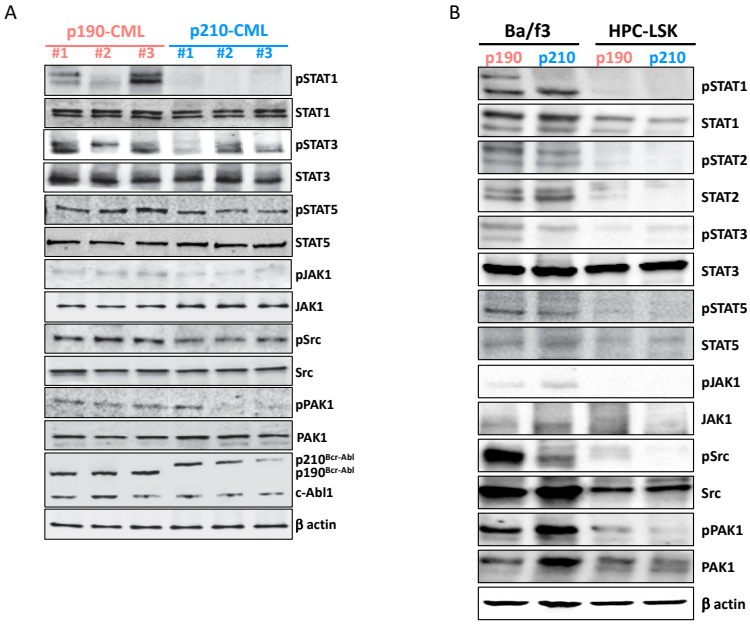


Figure 21. *Phosphorylation of key signaling molecules in p190/p210 CML patients (A) and cell lines(B).*

11. Identification of novel therapeutic options in high-risk CML patients (Studies II–III)

11.1. Drug sensitivity screening revealed increased sensitivities to glucocorticoids and to mTOR and BCL2 inhibitors in *RUNX1*-mutated BP-CML patients (Study II)

To identify a potentially effective targeted therapy for BP-CML patients, we screened diagnostic samples of 8 BP-CML patients using a library of 252 approved and investigational oncology drugs. Collectively, the BP-CML samples showed an increased sensitivity to CDK-, HDAC-, BCL2-, and mTOR- inhibitors in addition to TKIs. Other drug families that showed less pronounced sensitivities included MEK and Aurora inhibitors as well as glucocorticoids (Figure 22).

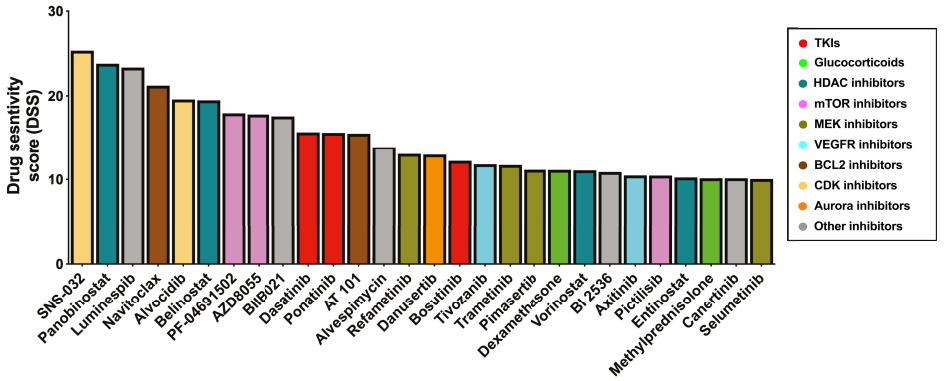


Figure 22. *Mean drug sensitivity score (DSS) of the top targeted drugs in BP-CML samples (n = 8).*

To investigate whether the transcriptional alterations associated with *RUNX1* mutations are reflected on the drug sensitivity profiles of BP patients, we compared DSS scores from *RUNX1*-mutated (n = 4) and *RUNX1* wild-type (n = 4) patients. Interestingly, the *RUNX1*-mutated samples showed an increased sensitivity to glucocorticoids, mTOR-, VEGFR-, and BCL2 inhibitors in comparison to *RUNX1* wild-type samples (Figure 23a). The differential sensitivity of the above-mentioned drugs became more remarkable when the analysis was limited to myeloid-BP samples in order to exclude phenotypic variations. The transcriptional data revealed overexpression of genes encoding targets for some of the identified drugs, such as *NR3C1* (glucocorticoid receptor). Moreover, we noticed an association of the *RUNX1* mutation type and drug sensitivity pattern, where a patient with a stop-gain mutation exhibited an increased sensitivity to glucocorticoids and mTOR inhibitors and a reduced sensitivity to navitoclax in comparison to patients with missense mutations. The number of samples is, however, limiting.

Subsequently, we investigated the potential synergistic effect of various combinations of identified drugs with TKI in enhancing the killing of *RUNX1*-mutated blasts. We screened samples from 4 *RUNX1*-mutated and 2 *RUNX1* wild-type patients using combinations of imatinib with one of the following drugs—axitinib, cobimetinib, dexamethasone, everolimus, or venetoclax—in a dose-dependent manner. We identified the imatinib-dexamethasone combination and, to a lesser degree, the imatinib-cobimetinib and imatinib-venetoclax, as potentially synergistic combinations with enhanced blast killing. It is worthy to note that one *RUNX1*-mutated patient (marked in the figure as mut1) also carried an ABL1-KD T315I gatekeeper resistance mutation that renders blasts resistant to imatinib and, hence, no synergy was detected (Figure 23b)

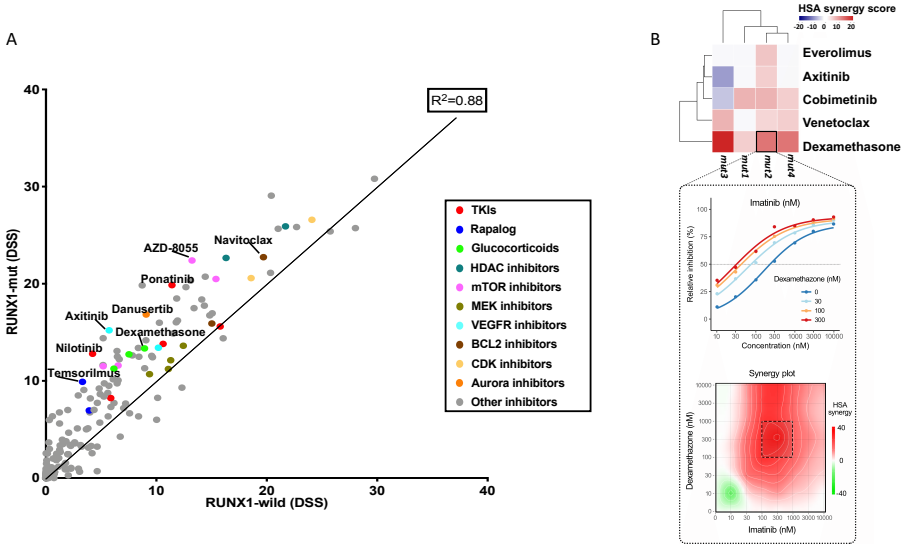


Figure 23. *(A) Comparison of DSS between the RUNX1-mutated and RUNX1 wild-type BP-CML samples. (B) Heatmap of synergy scores when combining imatinib with each of the selected drugs. An example of the inhibition cure with IC_{50} and synergy plot is highlighted.*

11.2. Cell lines with *RUNX1* mutations shared drug sensitivity profile alterations with *RUNX1*-mutated patients (Study II)

To confirm the specificity of the identified drug sensitivities to *RUNX1* mutations, we also screened the drug sensitivity profiles of *RUNX1*-edited cell lines using a comprehensive library of 528 drugs. Ba/f3 cells with heterozygous *RUNX1* mutation (*RUNX1*$^{-/mut}$) showed a greater sensitivity to mTOR and VEGFR inhibitors in comparison to control Ba/f3 cells, which was consistent with patient DSRT profiles. Moreover, *RUNX1*$^{-/mut}$ demonstrated enhanced sensitivities to BET and CDK inhibitors, as well as selective resistance to IAPs inhibitors, which were not tested in patient samples (Figure 24a). Similar DSRT profile alterations were also seen in the *RUNX1*-edited K562 cell line. Introduction of a missense *RUNX1* mutation (p.R162K) into cell lines led to enhanced sensitivity to AZD8055, axitinib, and navitoclax, similar to *RUNX1*-mutated patient samples. Furthermore, a comparison of DSRT data from cell lines with different *RUNX1* mutations revealed mutation type-specific sensitivities

similar to those noted in patient samples, such as enhanced mTOR activity with nonsense mutations and navitoclax activity with missense mutations (Figure 24b).

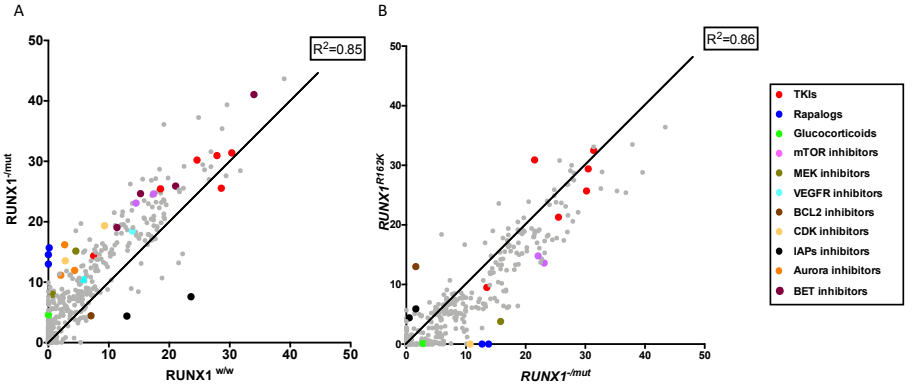


Figure 24. *Drug sensitivity profiles of the RUNX1-mutated cell line samples.*

11.3. CD19-CAR T-cells are promising effective immunotherapeutic option in *RUNX1*-mutated patients (Study II)

Given the characteristic upregulation and aberrant phenotypic expression of lymphoid markers—namely, CD19—in *RUNX1*-mutated myeloid-BP patients, we investigated the use of CD19-CAR T-cells as a potential form of immunotherapy for *RUNX1*-mutated BP patients. We used a 24-hour ex-vivo coculture assay to measure the cytotoxic activities of CD19-CAR T-cells against CD34+ blasts from *RUNX1*-mutated BP patients, both in presence and absence of imatinib, using flowcytometry. Interestingly, the CD19-CAR T-cells showed an effective cytotoxic activity against *RUNX1*-mutated blasts from a lymphoid BP patient, as well as a myeloid-BP patient, expressing aberrant CD19 on 25% of blasts. Even at low effector–target (E-T) of 1:8, the CD19-CAR T-cells were able to induce the killing of *RUNX1*-mutated blasts to a variable extent (13–50%). Cytotoxic activity of CD19-CAR T-cells at the ET ratio of 2:1 was superior to imatinib-induced killing of blasts from *RUNX1*-mutated BP patients, including a lymphoid BP-patient carrying an ABL1-KD T315I mutation (Figure 25). The cytotoxic activity induced by CD19-CAR T-cells was demonstrated to be specific, in contrast to the mock-targeting CAR T-cells. Furthermore, the combination of CD19-CAR T-cells with imatinib induced an enhanced cytotoxic activity in comparison to imatinib alone or to an imatinib-mock CAR T-cell combination.

The CD19-CAR T-cells showed an activated phenotype of both CD8+ and, to a lesser degree, CD4+ cells, which was evident by 1.5–4-fold increases in the expression of the CD69 activation marker upon coculture with *RUNX1*-mutated blasts. The CD19-CAR T-cells induced modest cytotoxicity against *RUNX1*-mutated blasts from two myeloid patients lacking a CD19 expression. The identified cytotoxic activities were,

however, superior to imatinib-induced killing in one patient as well as to mock-cell activity in both patients. As a control, we also used blasts from two *RUNX1* wild-type patients using the coculture assay. Only in a lymphoid BP patient the CD19-CAR T-cells were able to induce effective killing of blasts, while no activity was detected in the other myeloid BP sample.

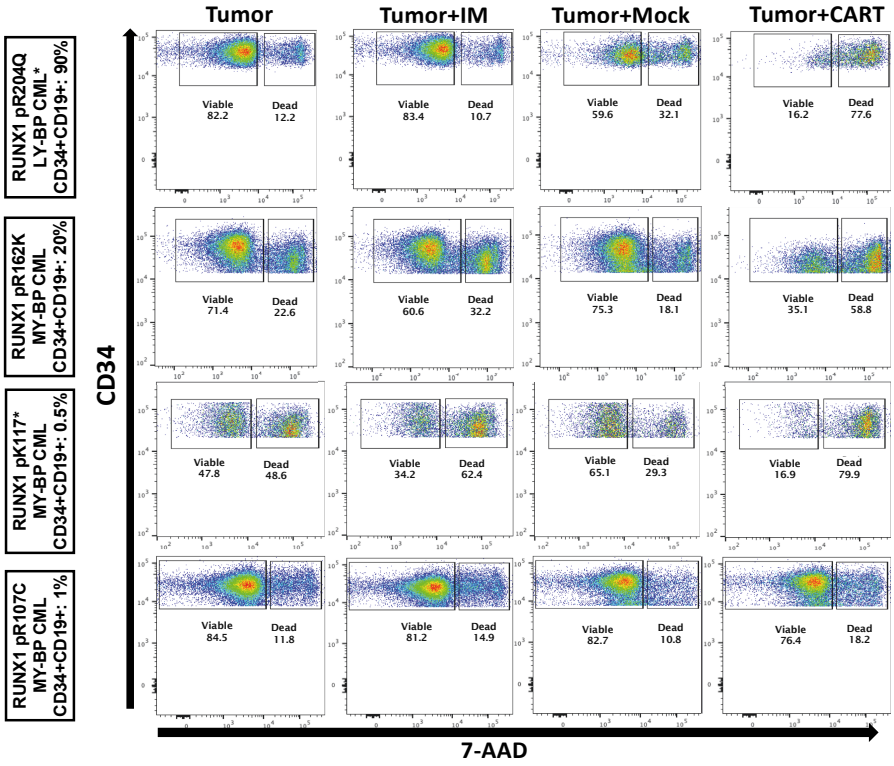


Figure 25. *CD19-CAR T-cells' cytotoxic activity against blasts of RUNX1-mutated patients. Staining for CD34 was applied to gate blasts and 7-AAD to indicate viability (dead cells are 7-AAD positive).*

11.4. Drug sensitivity profiling revealed increased sensitivities of the p190-CML cell lines to IAPs, PAK1, and Src inhibitors (Study III)

To investigate whether the transcriptional and signaling differences between BCR-ABL1 isoforms are translated into specific drug sensitivities, we screened the CML cell lines expressing either p190 or p210 isoforms using high-throughput drug testing. Generally, the two isoforms had an overlapping DSRT profile. In the Ba/f3 model (Figure 26a), the p190 cells showed enhanced sensitivities to IAPs inhibitors (LCL161, AT406, Birinapant), PAK inhibitor (FRAX486), AKT inhibitor (Triciribine), and glucocorticoids in comparison to the p210-expressing cells. Other less pronounced sensitive drugs in the p190 cells included rapalogs, MDM2 and JAK inhibitors, as well as TKIs with Src inhibitory activity, such as dasatinib and bosutinib.

On the other hand, navitoclax showed increased activity in p210 in comparison to p190 cells. It should be noted that the targets for some of the identified drugs were hyperphosphorylated in the p190 cells, such as PAK1 and Src molecules. In concordance with the Ba/f3 model, the p190-HPC-LSK cells showed an increased sensitivity to LCL161 and FRAX486 and a reduced sensitivity to navitoclax in comparison to p210-HPC-LSK cells (Figure 26b). Additionally, the p190 cells in both models demonstrated slightly increased sensitivity to in vitro IFNα treatment in comparison to p210 cells.

Next, we investigated the potential value of imatinib combinations with the identified drugs in targeting the p190-expressing cells. In both models, we screened the p190 cells using combinations of imatinib with LCL161, FRAX486, idasanutlin, dexamethasone, and IFNα. Imatinib-LCL161 and imatinib-FRAX486 were the most promising combinations, showing consistent additive effect in both models. Other combinations showed variable enhancing effects, which were more pronounced in HPC-LSK model.

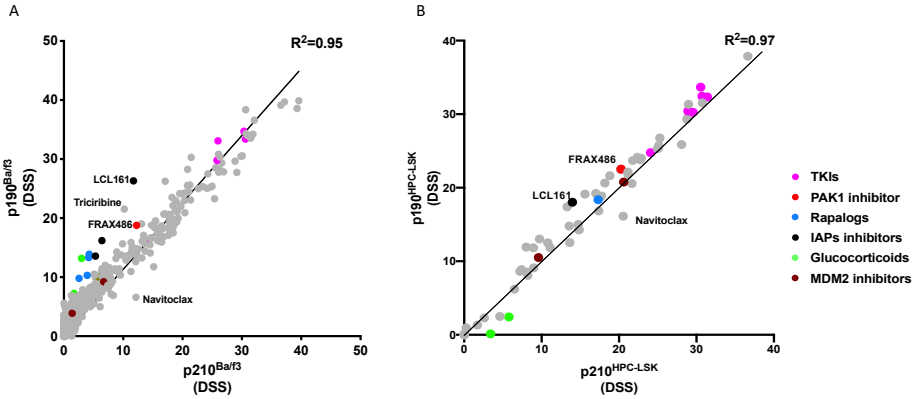


Figure 26. *DSRT profiles of p190- and p210-expressing cells in Ba/f3 (A) and HPC-LSK (B) models.*

11.5. Dasatinib, LCL161, and idasanutlin inhibit the clonogenic potential of the p190-HPC-LSK cells (Study III)

We also investigated the effect of the identified drugs alone and in combination with imatinib on the colony forming potential of HPC-LSK cells expressing either p190 or p210 isoforms (Figure 27). In general, p190-HPC-LSK produces higher colony counts in comparison to p210 cells. Additionally, p190 clones revealed more stem-like phenotype and increased tendency for lymphoid rather than myeloid differentiation, as evident by the increased expression of CD19 and the lowered CD11b expression than shown by p210 clones. Imatinib showed diminished activity in reducing colony counts in p190-HPC-LSK than in p210 cells, while dasatinib and FRAX486 could efficiently inhibit colony formation in both isoforms. Moreover, LCL161 and idasanutlin showed greater inhibitory effect of the clonogenic potential of p190- over p210-HPC-LSK cells. Furthermore, combinations of imatinib with LCL161,

FRAX486, or idasanutlin showed an additive effect in reducing colony counts of p190-HPC-LSK cells.

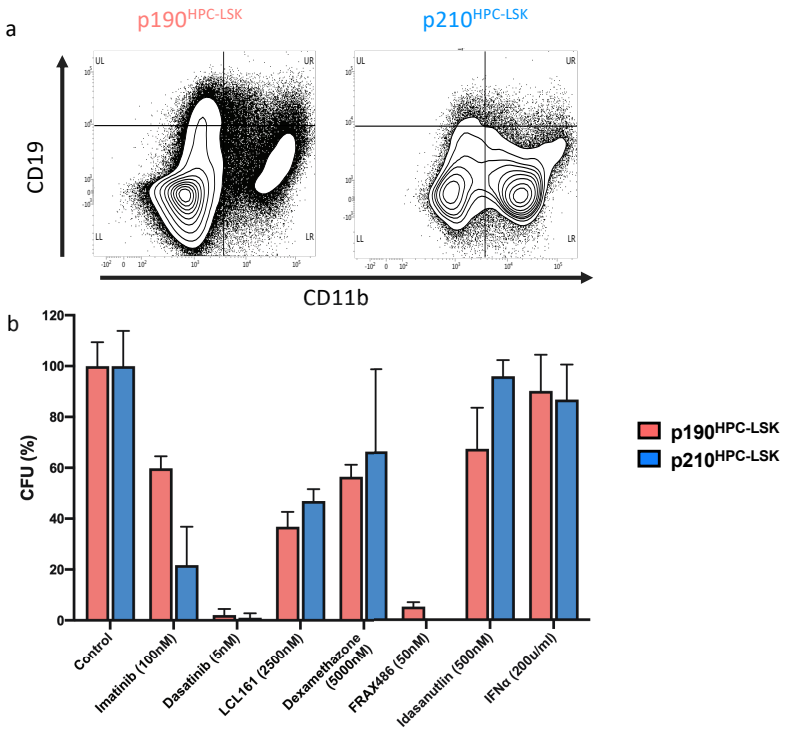


Figure 27. *Colony forming potentials of p190 and p210 HPC-LSK cells. a) Phenotyping of colonies from p190 and p210. b) Colony counts from CFA of p190 and p210 HPC-LSK cells.*

11.6. Validation of isoform-induced signaling and drug sensitivity differences in primary Ph+ALL samples (III)

Since fresh viably-frozen samples were not available for p190-CML patients, we were not able to investigate the drug sensitivity profile of primary CML cells. Instead, we used samples from a cohort of Ph+ALL patients (n = 10) to validate signaling and drug sensitivity findings from cell line models. Interestingly, the p190-Ph+ALL samples showed increased sensitivity to glucocorticoids, idasanutlin, bosutinib, and dasatinib in comparison to p210-Ph+ALL samples. LCL161 and FRAX486 showed, however, similar inhibitory profiles with both isoforms (Figure 28a). To investigate isoform-specific signaling in Ph+ALL samples, we tested the phosphorylation levels of STAT molecules as well as Src kinase. Comparable to p190-CML and cell line data, p190-Ph+ALL revealed increased phosphorylation levels of Src, STAT2, and STAT5 molecules, while STAT3 showed increased phosphorylation in P210 samples (Figure 28b).

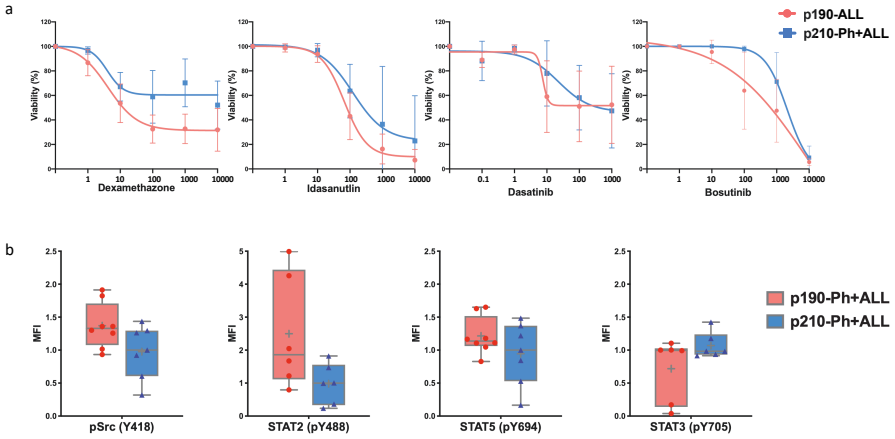


Figure 28. *Differential drug sensitivity (a) and phosphorylation profiles (b) of p190 and p210 in Ph+ALL patients. For DSRT, samples from five p190 and three p210 patients were used, while samples from five p190 and four p210 patients were used for phosphorylation study.*

12. Personalized medicine approach to BP-CML management (I)

Finally, we provided examples of the potential benefits of applying a personalized approach to integrating genomic and drug sensitivity data in the management of two BP-CML patients. At diagnosis, genomic characterization using WES and RNA sequencing, as well as DSRT testing, was done for the patients. The first patient was a lymphoid BP patient, carrying *ABL1* (p.T315I) and *RUNX1* (p.R177Q) mutations. RNA sequencing revealed a *RUNX1*-mutated transcriptional signature with dysregulation of HSCs, coagulation, and complement pathways. Ponatinib was the only active TKI in the DSRT data. Interestingly, the drugs described above to show specific sensitivity in association with *RUNX1*-mutations, such as mTOR and VEGFR inhibitors, were among the most active drugs. Building on these findings, the VEGFR inhibitor, axitinib, was used to treat the patient, in parallel with a short course of ponatinib. Axitinib treatment induced partial remission of the disease for 2 months, which was then followed by a relapse. Interestingly, WES of the relapse sample revealed elimination of the original clone and emergence of a relapse-specific clone with the *EZH2* mutation. In contrast to the diagnosis sample, transcriptional data revealed upregulation of IL8, CXCR1-signaling pathways in addition to fatty acid metabolism in the relapse sample. In concordance, DSRT data from the relapse sample revealed enhanced sensitivity to PF-3845 (fatty acid amide hydrolase inhibitor) and loss of sensitivity to *RUNX1*-related drugs which were active at diagnosis (Figure 29). The patient then proceeded to Allo-SCT.

The same integrative approach was applied to another BP patient with the myeloid phenotype. Genetic analysis revealed somatic mutations involving the *ILIR1* and

DOTL1 genes. Dasatinib was administered as a frontline treatment, and achieved a short period of partial remission before the disease relapsed. WES of the relapse sample identified acquired mutations in *ABL1* (p.T315I), *MSH6,* and *SETD1B* genes (Figure 29). RNA sequencing identified the *CBFB-MYH11* fusion gene, as well as upregulation of the *CBFB-MYH11* target genes, such as *EPX* and *PRG2* eosinophilic markers. In the DSRT results, ponatinib was the only active TKI. Axitinib was among the most sensitive drugs and was administered for 3 months in parallel with short cycles of CVAD chemotherapy and bosutinib. Treatment induced remission of the disease and the patient proceeded to Allo-SCT.

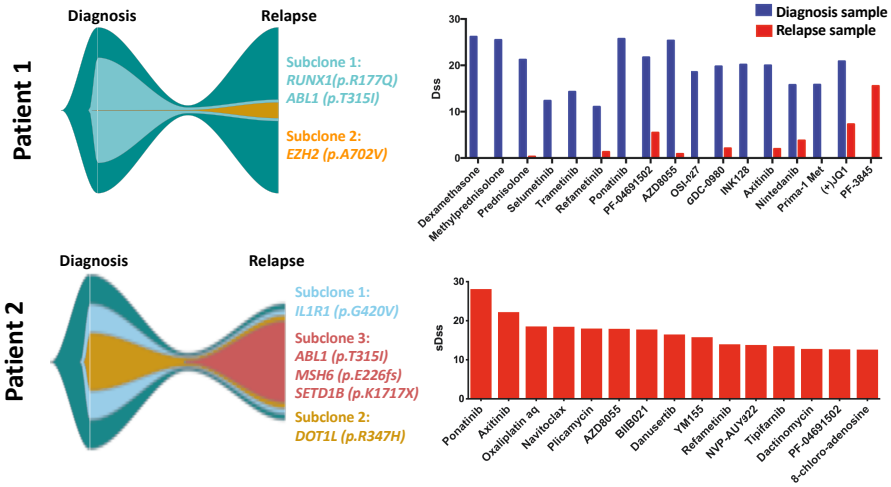


Figure 29. *Personalized approach in two index BP-CML patients. For each of the two patients a fish plot showing the clonal evolution in patient' samples as well as a waterfall of the most active drugs were shown.*

Discussion

Implementation of TKIs into CML management, as well as advances made in *BCR-ABL1* monitoring over the last two decades, have led to significant improvement of treatment outcomes in CML patients with nearly normal survival rates.[4,304] Some challenges, however, remain to be addressed in the TKI era, including: i) identification of novel biomarkers at initial diagnosis that can be used for risk stratification and frontline treatment selection, ii) discovery of the genetic and molecular mechanisms underlying treatment resistance and disease progression, and iii) identification of potentially efficient therapies that can be used in combination with TKI to improve the outcome of patients with poor prognosis, such as BP-CML patients. Such combination therapies can also be useful for targeting CML LSCs and, hence, for improving long-term free remission after treatment discontinuation.

The personalized medicine approach that integrates genomic data into risk stratification and treatment decisions has been successfully implemented in the clinical management of leukemia patients, such as AML and MPN patients,[248,305] Recent data suggest a potential predictive role of genetic profiling in CML, which can serve as a useful complement to the current clinical scoring system for risk stratification and selection of frontline treatment.[235,237] Another personalized medicine approach is that of ex-vivo high throughput drug testing, which has revealed potential benefits in guiding AML treatment choice.[240,251] This strategy has also been highlighted as a potentially useful approach in the poor prognosis BP-CML patients.[306] With acceptance of TFR as the new goal of CML management, exploring the clinical impact of mutational data on the outcome of TKI discontinuation, as well as identification of novel drugs targeting LSCs and allowing for an operational CML cure, remain key questions to be answered. Despite further studies being warranted to obtain more conclusive evidence, the genetic-based personalized approach is expected to soon be an important part of clinical CML practice.[242,307]

13.1. Somatic mutations in CP and BP CML

In study I, we characterized the genetic profiles of both CP and BP-CML phases. Within the CP group, we compared samples from patients with different TKI treatment responses and identified potential genetic biomarkers that were associated with treatment outcomes. Recently, there is a growing evidence regarding the potential contribution of somatic mutation to response heterogeneity in CP-CML patients.[308,309] In our data, somatic mutations found in cancer genes at diagnosis were associated with treatment outcomes in CP-CML. The SNV burden was generally higher in CP patients with poor responses in comparison to patients achieving molecular response to TKI treatment. The difference becomes more pronounced when restricting the SNV burden to target regions involving cancer-associated genes, highlighting the potential prognostic value of somatic mutations in CP-CML; however, it also indicates the presence of passenger mutations that are expected with elderly onset CML. It should

be noted that other studies showed a weak positive correlation of the mutational burden with the age of CML patients at diagnosis.[202,203] Similar weak correlation was also noted in our WES data but was, interestingly, abrogated by restricting the analysis to cancer-gene regions. Consistent with this finding, another study has reported a significant association between high mutational burden and imatinib resistance when the identified variants were further refined by applying a scoring system to rank the oncogenic potential of each variant.[202]

Despite *BCR-ABL1* fusion gene is the main event in CML pathogenesis, mutations in cancer-related genes are suggested to play an important role in the pathogenesis of different CML phases. In our study, mutations of cancer-associated genes were identified in 38% of the CP patients at diagnosis. Mutations involving epigenetic modifier genes represented the major fraction of the identified mutations in agreement with previous studies.[199,200,310] Our data revealed increased incidence of epigenetic modifier variants in poor responders in comparison to patients achieving MMR on TKI treatment, which is in concordance with studies that have reported the association of epigenetic modifier mutations with inferior response to TKI treatment.[199,311] A recent study has found that a poor prognostic effect of epigenetic modifier mutations is observed in imatinib treated patients but is abrogated in patients treated with 2G-TKIs, suggesting that CP patients with variants in the epigenetic modifier genes could benefit from assignment to frontline 2G-TKI treatment.[201] Among epigenetic modifier genes, *ASXL1* was, by far, the most frequently mutated gene in CP patients in our cohort—in agreement with a recent review of current genetic knowledge in CML.[8] Interestingly, the *ASXL1* mutations have recently been reported as predictors of TKI treatment resistance, especially to imatinib.[235,312]

Our analysis revealed an accumulation of mutations in the BP-CML samples, which showed a significantly higher SNV burden in comparison to CP patients. We have investigated the mutational signature profiles of CML patients in order to identify common, as well as phase-specific, active mutagenic processes. In general, the mutational signature profiles of CML patients showed an enrichment of variants, from aging-related signature 1 and DNA-MMR signatures (6 and 15), in line with the reported activity of *BCR-ABL1* in inhibiting DNA repair.[313] BP samples showed specific enrichment of variants from DNA-DSBR signature 3 and alkylating agent signature 11, in addition to increased contribution of signature 1 to the total profile, in contrast with CP samples. Moreover, mutational signature profiles revealed a similarity between AML and BP-CML, suggesting that additional mutagenic mechanisms are activated during disease progression, which represent a potential target for treatment.[314] Additionally, we identified potentially novel signatures in CML samples, such as UV-related signature 7, which was more enriched in the CP samples and has been described as a potential mutagenic mechanism in B-ALL.[170] Somatic hypermutation signature 9 was also noted in samples from BP and poor responder CP CML patients. This signature has been linked to illegitimate RAG

enzyme activity in ALL patients[315] and, recently, in lymphoid-BP patients.[316] Interestingly, a recent study has reported a novel CML-specific signature that was enriched in progression-associated variants and revealed specific enrichment in BP-CML in comparison to other leukemias.[210] In comparison to large scale studies in solid tumors and other leukemias, such as CLL and AML, few studies with limiting cohort sizes have explored the mutational signatures in CML. Hence, further studies involving larger cohorts are needed to characterize the active mutational processes in CML in different phases, which would enable better understanding of the BP-CML pathogenesis.

Our data demonstrated the presence of at least one cancer-associated gene mutation in 85% of the BP-CML patients. As expected, the ABL1-KD mutations were the most common mutations (35% of samples), which is in agreement with other genetic studies of BP-CML in the TKI era.[8,317] Recurrent mutations in the BP samples also included known leukemia-associated mutations, such as *ASXL1*, *RUNX1* and *BCOR* genes, as well as *IKZF1* deletions, in concordance with previous studies.[205,207,210] Interestingly, these mutations—except for the *ASXL1* mutations—are infrequent in the CP samples in which they are associated with poor responses,[8,205] suggesting that these mutations have a driver role in disease progression. In line with previous studies,[205,212] we noted an interesting association of *IKZF1* deletions with the lymphoid phenotype and of *ASXL1* mutations with the myeloid phenotype. Other leukemia-associated mutations, involving *BCORL1, SETD2, MSH6,* and *SETD1B* genes, were identified in individual cases. Mutations of some of these genes, such as *BCORL1* and *SETD1B*, were also highlighted in comparable frequencies by other BP-CML studies.[205,210] Among recurrently mutated genes, some were novel to be reported in BP-CML patients, such as *BRD3* and *JAK3*, which have a known role in other leukemias.[318,319] On the other hand, some genes reported to be recurrently mutated in BP by individual recent reports, such as *UBE2A*[208] and *WT1*,[210] were not identified in our cohort—most probably due to the limiting cohort size. Thus, further studies are warranted to improve the characterization of the BP-CML mutational landscape.

Another class of somatic mutations that was demonstrated to be associated with advanced phase CML is that of fusion genes, which have an established driver role in acute leukemia.[215] Early cytogenetic studies have identified progression associated translocations, some of which encode for known leukemia-associated fusions, such as the inv(16) encoding *CBFB-MYH11* fusion.[220] Using RNA sequencing, we identified fusion genes, other than *BCR-ABL1*, in the majority of BP but not in any CP samples. Among the identified fusion genes, there were many leukemia-associated fusions, such as *CBFB-MYH11*, as well as fusions involving leukemia-associated genes, such as *RUNX1* and *MRC1*. In concordance, a recent study applying RNA sequencing to BP samples has also highlighted fusion genes as progression associated events.[205] Fusion genes were detected in about half of the BP samples including *MLL* rearrangements, *CBFB-MYH11*, and rearrangements involving *RUNX1, MECOM,* and

PAX5 genes.[205] In general, RNA sequencing is an efficient tool for identifying fusion genes.[320]

Through an analysis of serial samples from CP patients under TKI treatment, we have demonstrated different patterns of mutational dynamics associated with different treatment outcomes. Persistence of ancestral clones and acquisition of cancer-associated mutations were associated with poor response to TKI treatment and disease progression. We also identified preleukemic and Ph-negative clonal mutations, mostly involving epigenetic modifier CHIP genes, such as *DNMT3A* and *TET2*, that were associated with variable outcomes. In general, our data is in agreement with the results obtained by Kim et al.,[199] who have systematically analyzed serial samples from 100 CP patients and identified similar mutational dynamic patterns. Acquisition of mutations, including ABL1-KD mutations and mutations in cancer-associated genes such as *RUNX1, UBE2A,* and *IKZF1*, have commonly been reported as progression-associated events.[205,208,210] Other studies have also reported that the clearance of diagnostic phase mutations was associated with successful TKI treatment.[201,203] In these studies, emergence of CHIP mutations (e.g., *TET2* and *DNMT3A*) in remission samples were similarly reported, suggesting its preleukemic/Ph-negative origin.

Given the elderly onset of CML and the frequent incidence of CHIP-related mutations in CP-CML, the contribution of CHIP to CML pathogenesis and treatment responses represents an interesting area for further investigations. Preleukemic mutations have been suggested to precede and cooperate with BCR-ABL1 in a multistep model of leukemia pathogenesis.[321] Furthermore, CHIP-related mutations have frequently been identified in T-cells and remission samples from CP patients, where they have also been identified in diagnostic leukemic samples in many cases, suggesting their preleukemic nature.[199,201,204] On the other hand, the frequencies of CHIP-related mutations in CML differ from CHIP profiles in healthy elderly as well as AML samples. In contrast to the observed low frequency of *DNMT3A* mutations in CML,[322] which were also noted in our study, *DNMT3A* mutations are the most frequent CHIP-related mutations in healthy elderly and AML patients. Although *ASXL1* mutations were the most frequent CHIP-related mutations in CML, the mutational profiles of childhood and young adult CML patients revealed a high frequency of *ASXL1* mutations,[323] suggesting that *ASXL1* mutations in CML are not entirely a CHIP-related phenomenon. Moreover, CHIP mutations were associated with an increased risk of cardiovascular diseases.[190] Cardiovascular events are among the adverse effects of some TKIs, such as nilotinib and ponatinib.[324] A recent study[325] has reported a higher frequency of CHIP mutations in remission samples of nilotinib treated patients who suffered from cardiovascular events than in patients with no events.

Transcriptional profiling of CML and healthy HSCs samples highlighted CML-associated transcriptional reprogramming as well as potential CML markers. In agreement with previous reports,[167,225,326,327] the genes that are suggested to be CML

markers, such as *RXFP1, CD25, CD26, PRAME,* and *PIEZO2*, were remarkably upregulated in CML samples. We also demonstrated the upregulation of immune checkpoint pathways, including CTLA4 and PD1 pathways in CML, which represent potential immune evasion mechanisms in CML.[158] Other dysregulated pathways between CML and healthy HSCs included the downregulation of TNF, inflammation, TP53-related, and neutrophil degranulation pathways, which is in line with previous reports.[167] We also identified progression-associated transcriptional regulations by comparing gene expression data from CP and BP samples. Several cytokine-activated JAK/STAT pathways, including IL2/JAK/STAT5 and IL6/JAK/STAT3, were upregulated in association with disease progression, consistent with the findings of a recent study.[210] Other progression-associated dysregulated pathways included the KRAS, ERK/MAPK, and HDAC pathways, which represent potential targets for targeted therapy.[130,137]

In the final part of study I, we provided examples of using a personalized approach in CML management by describing the case scenarios of two BP-CML patients in which genetic and drug sensitivity data were incorporated into clinical decisions. The mutational profile of the first patient revealed *RUNX1* (p.R177Q) and *ABL1* (p.T315I) mutations at diagnosis. In concordance with genetic data, drug screening pinpointed the *ABL1*-T315I inhibitor ponatinib, as well as *RUNX1*-mutation related active drugs like glucocorticoids,[328] mTOR,[329] and VEGFR inhibitors.[330] The patient was treated with the VEGFR inhibitor, axitinib, and achieved partial remission. Relapse was associated with the eradication of the original clone and loss of sensitivity to the administrated drug as well as other active drugs in the diagnosis sample. Another clone that was marked with an *EZH2* mutation and a downregulation of EZH2/PRC2 complex target genes[331] had expanded, leading to the relapse. The second patient scenario showed similar results, where the same integrative approach identified *ABL1* (p,T315), *MSH6* (p.E226fs), and *SETD1B* (p.K1717X), pinpointing axitinib to be an active drug. The axitinib treatment was successfully associated with remission. Further investigations are warranted to overcome short-term responses to DSRT-based targeted therapy caused by expansion of resistant clones, which eventually leads to relapse. Strategies combining targeted therapy with other approaches, such as immunotherapy or chemotherapy, may enable efficient eradication of leukemic cells with further incorporation of the personalized approach in clinical care.

13.2. *RUNX1* mutations in BP CML

Given the high frequency of *RUNX1* mutations in BP-CML,[8] as well as the association of *RUNX1* mutations with distinct criteria in other leukemias such as AML,[332,333] we selected *RUNX1* mutations to serve as a model driver mutation in CML progression and investigated its potential impact on CML management in study II. *RUNX1* mutations were identified in 35% of the BP-CML patients and were evenly distributed between the lymphoid and myeloid phenotypes. All identified mutations were located in the RUNT domain, in line with previous reports,[334,335] and have been shown to

affect protein dimerization or DNA binding.[336] The driver role of the *RUNX1* mutation was best shown in a patient with matched CP and myeloid BP samples, where the *RUNX1* mutation was the sole leukemia-associated mutation in both phases. Progression to BP was associated with the loss of the *RUNX1* wild-type allele. This agrees with another study that reported the presence of the *RUNX1* mutation in LSCs from both the CP and BP of a lymphoid BP patient.[167]

We have identified a novel mechanism by which *RUNX1* mutations contribute to mutagenesis in BP-CML through aberrant activation of the AID/RAG axis, not only in lymphoid BP but also, interestingly, in myeloid BP samples with *RUNX1* mutations. The *RUNX1* gene is important for the physiological RAG activity in T-cell development.[337] Additionally, the off-target activity of the AID/RAG axis has been identified as a main mutagenic driver in *ETV6-RUNX1* ALL.[315] In our data, *RUNX1* mutations were associated with enrichment of the SHM signature-9 variants, related to AID/RAG activity in BP samples. Furthermore, the canonical RSS motif of the RAG enzyme was enriched in sequences spanning the SV breakpoints in *RUNX1*-mutated samples in comparison to *RUNX1* wild-type samples. Moreover, the genes encoding several components of the AID/RAG axis were specifically upregulated in the *RUNX1*-mutated samples. We identified RAG-RSS around an *IKZF1* deletion, highlighting the contribution of RAG activity to the aberrations affecting known leukemia-genes. AID activity has been suggested as a mechanism of drug resistance and lymphoid progression in CML.[338] A recent study has highlighted the central role of RAG off-target activity in CML progression to lymphoid BP.[316] In line with our data, they reported a *RUNX1*-mutated myeloid BP patient as an outlier of other myeloid BP samples, with a high RAG expression and an aberrant lymphoid antigens expression.

The characterization of *RUNX1*-mutated BP-CML patients revealed several genomic and phenotypic similarities with *RUNX1*-mutated AML, underscoring a specific signature of *RUNX1* mutations across leukemias. The mutational landscape of *RUNX1*-mutated BP-CML revealed coexisting mutations of the *PHF6* and *BCORL1* genes, as well as deletions of the *IKZF1* genes, comparable to *RUNX1*-mutated AML.[332,339] Transcriptional profiling revealed the upregulation of HSCs, lymphoid, and pDCs transcription factors and markers in association with *RUNX1* mutations in BP-CML, similar to *RUNX1*-mutated AML.[340–342] In line with gene expression profiles, the *RUNX1*-mutated myeloid BP-CML samples frequently showed aberrant expression of lymphoid antigens, such as CD19 and CD7, which is consistent with the phenotypic criteria of AML patients with *RUNX1* mutations and translocations.[342,343] Furthermore, a recent study has described a provisional AML entity—"AML with pDC differentiation," that frequently shows *RUNX1* mutations and cross-lineage antigen expression.[344] In agreement, we demonstrated a notable *RUNX1*-mutated upregulation of key pDCs transcription factors—namely, *IRF8*—and enrichment of the pDC population in BP-CML patients. Moreover, the *RUNX1*-mutated BP-CML

samples revealed a downregulation of megakaryocytic TFs and the coagulation pathway, in line with the reported role of *RUNX1* mutations in FPD/AML pathogenesis.[345]

The common transcriptional and phenotypic signatures of mutated *RUNX1* between BP-CML and AML were further reflected in drug sensitivity profiles. We demonstrated specific sensitivity of the *RUNX1*-mutated BP samples to glucocorticoids, mTOR, VEGFR, and BCL2 inhibitors, previously reported to specifically inhibit the *RUNX1*-mutated AML.[328–330,346] Interestingly, *RUNX1* mutations type and the residual activity of wild type *RUNX1* were correlated to glucocorticoid sensitivity in *RUNX1*-mutated AML patients,[328] in line with the variation of drug responses in our data that was noted in association with different *RUNX1* mutation types. Furthermore, transcriptional data underscored the potential mechanisms of some of the identified drug sensitivities, such as the upregulation of the glucocorticoid receptor, agreeing with the findings for *RUNX1*-mutated AML.[328] Moreover, drug sensitivity data of cell line models identified other *RUNX1*-mutated selective drugs, such as BET and CDK inhibitors, in accord with recent studies on the *RUNX1*-mutated AML.[238,347] It is worthy to note that, in one of the two index cases that were presented in study I, the VEGFR inhibitor, axitinib, could efficiently eradicate the dominant *RUNX1*-mutated clone at diagnosis by the time of relapse, which highlights the selective sensitivity of the *RUNX1*-mutated clone to axitinib. Combinations of imatinib with selected drugs, such as dexamethasone and venetoclax, were associated with the enhanced killing of *RUNX1*-mutated blasts, which represents a promising strategy for future clinical trials in BP-CML, namely *RUNX1*-mutated patients.

Another translational novel finding in our study was the potential effectiveness of the CD19-CAR T-cell immunotherapy in killing the *RUNX1*-mutated BP-CML blasts. CAR T-cell therapy is a promising immunotherapeutic approach that has successfully been implemented in cancer management to target various targets,[348] such as the CD19-CAR T-cells treatment of B-cell malignancies.[349] In line with the identified association of the *RUNX1* mutations with the aberrant expression of CD19 in some BP-CML patients, we demonstrated a strong ex-vivo cytotoxic activity of CD19-CAR T-cells against *RUNX1*-mutated blasts that harbor the ABL1-KD T315I mutation in a sample from a lymphoid BP patient. Potent CD19-CAR T-cells cytotoxic activity was similarly identified against blasts from a myeloid BP patient, expressing CD19 on about 25% of blasts, highlighting the potential power of CAR T-cell therapy in management of BP-CML. In agreement with our findings, a recent case report described the ability of CD19-CAR T-cells to induce remission in an AML patient with the *RUNX1-RUNX1T1* fusion and an aberrant CD19 expression.[350] Given the reported benefits of combining CAR T-cell therapy with targeted therapy for inducing higher response rates,[351] we also investigated the potential value of combining the CD19-CAR T-cells with TKI treatment. Interestingly, combining CD19-CAR T-cells

with imatinib demonstrated enhanced killing of *RUNX1*-mutated blasts. Such a combined therapy approach would potentially reduce the possibility of CD-19 negative relapse that has previously been reported with CD19-CAR T-cell treatment in ALL.[352]

13.3. p190 BCR-ABL1 isoform in CP CML

We continued to investigate the potential impact of genomic data on CML management in study III by characterizing specific features of CP-CML patients who express the p190-BCR-ABL1 isoform. For that purpose, we applied genomic, phospho-signaling, and drug sensitivity profiling in order to study the differences between the p190 and p210-BCR-ABL1 isoforms in CML patients as well as in hematopoietic progenitor cell line models. CML patients solely expressing p190-BCR-ABL1 failed to achieve molecular responses or showed a primary resistance to frontline imatinib treatment, in line with previous clinical studies.[141,143,353] WES data from p190-CML patients revealed the absence of ABL1-KD mutations, suggesting that the mechanism underlying imatinib resistance is related to the p190 expression instead. Furthermore, the identified variants in p190-CML patients showed frequent involvement of epigenetic modifier genes, such as *ASXL1* and *DNMT3A*, in a manner comparable to the default landscape of CP-CML. Distinct hematological features, such as monocytosis, absence of basophilia, and presence of ACAs at diagnosis—which have previously been described to associate with the p190 expression in CML[7,143,354]—were also noted in our study.

By applying transcriptional and phospho-signaling analyses, we identified IFN/STAT1 as a key downstream pathway in the p190 cells from CML patients as well as cell line models. Interestingly, previous studies comparing the phosphorylation activities of p190 and p210 isoforms in cell line models have reported isoform-specific phosphorylation activities on STAT molecules, where p210 is associated with increased phosphorylation levels of STAT3 and STAT5, while p190 is associated with STAT1 and STAT6 hyperphosphorylation.[46,355] We provided novel findings of p190-specific activation of the IFN/STAT1 pathway in p190 CML patients, including transcriptional upregulation of IFN pathway and IFN downstream target genes as well as increased phosphorylation of STAT1 molecules. Moreover, we demonstrated similar transcriptional and phosphorylation activation of the IFN/STAT1 axis in CML progenitor cell line models. We also provided phenotypic evidence of IFN/STAT1 activity in the HPC-LSK model, where p190 cells demonstrated increased expression of sca-1 and Ly6c antigens, previously linked to IFN/STAT1 signaling.[356] STAT1 has been described as a potential direct target for p190 tyrosine kinase activity and was specifically enriched in the p190 interactome.[46,47] STAT1 hyperphosphorylation has additionally been linked to the activation of IFN signaling.[357] Activation of IFN/STAT1 signaling has been reported to correlate with sensitivity to IFNα treatment.[358] STAT1 signaling mediates the antiproliferative activity of IFNα therapy[359] and is critical for IFNα sensitivity in CML cells.[360] Together with the drug

sensitivity findings in p190 cell lines that show consistent—however modest—in vitro sensitivity to IFNα treatment, our results suggest that IFNα treatment is a potential therapeutic option to be further investigated for p190-CML patients.

We were also able to identify additional targetable signaling molecules, such as Src and PAK1 kinases, which showed specific hyperphosphorylation in p190-CML cells. Drug sensitivity profiles of patient samples, as well as cell line models, revealed specific sensitivity of the p190 cells to Src and PAK inhibitors. In line with our data, Src has been described to be enriched in the p190-interactome in cell line models,[46] while Src activity has been described to be critical for leukemogenesis of p190-ALL.[361] Furthermore, the broad spectrum TKI, dasatinib, that exerts Src inhibitory activity[362], has been described to inhibit Ph+ALL cells more than imatinib.[363] PAK1 has also been reported to play an important role in the pathogenesis of BCR-ABL1-induced leukemias.[364] Combining imatinib with PAK inhibitors has been demonstrated to have a synergistic inhibitory effect targeting CML cells.[365]

In line with transcriptional data highlighting the dysregulation of apoptosis pathways in p190-CML, drug screening in cell line models revealed isoform-specific responses to different families of apoptotic modulator drugs. The p190 cells demonstrated an increased sensitivity to IAPs inhibitors/SMAC mimetic and MDM2 antagonists, as well as a reduced sensitivity to BCL2 inhibitors in comparison to p210 cells. IAPs inhibitors suppress antiapoptotic molecules, such as cIAP1, cIAP2, and Survivin, and have recently been reported as potentially effective drugs in CML.[366,367] Additionally, p190-BCR-ABL1 was described as cooperating with Src signaling to activate the γ-catenin/MYC pathway, resulting in overexpression of Survivin in Ph+ALL.[368] This activated cascade was specific to p190 in comparison to p210, which potentially explains the enhanced sensitivity of p190 cells to IAPs inhibitors. Moreover, the activation of IFN signaling can sensitize cells to IAPs inhibitors. We demonstrated an enhanced expression of the IFN-induced proapoptotic XAF1 molecule, which has been described to form a complex with the XIAP molecule with further degradation of IAPs.[369] On the other hand, IAPs inhibitors activate IFN signaling in tumor cells,[370] which can further prime p190 cells for apoptosis. Additionally, p190 leukemic cells have been reported to show specific activation of the Vav3/Rac2/PAK/pBAD pathways in contrast to p210 cells,[371] which may partly explain their reduced sensitivity to BCL2 inhibitors.

Conclusions and future directions

This thesis provided novel insights and elucidated the molecular pathogenesis underlying high-risk CML groups.

- Somatic mutations in cancer genes (other than BCR-ABL1) are contributing to the pathogenesis of different CML phases. In CP, mutational burden and mutations in epigenetic modifier genes represent potential predictors of treatment outcomes.
- In BP, accumulation of mutations in cancer genes, including SNVs and fusion genes, in addition to transcriptional dysregulation of key signaling pathways, such as JAK-STAT pathway, are playing driver roles in disease progression, and represent potential targets for targeted therapy.
- Mutational signature analysis highlighted the potential contribution of novel mechanisms in CML pathogenesis such as AID/RAG off-target activity and UV-related aberrations.
- *RUNX1* mutations are recurrent events in BP-CML associated with distinct phenotypic and genetic characteristics and activation of mutagenic AID/RAG axis.
- Phenotypic and transcriptional features of *RUNX1*-mutated BP-CML were comparable to the features of *RUNX1*-mutated AML patients, and were translated into specific drug response profile and sensitivity to CD19 CAR T-cell therapy.
- p190-CML revealed transcriptional and signaling activation of specific pathways, such as the IFN/STAT1 and apoptotic pathways, and also activated the Src and PAK1 key signaling molecules, which was associated with increased sensitivity to therapies targeting these pathways.
- A personalized approach that incorporates genetic and drug sensitivity data was highlighted as a potential strategy in the management of BP-CML patients.

The results provided novel proofs of the impact of somatic mutations on clinical outcomes of CML patients. Despite the central role played by BCR-ABL1 in CML pathogenesis, somatic mutations in other cancer-related genes contribute to the clinical heterogeneity of CML under TKI treatment. Further evidence about the importance of genetic data in different CML phases was obtained by characterizing the role of *RUNX1* mutations in BP-CML as well as the role of p190-BCR-ABL1 isoforms in CP-CML. One limitation of this project, which is present in other genetic CML studies, was the scarcity of available samples from BP-CML patients. Further studies are, hence, warranted in order to more comprehensively characterize the mutational spectrum of CML patients as well as to establish the prognostic role of somatic mutations in CML. Large-scale studies would also enable identification of driver mutations in BP-CML, such as *RUNX1,* and provide a higher resolution look at the impact of these mutations and identify potentially effective targeted therapies. Additionally, such studies would allow better characterization of rare CML patients groups of high risk, such as p190-CML, with potentials to improve management of these patients by, for example, assigning them to frontline dasatinib treatment.

Small cohort sizes, scarcity of available samples, heterogeneity of the applied sequencing methods as well as variations of the data analysis approaches, are challenging issues which are present in studies of CML genetics, to date, and hinder better understanding of the data. Meta-analysis of the previously published genetic data of CML patients is one way to provide a better overview of the mutational landscape of CML and an accurate estimation of the prognostic value of somatic mutations. In addition, international collaborative work, such as the "iCMLf Genomics Alliance," would enable better use of genomic resources from different local CML research projects that lead to development of a genomic risk score with potential clinical benefits. Additionally, DNA methylation profiles of CML patients at different phases remain to be characterized; given the frequency of mutations involving epigenetic modifier genes.

Another factor to be investigated in future work is the clinical relevancy of the identified mutations to CML prognosis. Functional studies, including modeling of somatic mutations by gene editing techniques like CRISPR technology, can provide deeper insights into the pathogenic role of somatic mutations in CML. Validation of the impact of specific gene mutations, such as mutations in epigenetic modifier and leukemia-associated genes, on treatment outcomes and progression will facilitate the design of gene panel sequencing that can be incorporated into routine clinical care for CML patients. By shifting the goal of CML management toward TFR achievement, the impact of somatic mutations on the chance of achieving TFR represents another interesting future direction. Mutations in epigenetic modifier genes, as well as CHIP-related mutations, were identified in low frequency in optimal responder CML patients. The outcome of TKI discontinuation in such patients remains to be investigated.

Given the potentially promising targeted therapies identified via ex-vivo drug testing, further research is warranted to enable the clinical translation of these results. Use of the mouse model is an efficient method for studying the in-vivo effects of candidate drugs identified by ex-vivo DSRT. Moreover, clinical trials would then be the subsequent step for successful drug candidates. International networking is still required to overcome the challenge of a small number of BP patients in local centers. Another concern of the application of targeted therapy through a personalized approach is the clonal heterogeneity of CML, which underlies treatment resistance and disease relapse. Combination of targeted therapy with other synergistic drugs or immunotherapy could, potentially, enable efficient eradication of leukemic cells and minimize the development of resistant clones. Recently, a computational approach that combined scRNA sequencing data with ex-vivo DSRT data enabled the prediction of clone-specific drug sensitivities and the identification of potentially synergistic patient-specific drug combinations.[372]

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