Targeting residual malignant cells in myeloid leukemia

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To my family

ABSTRACT

Malignant cells persisting during treatment prevent cure in many patients with myeloid leukemia. In acute myeloid leukemia (AML), the failure to eradicate the leukemic clone during conventional chemotherapy is associated with leukemic relapse, mostly with dismal survival outcome. Chronic myeloid leukemia (CML) is often successfully treated with targeted tyrosine kinase inhibitors (TKI). However, persisting treatment-resistant leukemic stem cells (LSC) put the patients at risk for acquired TKI resistance, relapse, or disease progression. This thesis encompasses studies ultimately aimed at facilitating the elimination of residual malignant cells in myeloid leukemia. AML is a heterogeneous disease in which subpopulations of patients may benefit from distinct treatment approaches. In **papers I and II**, we identified younger patients in first complete remission with chemotherapy-sensitive, normal karyotype AML (without *FLT3* mutation) as a new target group that may benefit from relapse-preventive immunotherapy with histamine dihydrochloride (HDC) and low-dose interleukin 2 (IL-2). In this group of patients, HDC/IL-2 may help prevent the expansion of residual leukemic cells and thus improve the chances of long-term relapse-free survival. In **paper III**, we performed an unprecedently detailed multiomic single-cell characterization of the $CD34^+$ stem and progenitor cell (SPC) compartment in CML bone marrow and compared it to that of healthy bone marrow. Through development of a method allowing detection of pathognomonic *BCR-ABL1* expression at the single-cell level, we identified a group of LSC displaying a TKI-resistance phenotype and defined novel expression patterns within this group of cells, including expression of von Willebrand factor and TIM3. Additional findings carried implications for the understanding of differences between leukemic and normal hematopoiesis and the phenotypic definition of CML LSC. **Paper IV** addressed effects of cytoreductive hydroxyurea (HU) treatment on CML SPC. The results revealed HU-induced hemoglobin expression in erythrocyte progenitors and signs of treatment-induced S phase arrest at all maturation stages within the CML SPC compartment. Taken together, the results presented in this thesis may have implications for future relapse-preventive treatment decisions in AML and studies of the TKI-resistant LSC population in CML, thus contributing to the targeting of residual disease in the two primary forms of myeloid leukemia.

Keywords: acute myeloid leukemia, chronic myeloid leukemia, histamine dihydrochloride, hydroxyurea, leukemic stem cells

SAMMANFATTNING PÅ SVENSKA

Leukemi är ett samlingsnamn för en grupp maligna sjukdomar som kännetecknas av onormal expansion av blodceller av varierande mognadsgrad i blod och benmärg. Vid myeloisk leukemi liknar den expanderande cellklonen de celler som normalt ger upphov till röda blodkroppar, blodplättar eller celltyper inom det medfödda immunförsvaret (såsom monocyter och granulocyter). De två huvudtyperna av myeloisk leukemi är akut myeloisk leukemi (AML) och kronisk myeloisk leukemi (KML).

AML är en heterogen och allvarlig form av leukemi. Trots att majoriteten av patienterna svarar väl på inledande cellgiftsbehandling kommer sjukdomen i många fall tillbaka, med dålig prognos för långtidsöverlevnad. De kliniskt utmanande återfallen tros härröra från ofullständig eliminering av leukemiceller under cellgiftsbehandlingen, och utveckling av metoder för att bli av med de kvarvarande cellerna är därför angeläget. En möjlig strategi för att åstadkomma detta är genom immunterapi, d.v.s. behandling som syftar till att förbättra immunförsvarets möjligheter att känna igen och döda kvarvarande leukemiceller. Vid AML har immunterapeutisk behandling med histamindihydroklorid (HDC) och interleukin-2 (IL-2) efter avslutad cellgiftsbehandling visat sig ha återfallsförebyggande effekt. Det är dock möjligt att vissa AML-patientgrupper har större nytta av HDC/IL-2-behandling än andra, och analys av behandlingsutfall i patient-subgrupper kan följaktligen leda till att behandlingen kommer rätt individer till gagn. **Delarbete I och II** syftade till att identifiera vilka patientgrupper som har störst nytta av HDC/IL-2 behandling vid AML, med fokus på skillnader i inledande svar på cellgiftsbehandling (**delarbete I**) och genetiska avvikelser i de leukemiska cellerna (**delarbete II**). Avhandlingens resultat indikerar att återfallsförebyggande behandling med HDC/IL-2 bör ges till individer med leukemiceller utan kromosomavvikelser som uppnår remission efter en cellgiftsbehandlingscykel.

Avhandlingens andra del rör en mindre aggressiv släkting till AML, KML. KML-patienter behandlas ofta framgångsrikt med tyrosinkinashämmare, men behandlingen efterlämnar ofta resistenta leukemiska stamceller (LSC), som åter kan orsaka sjukdom om behandlingen avslutas, eller i vissa fall ge upphov till behandlingsresistens eller sjukdomsprogression. Även vid KML är det således av intresse att identifiera behandlingar för att avlägsna kvarvarande leukemiceller. Ett viktigt fält inom nutida cancerforskning är målinriktad behandling; att utnyttja faktorer som skiljer cancercellerna från deras friska motsvarigheter för att selektivt döda de maligna cellerna utan att skada närliggande friska celler. Detta

angreppssätt kräver dock ingående förståelse av cancercellerna och deras utmärkande attribut.

För att öka förståelsen för KML-LSC och identifiera möjliga angreppspunkter för framtida målinriktad behandling genomfördes i **delarbete III och IV** detaljerade gen- och proteinuttrycksanalyser på encellsnivå där KML-stamceller jämfördes mot friska stamceller. Resultaten i **delarbete III** tydde på skillnader i utmognadsmönster mellan friska och leukemiska stamceller. Därutöver fann vi att KML-LSC uttryckte högre nivåer av von Willebrands faktor och TIM3 än friska stamceller. Dessa markörer har således potential att i framtiden fungera som biomarkörer för KML-LSC som skulle kunna användas för riktad analys av KML-LSC eller för målinriktad behandling. I **delarbete IV** visades att inledande hydroxyureabehandling, som många patienter får i väntan på KML-diagnos, påverkar proportionerna av olika omogna celltyper, vilket kan beaktas i framtida studier av KML-stamceller.

Sammanfattningsvis har avhandlingens delarbeten avsett att bidraga till en ökad kunskap inom områden av relevans för eliminering av behandlingsresistenta celler vid myeloisk leukemi. Avhandlingen har utmynnat i (i) identifikation av en AML-patientgrupp som sannolikt drar nytta av återfallsförebyggande behandling med HDC/IL-2, och (ii) djupare förståelse för den leukemiska stamcellspopulationen i KML, som i framtiden skulle kunna ligga till grund för LSC-riktad behandling.

LIST OF PAPERS

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

I. **Nilsson MS**, Hallner A, Brune M, Nilsson S, Thorén FB, Martner A, Hellstrand K. Complete remission after the first cycle of induction chemotherapy determines the clinical efficacy of relapse-preventive immunotherapy in acute myeloid leukaemia.
Br J Haematol. 2020;188(4):e49-e53.

II. **Nilsson MS**, Hallner A, Brune M, Nilsson S, Thorén FB, Martner A, Hellstrand K. Immunotherapy with HDC/IL-2 may be clinically efficacious in acute myeloid leukemia of normal karyotype.
Hum Vaccin Immunother. 2020;16(1):109-111.

III. **Nilsson MS***, Komic H*, Sheybani Z, Paul S, Rolfson O, Hellstrand K, Wennström L, Martner A#, Thorén FB#. Multiomic single-cell analysis of the $CD14^{-}CD34^{+}$ HSPC compartment in chronic myeloid leukemia identifies von Willebrand factor and TIM3-expressing *BCR-ABL1*$^{+}$ leukemic stem cells in aberrant myeloid-biased hematopoiesis.
In manuscript.
*,# Authors contributed equally

IV. Komic H*, **Nilsson MS***, Wennström L, Thorén FB#, Martner A#. Single-cell proteo-transcriptomic profiling of leukemic stem and progenitor cells in patients receiving cytoreductive hydroxyurea in early-phase chronic myeloid leukemia.
In manuscript.
*,# Authors contributed equally

Additional publications not part of this thesis:

S1. Sander FE, **Nilsson M**, Rydström A, Aurelius J, Riise RE, Movitz C, et al. Role of regulatory T cells in acute myeloid leukemia patients undergoing relapse-preventive immunotherapy.
Cancer Immunol Immunother. 2017;66(11):1473–1484.

S2. Bernson E, Hallner A, Sander FE, Nicklasson M, **Nilsson MS**, Christenson K, et al. Cytomegalovirus serostatus affects autoreactive NK cells and outcomes of IL2-based immunotherapy in acute myeloid leukemia.
Cancer Immunol Res. 2018;6(9):1110–1119.

S3. Kiffin R, Grauers Wiktorin H, **Nilsson MS**, Aurelius J, Aydin E, Lenox B, et al. Anti-leukemic properties of histamine in monocytic leukemia: the role of NOX2.
Front Oncol. 2018;8:218.

S4. Dolatabadi S, Jonasson E, Lindén M, Fereydouni B, Bäcksten K, **Nilsson M**, et al. JAK-STAT signalling controls cancer stem cell properties including chemotherapy resistance in myxoid liposarcoma.
Int J Cancer. 2019;145(2):435–449.

S5. Grauers Wiktorin H, **Nilsson MS**, Kiffin R, Sander FE, Lenox B, Rydstrom A, et al. Histamine targets myeloid-derived suppressor cells and improves the anti-tumor efficacy of PD-1/PD-L1 checkpoint blockade.
Cancer Immunol Immunother. 2019;68(2):163–174.

S6. Rugwizangoga B, Andersson ME, Kabayiza JC, **Nilsson MS**, Ármannsdóttir B, Aurelius J, et al. IFNL4 genotypes predict clearance of RNA viruses in Rwandan children with upper respiratory tract infections.
Front Cell Infect Microbiol. 2019;9:340.

S7. Johansson J, Kiffin R, Aydin E, **Nilsson MS**, Hellstrand K, Lindnér P, et al. Isolated limb perfusion with melphalan activates interferon-stimulated genes to induce tumor regression in patients with melanoma in-transit metastasis.
Oncoimmunology. 2019;9(1):1684126.

CONTENT

ABBREVIATIONS

ALL	Acute lymphoblastic leukemia
Allo-hSCT	Allogeneic hematopoietic stem cell transplantation
AML	Acute myeloid leukemia
AP	Accelerated phase
APL	Acute promyelocytic leukemia
ATO	Arsenic trioxide
ATRA	All-trans retinoic acid
BC	Blast crisis
BM	Bone marrow
BMM	Bone marrow microenvironment
CAR T cell	Chimeric antigen receptor T cell
CBF	Core binding factor
CCyR	Complete cytogenetic response
CHIP	Clonal hematopoiesis of indeterminate potential
CLP	Common lymphoid progenitor
CML	Chronic myeloid leukemia
CMP	Common myeloid progenitor
CP	Chronic phase
CR	Complete remission
CR1	First complete remission
CSC	Cancer stem cell
DMR	Deep molecular response
DNA	Deoxyribonucleic acid

dNTP	Deoxyribonucleoside triphosphate
ELN	European LeukemiaNet
EMA	European Medicines Agency
FAB	French-American-British
FACS	Fluorescence-activated cell sorting
FDA	Food and Drug Administration
FISH	Fluorescence in situ hybridization
GMP	Granulocyte-monocyte progenitor
GO	Gemtuzumab ozogamicin
GvHD	Graft versus host disease
GvL	Graft versus leukemia
H2R	Histamine H2 receptor
HDC	Histamine dihydrochloride
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cells
HU	Hydroxyurea
IFN-α	Interferon alpha
IL	Interleukin
ITD	Internal tandem duplication
LFS	Leukemia-free survival
L-IC	Leukemia-initiating cell
LMPP	Lymphoid-primed multipotent progenitor
LSC	Leukemic stem cell
LT-HSC	Long-term hematopoietic stem cell

MDS	Myelodysplastic syndrome
MEP	Megakaryocyte-erythrocyte progenitor
MMR	Major molecular response
MNC	Mononuclear cell
MPP	Multipotent progenitor
MRD	Minimal residual disease
mRNA	Messenger RNA
NK cell	Natural killer cell
NOX2	NADPH oxidase 2
NSG	NOD-*scid* IL2Rg^{null}
OS	Overall survival
PB	Peripheral blood
PCA	Principal component analysis
PCR	Polymerase chain reaction
PBMC	Peripheral blood mononuclear cell
RNA	Ribonucleic acid
RNAseq	RNA sequencing
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
scRNAseq	Single-cell RNA sequencing
scRT-qPCR	Single-cell RT-qPCR
SPC	Stem and progenitor cells
ST-HSC	Short-term hematopoietic stem cell

TFR	Treatment-free remission
TKI	Tyrosine kinase inhibitor
Treg	Regulatory T cell
TRM	Treatment-related mortality
UMAP	Uniform manifold approximation and projection
UMI	Unique molecular identifier
WHO	World Health Organization

PREFACE

Leukemia is a heterogeneous group of malignancies, collectively characterized by abnormal proliferation and accumulation of certain hematopoietic cell types in blood and bone marrow. Depending on the developmental lineage of the expanding cells and disease severity, leukemias are principally classified as lymphoid or myeloid, and chronic or acute. While chronic leukemias tend to progress slowly and maintain some degree of normal blood function, acute leukemias are characterized by an explosive overgrowth of immature cells unable to generate or perform the functions of mature blood cells, thus rapidly creating deficiencies in indispensable blood cell functions.

The treatment of leukemia (and many other cancer types) was revolutionized with the introduction of chemotherapy, first reported in the 1940s (1,2). In view of cancer at its core being characterized by abnormal rapid cell growth, chemotherapeutic agents aim to interfere with cell division. The stress induced in dividing cells through the actions of chemotherapy may result in their death, thus actively targeting and reducing the number of cancerous cells. Although chemotherapy remains a cornerstone in leukemia treatment until this day, it is inherently limited in that cell growth is not unique to malignant cells. Consequently, there is a fine line between efficient eradication of leukemic cells and toxicity to neighboring normal tissues, effectively placing a biological ceiling to the achievable dose and duration of chemotherapy. Malignant cells left behind at the inevitable treatment cessation are likely to cause leukemic relapse, with poor outlook for long-term survival.

Starting in the 1980s, the field of cancer medicine thus began to shift towards targeted therapy - finding vulnerabilities specific to the cancer cells and exploiting these for therapeutic targeting with minimal collateral damage to neighboring healthy cells. Inevitably, such an approach requires a deep understanding of the cancer cell itself. One of the first successful examples, and a striking proof-of-concept for targeted therapy, was the development of the tyrosine kinase inhibitor (TKI) imatinib following the discovery of the disease-causing BCR-ABL1 oncoprotein in chronic myeloid leukemia (CML) (3,4). Since its introduction, imatinib and later generation TKIs have completely transformed CML patient care and prognosis (5). However, an increasing body of evidence points to the persistence of TKI-resistant leukemic stem cells (LSC) during treatment. Thus, significant effort is currently put into finding features of the LSC that may allow also their targeted eradication and the ultimate cure of CML.

A different angle to targeted therapy is attempts at utilizing the body's own cancer cell recognition and killing machinery – the immune system – to eradicate residual leukemic cells (6). In acute myeloid leukemia (AML), this is exemplified by the use of histamine dihydrochloride (HDC) and low-dose interleukin-2 (IL-2) for relapse-preventive treatment following the completion of chemotherapy (7). Since AML, unlike CML, is a genetically and morphologically heterogeneous disease, it is conceivable that certain groups of patients carry leukemic clones that are more susceptible to HDC/IL-2-induced immune-mediated eradication than others. Identifying such patient groups to enable better-informed treatment decisions is an important aspect of current cancer therapy.

This thesis is focused on different aspects of importance for targeting the therapy-eluding residual malignant cells in myeloid leukemia. In AML (**papers I and II**), we aimed to contribute to the identification of patient groups in which HDC/IL-2 may allow immune-mediated targeting of residual leukemic cells. In hopes of revealing future targeting possibilities, **papers III and IV** instead focused on contributing to the basic understanding of the TKI-resistant LSC in CML.

INTRODUCTION

HEMATOPOIESIS

Although not visible to the naked eye, blood is a complex liquid connective tissue, essential to life in more ways than one. Suspended in a matrix of blood plasma are a range of cells and proteins that collaborate to carry out three broad functions: (i) transportation of oxygen, carbon dioxide, nutrients, waste and hormones to and from body cells, (ii) regulation of pH and body temperature, and (iii) protection against excessive blood loss and disease (8). While some of these functions mainly depend on the liquid nature of the tissue (i.e. nutrient/waste transportation and pH/temperature regulation), others are carried out by functionally specialized cells or cell fragments circulating throughout our bodies. Among these, erythrocytes (red blood cells) are responsible for gas transportation, thrombocytes (platelets) are involved in the process of hemostasis upon blood vessel injury, and leukocytes (white blood cells) make up the cellular portion of the immune system that provides protection against infection. The leukocyte group can be further divided into granulocytes (neutrophils, eosinophils, basophils, and mast cells), monocytes and lymphocytes (T cells, B cells and natural killer cells). Of relevance to this thesis work, subsets of leukocytes – i.e. cytotoxic lymphocytes: $CD8^+$ T cells and natural killer (NK) cells – additionally play a role in the surveillance, recognition and elimination of abnormal cells. This is e.g. evidenced by increased cancer susceptibility in mice lacking T and NK cell effector functions (9,10), improved survival rates for patients with tumor infiltration of these cell subsets in various solid cancer types (11–14), and reduced cancer risk in individuals with medium to high lymphocyte cytotoxic activity at baseline in an 11-year follow-up study (15).

The majority of the mature blood cells have relatively short life-spans, necessitating their continuous renewal (8). This is accomplished through the process of hematopoiesis, which, in adults, primarily takes place in the bone marrow. In the current consensus view of hematopoiesis, rare multipotent hematopoietic stem cells (HSC), able to give rise to all differentiated blood cells, reside at the top of a hierarchical structure. The generation of mature blood cells from HSC involves gradual changes to the expression of genes involved in hematopoietic lineage commitment, meaning that the process of differentiation effectively progresses through a set of intermediate cell types of increasing maturity, collectively termed hematopoietic progenitors (16). Unlike downstream

progenitors, the HSC by definition carry self-renewal capacity, enabling the maintenance of the pool of undifferentiated cells throughout life (17).

Some of the first definitive signs pointing to the existence of a multipotent HSC population came from studies were irradiation-induced bone marrow failure in mice could be rescued by injection of unirradiated rat bone marrow cells (18,19). Building on this finding, HSC are traditionally defined by their capacity to reconstitute all major blood cell types in various types of immunocompromised mice. A few decades later, the study of hematopoietic stem and progenitor cells (HSPC) was advanced with the development of flow cytometry and fluorescence-activated cell sorting (FACS), enabling the definition and isolation of specific hematopoietic stem and progenitor cell populations by surface protein expression, and subsequent functional assessment of isolated cells. Seminal work demonstrating the viability of positive/negative selection of mouse bone marrow HSC based on surface marker expression was reported in 1988 (19). In the following years, experiments employing similar principles to the characterization of hematopoietic progenitors (20–23) gave rise to the hematopoiesis model depicted in most textbooks to this day (referred to below as "the traditional model"). Today, the phenotypic compartment holding human HSC is believed to be $Lin^-CD34^+CD38^{-/low}CD45RA^-CD90^+CD49f^+$, as evidenced by single-cell transplantation to NOD-*scid* $IL2Rg^{null}$ (NSG) mice (24). Similarly, the collection of relatively immature HSPC are thought to reside within the $CD34^+$ compartment (25).

THE TRADITIONAL MODEL

Starting in the flow cytometry-based immunophenotyping era, hematopoiesis has been modeled in a stepwise, tree-like structure, where differentiation involves the progression through discrete sets of multipotent, oligopotent, bipotent, and eventually unipotent, progenitors. By the end of the last century, the top position of the hematopoietic tree was held by long-term HSC (LT-HSC), which sequentially gave rise to short-term HSC (ST-HSC) and multipotent progenitors (MPP), defined by decreasing self-renewal capacity and reduced or non-existent long-term engraftment potential in immunodeficient mice (26–28). Below the multipotent progenitors, an initial bifurcation between common lymphoid progenitors (CLP) and common myeloid progenitors (CMP) resulted in the early separation of differentiation trajectories for cells of myeloid and lymphoid cell lineages. In the myeloid lineage, subsequent branching steps gave rise to erythrocytes, thrombocyte-producing megakaryocytes, granulocytes, and

monocytes through megakaryocyte-erythrocyte progenitors (MEP) and granulocyte-monocyte progenitors (GMP), while CLP sequentially differentiated into B, T and NK cells (Figure 1A).

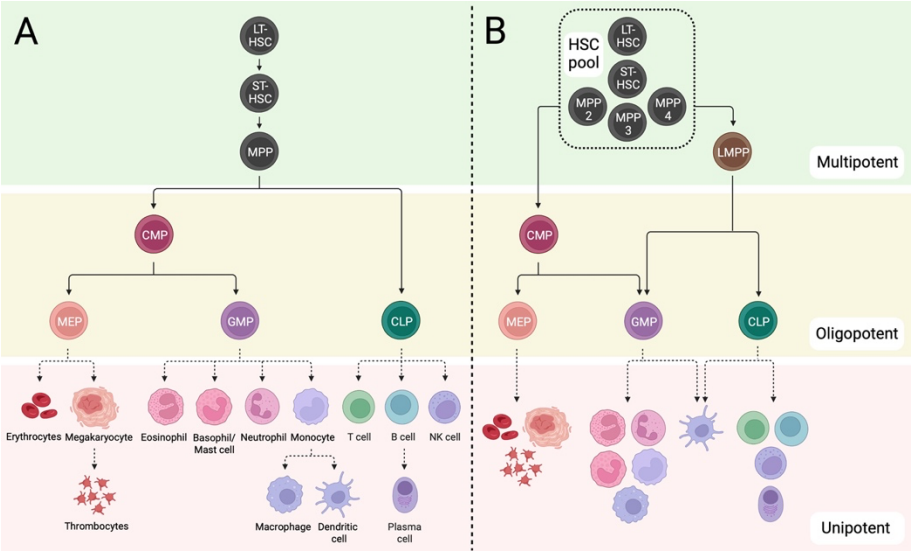

Figure 1. Immunophenotype-based models of hematopoiesis. (A) The traditional hematopoietic model. (B) An adapted model of hematopoiesis. For simplicity, various precursor cells downstream of the oligopotent progenitors have been omitted. LT-HSC, long-term hematopoietic stem cell; ST-HSC, short-term hematopoietic stem cell; MPP, multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte-erythrocyte progenitor; GMP, granulocyte-monocyte progenitor; LMPP, lymphoid-primed multipotent progenitor. Created with BioRender.com.

AN ADAPTED MODEL

Over subsequent decades, functional assessment of novel progenitor cell types based on the discovery of new cell surface markers resulted in several alterations to the original hematopoietic model. The Jacobsen group reported the existence of a lymphoid-primed multipotent progenitor (LMPP) population able to give rise to mature cell types downstream of CLP and GMP, but not those stemming from MEP, indicating that myeloid and lymphoid cell lineages may stay connected longer than previously believed (29). Additional revisions were founded in discoveries regarding heterogeneity within the MPP compartment, where three MPP subtypes were suggested based on distinct immunophenotypes and lineage biases (30). The consequent revisions to the hematopoietic tree (Figure 1B) suggested that lineage fate decisions may (i) occur higher in the hierarchy, and (ii) not be as binary, as previously thought.

THE CONTINUOUS MODEL

Although the concept of HSC-derived generation of mature blood cells through gradual differentiation remains intact, the specific details of hematopoiesis have undergone substantial scrutiny in recent years (27,28,31). Just like the introduction of immunophenotypic cell sorting once provided the foundation for the understanding of the blood cell forming process, the advent of high-throughput transcriptome-wide single-cell RNA sequencing (scRNAseq) technologies allowing multidimensional assessment of gene expression in thousands of individual HSPC is currently transforming hematopoiesis research. In view of regulation of gene expression lying at the core of lineage fate decisions and cell identity, scRNAseq-derived expressional snapshots of the HSPC compartment carry the potential for unprecedentedly detailed analysis of cell type heterogeneity as well as inference of differentiation routes among hematopoietic progenitors (28).

A growing body of evidence from scRNAseq studies indicates that the traditionally defined hematopoiesis model may be an oversimplification of HSPC complexity. The tree-like structures of the models presented in Figure 1 rest on functional analyses of immunophenotypically defined cell types, inevitably entailing assumptions of functional homogeneity within cell surface marker-defined populations. This has been challenged in contemporary single-cell studies, where traditionally defined progenitor cell types (e.g. LMPP and CMP) were found to be strikingly heterogeneous and consist of cells almost exclusively unipotent in mature cell output (24,32). A recent scRNAseq study further reported the presence of distinct neutrophil-primed and monocyte/dendritic cell progenitors within the traditionally defined GMP compartment (33). The presence of unipotent progenitors of varying cell fates within immunophenotypically defined progenitor populations may thus falsely have made them appear oligopotent in bulk analyses.

Unlike FACS-based studies of hematopoiesis, scRNAseq approaches do not inherently require the subjective definition of cell types based on cell surface marker expression. Instead, gene expression data from heterogeneous groups of cells can be used to create transcriptional maps, positioning each cell near other cells with similar expression patterns and further from cells that are transcriptionally different. Such an approach applied to the HSPC compartment thus allows tracing of hematopoietic differentiation trajectories based on gradual changes to the expression profiles of individual progenitor cells (Figure 2A).

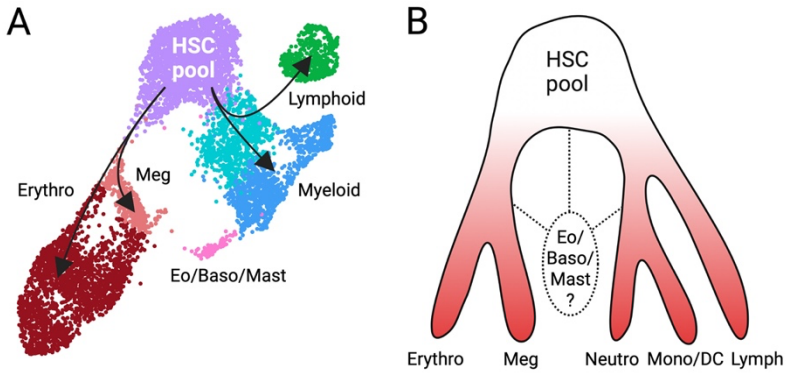

Figure 2. scRNAseq-based hematopoiesis. (A) A representative example of scRNAseq-based organization of HSPC based on gene expression in individual cells. (B) An illustration of the continuous model of hematopoiesis. Erythro, erythrocyte progenitors; Meg, megakaryocyte/thrombocyte progenitors; Eo/Baso/Mast, eosinophil-basophil-mast cell progenitors; Neutro, neutrophil progenitors; Mono/DC, monocyte-dendritic cell progenitors; Lymph, lymphocyte progenitors. Created with BioRender.com.

In recent years, several scRNAseq-based studies assessing transcriptional heterogeneity within the HSPC compartment have been reported (33–36). A central finding among these is the absence of discrete cell types among immature hematopoietic progenitors. Hence, hematopoiesis seemingly occurs through continuous, gradual advancement along differentiation lineages, rather than through stepwise progression via discrete progenitor cell types as suggested in traditional models. In addition, transcriptional priming events associated with lineage commitment may occur earlier than previously assumed; already at the stage of the most immature HSC there is evidence for lineage-bias and/or lineage-restriction. Several studies report an early bifurcation of megakaryocytic-erythroid and lympho-myeloid differentiation trajectories (33–35), thus additionally challenging the myeloid/lymphoid branching of the traditional hematopoietic tree. In the scRNAseq-revised model of hematopoiesis (Figure 2B), differentiation trajectories towards erythrocyte, megakaryocyte, eosinophil-basophil-mast cell, monocyte-dendritic cell, neutrophil and lymphoid progenitors are starting to emerge (28,33,34). However, the struggle to reconcile data from past and present studies is ongoing, and further refinements to the understanding of hematopoiesis are thus highly likely.

LEUKEMIA

Throughout life, random mutations accumulate in host genomes due to internal and external stress. While some occur through exposure to mutagenic substances or radiation, others are the result of occasional DNA replication errors during cell division. The first reports demonstrating the link between alterations to normal cellular genes and carcinogenesis were published in the 1970s (37,38). Since then, an accumulating body of research indicates that transformation from normal to cancerous cells involves sequential acquisition of mutations affecting genes with roles in cell proliferation, survival and apoptosis (39), ultimately unleashing a cell that will keep dividing without regard to the protective regulatory systems telling it to stop.

Cancer-causing mutations come in several different shapes, ranging from single base substitutions, segment deletions and insertions to more dramatic changes involving large-scale chromosomal rearrangements (deletions, duplications, inversions and translocations) (39). Mutational patterns vary greatly between cancer types. While some carry high mutational burden (e.g. lung cancer, colorectal cancer and melanoma), others are associated with relatively few genetic alterations (e.g. leukemia) (39,40). There is usually extensive heterogeneity also between patients diagnosed with the same type of malignancy (40). However, certain genetic abnormalities tend to recur among patients with the same cancer type, sometimes allowing for division of patients into subgroups of prognostic or therapeutic relevance.

By some estimates, the adult hematopoietic system produces 1.5-5 million blood cells each second to compensate the removal of aging cells (41,42). Extensive control mechanisms are thus in place to avoid catastrophic insufficiencies resulting from unbalanced production (41). However, the sheer number of cell divisions required to uphold hematopoietic function throughout life inevitably renders the hematopoietic stem and progenitor cells sensitive to random replication errors. Over time, the accumulation of genetic alterations may result in malignant transformation of hematopoietic subpopulations, leading to the development of different leukemic subtypes.

Leukemia, characterized by uncontrolled proliferation and accumulation of abnormal hematopoietic progenitor populations in blood and bone marrow, is a clear example of the consequences of unbalanced and insufficient blood cell production. The buildup of malignant cells typically interferes with normal hematopoiesis and the symptoms, e.g. ranging from fatigue and excessive bleeding to susceptibility to infection, reflect ensuing deficiencies in blood cells with roles in oxygen transportation (erythrocytes), clot formation (thrombocytes)

and immune-mediated protection against infection (leukocytes), respectively. As mentioned in the preface, leukemias are principally divided into subtypes based on the hematopoietic lineage of the transformed cells. Myeloid leukemia involves excessive proliferation of erythroid, megakaryocytic, granulocytic and/or monocytic progenitors, whereas primitive B and T cell populations are implicated in lymphoid leukemias. The latter are outside the scope of this thesis, which instead deals with the two main types of myeloid leukemia – acute myeloid leukemia (AML) and chronic myeloid leukemia (CML).

LEUKEMOGENESIS

In recent decades, multiple models have been proposed to describe how a single oncogenic mutation in a healthy cell eventually results in a full-blown malignancy. In 1976, Peter Nowell forwarded the clonal evolution theory (43), which proposes tumorigenesis as a process rooted in the principles of Darwinian natural selection. In this model, an initial non-detrimental oncogenic mutation in a single cell of origin creates a pre-malignant clone, which in subsequent generations of daughter cells continues to acquire genetic or epigenetic alterations that give rise to subclones with varying degrees of fitness. In the competition for space and resources in the malignant microenvironment, subclones that have acquired growth advantages prevail and may become dominant in subsequent generations, progressively enriching for more malignant and invasive properties (44,45). In agreement with this model, advances in genotyping capabilities have unveiled substantial genetic heterogeneity within the malignant clone of many cancer types (45,46), with co-existing subclones ready to expand with the emergence of new genetic lesions or changes to the microenvironment that alter the fitness of the respective subclones. Additional support in the context of leukemia comes from experience with patients with myelodysplastic syndrome (MDS). This malignant but less aggressive relative of acute myeloid leukemia may progress to AML through clonal selection following the acquisition of additional mutations (47). In addition, 10% of healthy individuals above the age of 65 reportedly display clonal expansion of cells carrying mutations associated with hematologic malignancy, e.g. including lesions in *DNMT3A, TET2* and *ASXL1* (48). This condition, referred to as clonal hematopoiesis of indeterminate potential (CHIP) increases the risk of subsequent development of hematologic malignancy approximately 10-fold, to around 0.5-1% annually (49).

More recently, findings demonstrating the existence of maturation hierarchies within malignant clones sparked the foundation of a new, or complementary, theory describing the process of tumorigenesis, commonly referred to as the cancer stem cell model (50,51). In this hypothesis, tumorigenesis is driven by a subpopulation of malignant cells with extensive self-renewal capacity, referred to

as a cancer stem cell (CSC), or in leukemia as a leukemia-initiating cell (L-IC) or leukemic stem cell (LSC). In resemblance with normal HSC and hematopoiesis, the rare CSC population sits at the top of a hierarchy of malignant progenitors and gives rise to cells of diminishing self-renewal capacity through differentiation (50). The initial data supporting the CSC model stemmed from transplantation experiments in AML, where only the $CD34^+CD38^-$ leukemic compartment comprised cells with unlimited self-renewal and ability to reconstitute the full malignant clone upon transplantation to immunocompromised mice (52). In agreement with the situation in normal hematopoiesis, the $CD38^+$ counterpart contrastingly lacked this capability. Subsequently, the clinical relevance of CSC has been demonstrated in several studies, e.g. indicating that aspects of stem cell frequency, activity and self-renewal correlate with survival outcomes in leukemia and other cancer types (53–55), and that CSC display decreased sensitivity to conventional cancer therapeutics compared with the rest of the malignant clone (51). Combined, the aforementioned characteristics have implicated CSC as likely sources of disease progression and malignant relapse, inciting significant effort aimed at their targeted eradication in various cancer types, including leukemia (56).

The clonal evolution and cancer stem cell models described above are not mutually exclusive. On the contrary, they are combined in the current, seemingly consensual, view of leukemogenesis. In agreement with the CSC model, pre-malignant and transforming lesions are believed to occur in hematopoietic precursor cells that already have, or through mutation acquire, self-renewal capacity, giving rise to L-IC/LSC maintaining and propagating the disease (57). At disease presentation, the LSC compartment may consist of varying numbers of LSC subclones (58), providing a substrate for subsequent Darwinian selection in line with the clonal evolution theory. Treatment insult provides a prime example of imposed selective pressure that may enrich for more or less prevalent pre-existing resistant subpopulations (44). Indeed, comparisons of the genetic heterogeneity at diagnosis and leukemic relapse have revealed reemergence of major or minor diagnosis subclones at relapse, often following the acquisition of additional genetic aberrations that may have conferred survival advantages (in line with the clonal evolution model) (59,60). Collectively, the above-mentioned findings reconcile the two tumorigenesis models by identifying the rare, self-renewing LSC population as the principal entity subjected to Darwinian selection and clonal evolution during leukemic progression. As such, LSC are currently believed to be of central relevance at all stages of disease, supporting the notion that their targeted eradication should be a goal of emerging anti-leukemic therapeutics.

ACUTE MYELOID LEUKEMIA

Acute myeloid leukemia (AML) is the most common form of acute leukemia, with western country annual incidences of around 3-5 cases per 100,000 individuals and a slight male predominance (61–64). The incidence increases with age, with a median age at onset of around 70 years (64–66). Recent data from the Swedish Acute Leukemia registry indicate 5-year survival rates of around 20%, a number that has not significantly improved in recent decades (64). However, the prognosis is highly age-dependent, with 5-year overall survival of >50% for patients below 50 years and <10% in patients above 70 (64). In AML, the malignant cells overcrowding the blood and bone marrow carry features of myeloid lineage progenitors. However, the abnormal leukemic cells, or blasts, are typically dysfunctional and halted in differentiation, thus incapable of generating mature blood cells (66). In addition, their accumulation disturbs the blood-forming ability of non-malignant cells within the hematopoietic system (66), causing the typical symptoms of acute leukemias.

Diagnosis of AML involves the assessment of differential cell counts and bone marrow morphology, as well as cytogenetic, mutational and immunophenotypic analysis of the leukemic cells (67). Decisive findings include the presence of ≥20% myeloid blasts among nucleated cells in peripheral blood or bone marrow, and/or detection of certain AML-specific cytogenetic abnormalities within the leukemic clone (68,69).

Studies of cellular hierarchies in AML have indicated that the leukemia-initiating capacity may lie in the $CD34^{+}CD38^{-}$ HSC compartment (52,70–72). The cell type of origin for AML was thus initially believed to be HSC. However, other studies have challenged this view, showing LSC capacity and acquisition of self-renewal capability in more mature phenotypic compartments (73–77). The cell acquiring the initial pre-malignant oncogenic lesions may thus be self-renewing HSC, or more mature hematopoietic progenitors that through mutation gain self-renewal capacity. Leukemic development in AML has been proposed to occur through the so called two-hit model (78), in which two classes of mutations collaborate in leukemogenesis. In this model, class I mutations to genes involved in signaling pathways confer proliferative and survival capacity, while class II mutations targeting myeloid transcription factors impose impaired differentiation (79). Together with other, more recently identified mutations (e.g. those involved in epigenetic regulation (72,80,81)), these are believed to result in the excessive proliferation and accumulation of leukemic cells that characterizes AML.

AML is genetically heterogeneous, with a range of karyotypic and mutational aberrations affecting disease manifestation and prognosis (67). However, compared to many solid tumors the number of genetic abnormalities per individual is relatively low, averaging at only 13 mutations out of which 5 are recurrently mutated (82). In a comprehensive effort by the Cancer Genome Atlas Research Network, the mutational status of 200 AML patients was characterized by genomic and transcriptomic sequencing (82). The results revealed the presence of recurrent mutations that could be classified as belonging to either of nine functional groups (Table 1). The identified recurrent genetic aberrations e.g. included gene fusions resulting from chromosomal translocations or inversions, gene segment duplications, deletions and single nucleotide substitutions altering gene function. Of relevance to **paper II**, malignant clones that maintain non-cancerous chromosomal integrity are referred to as carrying normal karyotype. Approximately 45% of AML patients carry leukemic clones of this type, where recurring mutations e.g. occur in *NPM1* (40-50% of patients), *FLT3* (30-40%), *DNMT3A* (30%), *IDH1/2* (10%) and *CEBPA* (10%) (83–86).

Table 1. Genes recurrently mutated in AML. Adapted from (82).

EPIGENETIC REGULATION		PROLIFERATION		DIFFERENTIATION		SPLICING	CELL DIVISION	
DNA methylation	Chromatin modifiers	Activated signaling	Tumor suppressors	Myeloid transcription factors	Transcription factor fusions	Spliceosome	Cohesin complex	NPM1
DNMT3A/B *DNMT1* *IDH1/2* *TET1/2*	*ASXL1* *EZH2* *KDM6A* *KMT2A*-fusions *KMT2A*-PTD *MLL-X* fusions *MLL-PTD* *NUP98-NSD1*	*FLT3* *KIT* *KRAS* *NRAS*	*PHF6* *TP53* *WT1*	*CEBPA* *RUNX1*	*CBFB-MYH11* *PICALM-MLLT10* *PML-RARA* *RUNX1-RUNXT1*	*U2AF* *SRSF2*	*SMC1/3* *STAG2* *RAD21*	*NPM1*

Since the 1970s, AML has been divided into subtypes based on morphological and/or genetic heterogeneity. The traditional French-American-British (FAB) classification system stratified AML into eight subtypes (M0-M7) according to morphological appearance of the dominant leukemic clone (87). Subsequent recognition of the molecular heterogeneity of AML prompted the development of the World Health Organization (WHO) system, which integrates clinical characteristics and genetic, immunophenotypic and morphological features for disease classification (88). With the increased acceptance of genetically defined subgroups having bearing on disease prognosis (89–92), the WHO classification system outperforms its FAB counterpart in terms of clinical relevance.

The prognosis at AML diagnosis differs significantly between patients. This is partly due to differences in aggressiveness of the disease itself, but also to varying treatment tolerability among individuals, impacted by factors such as comorbidities and age (67). As mentioned, the prognostic importance of certain genetic abnormalities within the leukemic clone has been increasingly recognized in recent decades, resulting in the development of AML-specific risk classification guidelines by the European LeukemiaNet (ELN) (92). An overview of the 2022 revision of the guidelines is presented in Table 2 (for specific details on which abnormalities that take precedence at cooccurrence, the reader is referred to the original paper; (92)). As indicated in the table, karyotypic abnormalities are not associated with better or worse prognosis *per se*. Instead, specific gene fusions resulting from chromosomal events may herald favorable, intermediate, or adverse risk depending on identity. Similarly, different mutational patterns are associated with differing risk within the group of cytogenetically normal AML. Hence, patients with mutated *NPM1* without concurrent *FLT3*-ITD have favorable risk, while patients with *FLT3*-ITD and wild type NPM1 have inferior prognosis.

Table 2. Genetic risk classification of AML. Adapted from (92).

RISK CATEGORY	GENETIC ABNORMALITY
Favorable	t(8;21)(q22;q22.1);*RUNX1-RUNX1T1* inv(16)(p13.1q22) or t(16;16)(p13.1;q22);*CBFB-MYH11* Mutated *NPM1* without *FLT3*-ITD bZIP in-frame mutated *CEBPA*
Intermediate	Mutated *NPM1* with *FLT3*-ITD Wild-type *NPM1* with *FLT3*-ITD (without adverse-risk genetic lesions) t(9;11)(p21.3;q23.3);*KMT2A-MLLT3* Cytogenetic and/or molecular abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23.3;q34.1);*DEK-NUP214* t(v;11q23.3);*KMT2A*-rearranged t(9;22)(q34.1;q11.2);*BCR-ABL1* t(8;16)(p11.2;p13.3);*KAT6A-CREBBP* inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2);*GATA2-MECOM(EVI1)* t(3q26.2;v);*MECOM(EVI1)*-rearranged −5 or del(5q), −7, −17/abn(17p) Complex karyotype* Monosomal karyotype** Mutated *ASXL1, BCOR, EZH2, RUNX1, SF3B1, SRSF2, STAG2, U2AF1, and/or ZRSR2* Mutated *TP53*

* ≥3 unrelated chromosome abnormalities in the absence of other class-defining recurring genetic abnormalities

** ≥2 distinct monosomies (excluding loss of X or Y) or one single autosomal monosomy in combination with at least one structural chromosome abnormality

THERAPEUTIC MANAGEMENT

Today, genetic mapping, risk stratification and identification of patients with high, intermediate and low-risk disease often impact on decisions concerning suitable therapeutic approaches (69). For example, a subgroup of AML defined by the t(15;17)(q22;q12) translocation and consequent *PML-RARA* fusion oncogene (acute promyelocytic leukemia; APL) receives treatment with all-trans retinoic acid (ATRA) and arsenic trioxide (ATO), which has drastically improved survival outcomes for this group of patients (93). Conversely, genetic factors associated with worse outcome in adverse risk patients may motivate the use of more aggressive treatment approaches (69). However, patient-specific performance status and comorbidities frequently restrict therapeutic strategies attainable, and certain patient groups (especially older individuals) may therefore be limited to palliative care.

Induction and consolidation therapy

Despite substantial improvements in the understanding of AML over the last 50 years, treatment has not changed dramatically for the majority of patients (67,69). For fit individuals (usually below 75 years of age), AML treatment with curative intent begins with intensive cytotoxic chemotherapy, administered in two phases: induction and consolidation.

The initial treatment given at diagnosis, referred to as induction chemotherapy, typically comprises 7 days of continuous infusion of cytarabine (in Sweden 5 days) along with an anthracycline (often daunorubicin or idarubicin) during the first 3 days ('7+3'; (94)). This initial phase of chemotherapy aims to induce complete remission (CR), defined as <5% blasts among nucleated bone marrow cells, absence of circulating blasts/extramedullary leukemia and normalized neutrophil and platelet counts (92). Hence, when blood counts have started to recover following the initial acute phase induced by the treatment, a bone marrow biopsy is performed and assessed for presence of residual AML (69). Patients that fulfill the above criteria have achieved CR, which marks the end of the induction phase of chemotherapy. Patients that do not attain CR after the first treatment cycle typically go on to receive further induction cycles, which may result in subsequent remission. Overall, induction chemotherapy brings about CR for around 60-85% of patients aged 60 and below (67). The corresponding numbers for patients above 60 are 40-60% (67). The lower degree of chemosensitivity in patients who need multiple cycles of induction chemotherapy to achieve CR has incited investigation into whether these patients (approximately 20-25%) are more likely to relapse or die from their disease. While studies indicated no difference in outcome for patients diagnosed in the 1980s and 1990s, clinical data obtained more recently imply improved survival outcomes for patients achieving CR after one induction cycle (95–97).

The next phase of treatment, consolidation, aims to eradicate remaining leukemic cells in CR and reduce the risk of relapse after the completion of chemotherapy. Consolidation treatment typically involves additional cycles of chemotherapy (usually 1-4), alone or followed by allogeneic stem cell transplantation (allo-hSCT) (67,69). As previously discussed, one of the main uses for AML risk stratification is treatment decisions, e.g. including whether a patient should be considered for allo-hSCT following initial chemotherapy. In the current Swedish AML treatment guidelines, allo-hSCT is the preferred choice for patients below 70 years with adverse or intermediate risk disease (69). During allo-hSCT, patients receive high-dose chemo/radiotherapy (conditioning) aimed at eradicating the majority of hematopoietic cells, followed by donor cell infusion allowing rescue and reconstitution of the hematopoietic system (67). The leukemia-targeting effects are believed to be two-fold: (i) related to the intensive chemotherapeutic regimen of the conditioning phase, and (ii) deriving from donor immune cell targeting of residual leukemic cells (known as graft versus leukemia; GvL) (98). However, even though allo-hSCT has curative potential, it is also associated with quality-of-life concerns and significant risk of serious complications, including development of graft-versus-host-disease (GvHD) and treatment-related death. Thus, AML patients with low-risk disease, that have relatively good chances of lasting remission after conventional chemotherapy, are typically not transplanted. However, inefficient removal of leukemic cells in initial rounds of chemotherapy carries prognostic significance in this group of patients, and allo-hSCT may thus be considered in these cases (i.e. when >2 induction cycles are required to attain CR and/or upon detection of residual leukemic cells in CR; referred to as minimal residual disease, MRD) (69,99).

The current chemotherapeutic regimen, at times supplemented with allo-hSCT, has remained a cornerstone in AML treatment since the 1970s (94,100). It is only recently that targeted therapies (e.g. including FLT3, IDH1, IDH2 and BCL2 inhibitors) have been added to the available therapeutic arsenal (57), allowing tailored treatment of certain patient groups. In Sweden, the 30% of patients that carry *FLT3*-mutated AML receive treatment with the FLT3 inhibitor midostaurin following each cycle of induction and consolidation chemotherapy, and patients with 'core binding factor' (CBF) AML (t(8;21);*RUNX1-RUNX1T1* or inv(16);*CBFB-MYH11*) receive gemtuzumab ozogamicin (GO; an anti-CD33 drug-conjugated antibody) in the first chemotherapy cycle (69). Other targeted therapies may be administered in the context of clinical trials. In patients unfit for intensive chemotherapy, prolonged survival may be achieved using frontline treatment with the hypomethylating agent azacitidine in combination with the BCL2 inhibitor venetoclax. In purely palliative cases, treatment instead usually aims at cytoreduction, e.g. comprising hydroxyurea or low-dose cytarabine (69).

Maintenance therapy

A significant reason for the poor survival rates associated with AML is that 50-70% of patients relapse despite initial successful achievement of complete remission (often within 2-3 years) (67,101,102). A hematological relapse is defined as the presence of ≥5% blasts in bone marrow, reappearance of circulating blasts, or development of extramedullary leukemia (92). Relapse is likely to be the result of inefficient eradication and subsequent expansion of residual leukemia, and/or clonal evolution of pre-malignant or malignant cells (103,104). Although a minority of patients may achieve secondary remissions following reinduction or allo-hSCT, long-term survival rates following leukemic relapse are often poor (67,105). In recent decades, reduced intensity conditioning schemes have allowed a wider indication for potentially curative allo-hSCT than previously achievable (106,107). Though this may have reduced relapse rates in a subgroup of patients, it has not yet been demonstrated in a randomized study (69). Nevertheless, many patients remain ineligible for allo-hSCT due to factors such as age, comorbidities, donor availability, and/or genetic risk, and thus mostly receive no further treatment post consolidation. Consequently, there is a pressing need for additional therapeutic strategies to maintain long-term remission in AML, especially for patients not eligible for transplantation.

Relapse risk reduction may conceivably be achievable through further treatment following the completion of conventional chemotherapy, an approach known as maintenance therapy (Figure 3). This is a not a new concept, but one that has been explored in numerous clinical trials over the years, with approaches e.g. involving continued chemotherapy, hypomethylating agents, immunomodulation and targeted therapy (104,108,109). However, inconsistent results and failure to produce benefit in terms of relapse incidence and/or overall survival have so far hampered its wide-spread use (109).

There is a consensus that the use of maintenance therapy is mainly motivated by data showing prolongation of overall survival, since exposure to additional anti-leukemic therapy may carry toxicities that reduce overall survival in certain patient groups (treatment-related mortality, TRM). Despite this belief, there is data indicating that leukemia-free survival (LFS) may be a statistically valid surrogate for overall survival (OS) in the context of remission maintenance in AML (110), and that failure to achieve significance in terms of OS should be distinguished from actual lack of impact on OS, which could be associated with TRM. The evaluation of OS in the context of AML maintenance therapy involves relatively long follow-up times, and may thus be confounded by events such as non-leukemia-related death or regional differences in relapse management (110).

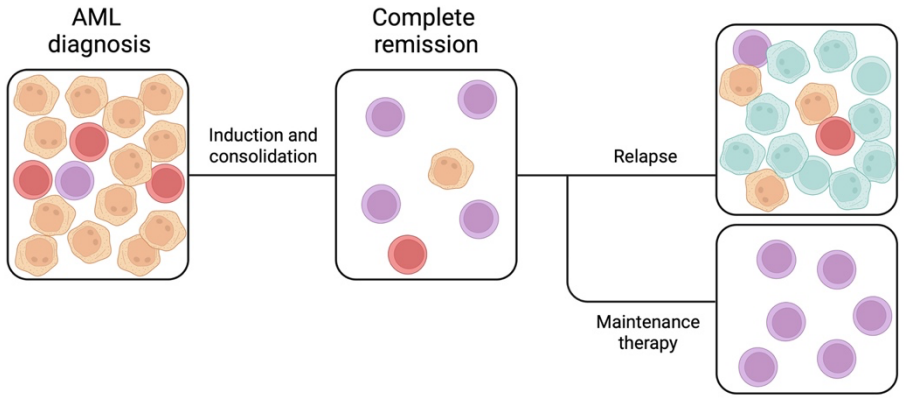

Figure 3. The concept of maintenance therapy in AML. Purple and red cells represent healthy HSC and AML LSC, respectively. Beige and turquoise cells represent leukemic blasts of different subclone identity. Created with BioRender.com.

In recent years, maintenance therapy using an oral formulation of azacitidine (CC-486/Onureg) was approved by American and European medicines agencies in view of data suggesting improvements in median leukemia-free and overall survival in patients ≥55 years in first complete remission (CR1) ineligible for allo-hSCT (111). However, the LFS and OS Kaplan-Meier curves for untreated and treated patients eventually merged (111), possibly indicating that the treatment delayed relapse rather than cured the patients (112). Also, there are concerns regarding the heterogeneity of consolidation pre-treatment within the patient group (20% had received no consolidation, others received 1-2 courses), which may suggest that the benefit of oral azacitidine lies in administration of continued chemotherapy to patients not able to receive sufficiently intensive therapy in the initial phase of treatment (109).

Another maintenance approach that recently has shown promise is targeted treatment with FLT3 inhibitors for patients carrying *FLT3* mutations. In 2017, the RATIFY trial indicated significantly improved event-free and overall survival for patients receiving midostaurin as maintenance therapy following the completion of chemotherapy (113). However, this study additionally involved FLT3 inhibition in conjunction with induction and consolidation chemotherapy, making it difficult to assess the relative benefit of FLT3 inhibition in the maintenance setting.

Although some other maintenance treatment approaches have shown benefit in terms of improved leukemia-free survival in AML (104,114), the only currently approved alternative to oral azacitidine and FLT3 inhibition, and the only relapse-preventive treatment currently recommended for maintenance consideration in

the Swedish AML treatment guidelines, is immunotherapy with low-dose interleukin-2 (IL-2) in conjunction with histamine dihydrochloride (HDC).

HDC/IL-2 TREATMENT IN AML

The results of numerous studies highlight the importance of aspects of immune cell function for the control of residual leukemia in the post-consolidation phase of AML (6,115). Inter alia, AML cells may reportedly express structures of relevance for cytotoxic lymphocyte surveillance (115), and both T and NK cells have been implicated in the GvL-related relapse risk reduction associated with allo-hSCT (116,117). Significant effort has thus been put into attempts at directing and/or supporting cytotoxic lymphocyte recognition and eradication of leukemic cells, in particular in CR, where the burden of leukemia is minimal (6). Such approaches include, but are not limited to, the use of monoclonal antibodies targeting leukemia-associated antigens, adoptive transfer of lymphocyte populations, or systemic treatment with immunostimulatory cytokines (6).

Interleukin-2 (IL-2) is an endogenous T cell-derived cytokine that initially was assumed to induce anti-leukemic effects due to its reported roles in T and NK cell differentiation, activation, expansion and cytotoxic activity (118). However, although monotherapy with IL-2 resulted in improved survival outcomes in a fraction of patients with metastatic renal cell carcinoma and melanoma, this approach yielded disappointment in terms of post-consolidation relapse prevention in several trials in AML (119–125). A possible explanation to the disappointingly low efficacy of IL-2 monotherapy as an anti-cancer treatment is the cytokine-induced expansion of regulatory T cells (Tregs). Hence, Tregs, that suppress cytotoxic effector cells, express high-affinity IL-2 receptors (CD25) and thus preferentially expand in response to exogenous IL-2 (126). Additional clues to the lack of efficacy of monotherapy with IL-2 in leukemia were provided by investigators in the 1980s and 90s (127–132), with data indicating that T and NK cells are sensitive to exogenous reactive oxygen species (ROS), and that IL-2-induced NK cell killing of leukemic cells (including AML blasts) is hampered by monocyte-derived production of immunosuppressive ROS. In these studies, the addition of histamine was found to rescue IL-2-induced antileukemic effects in the presence of ROS-producing monocytes, thus synergizing with IL-2 to eradicate leukemic cells. The effect of histamine was shown to occur via agonistic binding to histamine type 2 (H2R) receptors on the monocyte cell surface, resulting in inhibition of the ROS-producing myeloid NADPH oxidase NOX2. Overall, the above-mentioned findings implied that IL-2-induced cytotoxic lymphocyte targeting of leukemic cells may be impeded by immunosuppressive ROS in the malignant microenvironment, thus warranting investigation into whether the addition of histamine may unravel the relapse-preventive effect of IL-2 in AML.

A subsequent randomized phase III clinical trial (the 0201 trial) evaluated the relapse-preventive efficacy of combinatorial treatment with HDC and low-dose IL-2 in AML patients in CR not eligible for allo-hSCT (7). HDC/IL-2 therapy was given over 18 months in ten three-week treatment cycles with rest periods in-between. The results indicated significantly improved LFS for treated patients (especially for those in CR1 <60 years of age), but did not demonstrate significant efficacy in terms of OS (possibly due to the lower power of the OS analysis (110)). In contrast to monotherapy with IL-2 (in higher doses), the HDC/IL-2 treatment was well-tolerated, with 92% of non-relapsing patients adhering to the regimen throughout all ten cycles (7,108). The effects of HDC/IL-2 on LFS, which led to its approval for maintenance treatment of adult AML patients in CR1 within the European Union in 2008 (133), are shown in Figure 4.

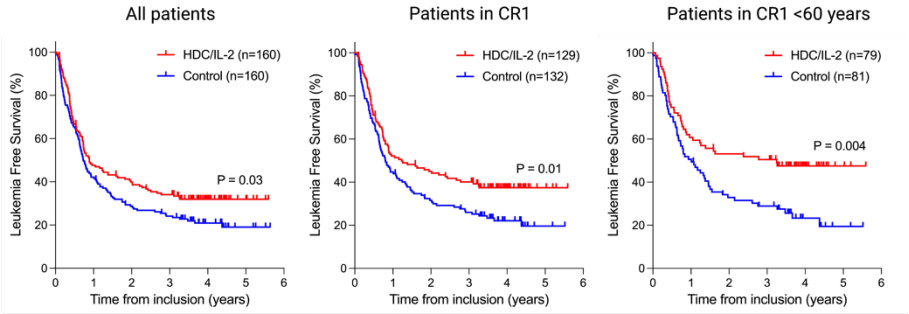

Figure 4. Results of the HDC/IL-2 phase III 0201 clinical trial. CR1, first complete remission. Created with Biorender.com.

Subsequent *in vitro* experiments and retrospective analysis of the 0201 trial data indicated that the effect of HDC/IL-2 was pronounced in AML patients with myelomonocytic and monocytic subtypes of leukemia (FAB M4/M5) in which the blasts themselves were found to express H2R and NOX2 and produce immunosuppressive ROS (134,135). Consequently, certain leukemic clones may be directly responsible for the immunosuppressive microenvironment in AML, and potentially be directly targeted by HDC. In addition, histamine has recently been shown to play a role in the induction of malignant and non-malignant myeloid differentiation (136–138), thus providing a supplementary mechanism potentially involved in the HDC/IL-2-mediated targeting of residual myeloid leukemia.

The final proof of the immunomodulatory effects of HDC/IL-2 in AML came from a single-armed phase IV trial (Re:Mission), in which significant induction of NK cells was observed during treatment cycles, and where aspects of immunity (e.g. including expression of activating receptors on NK cells and

treatment-induced memory to effector T cell transition) were found to correlate with treatment outcome (139–141). Of note, although immunosuppressive Tregs were induced during HDC/IL-2 treatment cycles, no association with clinical outcome was noted, and the Treg induction seemed blunted in later HDC/IL-2 treatment cycles (142). Along with the above-mentioned data, this pointed to the feasibility of immunostimulatory maintenance treatment with HDC/IL-2 for relapse prevention in AML.

CHRONIC MYELOID LEUKEMIA

Chronic myeloid leukemia (CML) is a less prevalent form of myeloid leukemia, with an incidence of 1-1.5 per 100,000 and a slight male predominance (143–146). In contrast to AML, CML is an unusually homogeneous malignancy. Discoveries in the 1960s, 70s and 80s (147–150) uncovered that virtually all patients carry leukemic clones with a reciprocal chromosomal translocation of chromosomes 9 and 22 (t(9;22)(q34;q11)), resulting in the molecular juxtapositioning of *BCR* on chromosome 22 and *ABL1* on chromosome 9 (Figure 5). The formation of the chimeric *BCR-ABL1* fusion oncogene generates a deregulated, constitutively active tyrosine kinase that drives the proliferation, survival and accumulation of myeloid cells at different stages of maturation in blood and bone marrow (151,152). Typical features associated with CML are presence of large numbers of morphologically and functionally normal granulocytes and granulocyte progenitors in blood (145,153), and/or splenomegaly (145,151). The final diagnosis of CML is made based on positive identification of the t(9;22) translocation by cytogenetic, fluorescence in-situ hybridization (FISH) and/or reverse transcription polymerase chain reaction (RT-PCR) analysis of blood or BM samples (145). About 95% of patients carry *BCR-ABL1* transcripts with fusions between *BCR* exon 13 or 14 and *ABL1* exon 2 (e13a2 and e14a2, respectively) (154).

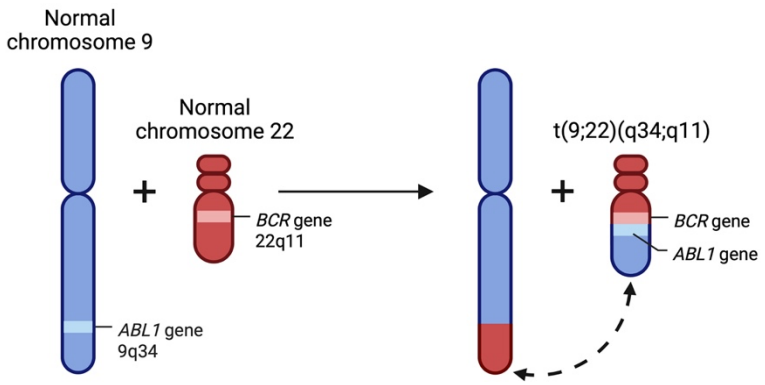

Figure 5. The t(9:22)(q34;q11) reciprocal chromosomal translocation giving rise to the BCR-ABL1 oncogene. Adapted from (155) using BioRender.com.

Most CML patients (>90%) are diagnosed in the chronic phase (CP) of the disease (156,157), at which point approximately 50% are asymptomatic (145,156,157). Symptoms that do occur commonly relate to anemia or splenomegaly (e.g. fatigue, weight loss, abdominal fullness and/or early satiety), and less frequently to thrombocyte dysfunction (thrombosis, bleeding), or leukostasis (dyspnea) (157). Although CML is less aggressive at presentation than AML, it is far from benign.

Without treatment, the disease will invariably, through acquisition of additional mutations and clonal evolution, progress to a fatal blast crisis (BC; typically over a 5-year time frame) (151,158–160). The BC phase of CML resembles an acute leukemia and is characterized by the loss of maturation and uncontrolled proliferation of leukemic blasts, which may bear resemblance to myeloid or, less often, lymphoid progenitors (146,157). A BC diagnosis is based on the presence of ≥20% immature myeloid blasts among nucleated cells in peripheral blood (PB) or bone marrow (BM), extramedullary blast proliferation or increased lymphoblasts in PB or BM (88). CML was long considered a triphasic disease, where an accelerated phase (AP) often preceded BC. However, in the most recent update to the WHO classification of hematolymphoid tumors (88), the AP definition was removed and replaced by an emphasis on features associated with high risk of progression to BC in chronic phase.

THERAPEUTIC MANAGEMENT

Before 2001, CML diagnosis was associated with poor prospects of long-term survival (146). In the decades leading up to the new millennium, CP-CML treatment typically consisted of periodical chemotherapy with hydroxyurea or busulfan (146). However, although these therapies were able to induce hematologic responses and somewhat prolong survival, the majority of patients progressed after a median time of 3-4 years (161), with dismal prognosis. Allogeneic stem cell transplantation (allo-hSCT) became the first potentially curative treatment option in CML (162), and was later followed by the introduction of interferon alpha (IFN-α) treatment, which induced cytogenetic responses and improved outcomes in a subgroup of patients (146).

Around the turn of the century came the first reports of the development of a small-molecule tyrosine kinase inhibitor (TKI); imatinib, that targeted and blocked the kinase activity of the BCR-ABL1 protein (3). In the first clinical trial of the drug, 53 out of 54 patients treated with a high dose achieved a complete hematologic response within weeks (4). Subsequent studies showed that as long as the patients stayed on treatment they were often able to maintain remission long-term (163,164), providing a striking improvement over the treatment options that had thus far been available. In the coming years, the realization that some patients were, or eventually became, resistant to imatinib led to the development of second (dasatinib, nilotinib, and bosutinib) and third (ponatinib, asciminib) generation TKIs, which are often able to overcome resistance by higher potency and/or alternate binding mechanisms to the BCR-ABL1 protein (153,165,166). While dasatinib, nilotinib and bosutinib are effective against many of the commonly occurring *BCR-ABL1* kinase-domain resistance mutations, ponatinib and asciminib are the only ones able to target the challenging T315I mutation (albeit for ponatinib at the cost of a relatively high degree of

cardiovascular toxicity) (157,166–169). Both first and second generation TKIs are currently approved for frontline treatment of CP-CML patients at diagnosis (165). Frontline second generation TKI treatment is associated with faster and deeper responses than treatment with imatinib, but also with a higher degree of off-target toxicity, seemingly without additional survival benefit (157,170–172). Despite this, first line therapy with second-generation TKIs may be practiced for several reasons, e.g. including high-risk disease where faster responses may be preferable (145,157).

The response to TKI treatment in terms of leukemic burden is monitored by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) analysis of *BCR-ABL1* transcript levels in peripheral blood, converted to an international scale (IS) (165). At this scale, a 3 $\log_{10}$ reduction of *BCR-ABL1* level (i.e. ≤0.1%) compared to the international standard is referred to as a major molecular response (MMR; MR3) (173). Correspondingly, log reductions of 4 (≤0.01%) and 4.5 (≤0.0032%) represent deep molecular responses (DMR; MR4 and MR4.5) (173). Cytogenetic analysis of BM samples is used to assess early treatment responses. Patients that show no signs of residual cells with the t(9;22) translocation among at least 20 analyzed bone marrow metaphases are referred to as having achieved a complete cytogenetic response (CCyR) (145). Failure to reach prognostically important milestones (e.g. *BCR-ABL1*^{IS}≤10% at 3 months or CCyR at 12 months (157,174)) identifies patients at risk of treatment failure, and may trigger changes to more potent TKIs aiming to reduce the leukemic clone to low enough numbers to minimize the risk of progression (145).

Of relevance to this thesis, even after the advent of TKIs, hydroxyurea treatment remains relevant at CP-CML diagnosis. In patients presenting with high white blood cell or platelet counts, cytoreductive hydroxyurea treatment may be used to reduce the risk of leukostasis until definitive cytogenetic or molecular diagnosis and start of TKI treatment (145,157,165). Its mechanism of action relates to inhibition of an enzyme involved in the generation of deoxyribonucleoside triphosphates (dNTPs; ribonucleotide reductase). The subsequent reduced availability of dNTPs halts cells in the DNA-replicating S phase of the cell cycle through activation of the cellular replication checkpoint (175). Depending on concentration and time of exposure, the effects of hydroxyurea may be reversible or cytotoxic (175). Either way, the drug ultimately slows down cell proliferation, effectively reducing the abnormally high cell numbers associated with CML.

Today, a CML diagnosis is associated with excellent survival outcomes for most patients. At five years of TKI treatment, approximately 80% of patients diagnosed in the chronic phase are in MMR, a number that increases to 90% at the ten-year mark (57). Hence, proper use of the available arsenal of TKIs has resulted in close

to normal life spans for most patients (5). However, TKI resistance and intolerance are issues of concern in approximately 25% of CP-CML patients (57), and although disease progression is much rarer than it once was, a subgroup of patients presents with BC or develops BC following insufficient TKI efficacy (156,176,177), which still carries poor chances for long-term survival (178). For fit patients, BC treatment comprises AML- or acute lymphoblastic leukemia (ALL)-specific intensive chemotherapy in combination with later generation TKIs in attempts to induce remissions that may allow successful subsequent allo-hSCT (145,165). To prevent disease progression, allo-hSCT may also be considered in CP to patients with suboptimal response to at least two TKIs, or patients who do not respond to ponatinib within three months of treatment (145,165).

CML patients were originally believed to need lifelong TKI treatment, owing to the persistence of TKI-resistant leukemic stem cells that may expand and cause disease relapse upon treatment discontinuation (145,179). However, an increasing number of studies have demonstrated the feasibility of TKI discontinuation without leukemic relapse in carefully selected groups of CP-CML patients (180–184). In many of these studies, around 40-50% of patients have been able to sustain remission in the absence of TKI treatment after having been in stable DMR for >2 years (157). As daily TKI treatment is associated with high costs and toxicities in many patients (145,157), treatment discontinuation and the concept of treatment-free remission (TFR) has become the new goal of CML therapy (165,185). Criteria defining patients eligible for TFR attempts with maximal success rate is still an area of intense research. However, both treatment and DMR duration have been shown to significantly impact on TFR rates (186). This is reflected in the current Swedish TFR attempt guidelines that define eligible patients as those that have been on TKI treatment for at least 5 years, and maintained DMR (MR4 or MR4.5) for a minimum of two years (145). With these criteria, however, TKI discontinuation is only accessible to a minority of patients, prompting continued research into ways of eradicating residual leukemia in TKI-induced remission and improving TFR rates.

LEUKEMIC STEM CELLS

The definitive proof of the existence of a rare population of LSC with *in vitro* and *in vivo* stem cell properties in patients with CML was first published in 1999 (187), predating the advent of TKIs. In this study, the LSC population demonstrated a reversibly quiescent phenotype, which was suggested to explain why CML could not be cured by conventional chemotherapy. Since the introduction of TKIs, several studies have indicated that the resistance phenotype of the LSC also extends to BCR-ABL1-targeted therapy, thus suggesting that LSC are not dependent on BCR-ABL1 kinase activity for survival (188–192). In agreement,

LSC persistence during TKI treatment has repeatedly been demonstrated in CP-CML patients despite successful eradication of the bulk of the leukemic clone (193–195). As previously discussed, the residual LSC mostly do not expand unless TKI treatment is interrupted (196). However, the sustained presence of a cell type able to reconstitute the leukemic clone is a matter of concern in CML, especially as these rare cells may acquire additional mutations, potentially leading to disease progression (197), or TKI resistance and subsequent disease relapse (57). Previous studies have suggested that LSC may persist also in patients in successful treatment-free remission post treatment discontinuation, in view of the detection of *BCR-ABL1* DNA (198) or RNA (199), or LSC by flow cytometric analysis using assumed LSC cell surface markers (200). The lack of expansion of the LSC clone has then e.g. been proposed to reflect immune-mediated control or heterogeneity in terms of leukemia-initiating capacity among *BCR-ABL1*$^+$ LSC. However, these findings were recently partially problematized by the demonstration of *BCR-ABL1* DNA in the lymphocyte compartment (but never in granulocytes) in patients in TFR, pointing to the possibility that the *BCR-ABL1* persistence may derive from a long-lived population of *BCR-ABL1*$^+$ lymphocytes rather than LSC in some cases (201).

Since the t(9;22) translocation can be found in both myeloid and lymphoid cell types in CML patients (202), and *BCR-ABL1* expression is unable to induce self-renewal in murine hematopoietic progenitors (203), the cell of origin for CML, i.e. the cell that acquires the initial transforming *BCR-ABL1* translocation, has long been thought to be the immature, multipotent HSC (204). Although some studies have questioned this in view of data showing *BCR-ABL1* expression in patient endothelial cells (indicating that the *BCR-ABL1* translocation may have occurred in an HSC precursor; the hemangioblast) (205,206), CML LSC activity is currently widely recognized as belonging to the normal CD34$^+$CD38$^-$ HSC compartment (57,207). In recent years, significant effort has been put into the identification of additional cell surface markers that distinguish LSC from their healthy counterparts, with a view to use these for targeting approaches with minimal collateral damage to healthy cells, or for prospective FACS isolation of cell populations enriched for LSC activity for further biomolecular or functional characterization. Many LSC markers have thus been reported over the years, with expression patterns more or less restricted to the LSC population (208). In this context, the three arguably most promising ones to date are CD26 (209), CD25 (210) and IL-1RAP (211–213).

CD26 (*DPP4*) is a serine exopeptidase that is normally expressed on the surface of activated T cells (212). In the original paper defining CD26 as an LSC marker, only the CD26$^+$ lineage (Lin) negative cells from primary CML samples were able to induce *BCR-ABL1*$^+$ engraftment in immunodeficient NSG mice, while

Lin$^-$CD26$^-$ cells gave rise to multilineage engraftment of *BCR-ABL1*$^-$ cells (209). CD26 expression is reportedly mostly present within the CD34$^+$CD38$^-$ CML LSC compartment, and largely absent from healthy stem and progenitor cells (209,211,214). The peptidase activity of CD26 has been proposed to explain the abnormally large numbers of mobilized LSC and HSC in the peripheral blood of CP-CML patients at diagnosis (209,215). Hence, CD26-mediated degradation of the CXCL12 ligand, that normally contributes to BM sequestering of HSC through interaction with the CXCR4 receptor on the stem cell surface, results in a lower degree of LSC BM homing (216,217).

CD25 (*IL2RA*) normally makes up part of the IL-2 receptor complex on lymphocytes (218). However, in the CML LSC context the other IL-2 receptor components are seemingly not expressed, effectively rendering the cells unresponsive to IL-2 treatment (219). Among CD34$^+$CD38$^-$ cells, CD25 expression has been found to be exclusive to the leukemic cells, without corresponding expression on healthy stem cells (209,210). From a functional standpoint, CD25 expression seems to reduce LSC proliferative capacity and engraftment potential, and the finding that TKI treatment seems to downregulate CD25 expression on LSC has sparked interest in trying to induce it once again (210).

The first specific CML LSC surface marker to be reported was IL-1RAP, which serves as a co-receptor for the interleukin 1 (IL-1) receptor (213). Within the CD34$^+$CD38$^-$ compartment, its expression is reportedly restricted to *BCR-ABL1*$^+$ cells, and the percentage of IL-1RAP-expressing cells at CP-CML diagnosis seemingly correlates with the efficiency of TKI treatment (211). There is also data indicating that a higher proportion of cells within the CD34$^+$CD38$^-$ compartment expresses IL-1RAP compared with CD25, and that there is a higher correlation between the percentages of IL-1RAP and *BCR-ABL1* positive cells (211). However, other studies have shown high levels of IL-1RAP expression also within the CD34$^+$CD38$^+$ compartment, possibly making it a less specific immature LSC marker than CD25 and CD26 (209,220).

A variety of immunotherapeutic approaches are conceivable to target LSC based on cell surface expression, e.g. including monoclonal antibodies, antibody conjugates, bispecific antibodies, or chimeric antigen receptor (CAR) T cells. Some support for this approach in CML has stemmed from recent studies, e.g. showing the feasibility of utilizing IL-1RAP-directed CAR T cells for specific targeting of LSC *in vitro* and in a xenograft model *in vivo* (221). A similar approach was successfully attempted for targeting CD26-expressing cells, although this version was associated with a certain degree of fratricide, entailing toxicity to normal activated lymphocytes (222). In another study, a monoclonal antibody

against CD26 was coupled to a liposome carrying the BCL2 inhibitor venetoclax (214), which was previously shown to target CML stem and progenitor cells (223). Treatment with the immunoliposome formulation resulted in specific inhibition of cell growth and induction of apoptosis in $CD26^+$ cells *in vitro*, and decreased engraftment in a xenograft model of CML *in vivo* (214). If coupled with specific enough markers, immunotherapeutic strategies may thus allow specific LSC targeting with minimal off-target toxicity.

Another line of research focuses on aspects other than cell surface expression that may be essential for LSC and thus potentially targetable. Such approaches e.g. emphasize the importance of dysregulated intracellular signaling pathways (such as PI3K/AKT, JAK/STAT, WNT/β-catenin) and processes (autophagy, metabolism and epigenetics), as well as cell extrinsic factors that may support LSC resistance and survival, including the bone marrow microenvironment (BMM) (224). The BMM is currently believed to be key to LSC sustenance through multiple mechanisms (225). Among them, several studies have supported the importance of the CXCL12-CXCR4 axis. Both the bone marrow-produced CXCL12 ligand and the stem cell CXCR4 homing receptor are found at abnormally low levels in the CML BM. As previously mentioned, the lower CXCL12 levels are partially due to LSC expression of CD26, but have also been attributed to increased secretion of the CXCL12 antagonist granulocyte colony-stimulating factor (G-CSF) within the BM (226). In addition, BCR-ABL1 expression reportedly decreases the expression of CXCR4 on the LSC surface (227,228), which together with the lower CXCL12 levels in the BMM, contributes to the reduced BM retention of LSC at CP-CML diagnosis. However, TKI treatment reportedly enhances LSC CXCR4 expression, favoring their return to the BM, where LSC may reside in a quiescent state protected from treatment insult (227,229). Studies targeting various aspects of the CXCL12-CXCR4 axis in conjunction with TKI treatment have supported a role in LSC treatment escape, e.g. showing reduced CML cell growth and tumor burden *in vitro* and *in vivo* (229–231).

As previously alluded to, single-cell transcriptomic analyses hold promise for the characterization of heterogeneity in normal and malignant hematopoiesis that cannot be fully captured by traditional bulk approaches. In 2017, two groups independently reported results from single-cell transcriptional analyses of the $Lin^-CD34^+CD38^-$ compartment in CML (228,232). Both studies found significant heterogeneity within the classically defined $CD34^+CD38^-$ LSC compartment and concluded that only a subpopulation consisting of primitive and quiescent cells persisted during TKI treatment. In the Warfvinge study, the subpopulation that was enriched with TKI treatment was found to belong to the $Lin^-CD34^+CD38^{-/low}CD45RA^-cKIT^-CD26^+$ compartment (232), thus

potentially providing a phenotypical signature better capturing the true LSC. However, dating back to the early days of single-cell transcriptomics, both studies were limited to relatively low numbers of cells compared to what can be achieved using current high-throughput scRNAseq approaches. Hence, the possibilities for detailed characterization of the heterogeneity within the $CD34^+$ CML stem and progenitor cell (SPC) compartment (e.g. including differentiation hierarchies) have immensely improved over the last few years, leaving room for studies confirming and elaborating on these initial findings. Recent scRNAseq-based methods providing parallel transcriptomic and proteomic data from individual cells (multiomic methods) may be even more promising in this context, as they would enable straight-forward translation between the transcriptional and immunophenotypic profiles of identified LSC populations.

AIM

The overall aim of this thesis was to contribute to an increased understanding of (i) patient populations that may benefit from relapse-preventive treatment with HDC/IL-2 in AML, and (ii) the TKI-eluding LSC population in CML. Specific aims for each of the papers are listed below:

Paper I	To assess whether the efficacy of previous induction chemotherapy impacts on HDC/IL-2 treatment outcomes in AML
Paper II	To determine whether the presence of chromosomal aberrations in the leukemic clone has bearing on the efficacy of HDC/IL-2 in AML
Paper III	To further characterize the CML SPC and LSC compartments by multiomic analysis paired with *BCR-ABL1* detection at the single-cell level
Paper IV	To investigate whether/how cytoreductive treatment with hydroxyurea at chronic phase CML diagnosis affects the SPC compartment

MATERIALS AND METHODS

A general introduction to the data, patients and main analysis methods involved in **papers I-IV** is provided below. For further detail, the reader is referred to the methods sections of the respective papers.

CLINICAL TRIALS IN AML

Data from two clinical trials assessing the efficacy of relapse-preventive HDC/IL-2 treatment in AML were used for the analyses presented in **papers I and II**: the phase III 0201 and the phase IV Re:Mission trials. Both trials were approved by the ethical evaluation committees of the participating institutions, and all patients gave written informed consent prior to enrolment.

PHASE III TRIAL

The phase III trial (0201; NCT00003991; (7)) enrolled 320 AML patients (age 18-84; median 57 years) in first (CR1; n=261) or subsequent (CR>1; n=59) complete remission (CR) who were not eligible for allogeneic stem cell transplantation. At inclusion, patients had <5% blasts in normocellular bone marrow and were within three months of receiving chemotherapy or six months of achieving CR. Patients from 92 clinical centers around the world (11 countries) were stratified by country and CR status (CR1 vs CR>1) and randomized to achieve relapse-preventive treatment with HDC/IL-2 or standard of care (no treatment) after the completion of regular induction and consolidation chemotherapy. Treatment was administered by subcutaneous injection twice daily (0.5mg HDC, 16,400 IU/kg IL-2) in ten three-week cycles over a period of 18 months (Figure 6), or until unacceptable toxicity, relapse, or death. After the first three treatment cycles, the rest period between cycles was extended from three to six weeks. Surviving patients were monitored for a minimum of 36 months, with a median follow-up time at trial completion of 48 months.

The primary endpoint of the trial was assessment of the impact of HDC/IL-2 treatment on leukemia-free survival (LFS), defined as time from random assignment to relapse or death from any cause. Secondary endpoints included effects on overall survival (OS), LFS or OS in CR status subgroups as well as assessments of toxicity, safety, and aspects of quality of life.

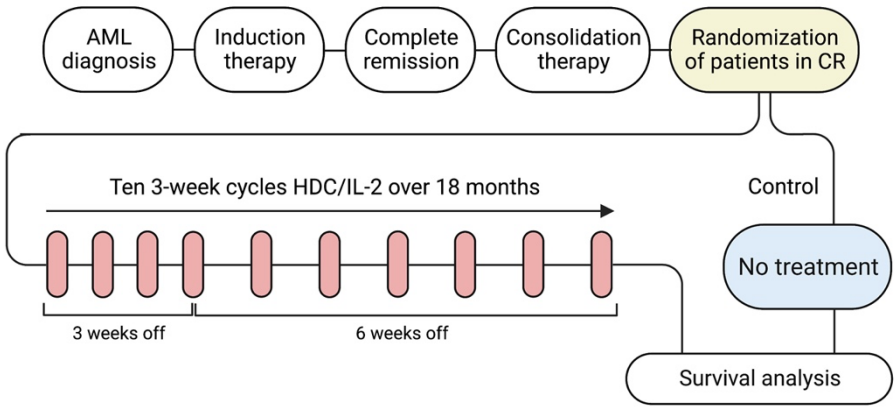

Figure 6. HDC/IL-2 phase III trial design. Created using BioRender.com.

Of relevance to the analyses in **papers I and II**, 203 out of 260 patients in CR1 (78%) had previously achieved complete remission after one induction chemotherapy cycle, whereas 57 patients (22%) had required more than one cycle (**paper I**). Among 225 CR1 patients with known karyotype, 128 (57%) had leukemic clones of normal karyotype and 97 (43%) carried chromosomal aberrations (**paper II**).

PHASE IV TRIAL

In the follow-up single-armed phase IV trial (Re:Mission, EPC2008-02, NCT01347996; (140)), 84 AML patients (age 18-79; median 61 years) in CR1 from 20 European centers received relapse-preventive treatment with HDC/IL-2 using a similar schedule and dosing as that employed in the phase III trial (Figure 6). Also in this trial, treatment was initiated within three months of completion of consolidation chemotherapy, or six months of CR achievement, in patients not eligible for allogeneic stem cell transplantation. Surviving patients were followed for a minimum of 24 months from enrolment.

The primary trial endpoint was assessment of the impact of HDC/IL-2 treatment on immune cell populations and minimal residual disease (MRD) monitoring, with secondary endpoints comprising LFS, safety and correlations between immune responses and MRD levels.

Of relevance for **paper II**, 44 out of 80 patients with available baseline karyotype data (55%) had normal karyotype. Out of the 36 normal karyotype patients genotyped for *NPM1* mutation, 18 (50%) were positive. Similarly, four out of 39 genotyped patients carried *FLT3*-ITD, out of whom three also had a co-existing *NPM1* mutation.

CELLS AND PATIENT MATERIAL

Papers III and IV involved the analysis of bone marrow (BM) and/or peripheral blood (PB) mononuclear cells (MNC) obtained from 21 chronic phase (CP) CML patients (age 25-75; median 53 years) diagnosed and treated at the Sahlgrenska University (Gothenburg, Sweden; n=20) and Uddevalla (Uddevalla, Sweden; n=1) hospitals. 17 of the 21 patients received cytoreductive hydroxyurea (HU) treatment prior to TKI treatment start and were thus included in the analyses presented in **paper IV**. In these analyses, the assayed PB/BM samples were obtained from seven treatment-naïve patients and from ten patients following initial HU treatment (4-19 days; median 9 days). Three of the HU-treated patients additionally had PB samples obtained before HU treatment, which allowed their use in paired analyses of HU effects within the same individual. A summary of the age and gender of all CML patients included in the two papers along with details on HU treatment and which analyses each patient was involved in is provided in the Appendix.

Follow-up BM samples were obtained three months into TKI treatment from ten of the 16 patients included in **paper III**. Two of the patients additionally had samples retrieved at seven months, giving a total of 12 follow-up BM samples in the study. **Paper III** also involved the analysis of healthy control BM samples. These were obtained from the femur of five patients undergoing hip replacement surgery at the Sahlgrenska University Hospital (Mölndal, Sweden).

To isolate MNC, PB and BM samples were subjected to Lymphoprep density gradient centrifugation. MNC samples with significant immature granulocyte contamination additionally went through magnetic bead CD15 depletion/CD34 enrichment or morphological MNC FACS sorting prior to cryopreservation. The sample processing procedure is summarized in Figure 7.

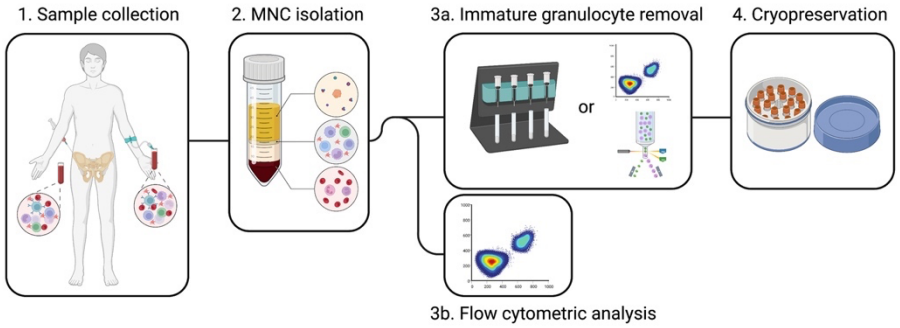

Figure 7. Processing of CML/healthy peripheral blood and bone marrow samples. Created with BioRender.com, using (233).

The studies were approved by the Regional Ethical Review board in Gothenburg, and all CML patients and healthy controls gave written informed consent prior to BM and/or PB retrieval.

FLOW CYTOMETRY

When combined with fluorescently labeled antibodies binding to specific extra- or intracellular proteins, flow cytometry enables high-throughput single-cell analysis of protein expression, cell size and granularity. Fluorescence-activated cell sorting (FACS) is an extension of the technique, where the flow cytometric analysis output allows for selection and sorting of cell populations of interest for further characterization.

In **paper IV**, flow cytometry was used to assess proportions of $CD34^+$ and $CD34^+CD38^-$ cells in PB MNC (PBMC) and BM MNC samples obtained before and after HU treatment prior to cryopreservation (Figure 7). In addition, FACS-based isolation of $CD14^-CD34^+$ cells preceded single-cell capture for the multiomic analyses in **papers III and IV**.

SINGLE-CELL MULTIOMIC ANALYSIS

As previously mentioned, high-throughput single-cell RNAseq approaches may enable unbiased analysis of the inherent heterogeneity within healthy and diseased cell samples, and thus improve the detailed understanding of hematopoiesis and leukemia. Traditionally employed bulk approaches to gene expression analyses inevitably yield averaged expression values, which may skew or conceal heterogeneity among cells.

Multiomic single-cell approaches carry additional benefit in that they provide coupled protein expression data. This can be beneficial in several ways. First, protein expression is more stable than mRNA expression, which suffers from dropouts due to transcriptional bursting and RNA degradation. As illustrated in Figure 8, this e.g. means that protein expression data often outperform mRNA data in terms of clarity when it comes to assessing phenotypes and annotating cell types in transcriptome-based analyses. In addition, multiomic analysis may allow the identification of cell surface markers for cell types defined using scRNAseq data, enabling prospective isolation and functional characterization. As such, multiomic single-cell analysis provides a much-needed bridge between scRNAseq and flow cytometry, which additionally may facilitate the interpretation of data obtained in the immunophenotypic and scRNAseq-based research eras.

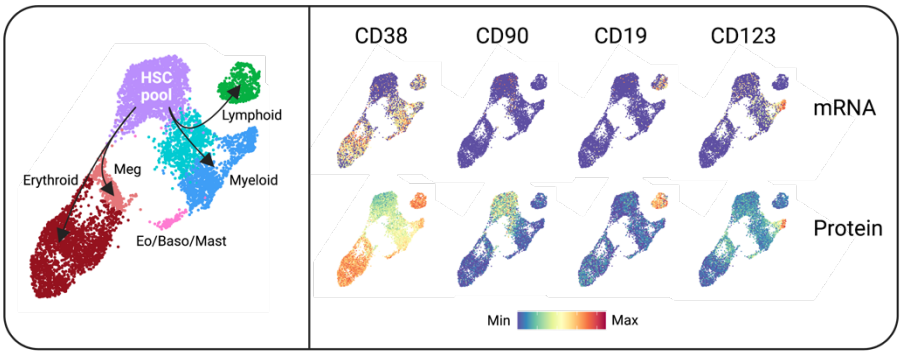

Figure 8. RNA vs protein expression of selected markers in single-cell multiomic analysis. Created using BioRender.com.

In **papers III and IV**, the BD Rhapsody Single-Cell Analysis System was used to assess gene and protein expression in single cells obtained from chronic phase CML patients and healthy controls. In **paper III**, we performed multiomic analysis of single $CD14^{-}CD34^{+}$ BM MNCs obtained from 16 CP-CML patients prior to TKI treatment start, 10 CP-CML patients three to seven months into TKI treatment and five healthy controls. The analyses involved a total of 58,682 diagnosis cells (out of which 11,247 additionally were $CD38^{-/low}$), 4,571 TKI follow-up cells (2,483 $CD38^{-/low}$) and 7,161 healthy BM cells (4,090 $CD38^{-/low}$). In **paper IV**, we additionally generated multiomic data for peripheral blood $CD14^{-}CD34^{+}$ MNCs obtained before and after HU treatment for two CP-CML patients.

BCR-ABL1 DETECTION

The only unequivocal marker of CML LSC is the expression of the disease-causing fusion oncogene *BCR-ABL1*. However, many concurrent high-throughput RNA sequencing approaches (including that utilized in this thesis) rely on 3' end capture of mRNA molecules through complementary binding to the poly-A tail, and only provide sequence information in its proximity. This makes reliable identification of *BCR-ABL1* transcripts impossible. For **paper III**, we thus set up a method allowing assessment of *BCR-ABL1* expression in single cells subjected to 3' end capture-based multiomic expression analysis (further described in the results and discussion section).

SEQUENCING DATA ANALYSIS

Following the generation of mRNA and protein unique molecular identifier (UMI) count matrices by the BD Rhapsody Targeted Analysis Pipeline, the multiomic data generated in **papers III and IV** was analyzed in R using standard

Seurat methodology (v4; (234)). Differences between S and G2/M cell cycle phase-related gene expression were regressed out of the data followed by mRNA expression-based principal component analysis (PCA), clustering and visualization using uniform manifold approximation and projection (UMAP). This methodology effectively reduces the dimensionality of the multiomic data and enables clustering and two-dimensional visualization of transcriptional similarity between cells.

STATISTICAL METHODS

In **papers I and II**, the logrank test was used to assess survival distribution differences between patient groups. Multivariable analyses were performed by Cox regression on covariates with p values below 0.1 in univariable analyses using SPSS Statistics (IBM). Analyses of aspects of induction chemotherapy and karyotype in relation to survival outcomes were performed *post hoc*.

In **papers III and IV**, differences in single-cell gene and protein expression were assessed by the non-parametric Wilcoxon Rank Sum test, only considering genes/proteins expressed in at least 25% of the cells in each group and filtering out |log2FC| values below 0.25. In analyses evaluating differential expression of the full set of genes/proteins, p values were adjusted for multiple testing using the Bonferroni method. The unpaired Mann-Whitney test was employed for unpaired comparisons of cell type proportions, and the paired Wilcoxon test for corresponding analyses in paired PBMC and BM MNC data (**paper IV**).

In **paper III**, The Pearson correlation coefficient was used to assess correlation between expressional patterns in the K562 cell line before and after changing the RT procedure to allow for *BCR-ABL1* detection.

In all analyses, two-tailed p values below 0.05 were considered significant.

RESULTS AND DISCUSSION

This thesis comprises data from four papers focusing on aspects of targeting residual malignant cells in myeloid leukemia. In the first two papers, retrospective analyses of HDC/IL-2 clinical trial data were carried out to identify AML patient groups where this treatment may allow the eradication of residual leukemia in the post-chemotherapy phase. For the other two papers, with focus on CML, we set out to provide a multiomic characterization of the stem and progenitor cell compartment that is believed to hold the treatment-resistant leukemic stem cells.

SUBGROUP EFFICACY OF HDC/IL-2 IN AML

In recent decades, the increased recognition of disease heterogeneity has birthed the concept of precision medicine; that each individual should receive treatment tailored to their specific disease risk and/or predicted response (235). In AML, disease and patient heterogeneity is present at many levels; from the genetic makeup and morphology of the leukemic cells to individual treatment responses and factors affecting treatment tolerability, such as age and underlying comorbidities. In line with this, several drugs targeted to specific populations of AML patients have been released in recent years (236). While the definition of target populations is relatively straight-forward for some therapies (e.g. first and later generation FLT3 inhibitors for patients carrying *FLT3* mutations), patient selection for optimal therapeutic benefit is more challenging for others. AML maintenance treatment with HDC/IL-2 is an example of the latter. A therapy that aims to stimulate immune eradication of the leukemic clone does not have an inherent target population *per se*. Instead, clinical experience may be used to identify patient groups of primary interest. In its phase III trial, HDC/IL-2 was assessed in a wide group of patients, comprising adults in first or subsequent CR, not eligible for allo-hSCT. The trial met the primary endpoint of improved LFS across all included patients, with pronounced efficacy for patients in CR1 (the current indication) and in patients below the age of 60 (Figure 4; (7)). The best responder group was later further refined by *post hoc* analysis to patients carrying leukemic cells of monocytic morphology, i.e. AML FAB M4/M5 (135), which is the recommended target group for HDC/IL-2 treatment in the Swedish AML treatment guidelines (69). However, with the field gradually turning away from the FAB classification system in favor of the more prognostically relevant WHO and ELN systems, the identification of additional HDC/IL-2 target populations has become increasingly important.

In this thesis, we aimed to define AML patient groups likely to benefit from relapse-preventive HDC/IL-2 treatment using current clinical parameters. To this

end, we performed retrospective analysis of the HDC/IL-2 phase III trial data under the current treatment indication (i.e. patients in first CR), focusing on response to initial chemotherapy (**paper I**) and genetic stratification relevant in the WHO/ELN classification era (**paper II**). A graphical abstract for this part of the thesis, including the main results, is shown in Figure 9.

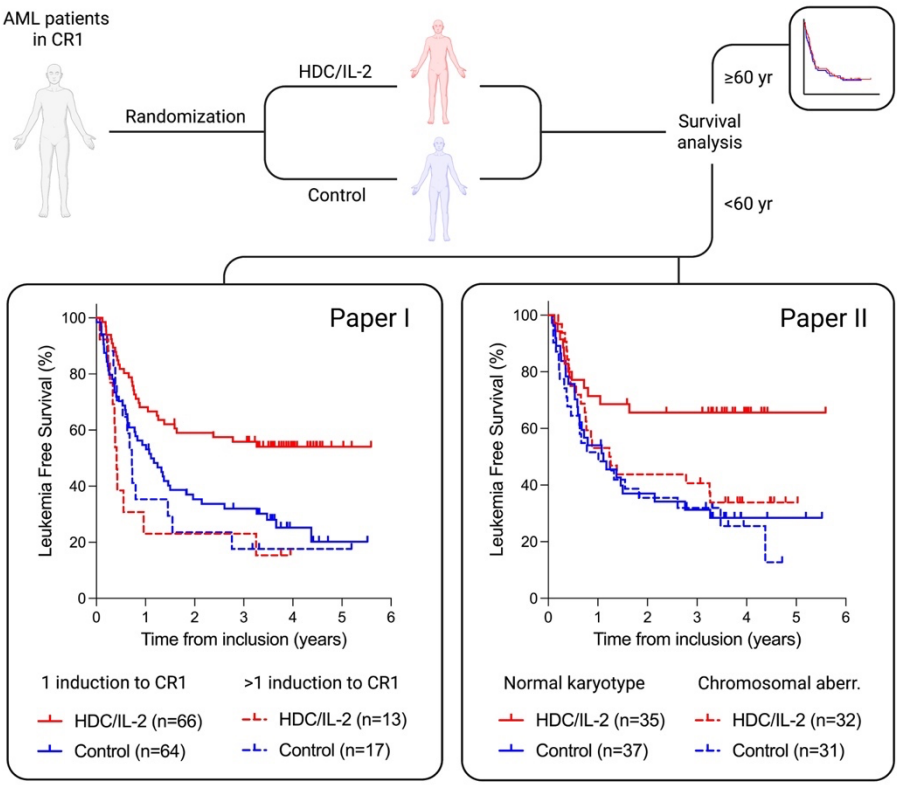

Figure 9. Graphical abstract of papers I and II. Created with BioRender.com.

In **paper I**, we found that HDC/IL-2 treatment primarily benefitted the group of patients achieving complete remission after one cycle of induction chemotherapy, but not those requiring ≥2 induction courses. Other contemporary studies support the importance of fast achievement of CR upon initial chemotherapy for AML outcomes (96,97), as well as for the outcome of allo-hSCT (237). There is also data supporting that rapid clearance of circulating blasts during initial treatment produces superior outcomes (238), collectively indicating that the chemosensitivity of the leukemic clone carries prognostic importance in AML. It is tempting to derive that efficient initial therapeutic reduction of malignant cells may be indicative of the amount of residual leukemia

present at the end of chemotherapy, when higher levels (as reflected by detection of minimal residual disease, MRD) are known predictors of poor survival outcomes (239). Since both HDC/IL-2 treatment and allo-hSCT are believed to rely on immune-mediated clearance of leukemic cells, a higher level of residual disease at treatment onset may shift the balance in favor of the leukemia, overwhelming the immune system and thwarting the treatments' relapse-preventive effects.

In **paper II**, the analysis revealed pronounced efficacy of HDC/IL-2 in patients with normal karyotype AML. As 65% of these patients carry mutations in *NPM1* and/or *FLT3*-ITD (240), the magnitude of the observed treatment effect spoke in favor of efficacy in patients with at least one of these aberrations. Dating back to the turn of the century, the phase III trial did not have genotyping data enabling correlation between treatment outcome and specific genetic lesions other than karyotype. However, using data from the subsequent single-armed phase IV Re:Mission trial, we concluded that treatment efficacy likely comprises patients with *NPM1* mutations, but not those carrying *FLT3*-ITD (alone or in parallel with *NPM1* mutation) (Figure 10).

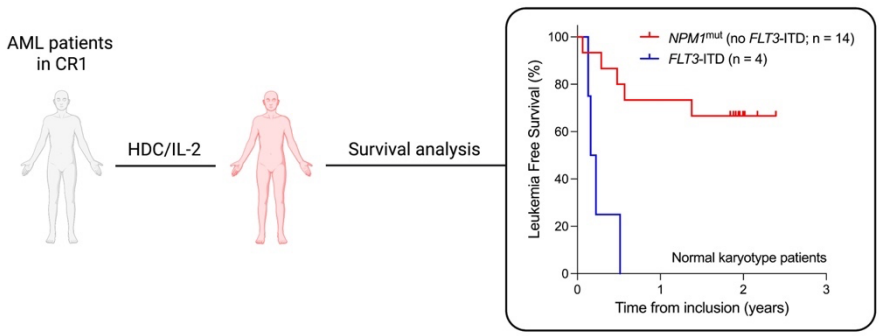

Figure 10. Outcome of HDC/IL-2-treated normal karyotype AML patients with NPM1 mutation (NPM1^{mut}) or FLT3-ITD in the single-armed phase IV Re:Mission trial. Created with BioRender.com.

While administration of relapse-preventive treatment with HDC/IL-2 to patients with *NPM1* mutation without concurrent *FLT3*-ITD may be more clinically relevant today than the previous FAB M4/M5 recommendation, the two may in fact be related. Among patients with M4/M5 AML, 77-90% reportedly carry *NPM1* mutations (241). The observed efficacy in this group of patients may thus, as previously discussed, relate to the leukemic phenotype, where direct inhibition of immunosuppressive ROS production (135) and maturation effects (138) may help explain the relapse-preventive effects of HDC/IL-2. In addition, patients with *NPM1* mutations are reportedly less likely to require more than one

induction chemotherapy cycle to achieve CR (97), possibly providing a bridge between the findings in **papers I and II**.

In both studies, the relapse-preventive efficacy of HDC/IL-2 was most pronounced in patients below 60 years of age. Although the reasons for this are not clear, one might speculate that the older patient group, due to tolerability concerns, may have received less intensive chemotherapy regimens, which in turn may have resulted in higher levels of residual leukemia at HDC/IL-2 treatment start. As mutations accumulate throughout life, it is also possible that there are differences in the underlying mutational profiles of the leukemic clones, which may entail a higher number of known or unknown intermediate or adverse risk aberrations in the older patients. Without genotyping data for the phase III trial patients this is, however, difficult to assess. Future studies may provide further insight into whether there are specific patient groups also above 60 years that may benefit from maintenance treatment with HDC/IL-2.

Taken together, the results from **papers I and II** support that relapse kinetics, e.g. affected by mutations in the leukemic clone and/or residual leukemia at treatment start, may impact on the efficacy of HDC/IL-2 in AML. Hence, less aggressive disease among normal karyotype patients (e.g. reflected by *NPM1* mutation without concurrent *FLT3*-ITD, or by a more chemosensitive leukemic clone) might give the immune system a better chance to, bolstered by the immunostimulatory efficacy of HDC/IL-2, eradicate residual leukemic cells and prevent relapse in the post-chemotherapy phase of AML. If so, future improvements to frontline AML therapy enabling reduced disease burden post consolidation therapy may allow HDC/IL-2 efficacy in a wider, yet distinct, group of patients.

MULTIOMIC CHARACTERIZATION OF CML SPC

CML has transformed from being an almost invariably fatal leukemia into a largely controllable chronic disease with most patients now approaching a normal life expectancy (5). However, TKI resistance, intolerance and blast crisis progression remain matters of concern, and due to the persistence of TKI-resistant LSC, only a subgroup of patients may discontinue TKI treatment without ensuing leukemic relapse. Therefore, an increased understanding of the TKI-eluding LSC population may pave the way to the development of targeted treatment options, potentially curing CML once and for all.

Since progressive alterations to gene expression in progenitor cells are associated with maturation into distinct hematopoietic lineages, single-cell RNAseq (scRNAseq) approaches are ideally suited for assessment of heterogeneity as well as differentiation patterns within healthy and leukemic bone marrow. As illustrated by Warfvinge *et al.* in 2017 (232), the traditionally defined $CD34^+CD38^{-/low}$ LSC compartment in CML houses significant heterogeneity in terms of cell types, suggesting that a redefinition of the LSC phenotype may be needed. However, due to low engraftment rates of primary CML samples in immunodeficient mice (57), the golden standard definition of HSC/LSC has been problematic, thus complicating the identification of CML LSC cell surface markers. For this type of analysis, scRNAseq approaches that involve parallel protein expression analysis (multiomics) are promising, as they provide a bridge between the transcriptional profile and surface protein expression of individual cells. Multiomic analysis of the $CD34^+$ stem and progenitor cell (SPC) compartment in CML may thus allow transcriptional back-tracing from more mature progenitors to a common ancestor (likely comprising the LSC), which through the paired protein expression data could be prospectively isolated and functionally characterized by traditional approaches.

This thesis comprises a detailed single-cell multiomic characterization of the $CD34^+$ CML SPC compartment at diagnosis, which was compared to those present during TKI treatment and in healthy BM in hopes of providing further insight that may ultimately help target the LSC population. In **paper III**, a method allowing parallel detection of single-cell *BCR-ABL1* expression was developed and used together with the multiomic data to characterize the CML BM SPC compartment in general, and the LSC in particular. In further studies (**paper IV**), we assessed the impact of cytoreductive hydroxyurea treatment on diagnosis CML SPC. The workflow for the multiomic analyses presented in **papers III and IV** is shown in Figure 11.

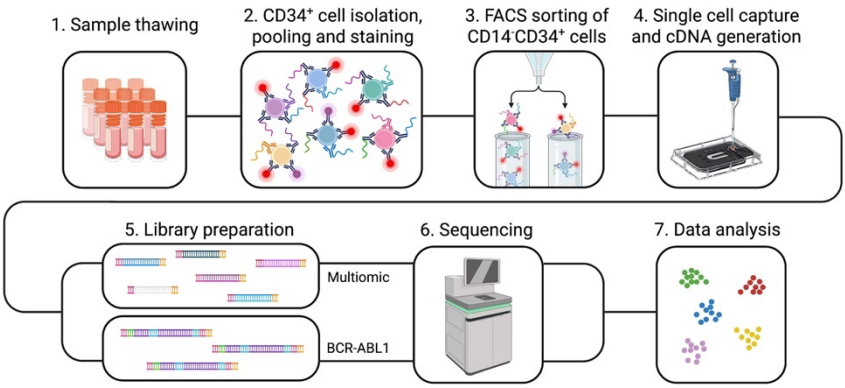

Figure 11. Multiomic analysis workflow. Created with BioRender.com.

Concurrent high-throughput 3' end capture-based scRNAseq approaches are typically not compatible with detection of *BCR-ABL1* expression, as they rely on identification of transcripts through sequences relatively close to the mRNA poly-A tail. For *BCR-ABL1* transcripts, this sequence is shared with *ABL1* transcripts, thus preventing their conclusive identification. In order to distinguish between healthy and leukemic cells within the CML BM, we thus adapted a previously described method (242) to bring the unique fusion point of the *BCR-ABL1* transcript closer to the poly-A tail and cell label, allowing identification of transcripts as well as their cell of origin in subsequent sequencing analysis. As illustrated in Figure 12, the adapted method involved a series of PCR reactions and PCR product circularizations, ultimately resulting in a shortened *BCR-ABL1* fragment compatible with short-read sequencing of the *BCR-ABL1* fusion point. In subsequent multiomic data analysis, the identification of *BCR-ABL1*-positive cells successfully distinguished healthy and leukemic stem cell clusters, thus providing additional support to our LSC classification.

In the first part of **paper III**, we assessed the cell type composition of the $CD34^+$ BM SPC compartment in 16 CML patients at diagnosis, and observed a distinct myeloid bias, with most patients displaying unproportionally high percentages of erythroid and relatively immature myeloid-biased progenitors, and lower proportions of HSC and lymphoid progenitors, compared with healthy SPC. This agrees with previous scRT-qPCR-based findings from the $CD34^+CD38^{-/low}$ compartment, which indicated relatively high proportions of certain myeloid and megakaryocyte/erythroid progenitors, but lower proportions of immature and lymphoid cells within the *BCR-ABL1*$^+$ fraction of the CML BM (232). In our analysis, the proportions to a higher degree resembled those of healthy BM after three months of TKI treatment, likely reflecting the recovering healthy hematopoiesis in well-responding patients.

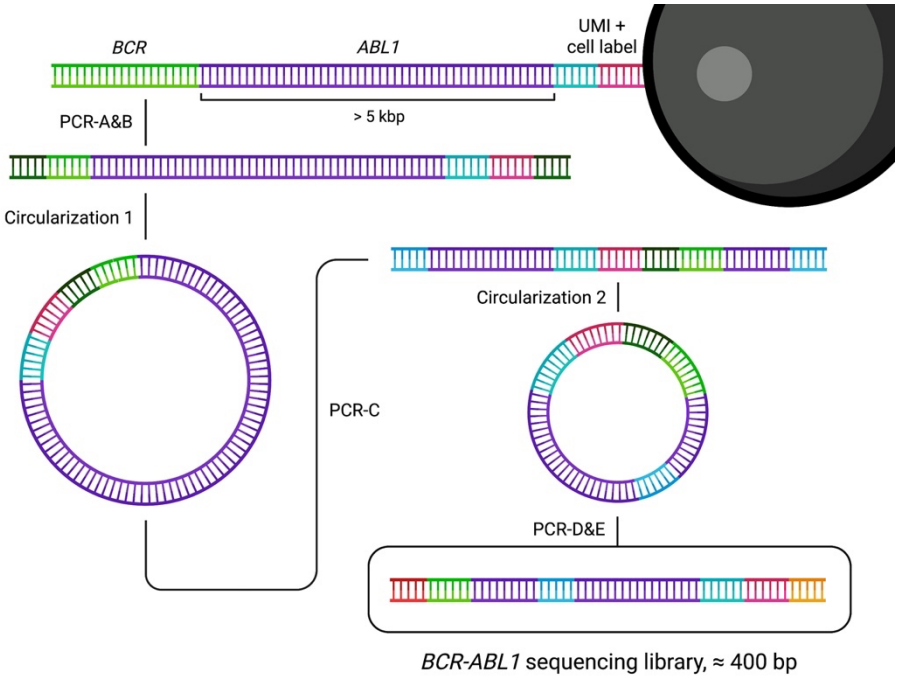

Figure 12. BCR-ABL1 sequencing library preparation. An overview of the method developed for detection of *BCR-ABL1* expression in cells with paired multiomic data in paper III. Circularization reactions relied on ligation of complementary PCR product ends. Sequencing adapters are shown in red and orange. UMI: unique molecular identifier. Created with BioRender.com.

As an add-on, the analysis of the CD34$^+$ compartment at CML diagnosis challenged the relatively recent scRNAseq-based model of hematopoiesis comprising an early bifurcation of lympho-myeloid and megakaryocytic-erythroid (Meg/Er) progenitors (33–35). While we did observe this early divergence in our analysis of healthy BM samples, the cell clustering patterns from the CML SPC compartment instead implied that differentiation to Meg/Er and other myeloid progenitors may occur through an immature common myeloid progenitor (Figure 13), as described in the original immunophenotype-based hematopoiesis models (Figure 1). In view of the current struggle to reconcile the immunophenotypically and transcriptionally defined hematopoiesis models, our findings thus point to the possibility that both models may have merit, and that specific differentiation patterns may depend on the conditions and cell types present within the individual bone marrow.

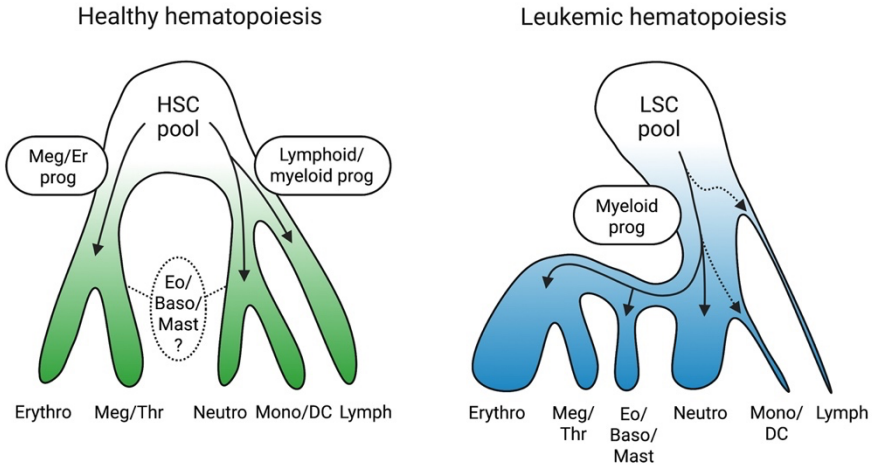

Figure 13. Healthy vs CML scRNAseq-based differentiation patterns. Hematopoiesis in CML seemingly progresses through an immature common myeloid progenitor population, generating large numbers of myeloid progenitors but little lymphoid output (as indicated by dashed arrows in the figure). Created with BioRender.com.

To achieve better resolution for analysis of CML LSC, we next turned our attention to the traditionally defined $CD34^{+}CD38^{-/low}$ CML LSC compartment through multiomic protein expression-based cell gating. In line with a previous report (232), we found substantial heterogeneity within the $CD34^{+}CD38^{-/low}$ compartment at CML diagnosis, albeit with a similar myeloid bias as that previously observed within the $CD34^{+}$ compartment. Arguably, the heterogeneity may have been lower with a stricter gate for CD38-negative cells, as the subsequently identified LSC (and HSC) were found to have the lowest CD38 expression within this compartment. This highlights the problems associated with bulk characterization of $CD34^{+}CD38^{-/low}$ CML "LSC", and the benefit provided by multiomic single-cell analysis. Using the multiomic data, we were able to identify the most immature subgroups of cells and focus our downstream analysis on these despite the generous CD38 gate, which would not have been possible in bulk analyses. Compared to the CML $CD34^{+}CD38^{-/low}$ compartment, the healthy counterpart was found to be less heterogeneous and contain a higher proportion of immature cells, thus skewing bulk data comparisons between $CD34^{+}CD38^{-/low}$ "HSC" and "LSC" even further. Hence, our data argue in favor of the use of strict gates for CD38 negativity when performing bulk analysis of CML LSC, and additionally identifies this as a factor that should be considered in the interpretation of previously obtained data.

In combined analysis of $CD34^{+}CD38^{-/low}$ cells from healthy, CML diagnosis and TKI follow-up samples, we identified four groups of cells displaying the traditional $CD45RA^{-}CD90^{+}$ HSC phenotype; two of which were found in healthy

BM and two that were not. Unlike the two populations of cells found in the healthy BM (HSC-I and HSC-II), those exclusive to the CML BM were found to express *BCR-ABL1* as well as the previously reported LSC markers CD25 and CD26, thus rendering it likely that these cell populations comprised the LSC. Out of the two LSC populations, one (LSC-I) matched the quiescent and $CD45RA^{-}cKIT^{-}CD26^{+}$ phenotype of cells previously described to persist during TKI treatment (232), whereas the other displayed a transcriptional profile bioinformatically associated with actively cycling cells (cells in G2, M or S phase of the cell cycle). In differential expression analyses between the presumably clinically relevant LSC-I population and HSC within healthy or CML BM, we identified previously unreported significant upregulations of von Willebrand factor (*VWF*) and the protein TIM3. Our findings additionally supported the previously reported downregulation of *CXCR4* in CML LSC compared with their healthy counterparts (228). However, also HSC within the CML diagnosis BM displayed abnormally low *CXCR4* expression. Furthermore, the HSC *CXCR4* expression was seemingly not normalized by TKI treatment, potentially indicating some lasting, BCR-ABL1 kinase independent effect on healthy cells within the CML BM. Figure 14 shows an overview of some of the results from the differential expression analyses.

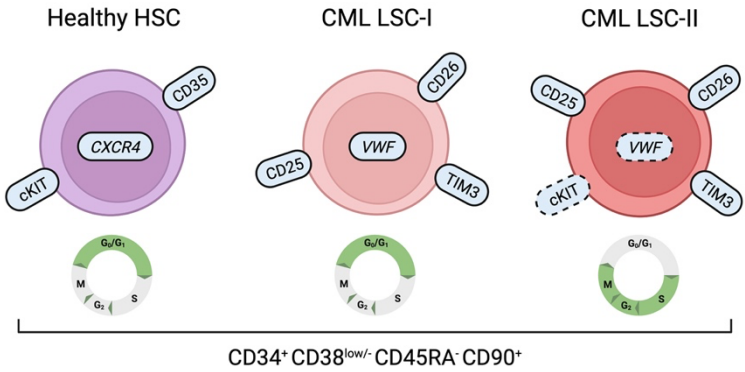

Figure 14. Healthy and leukemic stem cell populations and markers identified in the multiomic analysis. Dashed lines indicate lower expression level. Created with BioRender.com.

Previous studies implicate both *VWF* and TIM3 in myeloid-biased differentiation. *VWF* expression reportedly marks a subset of HSC predominantly producing myeloid output upon transplantation to mice (243,244), and TIM3 has been identified as a pan-myeloid marker in a recent multiomic single-cell analysis of the healthy $CD34^{+}$ HSPC compartment (35). Although little is known regarding these markers in the context of CML LSC, TIM3 has been reported to distinguish AML LSC from healthy HSC (245,246). In AML, TIM3 is reportedly involved in an autocrine loop supporting LSC self-renewal (247),

and has been explored as a therapeutic target for specific killing of AML LSC in preclinical and clinical trials (245,248). In a healthy context, TIM3 plays a role in immune regulation. Pertaining to this, a recent report by Irani *et al.* suggested that high expression of TIM3 on T cells at TKI cessation was associated with high risk of molecular recurrence in CML (249). The authors additionally reported upregulation of TIM3 gene expression in the CML LSC population using publicly available datasets and concluded that TIM3 targeting may carry dual efficacy in improving immune function as well as directly targeting the CML LSC to improve TFR rates upon TKI treatment cessation. In confirming consistent TIM3 CML LSC expression at the protein level, **paper III** thus supports and extends the aforementioned findings.

All in all, the results presented in **paper III** support the view of CML hematopoiesis as deriving from a relatively quiescent population of cells that carries a $CD34^+CD38^{-/low}CD45RA^-cKIT^-CD26^+TIM3^+$ phenotype, expresses *VWF*, and sits at the top of a differentiation trajectory of myeloid-biased leukemic progenitors within the $CD34^+$ and $CD34^+CD38^{-/low}$ compartments in the CML BM. Together with the previous report from Warfvinge *et al.* and studies reporting CD26 as an LSC marker in CML (200,232,250), our results additionally call to question whether the current $CD34^+CD38^{-/low}$ immunophenotype of CML LSC should be redefined to achieve higher specificity.

Although hydroxyurea (HU) is no longer indispensable in therapeutic management of CML, it is frequently used to reduce abnormally high white blood cell or platelet counts and reduce the risk of leukostasis prior to definitive diagnosis. Data from the pre-TKI era indicate that HU does not significantly affect the course of disease in CML (146,161), arguing against the drug being able to target or eradicate LSC. However, a detailed scRNAseq characterization of the effects of hydroxyurea on the CML SPC compartment had not been performed prior to the studies presented in **paper IV**. Since only a proportion of patients receive HU treatment, and since diagnosis samples for research purposes may be obtained prior to or after initial hydroxyurea treatment, potential effects on cell type proportions or the LSC phenotype may be of relevance for studies addressing heterogeneity within the SPC compartment at CML diagnosis.

By studying flow cytometric and multiomic data from paired blood samples obtained before and after HU treatment, as well as unpaired BM samples from HU-treated or -naïve patients (awaiting HU treatment), we found multiple HU-related effects, largely summarized in Figure 15. While HU treatment tended to reduce the proportion of $CD34^+$ cells among MNC in blood and BM, it resulted in a significant reduction in the proportion of $CD38^{-/low}$ cells within the $CD34^+$ BM compartment, likely reflecting the treatment's cytostatic effects. In studies of

the entire CD34+ compartment, both paired and unpaired multiomic analyses indicated enhanced proportions of relatively mature hemoglobin-expressing erythroid progenitors, which in line with previous reports may be the result of HU-mediated induction of nitric oxide, leading to activation of cyclic guanine monophosphate signaling and hemoglobin synthesis in erythroid cells (251).

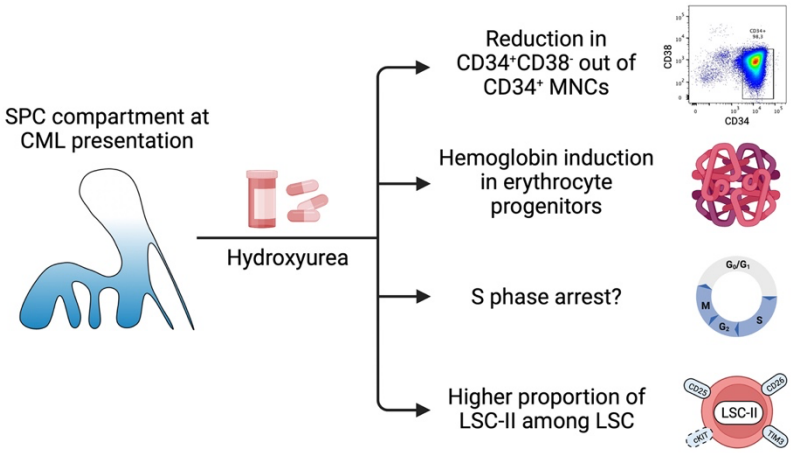

Figure 15. Effects of hydroxyurea treatment on the CD34+ SPC compartment at CML diagnosis. Created with BioRender.com.

The HU-treated CML SPC compartment was found to contain higher levels of cell subsets with proliferative S/G2/M phase-related gene expression than its treatment-naïve counterpart, which may seem counterintuitive. However, as a major effect of HU treatment is a reduction in the availability of dNTPs (175), the increased proportions of cells in S/G2/M phase may be indicative of higher numbers of cells arrested in, or progressing slowly through, the DNA-replicating S phase of the cell cycle. We observed this effect on all levels of the leukemic hierarchy, from more mature erythroid progenitors to relatively immature myeloid progenitors, and even within the LSC compartment, where HU treatment was found to increase the proportion of the S/G2/M-associated LSC-II population and decrease the proportion of LSC-I. These results thus imply that the cytoreductive effects of HU in CML involve leukemic cells at all SPC maturation levels.

In **paper III**, analysis of LSC proportions versus TKI treatment outcome indicated that patients who required highly potent ponatinib treatment to achieve CCyR had significantly higher proportions of LSC-II in their diagnosis BM samples, which we concluded may be indicative of a more proliferative LSC clone. However, this was complicated by the above-mentioned findings from **paper IV**,

suggesting that the LSC-II proportions may be increased by hydroxyurea treatment prior to sampling. Both of the ponatinib-treated patients had indeed received HU prior to BM aspiration, but we did not observe a correlation between time on HU treatment and the proportion of LSC-II among HU-treated patients. A higher proportion of LSC-II cells may thus still be indicative of a more proliferative LSC clone, as an increasing number of dividing cells involves increased proportions of cells that may end up progressing slowly through, or arrested in, the S phase during HU treatment.

In a final analysis addressing the LSC-I compartment, we performed differential expression analyses between LSC-I from paired blood samples obtained before and after HU treatment, as well as from paired blood and BM samples. These analyses did not reveal any significant impact on the LSC-I following HU treatment or depending on whether they had been obtained from blood or BM. In line with the pre-TKI data these results thus suggest that the most primitive, presumably mainly un-dividing, LSC-I population is unlikely to be affected by HU treatment. In addition, peripheral blood may be a valid alternative to BM for transcriptional, proteomic, or functional characterization of the LSC population at CML diagnosis.

CONCLUDING REMARKS

This thesis aimed at contributing to the understanding of aspects of importance for targeting leukemic cells that are left behind following conventional therapy in acute and chronic myeloid leukemia.

Based on current clinically relevant parameters, **papers I and II** focused on the identification of AML patient groups who might benefit from relapse-preventive HDC/IL-2 maintenance treatment for the eradication of leukemic cells escaping chemotherapy. Taken together, the results suggest that younger patients (<60 years) with chemosensitive AML of normal karyotype (without *FLT3*-ITD) may be considered for this therapeutic regimen.

Importantly, many patients within this new potential indication currently do not have other viable options for remission maintenance in the post-chemotherapy phase. Younger patients carrying *NPM1* mutation without *FLT3*-ITD in a normal karyotype context are e.g. not eligible for FLT3 inhibitor or oral azacitidine maintenance treatment due to their mutational profile and age, respectively. In addition, they are classified as favorable risk AML in the ELN 2022 risk stratification (92), and are as such typically not candidates for upfront allo-hSCT based on the risk-to-benefit analysis (in view of allo-hSCT-related morbidity and mortality). However, the long-term overall survival among patients with cytogenetically normal AML with *NPM1* mutations is in the range of 50% (84), highlighting the importance of remission maintenance also in patients of comparatively favorable risk.

In a subgroup of patients, HDC/IL-2 thus seems to fulfill an unmet need of relapse-prevention in the post-chemotherapy phase. However, the *post hoc* nature of the results presented should be emphasized, and future studies evaluating the efficacy of HDC/IL-2 in patient populations indicated in this thesis are warranted. Ideally, such studies would include a relevant control group and sequential MRD measurement to assess the impact of residual disease levels at treatment start and/or whether these cells can be removed by the immunotherapy.

In **papers III and IV**, we characterized the CML stem and progenitor compartment by multiomic analysis paired with *BCR-ABL1* expression detection at the single-cell level, to provide increased knowledge pertaining to the TKI-eluding LSC. The results in **paper III** indicated significant heterogeneity within the traditional CML LSC compartment, suggesting that the phenotypical definition of LSC as $CD34^{+}CD38^{-}$ cells may need revision. Notably, the method

set up for parallel detection of *BCR-ABL1* expression allowed distinction between leukemic and healthy cell populations within the CML BM, providing important support to our LSC identification. In line with a previous report, the LSC were found to display a $CD34^+CD38^{-/low}CD45RA^-cKIT^-CD26^+$ phenotype.

Within the LSC population, the analysis additionally revealed previously unreported upregulations of *VWF* and TIM3. These have both been associated with myeloid cell differentiation and may thus be implicated in the myeloid bias observed among SPC as well as in more mature compartments in CML. However, whether *VWF* and TIM3 are driving or passengers in the myeloid-biased differentiation remains to be explored. In addition, while the $CD34^+CD38^{-/low}CD45RA^-cKIT^-CD26^+$ LSC phenotype reportedly persists during TKI treatment (232), it is currently not known whether *VWF* or TIM3 expression is BCR-ABL1-dependent and as such would be affected by TKI treatment. Further studies are thus required to confirm and extend these findings. However, since TIM3 is a surface receptor, it may be included in FACS-based phenotypic identification of CML LSC.

In **paper IV**, multiomic characterization of the CML blood and BM SPC compartments before and after HU treatment revealed proportional shifts involving increased numbers of cells of different maturation levels and lineage displaying S/G2/M phase transcriptional patterns, presumably representing cells arrested in the S phase of the cell cycle. The observed proportional shifts imply that previous HU treatment is a factor that may be considered in the analysis of future studies addressing the CML SPC compartment at diagnosis.

In contrast, the expression pattern of the relatively quiescent LSC-I population was seemingly unaffected by HU, which may explain why the treatment does not alter the course of disease in CML. Finally, our results did not indicate substantial differences between blood and BM $CD34^+$ cell type proportions or LSC expression patterns, implying that the two sample sources may be used interchangeably in future LSC characterization studies.

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APPENDIX

Chronic phase CML patients included in papers III and IV.

Pat ID	Age	Gender	Cytoreductive HU treatment	Days of HU prior to BM sample for HU-treated patients	Paper III	Paper IV			
						Multiomic analysis		Flow cytometry	
					Multiomic analysis of BM samples	Paired PB pre and post HU	BM pre or post HU	Paired PB and BM	PB and BM pre or post HU
1	75	M	Yes	0	Yes	No	Yes	Yes	Yes
2	61	M	Yes	0	No	No	No	Yes	Yes
6	53	M	Yes	9	No	No	No	Yes	Yes
9	36	M	Yes	0	Yes	No	Yes	Yes	Yes
11	55	M	Yes	8	No	No	No	Yes	Yes
13	65	M	Yes	0	No	No	No	Yes	Yes
14	33	M	Yes	6	Yes	Yes	Yes	Yes	Yes
17	51	M	Yes	11	Yes	No	Yes	Yes	Yes
18	35	M	Yes	4	Yes	No	Yes	Yes	Yes
19	25	M	Yes	8	Yes	No	Yes	Yes	Yes
20	53	M	No	NA	Yes	No	No	No	No
23	72	M	Yes	8	Yes	Yes	Yes	Yes	Yes
24	29	M	Yes	19	Yes	No	Yes	Yes	Yes
25	44	M	No	NA	Yes	No	No	No	No
26	66	F	Yes	0	Yes	No	Yes	Yes	Yes
27	62	F	Yes	0	Yes	No	Yes	Yes	Yes
30	40	F	No	NA	Yes	No	No	No	No
31	59	F	No	NA	Yes	No	No	No	No
33	39	M	Yes	13	Yes	No	Yes	Yes	Yes
34	51	M	Yes	9	No	No	No	Yes	Yes
301	66	F	Yes	0	Yes	No	Yes	Yes	Yes

Abbreviations: Pat, patient; HU, hydroxyurea; PB, peripheral blood; BM, bone marrow.