Multiomic profiling of leukemic stem cells in myeloid leukemia: implications for immunotherapy

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Gothenburg 2024

Cover illustration: Heterogeneity among hematopoietic stem cells from human bone marrow by Hana Komic

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ISBN 978-91-8069-591-6 (PRINT)
ISBN 978-91-8069-592-3 (PDF)
http://hdl.handle.net/2077/79334

Printed in Borås, Sweden 2024
Printed by Stema Specialtryck AB

To my family
Mojoj dragoj porodici, kao zahvala za sve godine podrske i ljubavi

ABSTRACT
Despite the extensive array of therapies available for treating hematological malignancies, a major challenge in hemato-oncology lies in therapy resistance, which primarily is caused by residual resistant leukemic stem cells (LSC). In order to eliminate leukemic cells, it is crucial to understand the molecular mechanisms enabling their survival and to identify specific targetable markers for LSC to spare healthy cells. While tyrosine kinase inhibitors (TKI) provide a favorable prognosis for patients with chronic myeloid leukemia (CML), a fraction of patients suffers from disease progression or relapse upon treatment discontinuation. Acute myeloid leukemia (AML) on the other hand is a complex disease and despite development of novel therapies, survival prognosis remains poor. A common denominator for these diseases is the therapy-resistant LSC that propagate and cause a relapse. Hence, the main goal of this thesis was to identify novel targets on residual LSC by dissecting the heterogeneity among hematopoietic stem and progenitor cells (HSPC) in healthy and diseased conditions.

In **Paper I** we provide a comprehensive molecular map of early human HSPC differentiation. Detailed analyzes of immature cell compartments identified novel HSC markers, including CD273/PD-L2. Functional validation showed that $CD273^{hi}$ cells have a quiescent profile and delayed *in vitro* differentiation, compared to $CD273^{low}$ cells. Furthermore, we revealed changes in the distribution of the most immature cells and lineage differentiation propensities upon ageing. **Paper II** focused on detailed multiomic profiling of the $CD34^{+}$ cells in bone marrow samples of CML patients. The most important finding of this study was the detection of two novel LSC markers, von Willebrand factor (*VWF*) and TIM3. **Paper III** aimed at understanding the effects of short-term hydroxyurea (HU) treatment on HSPC in CML patients. The results implicate HU-induced increased the frequency of erythroid progenitors and accumulation of cell subsets with S/G2/M phase-related gene profile. With **Paper IV**, we aimed to investigate the impact of HLA-B genotypes on outcome during histamine dihydrochloride (HDC) and interleukin 2 (IL-2) immunotherapy. The HLA-B*44 allele, which is a weak ligand to the inhibitory NK cell receptor, KIR3DL1, was found to be associated with poor survival. Our results suggest that a strong ligand-receptor interaction induces enhanced NK cell function, which may result in better leukemia control and prolonged survival. In summary, the results from this thesis could serve as basis for a development of targeted treatment for TKI-resistant LSC in CML and relapse-preventive approaches in AML.

Keywords: hematopoiesis, HSC, LSC, CML, AML, immunotherapy

SAMMANFATTNING PÅ SVENSKA

Trots en omfattande terapeutisk arsenal för att behandla hematologiska maligniteter, förblir terapiresistens och återfall i sjukdom de största utmaningarna inom hemato-onkologin. För att eliminera de sista leukemiska stamcellerna (LSC) är det viktigt, dels att förstå de molekylära mekanismerna som möjliggör deras överlevnad, dels att identifiera specifika LSC-markörer som kan utnyttjas terapeutiskt utan att skada motsvarande friska celler.

Kronisk myeloisk leukemi (CML) har en god prognos tack vare tyrosinkinashämmare (TKI), men en del patienter drabbas av sjukdomsprogression eller återfall om de avslutar behandlingen. Akut myeloisk leukemi (AML) å andra sidan, är en komplex sjukdom, som trots utvecklingen av nya läkemedel är förknippad med dålig prognos. Det huvudsakliga målet med denna avhandling var således att identifiera nya målstrukturer på LSC genom att kartlägga heterogeniteten bland hematopoetiska stam- och progenitorceller (HSPC) i friska och sjuka blodceller.

I **Delarbete I** presenterar vi en omfattande molekylär karta över de mest omogna HSP-cellernas differentiering. Omfattande analyser påvisade nya HSC-markörer, såsom CD273/PD-L2. Funktionella valideringsförsök visade att $CD273^{hi}$-celler är vilande och uppvisar fördröjd *in vitro*-differentiering jämfört med CD273low-celler. Dessutom visar vi hur fördelningen av de mest omogna cellerna och differentieringsbenägenheten varierar med åldern. **Delarbete II** omfattar detaljerad multiomisk profilering av $CD34^{+}$ celler i benmärgsprover från CML-patienter. Den mest betydande upptäckten i denna studie var identifieringen av två nya LSC-markörer, von Willebrand factor (*VWF*) och TIM3, som båda är uppreglerade på TKI-resistenta LSC. **Delarbete III** syftade till att förstå effekterna av kortvarig hydroxyurea (HU)-behandling på HSPC hos CML-patienter. Resultaten indikerar att HU ger upphov till ökad andel av erytroida progenitorer och ansamling av cellsubgrupper med S/G2/M-fasrelaterad genprofil. I **Delarbete IV** ville vi undersöka hur HLA-B-genotypen påverkar utfallet av återfallsförebyggande immunterapi med histamindihydroklorid (HDC)- och interleukin-2 (IL-2). HLA-B*44, som kodar för en HLA-molekyl som uppvisar svag bindning till den inhiberande NK-cellreceptorn, KIR3DL1, var associerad med dålig prognos. Våra resultat visar att en stark ligand-receptor-interaktion ger förbättrad NK-cellsfunktion vilket underlättar eliminering av LSC och därmed bättre överlevnad.

Sammanfattningsvis kan resultaten från denna avhandling utgöra en grund för utveckling av riktad behandling för TKI-resistenta LSC vid CML och återfallsförebyggande metoder vid AML.

LIST OF PAPERS

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

I. **Komic H[*],** Schmachtel T[*], Simoes C[*], Yu W[*], Nilsson MS, Gonzales C, Jolly A, Rolfson O, Prosper F, Bönig HB, Paiva B, Thorén FB, Rieger MA. Continuous measures of early molecular steps in human bone marrow stem cell differentiation trajectories. *In manuscript.*

[*]Authors contributed equally

II. Nilsson MS[*], **Komic H[*]**, Gustafsson J, Sheybani Z, Paul S, Rolfson O, Hellstrand K, Wennström L, Martner A[#], Thorén FB[#]. Multiomic single-cell analysis identifies von Willebrand factor and TIM3-expressing *BCR-ABL1*$^{+}$ CML stem cells. bioRxiv 2023.09.14.557507; doi: https://doi.org/10.1101/2023.09.14.557507

[*, #] Authors contributed equally

III. **Komic H[*],** Nilsson MS[*], Wennström L, Hellstrand K, Thorén FB[#], Martner A[#]. Single cell proteo-transcriptomic profiling reveals altered characteristics of stem and progenitor cells in patients receiving cytoreductive hydroxyurea in early-phase chronic myeloid leukemia. *Submitted.*

[*, #] Authors contributed equally

IV. **Komic H[*],** Hallner A[*], Hussein BA, Badami C, Wöhr A, Hellstrand K, Bernson E, Thorén FB. HLA-B[*]44 and the Bw4-80T motif are associated with poor outcome of relapse-preventive immunotherapy in acute myeloid leukemia. *Cancer Immunol Immunother.* 2023; 72(11):3559-3566

[*]Authors contributed equally

CONTENTS

ABBREVIATIONS

Allo-SCT	Allogeneic stem cell transplantation
AML	Acute myeloid leukemia
BM	Bone marrow
BP CML	Blast crisis CML
CH(IP)	Clonal hematopoiesis (of indeterminate potential)
CML	Chronic myeloid leukemia
CP CML	Chronic phase CML
FAB	French-American-British system
HDC	Histamine dihydrochloride
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cells
HU	Hydroxyurea
IL-2	Interleukin-2
LMPP	Lympho-myelo primed progenitor
LSC	Leukemic stem cell
MDP	Myeloid progenitor
MPP	Multipotent progenitor

MKP/ERP	Megakaryocytic/erythroid progenitor
mRNA	Messenger RNA
NK cell	Natural killer cell
PB	Peripheral blood
RNAseq	RNA sequencing
TKI	Tyrosine kinase inhibitor
UMAP	Uniform manifold approximation and projection
WHO	World Health Organization

1 INTRODUCTION

1.1 HEMATOPOIESIS

The blood is an organized and complex tissue consisting of plasma, red blood cells (RBC) and white blood cells (WBC). It plays a vital role in the functioning of the human body and is responsible for transport of oxygen, nutrients, hormones and waste products, protection by eliciting immune responses, homeostasis, hemostasis and communication and signaling.

Hematopoiesis is a continuous regenerative process of cell division and differentiation, lineage choice and maturation, in which all blood cell lineages are replenished from a pool of self-renewing hematopoietic stem cells (HSC) in the bone marrow (BM) (1,2). Despite the huge diversity with regards to cell function and morphology, most adult immune and blood cells originate from multipotent hematopoietic stem cells (HSC). Additionally, these cells have ability to generate new HSC in a cell division, and the whole process is known as self-renewal (3,4).

The human immune system comprises cells, tissues and molecules that provide resistance to microbial infections. However, an inadequate immune response may result in inflammatory and autoimmune diseases with severe morbidity and mortality. Host defense mechanisms consist of a) innate and b) adaptive immunity, where innate immunity is a “first-line” defense, rapid immune response, initiated within minutes or hours after encountering a pathogen and has no or limited immunologic memory. The main role is a quick recruitment of immune cells to sites of infection and inflammation by cytokine and chemokine production. Majority of innate immunity tasks are performed by myeloid lineage cells (phagocytes, dendritic, mast, basophils, eosinophils), with the exception of natural killer (NK) and innate lymphoid cells that belong to the lymphoid lineage.

Adaptive/acquired immunity on the other hand is a second line of defense, antigen-dependent and antigen-specific, and acts in an organized and specialized yet delayed way. It adapts to the ongoing infection and initializes the production and differentiation of pathogen-specific lymphocytes (B and T) in order to provide an efficacious defense. The main hallmark of adaptive immunity is the memory capacity which provides a more rapid and efficient immune response by the host upon subsequent exposure to the same antigen.

These two defense lines are not mutually exclusive, they rather complement each other. The main effector cells discussed in this thesis are NK cells, and their role in eradication of leukemia will be discussed below.

1.1.1 HEMATOPOIETIC STEM CELL (HSC)

The term *stem cell* was first used by Ernst Haeckel in 1868, when referring to the primordial unicellular organism giving rise to different forms of multicellular organisms. From this time on, the hematopoiesis has been described like a tree-like model, with multipotent stem cells giving rise to their progeny, in a process of organized branching steps (5,6). Less than 100 years later, Till and McCulloch, two pioneers in the studies of *in vivo* blood system regeneration, proposed the so called "stem cell" concept. They observed colonies of cells formed in the spleen of mice, ten days after syngeneic bone marrow (BM) cell transplantation, showing that donor BM cells were able to both self-replicate and generate different types of myelo-erythroid cells (7–10). These findings served as a basis for the HSC-defining criteria, where *multipotency* represents the ability to proliferate and differentiate into more than ten mature blood cell types, while the ability to give rise to identical daughter HSC without differentiation is defined as *self-renewal capacity*. A balance between self-renewal and differentiation ensures that HSC continuously provide differentiated progenitors as mature blood cells are mostly short lived, and at the same time maintain the HSC pool (3). Even though the mature cell production rate is very high (>1M cells/second) in the adult human body, the majority of the HSC pool does not divide very often and usually resides in the G0 phase under homeostatic conditions (11,12). The importance of the balance between self-renewal and differentiation is easily understood in a disease setting, e.g. when progenitor cells do not differentiate into mature cells leading to a potential preleukemic progression or in cases where HSC differentiation is not followed by a loss of self-renewal capacity (13,14). As previously stated, HSC have multipotent potential, however all cell types do not have the same differentiation potential (Table 1).

Table 1. Nomenclature system for the differentiation potential. Adapted from (3)

Term	Differentiation potential	Examples
Toti-potent	All embryonic and extraembryonic tissues	Zygote
Pluri-potent	All embryonic tissues	Inner cell mass, ICM
Multi-potent	All lineages of a specific tissue/organ	HSC
Oligo-potent	Several lineages of a tissue/organ	Common myeloid progenitor (CMP), common lymhoid progenitor (CLP)
Uni-potent	Single lineage of a tissue/organ	Macrophage progenitor

HSC are the most-studied adult human stem cells, due to a few favorable properties. Firstly, they can be easily isolated without too much stress as peripheral blood (PB) cells isolation protocol is minimally invasive, and provides millions of cells. Secondly, these cells tolerate flow cytometry, which allowed early identification and characterization of surface protein markers involved in self-renewal, clonogenicity, and lineage potential; these findings collectively lead to a successful prospective enrichment of distinct HSC populations (15–20). Lastly, single HSC grows into colonies in optimal culturing conditions, allowing for functional *in vivo* tests. Thus, the identity of HSC can be determined by showing their abilities of long-term self-renewal and multilineage differentiation (21). Differentiation of HSC is accompanied with a successive loss of self-renewal and multi-lineage potential, resulting in lineage-specific functions and phenotypes (22). Different growth factors and cytokines, such as colony-stimulating factors (CSF), interleukins, and erythropoietin (EPO), are involved in the proliferation, differentiation, and maturation processes (23,24).

Even though the loss of self-renewal capacity correlates with priming of the lineage programs at the cellular level, the HSC transcriptional profile is determined by different metabolic and cellular properties, which is not necessarily linked directly with multipotency (25–29). So far, three phenotypically and functionally different HSC subsets have been reported in humans: long-term HSC (LT-HSC), short-term HSC/MPP (ST-HSC/MPP) and $CD34^-$ LT-HSC. LT-HSC are described as Lin-$CD34^+CD38^-CD45RA^-CD49f^+CD90^{+/-}Rho^{lo}$, have high self-renewal capacity, and long G0 exit. On the other hand, cells that instead display a short G0 exit, low self-renewal capacity and produce all differentiated cell types are called ST-HSC/MPP (30,31). The most quiescent cells, with high self-renewal capacity are $CD34^-$ LT-HSC and their phenotype is currently described as $Lin^-CD34^-CD38^-CD93^{hi}$ (32). It is important to mention that the LT vs ST HSC classification depends on the repopulation ability, with LT-HSC being able to repopulate for at least

16 weeks in primary transplantation settings (18,22,30,33,34), while ST-HSC show transient engraftment but produce all differentiated cell types (30,33,35).

CDK6 protein expression has been reported as one of the main G0/quiescence exit markers in human HSC subsets. LT-HSC do not express CDK6 on their surface and thus have the lowest division rate, with 5–6 hour delay to G0 exit (31). Human HSC have been shown to have mechanisms for propagation prevention upon damage (36,37), thus G0 exit might be a key factor in repair and fate decision of LT-HSC upon stress exposure. ST-HSC show upregulation of CDK6, which quickly pushes them into the cell cycle after mitogenic stimulation. Thus, the CDK6 expression level seems to be an underlying mechanism of heterogeneity among quiescent stem cells.

1.1.2 MODELS OF HEMATOPOIESIS

The classical model

According to the classical hematopoiesis model, HSC pass through a series of well-defined progenitor stages during the lineage commitment process, Multipotent progenitors (MPP) are the first progeny of HSC, with a limited self-renewal capacity and full lineage potential (4). MPP give rise to oligopotent progenitors, which have the highest proliferative capacity. According to this model the separation between common myeloid (CMP) and lymphoid progenitors (CLP) represents the first lineage separation (3,15,19). CLP then generates B and T lymphocytes and NK cells, and the CMP, gives rise to the granulocyte-macrophage progenitor (GMP) and the megakaryocyte-erythroid (MkE) progenitor (15,19,38). However, a direct differentiation pathway from HSC to megakaryocytic progenitors (MKP) has been proposed (39–41) (Figure 1A).

The classical model describes HSC as a relatively homogeneous population, but a growing body of data questions this view and points towards the existence of multiple HSC populations with different lineage biases that gradually lose plasticity and become lineage committed (42). Additionally, recent studies suggested that phenotypically defined HSC pool harbors cells with high molecular and functional heterogeneity. Studies have also suggested that the HSC compartment includes lineage-restricted cells with high self-renewal potential questioning the current HSC definition (40,41,43). These alternative hematopoiesis models are presented below.

The early split or adapted model

The first alternative model that gained broad acceptance in the field was the ***early split model***. It was proposed after the first scRNA wave, combined with lineage tracing experiments in mice, and single-cell *ex vivo* differentiation studies in both mice and humans. These methods allowed for deeper characterization of hematopoietic cells, providing information on a single cell level, revealing heterogeneity within previously assumed homogenous cell subsets (40,43–47). Thus, these studies suggest that separation of lineages takes place earlier than originally thought, with unipotent cell differentiation happening directly from MPP (Figure 1B).

On a similar note, an ***adapted*** model has been proposed, in which the HSC pool is divided into LT-HSC, ST-HSC and MPP. This division reflects the heterogeneity of the pool both in terms of self-renewal and differentiation potential. In this model, CMP and CLP branches remain connected via the LMPP population (6).

The continuous (Waddington-like) model

Further scRNAseq development allowed the reconstruction of developmental trajectories based on gene-expression data only, showing that HSC lineage priming is not as hierarchically organized as it was assumed. Velten et al. performed mapping of early HSC differentiation towards lineage commitment, using the integration of transcriptomic and functional studies at single-cell resolution along with flow cytometry. According to this model, HSC gradually and continuously acquire lineage-committed transcriptomic states, with progenitor cells representing transitory states within a "Continuum of LOw primed UnDifferentiated hematopoietic stem- and progenitor cells" (CLOUD-HSPCs) (Figure 1C) (43). Similar findings were reported in other studies, and the common model of gradual acquisition of the lineage-committed transcriptomic profiles is called ***Continuous Waddington-like model*** (43,48).

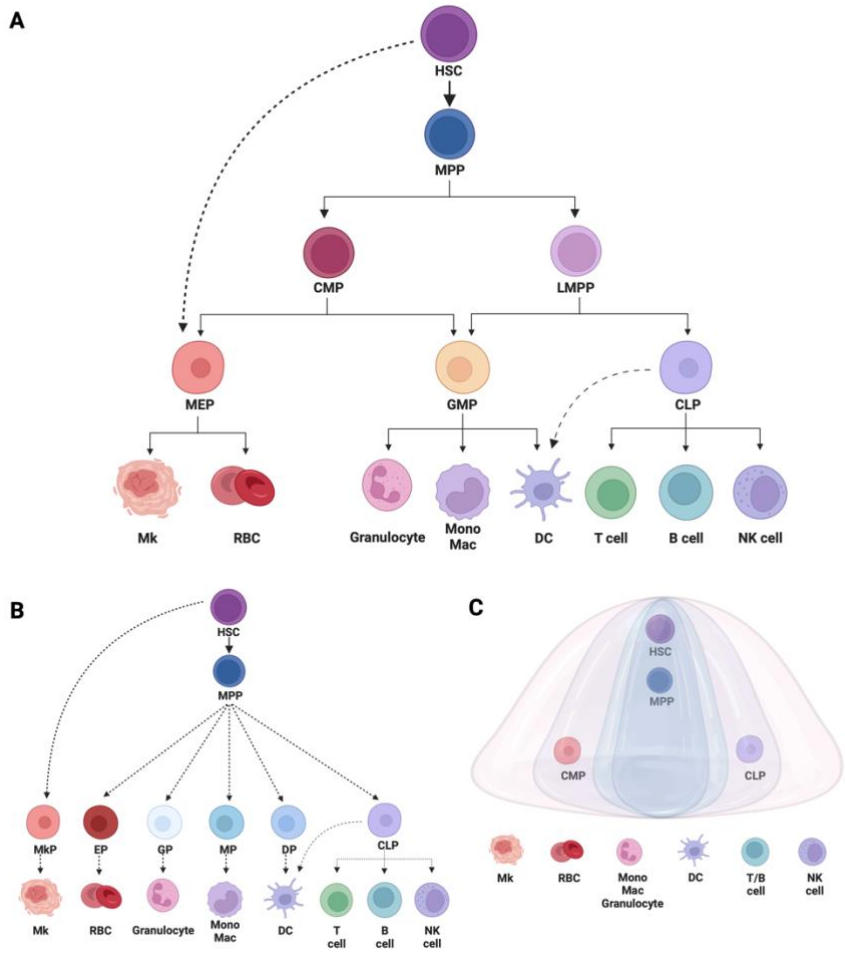

Figure 1. Models of HSC Lineage Commitment (A) Classical model, (B) Early-split model, (C) Continuous Waddington-like model. HSC: hematopoietic stem cell, MPP: multipotent progenitor, CMP: common myeloid progenitor, CLP: common lymphoid progenitor; MEP: megakaryocyte-erythroid progenitor; GMP: granulocyte-monocyte progenitor, LMPP, lymphoid-primed multipotent progenitor, Mk: megakaryocyte, RBC: red blood cell, Mono: monocyte, Mac: macrophage, DC: dendritic cell. Adapted from(42). Created with BioRender.com.

1.1.3 CURRENT HSPC RESOLUTION

Even though the abovementioned studies dramatically changed the understanding of the field of hematopoiesis, the HSPC compartment still requires deeper profiling. Regardless of the identification of novel surface markers of human HSC for their prospective isolation from different sources, the HSC fraction remains relatively impure and heterogeneous. Isolating human HSC with long-term blood repopulation capacity still lags behind achievements in the murine system. Nowadays, a purity of approximately 10% of true HSC is possible using the surface marker profile of $CD34^{+}CD38^{-}Lin^{-}CD45RA^{-}CD90^{+}CD49f^{+}$.

In the past few years, multiomic (proteo-transcriptomic, CITEseq) analysis has become an established method for resolving heterogeneity in complex cell compartments, including the bone marrow (BM) hematopoietic blood cell system. However, previous attempts - also in large-scale efforts – have fallen short of resolving cellular heterogeneity in very rare cell types, such as HSC. A contributing reason for the failure is the fact that these cells are commonly relatively transcriptionally inactive (49). Many studies have focused on whole-transcriptome analysis (WTA), but a recent study by Mair et al. showed that a targeted approach was beneficial when looking at low-abundance transcripts, and it works fine with lower sequencing depth that required for WTA (50).

Triana et al. generated the hitherto most extensive reference maps of human blood and BM samples in 2021, including up to 197 surface markers and WTA (51). Phenotypes for progenitor cell types are provided in Table 2. However, they did not specifically focus on the heterogeneity of the most immature cell subsets. Findings in **paper I** in this thesis may provide one of the most detailed and most comprehensive analysis of HSC and early progenitors to date, including the earliest trajectory branching and fate decision points.

Table 2. Progenitor cell phenotypes proposed by Triana et.al. Adapted from (51)

HSC & MPP	$CD34^{+}$, $CD38^{-}$, $CD133^{+}$, $CD45RA^{-}$, $CD49b^{+}$
Erythro-myeloid progenitors (EMP)	$CD34^{+}$, $CD38^{-}$, $CD133^{-}$, $CD45RA^{-}$, $CD4^{-}$, $CD49b^{dim}$
Early erythroid progenitor	$CD34^{+}$, $CD38^{+}$, $CD133^{-}$, $CD45RA^{-}$, $CD49^{dim}$, $CD326^{+}$, $CD98^{+}$
Late erythroid progenitor	$CD34^{+}$, $CD38^{+/-}$, $CD133^{-}$, $CD45RA^{-}$, $CD49b^{dim}$, $CD326^{++}$, $CD98^{++}$, $CD235^{+}$
Megakaryocyte progenitor (MkP)	$CD34^{+}$, $CD38^{+/-}$, $CD133^{-}$, $CD45RA^{-}$, $CD49b^{hi}$, $CD61^{+}$
Eosinophil/Basophil progenitor (EoBaso)	$CD34^{+}$, $CD38^{+}$, $CD133^{-}$, $CD45RA^{-}$, $CD49b^{-,}$ $CD61^{-}$, $CD326^{-}$, $CD33^{+}$
Lympho-myeloid prog	$CD34^{+}$, $CD38^{dim}$, $CD133^{+}$, $CD45RA^{dim}$, $CD49b^{+}$, $TIM3^{dim}$, $CD117^{dim}$
Early promyelocytes	$CD34^{+}$, $CD133^{+}$, $CD45RA^{+}$, $CD117^{+}$, $CD33^{+}$, $CD11a^{-}$, $CD11b^{-}$
Late promyelocytes	$CD34^{+/-}$, $CD133^{+/-}$, $CD45RA^{+/-}$, $CD117^{+/-}$, $CD33^{+}$, $CD11a^{+}$, $CD11b^{-}$
Plasmacytoid dendritic cell progenitors	$CD34^{+}$, CD38+, $CD133^{+}$, $CD45RA^{dim}$, $CD33^{dim}$, $CD123^{+}$, $CD98^{+}$, $TIM3^{dim}$, $CD25^{dim}$
Plasmacytoid dendritic cells (pDC)	$CD34^{dim}$, $CD38^{+}$, $CD133^{-}$, $CD45RA^{+}$, $CD33^{-}$, $CD123^{+}$, $CD98^{+}$, $CD61^{+}$, $CD141^{+}$, $CD4^{+}$, $CD10^{dim}$
Pre-pro B cells	$CD34^{+}$, $CD38^{dim}$, $CD133^{dim}$, $CD45RA^{dim}$, $CD10^{dim}$, $CD81^{-}$, $CD9^{-}$, $CD19^{-}$, $CD20^{-}$, $CD24^{-}$
Cycling pro-B and pre-B cells	$CD34^{+}$, $CD38^{+}$, $CD133^{dim}$, $CD45RA^{dim}$, $CD10^{+}$, $CD19^{dim}$, $CD81^{+}$, $CD98^{+}$, $CD24^{dim}$

1.2 DEVELOPMENT OF MALIGNANCIES

Carcinogenesis represents a series of events during which a normal, healthy cell, gains cancerous features. Cancer cells have certain key characteristics, which are summarized in the hallmarks of cancer, allowing better understanding of the underlying mechanisms of the disease. The hallmarks are shown in Figure 2 (52–54).

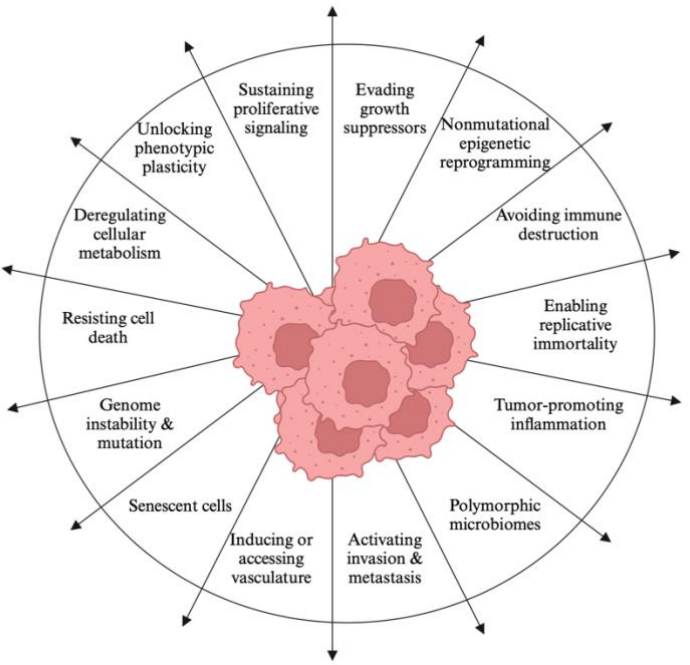

Figure 2. Hallmarks of cancer. Adapted from (54) using Biorender.

1.2.1 AGING

With age, HSC experience a decline in their self-renewal and regenerative potential, leading to high increase of cellular dysfunction (55). Since many diseases, including hematological malignancies, are strongly associated with age, it is important to understand how and why HSC age, ultimately leading to development of strategies with potential clinical benefits. In the future, it might be possible to not only prevent or delay, but also to reverse certain aspects of aging (55). This is especially important in the leukemia settings for elderly, as development of novel treatments is commonly mostly beneficial to the younger population (56). Even though aging and cancer seem to be opposite processes

at first, the general cause of aging seems to be the accumulation of cellular damage, which may provide aberrant advantages to certain cells. Thus, these two phenomena could be described as different presentations of the same underlying process. Drivers of the aging process can be both intrinsic and extrinsic (niche, environment), with intrinsic factors thought to be the major contributor. In analogy to cancer, cellular and molecular hallmarks of aging were proposed in 2013, and they are shown in Figure 3 (57). On the other hand, it is important to mention that the BM of elderly people has higher number and frequencies of HSC, but the cells overall have a reduced regenerative capacity compared to younger BMs (58–60). Furthermore, aged HSC display a myeloid-biased differentiation, compared to the lymphoid bias shown in younger healthy BMs. This is of relevance for **paper I** in this thesis.

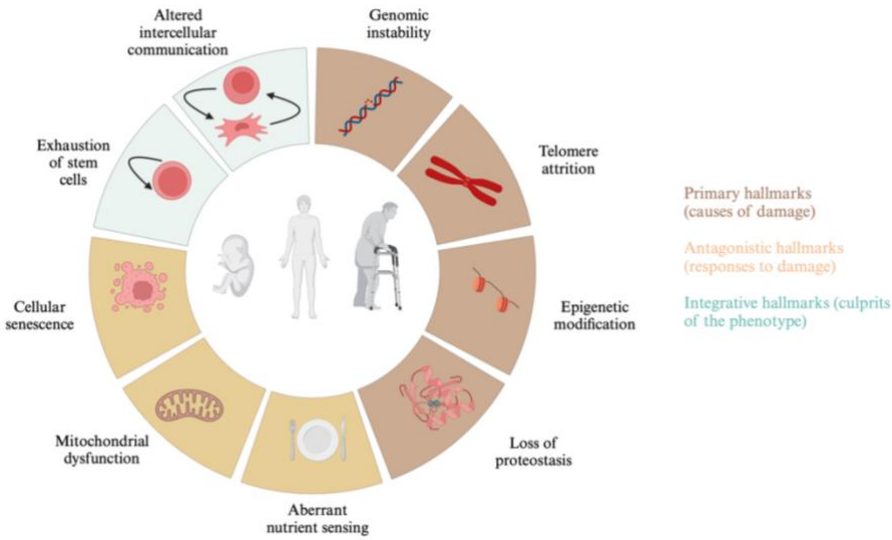

Figure 3. Hallmarks of aging. Adapted from (57) using Biorender.

1.2.2 CLONAL HEMATOPOIESIS

Clonal hematopoiesis (CH) is characterized by the expansion of HSC and its progeny from a single mutated stem cell, thus the name clonal. It is highly prevalent in the older population due to the increasing number of somatic mutations and is associated with an increased risk of myeloid leukemias. According to the estimates, human body has 50,000 to 200,000 HSC. A 70-year-old person is expected to harbor approximately 70 mutations per gene in at least one stem cell (55,61,62).

The clonal origin of a disease was among first detected in chronic myeloid leukemia (CML), which serves as a prototypical example of CH. Additional evidence supporting the existence of pre-malignant clonal expansions in healthy individuals was obtained in studies of AML patients in post-treatment remission. The initial proof came from an AML case study where an *AML1/ETO* translocation, a driver mutation of the leukemia, was detected also among seemingly healthy HSC and mature hematopoietic cells in remission samples (63). Later studies of DNA sequencing of HSC from AML remission samples confirmed pre-malignant CH being a general AML finding, as cells with one single driver mutation were frequently present (64–66).

CH-associated mutations include genes frequently mutated in leukemia, such as *DNMT3A*, *TET2* and *ASXL1* (epigenetic regulation), *SF3B1* and *SRSF2* (splicing) and *TP53* and *PPM1D* (apoptosis) (67). Conversely, clonal hematopoiesis of indeterminate potential (CHIP) is related to the presence of somatic mutations in individuals without evidence of disease. CHIP is age-dependent, with a rate of <1% in people younger than 40, and approximately 20% in the population above 70 years of age (61,68).

1.2.3 LEUKEMIC STEM CELL (LSC)

During the last 20 years, special focus was put on characterization of leukemic stem cells (LSC) and understanding their persistence. Multiple strategies have been proposed to enable LSC eradication (69,70). A majority of those imply targeting LSC-associated cell-surface markers, but additional strategies include: (a) regulation of signalling pathways, (b) interaction with the bone marrow microenvironment (niche), (c) leveraging of immune system (71).

Interestingly, the first evidence of LSC in CML was discovered prior to the targeted treatment introduction (tyrosine kinase inhibitor, TKI) (72), and later complemented with findings of LSC being deeply, but reversibly, quiescent subset of leukemic cells (73). These cells have been shown to be resistant to TKI-induced apoptosis, thus not killed off by treatment (74). Furthermore, normal hematopoiesis seems to be restored by reducing the proliferative capacity of CML progenitors during TKI course (75). Further studies led to a consensus stating that even though some chronic phase (CP) CML patients are on TKI and reach remission, they are not completely cured, and LSC presence in BM causes residual disease burden ("LSC persistence"). However, LSC persistence is believed to have a "low mutator" phenotype, which could explain why most CML patients respond to TKI and do not progress to blast phase (BP).

Functional characteristics of LSC include: a) higher engraftment capacity in immunocompromised mice compared to $CD34^+$ cells (76), b) self-renewal capacity, c) resistance to apoptosis (74,77), d) impaired DNA damage repair mechanisms (78).

The immunophenotype of LSC is very similar to HSC, with both cells types residing in $Lin^-CD34^+CD38^{-/low}$ stem cell compartment of BM (79,80). Importantly, the proportion of LSC varies a lot between patients. Hence, isolation of LSC from their normal counterparts remains one of biggest challenges in the field and a significant bottleneck to cure. IL-1RAP (81) and CD26 (82) have been have been suggested as CML LSC markers. A few other markers have been reportedly upregulated on CML LSC compared to lack or very low expression on HSC, including CD25, CD56 and CD93 (83,84). The phenotype of TKI resistant, quiescent LSC with primitive molecular signature is defined as $Lin^-CD34^+CD38^-CD45RA^-cKIT^-CD26^+$ (85). Just recently, CD35 was reported to be a unique cell-surface marker of HSC (86,87). Thus, Warfvinge et al. showed that combination of CD26 and CD35 allowed efficient distinction between HSC (*BCR-ABL1,*$CD26^-CD35^+$), and LSC (*BCR-ABL1*$^+$, $CD26^+CD35^-$) (87).

It has been shown that AML patients have at least two co-existing distinct $CD34^+$ LSC populations: a) $Lin^-CD34^+CD38^-CD90^-CD45RA^+$ (LMPP like), b) $Lin^-CD34^+CD38^+CD123^+CD45RA^+$(GMP-like) (88). AML and CML LSC share a lot of cell-surface markers (Table 3). One of the surface markers reported only in AML LSC is TIM3. It was shown that TIM3 expression allows separation of residual HSC from leukemic cells (including LSC) in combination with CD99 (66,89). This is highly relevant for **paper II** in this thesis, as we report TIM3 upregulation on CML LSC for the first time. 3 shows a compilation of all markers reported to be upregulated on LSC in CML and AML up to date (81,84,90–105).

Table 3. Stem cell markers. Adapted from (105)

Marker	Description	Gene	HSC	LSC
CD9	Leukocyte antigen MIC3	*CD9*	+	CML, AML
CD19	B-lymphocyte surface antigen B4	*CD19*	-	CML, AML
CD25	α-chain of the high-affinity interleukin 2 receptor	*IL2RA*	+/-	CML, AML
CD26	Serine exopeptidase S9B family member	*DPP4*	+/-	CML, AML
CD30	TNF receptor superfamily member 8	*TNFRSF8*	+/-	CML, AML
CD32	FCGR2A, FCGR2B, FCGR2C	Fc-γ receptor II		AML
CD33	Myeloid cell surface antigen	*CD33*	+/-	CML, AML
CD34	Hematopoietic progenitor cell antigen CD34	*CD34*	+	CML, AML
CD36	Thrombospondin receptor	*CD36*		CML
CD41	Integrin subunit alpha 2b	*ITGA2B*	+	CML
CD44	Hematopoietic cell E- and L-selectin ligand	*CD44*	+	CML, AML
CD46	Trophoblast-lymphocyte cross-reactive antigen	*CD46*	+	CML
CD47	Receptor for SIRPα	*CD47*	+/-	BP-CML, AML
CD49f	Integrin subunit alpha 6	*ITGA6*	+	CML
CD52	Campath-1 antigen	*CD52*	+/-	BP-CML, AML
CD56	Neural cell adhesion molecule 1	*NCAM1*	-	CML, AML
CD82	TSPAN27	Tetraspanin-27		AML
CD90	Thy-1 cell surface antigen	*THY1*	+	CML, AML
CD93	Complement component 1 Q subcomponent receptor 1	*CD93*		CML, AML

CD96	T cell surface protein tactile	*TACTILE*		AML
CD97	Adhesion G protein-coupled receptor E5	*ADGRG5*		AML
CD99	T cell surface glycoprotein E2	*MIC2*		AML
CD103	Integrin subunit αE	*ITGAE*		AML
CD105	Endoglin	*ENG*	+	CML
CD114	Colony stimulating factor 3 receptor	*CSF3R*	+/-	CML, AML
CD117	KIT proto-oncogene	*KIT*	+	CML, AML
CD123	Interleukin 3 receptor subunit α	*IL3RA*	+/-	CML, AML
CD133	Prominin 1	*PROM1*	+	CML, AML
CD135	Fms related receptor tyrosine kinase 3	*FLT3*	+/-	CML, AML
CD151	Platelet-endothelial tetraspan antigen	*CD151*	+	CML
CD164	Sialomucin	*CD164*	+	CML
CD184	C-X-C motif chemokine receptor 4	*CXCR4*	+	CML, AML
CD200	Antigen identified by monoclonal antibody MRC OX-2	*CD200*	+	CML
CD274	Programmed cell death 1 ligand 1	*CD274*	+/-	CML, AML (induced by IFN-γ exposure)
CD371	type lectin domain family 12 member A	*CLEC12A*	-	CML, AML
IL1RAP	Interleukin 1 receptor accessory protein	*IL-1RAP*	+/-	CML, AML
TIM3	Hepatitis A virus cellular receptor 2	*HAVCR2*		AML

Initial single-cell CML studies have demonstrated the heterogeneity of LSC subpopulations in CML, both when it comes to molecular patterns and cell-surface antigen expression (85,106). Therefore, it is important to keep in mind that target-specific treatments may not be efficacious in all patients, as some cells might avoid killing mechanisms and survive (71).

Some researchers have focused on BCR-ABL1 kinase-independent targets and pathways, which could be an additional or complementary strategy of CML LSC eradication. Dysregulation of several signaling pathways has been reported to date, including JAK/STAT, PI3K/AKT/FOXO, Wnt- β-catenin, Hedgehog and Notch signaling. Additionally, some cell-intrinsic process such as autophagy, epigenetic alterations, autocrine factors and metabolic changes have been investigated (71).

The cross-talk between the CML LSC and the bone marrow microenvironment (BMM, niche) is mediated by diverse molecules via paracrine and autocrine mechanisms. The interaction is involved in self-renewal, survival, expansion and distribution of LSC (107). These cells are capable of hijacking BMM to further favor leukemic cells over normal hematopoiesis. The key axis in HSC and LSC homing is the interaction between chemokine SDF-1 (CXCL12) and its receptor, CXCR4. LSC have reduced migratory response to SDF-1 and BMM adhesion which could be explained by upregulation of CD26. It has been shown that CD26 enzymatically digest the N-terminus of SDF-1, causing its inactivation and resulting with LSC mobilization (108). TKI treatment increases the levels of CXCR4 resulting with back-homing of LSC to BM leading to quiescence and TKI-resistance (107,108). CXCR4 has been reported as an upregulated marker on HSC compared to LSC (111).

1.3 CHRONIC MYELOID LEUKEMIA

Chronic myeloid leukemia (CML) is a clonal myeloproliferative neoplasm (MPN) characterized by a balanced translocation t(9;22), leading to a formation of the Philadelphia chromosome and the *BCR-ABL1* fusion gene (110). It is a stem cell cancer starting in the HSC, where the BCR-ABL1 fusion oncoprotein has constitutive tyrosine kinase activity, leading to proliferation and accumulation of malignant cells of different maturation stages in blood and bone marrow. This process can simply be described as a transformation of the HSC into a *BCR-ABL1*-expressing LSC (111).

The Philadelphia chromosome, a main CML hallmark, was discovered by Nowell and Hungerford in 1960, when they observed an unusually small chromosome 22 in malignant leukocytes of CML patients. This finding constituted the initial evidence for a genetic link to cancer (114), further supported by Janet Rowley's discovery of the *BCR-ABL1* translocation in 1973. Comparing the conventional chromosomal preparations with novel staining methods, she observed that additional material on chromosome 9 (9q+) corresponded to the amount of material missing from Ph chromosome (22q-), thus suggesting the potential translocation (115). Today, it is known that a reciprocal translocation occurs between long arm of Abelson (*ABL1*) gene on chromosome 9 to the long arm of chromosome 22, which is known as the Breakpoint Cluster Region *(BCR*) [t(9;22)(q34;q11)] (116) (Figure 4). The fusions occur between exon 13 or 14 in BCR and exon 2 in ABL1 (e13a2/b2a2 and e14a2/b3a2, respectively) or both (117), which generates a fusion transcript that is translated into a 210kD protein, p210 BCR-ABL.

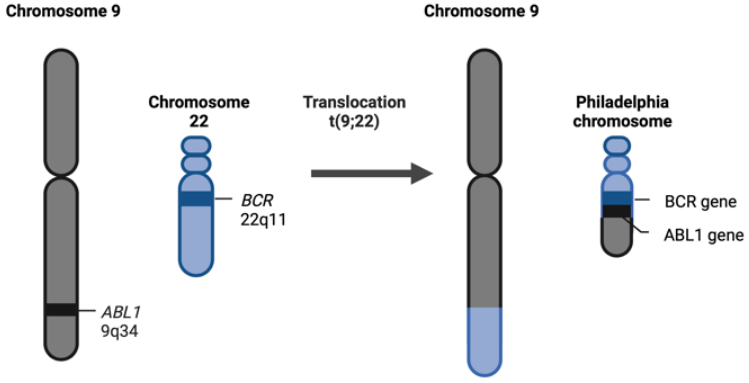

Figure 4. Philadelphia chromosome. Created by Biorender.

1.3.1 EPIDEMIOLOGY

CML is responsible for around 15% of all newly diagnosed adult leukemia patients (118). The incidence of CML is dependent on the patient's age, gender, and geographical region, with a range from 0-4-1.75/100,000 people in different countries (119–121). Children are rarely diagnosed with CML (0.7/million children/year), however the incidence increases with age (122). Males are more often diagnosed than females with the male/female ratio of 1.2–1.7 (119,123,124). Additionally, there is no reliable evidence for incidence change during the past few decades (125).

Swedish CML registry
The incidence in Sweden is 0.9–1/100,000 people per year, corresponding to approximately 100 new cases every year. Male to female ratio is 1.4:1, and similarly to worldwide data, gender difference is more apparent in aged patients. The median age at diagnosis is 59 years, with one in six younger than 40 (119). The prevalence is steadily increasing, mainly due to the greatly improved survival in CML after the introduction of TKI, but also due to increasing life expectancy in the normal population (126). In 2012, the prevalence was 11.9/100,000 people (127), and the estimated prevalence for 2030 is 18/100,000.

1.3.2 DISEASE MANIFESTATION AND DIAGNOSIS

About half of the CML patients have no syptoms, and are often diagnosed while undergoing a routine physical examination or via blood tests. The most common CP symptoms from anemia- and splenomegaly-related (fatigue, left upper quadrant fullness/pain, loss of weight, general discomfort and night sweats. Some of the less common symptoms include: bleeding due to low platelet count and/or platelet dysfunction and thrombosis (thrombocytosis and/or marked leukocytosis). Splenomegaly is found in approximately half of the cases and represents the most consistent physical sign (116).

According to the World Health Organization (WHO) revision of myeloid neoplasms and acute leukemia from 2016, *BCR-ABL1*$^{+}$ CML belongs to a subcategory of MPN (128). The clinical course of untreated disease consists of initial indolent chronic phase (CP), followed by a blast crisis (BC), associated with a poor prognosis. The newest WHO update from 2022 put bigger emphasis on the high-risk factors responsible for the disease progression and TKI resistance (129).

BP is presented with acute leukemia signs (myeloid in 60%, lymphoid in 30%, megakaryocytic or undifferentiated in 10% of cases) with worsening constitutional symptoms, bleeding, fever and infections (116). Current BP criteria include: a) ≥20% myeloid blasts in the blood or BM; or (b) extramedullary proliferation of blasts; or (c) increased lymphoblasts in PB or BM (128).

The diagnostic workflow is rather simple and standardized, and includes the detection of Ph presence by routine cytogenetics, fluorescence in situ hybridization (FISH) or real time PCR (RT-PCR). RT-PCR can be qualitative (presence/absence of the translocation) or quantitative (RQ-PCR, number of *BCR-ABL1* copies (130–132), relative to a standardized baseline. Most frequent additional chromosomal changes such as trisomy 8, isochromosome 17, additional loss of material from 22q or double Ph chromosome are detected in approximately 10-15% of CML patients (116).

1.3.2.1 TREATMENT RESPONSES

The main goal of CML treatment is the normalization of blood counts, along with the eradication of *BCR-ABL1*$^+$ cell. During the treatment course, the response is being measured by BM cytogenetics, RQ-PCR as well as speed of reduction of leukemic cells/transcript. Three main response categories are hematologic, cytogenetic and molecular. The quantification of molecular response is based on the *BCR-ABL1* transcript number relative to a standardized baseline. The development of international scale (IS) through "International Standardization" process allowed comparison of molecular response between laboratories (133,134).

Comparisons of BM cytogenetics and transcript levels in PB show good correlation, e.g. *BCR-ABL1* (IS) ≤10% equivalent to a major CyR (MCyR) and a *BCR-ABL1* (IS) ≤1% equivalent to a CCyR. Major molecular response (MMR/MR3) is defined as 3-log reduction (0.1% *BCR-ABL1*), while deep molecular remission (DMR) includes MR4 to MR5 (at least 4-log reduction, 0.01% *BCR-ABL1*) with the familiar TKI. In cases of durable DMR, patients could be offered to discontinue the treatment and potentially achieve the ideal goal, treatment-free remission (TFR). Different response categories are shown in Figure 5.

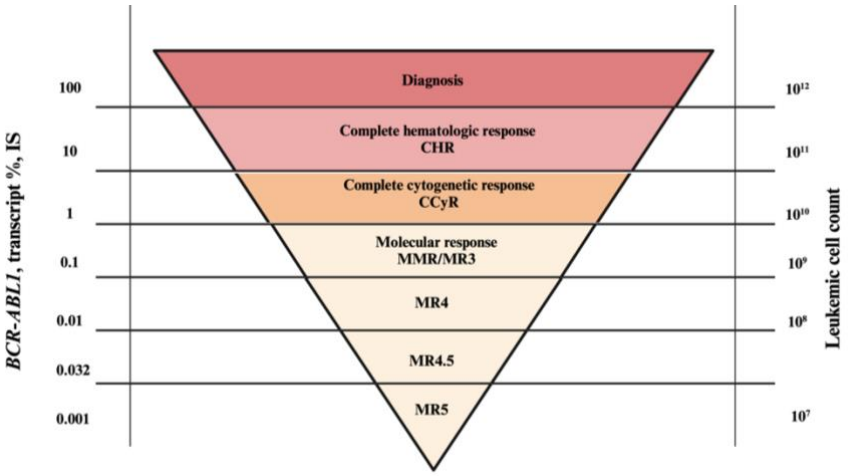

Figure 5. Patient response in CML. CHR = 1log reduction, CCyR = 2log reduction, MMR/MR3 = 3log reduction, MR4-MR5 (DMR).

1.3.3 TREATMENT

The main goal with the CML treatment approaches is to improve survival of the patients to a level comparable to the normal population. The second goal, beneficial to a fraction of patient, is the achievement of the durable DMR, which could further enable the discontinuation of treatment and potential TFR (135,136).

CML patients were initially treated with chemotherapeutics busulfan (alkylating agent) and hydroxyurea (HU, cytoreductive agent), which did not seem to be altering the course of the disease (137–139). In the late seventies, allogeneic BM or stem cells transplantation was suggested as the only potential curative treatment, however it came with significant mortality, morbidity and only young patients were eligible (140). Treatment with interferon-α (IFN-α) induced hematologic and cytogenetic remission as well as overall survival improvement (141). It caused a lot of side-effects, but nevertheless remained standard of care in CML treatment in combination with cytarabine along allogenic stem cell transplantation (allo-SCT) until TKI emergence in 2001.

Interestingly, HU treatment is used even after TKI introduction in cases when definitive diagnosis is pending or to decrease the WBC counts and mitigate

hyperleukocytosis symptoms. The mechanism of action relies on the inhibition of ribonucleoside diphosphate reductase, hence resulting in a reduction of the accessible deoxyribonucleotides necessary for DNA replication. As a result, hydroxyurea exerts cytotoxic effects on rapidly dividing cells, including malignant WBC (65). Studies have shown that there was no additional benefit of combining TKI and HU (142,143) nor that the unsuccessful pretreatment of HU before TKI have an impact on the course of the disease (144). HU is of special interest for **Paper III** included in this thesis, where we suggest the effects of HU on the LSC and progenitor cells in CML patients, both in blood and bone marrow. CML patients treated at Sahlgrenska University Hospital were treated with short-term HU if a) waiting for the diagnosis confirmation or b) when white blood cell (WBC) counts were too high hence TKI treatment could not be administered at baseline.

1.3.3.1 TYROSINE KINASE INHIBITORS (TKI)

Regardless of the opposing views on whether *BCR-ABL1* is the only genetic hit, it is the main disease driver, simply proven by the remarkable efficacy of TKI treatment. Two inhibitory classes have been suggested, based on the structural and functional features of *BCR-ABL1*, including ATP-competitive (TKI) and allosteric inhibitors. TKI bind to the kinase domain in the cleft between the N-terminal lobe and the C-terminal lobe (TKI) (145).

The development of tyrosine kinase inhibitors (TKI), compounds that suppress the abnormal tyrosine kinase (TK) activity of the BCR-ABL1 fusion protein, completely revolutionized the CML field. From a fatal disease with only 20% of patients >65 years old achieving a 5-year overall survival(OS), CML is nowadays a chronic disease and most patients have a life expectancy very similar to the general population (146,147). Almost 80% of patients diagnosed with CP-CML have achieved disease control after 5 years of TKI therapy and approximately 40% of them remain in TFR after TKI discontinuation (148,149). However, TKI treatment is not always efficacious, with 25% of CP-CML cases being resistant or intolerant to the drugs. It is more common in BP-CML or in cases where patients progressed from CP to BP during a TKI treatment (almost 100%) (150). Therefore the concept of “functional” or “operational cure” has been proposed (151), describing the lack of progression, resistance and disease symptoms. It was first believed that TKI would be to be administered life-long. However, clinical trials demonstrated that 40-60% patients reach DMR, described as a lasting reduction or clearance of remaining *BCR-ABL1* transcripts. These studies served as basis for the initiation of the TKI stop trials, where patients quit TKI after several years with the aim to stay in TFR without relapsing (152–154).

First generation STI-571, later named imatinib mesylate was the first TKI ever to receive the FDA approval for the CML CP patients in 2001. It competes with ATP at the ATP-binding site on the kinase, thus inhibiting phosphorylation of proteins involved in downstream pathways (155). The International Randomized Study of Interferon and STI571 (IRIS) represent the landmark clinical trial for TKI in CML treatments showing significantly improved outcomes for patients treated with imatinib in comparison to IFN-α and cytarabine (156).

Second generation Despite all imatinib-related benefits, some patients were intolerant or developed severe side effects, underscoring the need for additional treatment options. The second generation of TKI are generally more potent than imatinib, but induce more side effects, thus patients have to be monitored carefully. Dasatinib was the first 2nd generation TKI approved in 2006, 350 times more potent and provides inhibition of Src family of kinases (157–160). Nilotinib has 30-50 times more potency, and most importantly, targets the majority of imatinib-resistant mutations, with exception of T315I. It has less off-target effects, but serious cardiovascular events were reported in some patients. Bosutinib was initially approved for CML patients that showed intolerance or resistance to other available TKI.

Third generation A fraction of patients was not responding to first two generations of TKI and had a very poor outcome, mostly due to the T315I mutation. Ponatinib is a pan-BCR-ABL1 inhibitor with activity against all BCR-ABL1 mutations, including T315I (138).

Furthermore, the first allosteric inhibitor, **asciminib**, was approved by FDA in 2022 for patients who failed two lines of treatment or with T315I mutation. It is highly specific and potent against BCR-ABL1, targets majority of kinase domain mutations, and has potential to combined with ATP-competitive TKI (118).

One of the main goals in the past years has become TFR, unfortunately only around 20–40% CML patients can stop TKI based on current criteria. The rate of successful TFR is approximately 50% (135,161–164). Hence, a fraction of CML patients is required to continue long-term TKI treatment.

1.3.3.2 ALLO-SCT

The introduction of TKI significantly reduced the number of patients undergoing allo-SCT, nevertheless it is expected that more transplantations

will be needed with the increase of the disease prevalence. It is an important treatment option for patients who progressed after at least two TKI, and those with T315I mutation after failing to respond to ponatinib. Patients who have lower CML burden due to the TKI might have a better post-transplantation outcome (165,166).

1.3.3.3 NOVEL IMMUNE APPROACHES

Immunologic surveillance of residual LSC has been suggested as the key factor for TFR maintenance. Immune cells are thought to be efficacious in limiting the LSC clone and sustaining TFR if LSC counts are below threshold after treatment. Multiple studies have reported defects in the function of immune system in CML patients at the diagnosis (167,168) preventing the anti-leukemia response by the host (169). A dysregulation of both innate and acquired immune cells has been reported in CML at diagnosis, including reduction in cell count and cytotoxicity of natural killer (NK) cells (170), $CD8^+$ cytotoxic T-cells (CTLs), and upregulation of leukemia-associated antigens (LAAs), such as Wilm's tumor-1 (WT1), proteinase-1 (PR1) and preferentially expressed antigen of melanoma (PRAME) (171). Additionally, the microenvironment of CML cells exerts immunosuppressive feature, such as an increase of regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) or upregulated expression of programmed death-1 (PD-1) on CD4/CD8 T cells.

Immunotherapy has become a hot-topic during the past years, as it might serve as a potent strategy for eradication of residual LSC population. Different immunotherapeutic approaches have been tested in CML so far, including: IFN-α treatment, monoclonal antibodies (anti-CTLA-4, anti-PD1/PDL1), vaccines targeting leukemia-associated antigens, chimeric antigen receptor T-(CAR-T) cells and bispecific antibodies (169,171). Upon the IL-1RAP detection on CML LSC, an anti-IL-1RAP antibody was used for antibody-depended cell mediated cytotoxicity (ADCC) targeting $CD34^+CD38^-$ cells (81).

An immunotherapeutic regimen consisting of histamine dihydrochloride (HDC) and low-lose interleukin 2 (IL-2) (HDC/IL-2) has shown very promising results in the post-consolidation treatment of AML. Previously published results suggest NK cells as key players in the AML clearance during HDC/IL-2 immunotherapy. Our group has started a phase I/II trial to test feasibility of combining TKI with HDC/IL-2 CML patients in MMR. If promising, the ultimate goal would be to prepare patients for the TKI stop where they could continue with HDC/IL-2. In this trial, HDC/IL-2 is

administered twice a day for 21 days followed by equally long resting period. In total, patients receive 3 treatment cycles. More details about the mechanism of action and previously published results will be discussed in the following section.

1.4 ACUTE MYELOID LEUKEMIA

Acute myeloid leukemia (AML) is a genetically and morphologically heterogenous disease characterized by proliferation and infiltration of bone marrow and peripheral blood by immature myeloid cells (172). The main cause of AML are genetic changes on DNA of myeloid precursors, leading to the continuous proliferation of abnormal immature cells/leukemic blasts which disrupt normal hematopoiesis. AML is primarily a disease of elderly, with 80-90% of cases detected in patients >65 years of age, however 15-20% of cases are detected in children, thus AML is often associated with early childhood or late adulthood.

The initial disease classification was proposed by French-American-British (FAB) system, using maturation stage of leukemic cells as main criteria for the definition of morphological subtype (M0-M7)(173). However, it was not sufficient for prognosis or choice of treatment, with the exception of M3 subtype. Thus, WHO established a classification system in 2008, relying on the genetic aberrations in AML (174). Similar to other malignancies, classification has been changed over the past years, mostly due to the development of novel techniques that allow better and deeper understanding of the mechanisms driving the disease onset, progression and survival. According to the latest 5th WHO classification from 2022, AML with defining genetic abnormalities is completely separated from AML defined by differentiation. One of the key changes was the elimination of the 20% blast requirement for AML types with defining genetic abnormalities (except for *BCR-ABL1*$^{+}$ AML and AML with *CEBPA* mutation). Additionally, a section of AML with other defined genetic alterations has been established. All taken together, classification keeps relaying on the integration of clinical, molecular, biological, immunophenotypic and pathologic parameters (129).

AML is often associated with cytogenetic changes, such as deletions, translocations, inversions, monosomy and trisomy. Approximately 60% of patients have some cytogenetic alterations, and are often referred as aberrant/complex karyotype. Most commonly mutated genes in AML are *NPM1* and *FLT3*. Other mutations are often detected in genes involved in epigenetic regulation, cell division, proliferation, differentiation and splicing (175).

Cytogenetic aberrations and gene mutations are used for risk stratification, and according to 2022 European LeukemiaNet (ELN) genetic risk classification at diagnosis, there are three main categories: favorable, intermediate and adverse (176). Some changes, such as t(8;21), inv(16), t(16;16) or *NPM1* mutation are

associated with favorable risk. *FLT3* mutations are nowadays associated with intermediate, while t(6;9), t(9;22), inv(3) and mutated *TP53* represent high risk AML at diagnosis (176).

1.4.1 EPIDEMIOLOGY

Age-adjusted incidence of AML is around 4.3 per 100,000 person-years in US, Canada, Australia, UK as well as the rest of Europe (177,178). The lowest incidences are reported in Asia and South America. Since AML is primarily a disease of older adults, incidence is higher in patients >65 years old (20.1 per 100 000) compared to patients <65 (2.0 per 100 000). The median age at diagnosis is 68 years.

Studies show that males are 1.2–1.6 times more likely to develop AML during their lifetime (178). The increased risk for males is first detectable after 50 years of age, and the difference seems to become even higher with the age. The prevalence is 19 per 100 000 population in the US, and no difference in prevalence has been found between genders until age 65, with females having a lower prevalence in later age (178).

Sweden
Approximately 400 adults are diagnosed with AML in Sweden each year, corresponding to an incidence of 3–4 cases per 100,000 per year. The incidence is higher with increased age and the median age at diagnosis is 72 years. The prevalence is 13.7 per 100,000 inhabitants. In older age, men have a greater risk of developing AML (179,180).
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1.4.2 DISEASE MANIFESTATION AND DIAGNOSIS

AML symptoms usually develop very quickly and often include: abnormal complete blood counts, fatigue, night sweating, low-grade fever, nausea, bruising/bleeding, infections and pain in the skeleton (cause by the expanding BM). Leukemic cells infiltrate BM and block the proliferation of normal HSC, causing anemia, granulocytopenia and thrombocytopenia. If untreated, the disease has a rapid course and leads to death within weeks or months.

The diagnostics consists of morphologic examination of BM and blood smears, immunophenotyping by flow cytometry, cytogenetics and molecular analyses and medical history (176,181). Disease primarily develops in BM, and in most cases, blasts quickly spread to peripheral blood. By definition, AML used to

be a disease with blasts counts >20% in BM or PB. However, recent update by WHO considers >10% blasts presence sufficient for AML diagnosis, with some exceptions. In some instances, AML can also spread to lymph nodes, spleen liver, or central nervous system (CNS).

The hierarchical cellular organization of AML involves a tiny fraction of LSC at the apex of hematopoiesis, capable of initiating and maintaining the long-term disease in immunodeficient mice (79,182–184). Detailed studies of LSC characterization showed that these cells exhibit self-renewal, relative quiescence, resistance to apoptosis, and increased drug efflux, making them less sensitive to the conventional treatments aimed at blocking bulk proliferation (185). The cancer stem cell model suggests that eradication of LSC is a prerequisite for achieving long-term remissions. Development of genomic methods allowed detection of preleukemic HSC in AML patients. These cells do not cause leukemia *in vivo*; however, they carry early competitive driver mutations, usually detected in genes involved in epigenetic regulation (64–66). Some of the leukemia-initiating mutations happen early during leukemogenesis, (*DNMT3A*, *TET2* and *ASXL1),* while mutations in *FLT3*, *RUNX1 and NRAS* occur during later stages (176).

Secondary AML (s-AML), on the other hand, is developed due to the DNA damage caused by prior exposure to radiotherapy or chemotherapy (therapy-related, t-AML) or as a transformation from other hematologic malignancies, such as myelodysplastic syndrome (MDS) or MPN. Approximately 10–30% of all AML are secondary, however, the real situation might be different as many AMLs can arise from undiagnosed MDS (186).

1.4.3 TREATMENT

Due to the complexity and heterogeneity of the disease, as well as its acute nature, prompt therapeutic intervention is needed. As with any other disease, the main goal is to achieve better leukemia-free survival (LFS) and OS, especially for the elderly patients. Regardless of the huge developments during the last years both in disease characterization and therapeutic approaches, the backbone of the AML treatment remains the same and consists of a) induction chemotherapy, b) consolidation chemotherapy and in some cases c) maintenance treatment.

AML patients start with ***induction chemotherapy*** as soon as the diagnosis is established, preferably the same day, preventing any delays. Retrospective

analyses showed that the final outcome of the treatment is adversely affected when the treatment was started beyond five days from diagnosis (187).
The intensive chemotherapy typically involves 7 days of cytarabine (reduced to 5 days in Sweden) and 3 days of anthracyclines (usually daunorubicin or idarubicin, widely known as "7 + 3"(188). Many studies focused on patients up to the age of 50–55 years, with five-year survival around 40–45%, compared to only <10-15% when analyzing patients older than 60 (189,190).

The main aim of the intensive initial treatment is to kill leukemic cells and retrieve normal cell production, by achieving complete remission (CR) before switching to consolidation treatment. According to the previous ELN guidelines, CR is achieved in 60-80% of patients below 60 years (191), compared to 40-60% of older patients (172,181,192). Latest guidelines from 2022 define CR as a) <5% of blasts in BM, b) no circulating blasts, c) absence of extramedullary disease, d) peripheral blood reconstitution (absolute neutrophil counts, ANC $\geq 1.0 \times 10^9$/L, platelet count $\geq 100 \times 10^9$/L) (176). If patients do not achieve CR after one round of induction chemotherapy, they might undergo additional round(s).

During the past 6-7 years, the treatment arsenal has diversified, allowing better and more personalized approaches, especially for patients with severe forms of AML. Midostaurin, an oral multikinase inhibitor, is now added to danorubicin-cytarabine as a first-line treatment for patients with *FLT3* mutations. Patients with intermediate or favorable risk karyotypes are treated with gemtuzumab-ozogamicin/mylotarg (anti-CD33 drug-conjugated antibody), in combination with "7+3" (193–196). Venetoclax, a BCL-2 inhibitor, has been approved for the treatment of elderly AML patients in combination with azacytidine (hypomethylating agent) or low-dose cytarabine (197).

Once CR is achieved, patients start with so called ***consolidation chemotherapy***, usually by administering high dose of cytarabine for 1-4 cycles, with or without supplementation of anthracycline (172,198). However, lack of survival benefit and significantly increased toxicities support the use of intermediate cytarabine dose (199–201). The main aim of consolidation chemotherapy is to prevent the disease relapse; thus, some patients undergo allo-SCT. Allo-SCT is the most efficient relapse prevention method, but unfortunately not suitable for all AML patients. It is a preferrable option for fit, <70 years old patients with intermediate or adverse risk, if relapse incidence is 35-40% (202). However, this strategy brings risk of non-relapse related mortality (NRM) making the OS benefit slightly offset. One out of five fit and transplanted AML patients develops NRM (203). Additionally, even though the number of allo-HSC increases by 10% each year (204–206) only a minority of AML patients undergo this procedure, due to age, toxicities caused by prior

treatment, no successful CR, early relapse or comorbidities (191,207). Hence, high relapse rates are expected in adverse risk or elderly patients ineligible for allo-SCT and receiving intensive chemotherapy. Elderly AML patients have low tolerance to the chemotherapy, mostly due to a complex disease biology, often associated with complex karyotype, *TP53* mutation (20%), and a high incidence of s/t-AML (20 to 30%). Based on the Surveillance, Epidemiology, and End Results Program (SEER) data from 2010 to 2017, 5-year survival in patients 60-69 years old was 18%. However, the survival rate was only 4% in patients older than 70 years old, clearly indicating the unsatisfactory outcome in elderly patients (197).

Post-chemotherapy phase is often associated with a 50-60% relapse rate, including approximately one third of patients who underwent allo-SCT (169, 211–213), emphasizing the need for relapse prevention strategies. During the last few years, ***maintenance treatment*** has been discussed a lot. The idea is to treat patients for an extended, yet time-limited course, by administering a minimally toxic drug. Since a few years, Onureg, an orally administered azacytidine, has been approved as maintenance treatment for patients who achieve CR1, and cannot undergo allo-SCT. In one of the studies, the drug was administered to patients ≥55 years old for 14 days in 28-day cycles, and showed improved median OS and reduction of the relapse (211). Unfortunately, the study had some limitations, including no data on Onureg effects on younger patients, minority of included patients had adverse-risk cytogenetic profile, and different number of consolidation chemotherapy cycles among included patients (176), thus it is hard to draw more generalized conclusions. Additionally, midostaurin has shown to increase OS when given as a maintenance treatment for *FLT3* mutated patients. However, the RATIFY study included only patients who received the same drug during both induction and consolidation chemotherapy, thus the effects of midostaurin as a solo maintenance treatment are not fully resolved (212).

A regimen combining HDC and low dose IL-2 (HDC/IL-2) developed in our lab has shown a great efficacy as a post-consolidation treatment in AML and is further discussed in the next section.

NK CELLS IN CONTROL OF AML

NK cells are innate lymphocytes with cytotoxic and cytokine-producing effector functions. These cells distinguish normal/healthy "self" cells from aberrant cells (infected or transformed cells) (213). While T lymphocytes require a recognition of antigens presented on antigen presenting cells to expand and differentiate into cytotoxic effector cells, the NK cell detection system is regulated by the engagement of activating and inhibitory receptors. The interplay between these receptors determines whether NK cells exert cytotoxic effects and/or produce cytokines during the effector phase of activation (TNF-α and IFN-γ) (214). The inhibitory receptors control self-recognition by human NK cells and include the Killer Immunoglobulin-like Receptors (KIRs), the Natural Killer Group 2A (NKG2A) and Leukocyte immunoglobulin-like receptor subfamily B member 1 (LILRB1) (213). Ligands for inhibitory NK cell receptors are HLA class-I molecules expressed on surface of all nucleated cells. If the encountering cells have absent or reduced MHC class I-expression ("missing self"), inhibitory signals will be lost and NK cells will be more prone to exert their cytotoxic activity towards the target cells. Activating receptors trigger NK cell activation upon binding to ligands upregulated on infected or stressed cells. Some of the most important activating receptors are: natural cytotoxicity receptors (NCRs), NKp30, NKp44 and NKp46, and NKG2D, 2B4, and DNAM-1(215).

Around 10% of all lymphocytes in human PB are NK cells. There are two main NK cell subsets based on phenotype and functional features: $CD56^{dim}$ and $CD56^{bright}$. Vast majority, around 90% of PB NK cells are $CD56^{dim}CD16^{+}$ with mainly cytotoxic activity, but they can also produce cytokines in response to target cells. NK cells in lymph nodes and tonsils are mostly $CD56^{bright}CD16^{-}$ and their main effector function is production of cytokines, such as interferon (IFN)-γ in response to interleukin (IL)-2, IL-12, IL-15 and IL-18 (216). NK cells undergo a continuous process of education, in order to gain and maintain functional competence.

As already mentioned, the most challenging part of AML is a post-CR relapse prevention. The curative potential of allo-SCT and eradication of leukemic cells by immune cells, paved the way for additional immunotherapeutic approaches. Some examples are: CAR-T, CAR-NK, multifunctional engagers, monoclonal antibodies targeting LAA or LSC or immune checkpoints, or immunostimulatory cytokine treatment (217). AML is associated with dysfunction of NK cells, primarily as downregulation of NCRs and impaired *in vitro* cytotoxic effects towards leukemia, correlating to poor overall survival (218).

HDC/IL-2 in AML treatment IL-2 is a cytokine produced by T cells that stimulates activation and differentiation of T cells and induces proliferative and cytotoxic functions of NK cells (219,220). Recombinant IL-2 has shown an augmented response to cancer cells, with preclinical studies further supporting IL-2 therapy against leukemia. FDA approved the use of IL-2 monotherapy in metastatic melanoma and renal cell carcinoma back in 1992 (221) showing remarkable survival outcomes in a fraction of patients (222). However, IL-2 post-consolidation monotherapy showed no significant benefit in relapse prevention in AML (223–227). A lack of strong anti-leukemic response could be due to reactive oxygen species (ROS) that hamper T and NK cell functions (228). Several types of myeloid cells, such us monocytes and neutrophilic granulocytes, produce ROS (229). Almost 30 years ago, Brune et al. combined IL-2 treatment with histamine observing the restored anti-leukemic effect, with NK cells successfully killing AML blasts regardless of presence of ROS-producing monocytes (230). The mechanism of action is simply described as inhibition or reduction of ROS production by myeloid NADPH oxidase (NOX2), by binding to histamine type 2 (H2R) receptors on the surface of myeloid cells (229). These findings subsequently lead to the development of the HDC/IL-2 regimen and a first AML phase I/II trial, demonstrating clinical feasibility and safety (231). Based on these findings, a randomized phase III trial was initiated, and 320 patients in post-consolidation CR were randomized to standard of care or treated with HDC/IL-2. The treatment was administered in ten three-week cycles with resting periods inbetween. The results showed a significantly superior LFS in treated patients compared to the control arm (232). However, no significant benefit was detected in terms of OS (233). HDC/IL-2 maintenance treatment in post-consolidation phase has been approved in European Union countries since 2008. A phase IV trial (Re:Mission trial) including 84 patients in CR1 showed immunomodulatory effects of HDC/IL-2. The treatment was found to induce NK cell expansion and upregulation of NK cell activating receptors, NCRs (234). Even though the induction of immunosuppressive Tregs was noted during the initial cycles, there was no correlation with clinical outcome. (235). **Paper** IV discusses the eradication of residual LSC for a subgroup of AML patients enrolled in the Re:Mission trial.

2 AIM

The overall aim of the thesis was to characterize healthy hematopoietic stem cells as well as leukemic stem cells in order to find novel strategies to target residual myeloid leukemia. The specific aims for each of the papers are listed below:

Paper I	To understand the earliest steps of differentiation in human hematopoiesis using multiomic technology
Paper II	To further dissect LSC and stem and progenitor cells in CML by combining single-cell multiomic and concomitant *BCR-ABL1* detection.
Paper III	To investigate to what extent short-term cytoreductive hydroxyurea treatment affects the stem and progenitor cells in early phase CML
Paper IV	To determine whether different HLA-B genotypes have an impact on the outcome of HDC/IL-2-based post consolidation immunotherapy in AML

3 PATIENTS AND METHODS

3.1 SAMPLE COLLECTION

Mononuclear cells (MNCs) have been collected from blood or bone marrow samples, and isolated **Papers I** and **II** include healthy control bone marrow samples obtained during the hip replacement surgeries (n=15, n=5, respectively), in combination with peripheral blood samples. In **Papers II** and **III**, cells from 16 patients in chronic phase CML were collected from peripheral blood and bone marrow aspirates from posterior iliac crest at Sahlgrenska Univeristy Hospital. Samples used for **papers I-III** have been additionally enriched for immature cell content using FACS sorting, CD34+ positive isolation or CD15 depletion. using density gradient centrifugation using Lymphoprep (Stemcell Technologies). **Paper IV** presents the results of an open-label single-arm phase IV study (Re:Mission, NCT01347996) using cells from 84 patients in the first CR with *de novo* or secondary AML as well as leukopacks from healthy blood donors used for NK cells isolation.

Written informed consent were collected from participants in all studies prior to enrollment and we obtained ethical permits by the Ethical committees in all participating regions. All studies were conducted according to the principles of the Declaration of Helsinki.

3.2 KIR/HLA GENOTYPING

In **paper IV** DNA extraction from whole blood was performed by either by Roche MagNAPure 96 or Qiagen® DNeasy Blood & Tissue kits. HLA-B and -C allele genotyping was done by LABType SSO Class I Locus Typing Tests from One Lambda. Complementary KIR ligand typing for HLA-A Bw4 was performed using the Olerup SSP KIR HLA Ligand kit. Detection of six major KIR3DL1 alleles utilized PCR primers described by Boudreau et al. (236).

3.3 FLOW CYTOMETRY

Flow cytometry was used in different extent for all studies. In **papers I-III**, flow cytometry analyses were performed at the sample processing time, to allow for the cell characterization. Additionally, it was used for the functional experiments on CD273 in **paper I**. Fluorescence-activated cells sorting (FACS) was used for cell purification and enrichment prior to the cell-capture in **papers I-III**. In **paper IV**, it was used to determine the expression level of KIR3DL1, HLA-B and HLA-Bw4 in 80I/T versus 80 T donors, assessed by gating and calculating median fluorescent intensity (MFI). All collected data was analyzed in FACSDiva (v.8.0.1 or later) and FlowJo (v.10.8.1) software (BD Biosciences).

3.4 FUNCTIONAL ASSAYS

3.4.1 FUNCTIONAL EXPERIMENTS WITH CD273

Functional experiments with FACS sorted HSPCs ($lin^-CD34^+CD38^-CD273^+$ or $lin\text{-}CD34^+CD38^-CD273^-$) were used for a) differentiation status determination by flow cytometry, b) colony formation and lineage distribution, c) tracking of fate of progenies in the third cell generation using The Tracking Tool, TTT in **paper I** (237). Additionally, protein expression was measured by WES system, using proteins that were found upregulated in the multiomic dataset. Transcriotomics analysis was performed comparing $CD34^+CD38^-CD273^+$ and $CD34^+CD38^-CD273^-$ cells, paired-end sequencing (2x50bp), after RNA Isolation for Sequencing miRNeasy Kits (Qiagen), low-input.

3.4.2 NK CELL ASSAYS

To assess effector functions in **paper IV**, non-stimulated NK cells or NK cells pre-stimulated or with IL-2 overnight (Proleukin, Novartis Pharmaceuticals), were exposed to target cells (K562 cells line) in the presence of anti-CD107a-BUV395 (H4A3, BD Biosciences) for 4 hours. Additionally, an intracellular cytokine assay was performed using the same set-up, adding Brefeldin A (GolgiPlug, BD Biosciences) after one hour. The cells were later fixed and permeabilized by BD Cytofix/Cytoperm (BD Biosciences) and IFN-γ and TNF-α levels were determined by flow cytometry.

3.5 scCITEseq/multiomic experiments

BD Rhapsody platform, a 3' end capture method, was used for simultaneous detection of gene and protein expression at a single-cell level in **papers I-III**. The method allows for sample multiplexing and detection of protein expression, by using oligo-conjugated antibodies that have a PCR handle, and poly-A tails. Live, single cell suspensions were obtained by FACS sorting, by isolating immature cell compartment ($CD34^+$ and $CD34^+CD38^-$). Cells were further stained with oligo-conjugated antibodies (AbSeqs: protein expression and SampleTags: multiplexing), allowing for the detection of cell surface protein, and subjected to BD Rhapsody single cell capture protocol. In principle, cells were loaded onto a cartridge with 250 000 wells, ensuring the low percentage of multiples/well, followed by addition of cell-capture beads with poly T tails (complementary binding of poly A tails from mRNA and oligo-conjugated antibodies). After cell lysis and reverse transcription, libraries were generated. Both protein (51 proteins) and gene panels (597 genes) were custom-made designed to distinguish different stem and progenitor populations (HSC and progenitor genes/proteins, cell cycle and clonal hematopoiesis genes, LSC genes/proteins, redox and NK cell related genes etc). In total, 62 000 high quality CD34+ and CD34+CD38- cells were sequenced in **paper I** and 70 000 cells from chronic phase CML patients in **papers II and III**.

3.6 *BCR-ABL1* detection method

Since both HSC and CML LSC are $CD34^+$ and $CD38^{-/low}$ by cell surface expression, their definitive distinction within the leukemic BM requires *BCR-ABL1 detection* within the LSC. A disadvantage with 3' RNA-seq strategies is that the sequencing only captures the sequence a few hundred base pairs from the poly-A tail, while *BCR-ABL1* fusion point is located approximately 5.4 kb from the poly-A tail. To allow its detection, we performed a series of PCR amplifications and circularizations of the amplified products, removing the majority of the *ABL1* sequence and shortening the *BCR-ABL1* transcript. In our adapted workflow, we first used the cDNA for the standard scCITEseq library preparation, and then re-used the same cDNA-carrying beads for *BCR-ABL1* specific library preparation in a separate set of reactions. After parallel sequencing of the two libraries, the two datasets were merged on the basis of cell labels.

3.7 SEQUENCING DATA ANALYSIS

FASTQ files generated in **papers I-III** were processed using the BD Rhapsody Targeted Analysis Pipeline (v1.10.1 and 1.10) on the Seven Bridges Genomics platform (https://www.sevenbridges.com), generating single-cell gene and protein expression matrices based on reference files. Data analysis was performed in R (v. 4.2.0) using the Seurat package (v. 4.3.0). mRNA expression data were normalized using Log transform for mRNA and CLR for AbSeq. The samples were integrated with the CCA method available in Seurat v4.3.0. mRNA was used for the principal component analysis (PCA), using it for the additional dimensionality reduction. Finally, visualization of the clusters was accomplished using Uniform Manifold Approximation and Projection (UMAP). Slingshot and TradeSeq association test were used in **paper I**, to allow for the trajectory analysis and early on gene changes.

3.8 STATISTICS

Analyses were performed in Prism v. 9 or later (GraphPad Software), Rstudio (v. 2022.07.1+554; R (v. 4.2.1)) or IBM SPSS Statistics (v. 29). Differential gene/protein expression analyses in **Papers I-III** were assessed by Wilcoxon Rank Sum test in R using Seurat's FindAllMarkers and FindMarkers functions, as well as. Differences in cell type proportions were assessed in GraphPad Prism using the two-way ANOVA test (**Paper I**), and unpaired Mann-Whitney (comparisons of blood and BM) and paired Wilcoxon test (blood before and after treatment) (**Papers II-III**). Pseudobulk differential gene expression in paper II was performed by DESeq2 R package v1.40.2.

In **paper IV**, the Mantel-Cox logrank test was used to for survival comparison between patient groups. Mann–Whitney test was used to compare responses in functional experiments. Univariate and multivariate Cox regression analyses were used for the analysis of the impact of HLA-B and HLA-A alleles on LFS.

Additional information
For detailed description of each experiment, the reader is referred to the materials and methods of each included article.

4 RESULTS AND DISCUSSION

Multiomic profiling of most immature cell compartments in normal and chronic myeloid leukemia hematopoiesis

Human hematopoietic stem/progenitor cells (HSPC) are identified by expression of the CD34, and lack of expression of lineage/mature hematopoietic marker (Lin^-). However, the $CD34^+Lin^-$ cells do not comprise a homogenous population of cells and even though our understanding of the differential molecular signatures and functions within this subset has increased during the last years, a more thorough characterization is highly warranted.

Recent advances in the scRNAseq field lead to a number of studies focusing on human bone marrow (BM) cells under healthy and diseased conditions (43,51,238–240). However, information regarding the heterogeneity among most immature HSC and early steps of differentiation is still scarce. Hence, we decided to combine freshly isolated, cryopreserved cells, a targeted approach with a selection of genes and proteins chosen to comprise relevant markers for HSC and LSC biology and sequencing to allow single-cell analyses of also relatively transcriptionally inactive HSCs and LSCs. With this set up we characterized BM cells at the single cell level from healthy individuals and chronic myeloid leukemia patients, with an emphasis on stem and progenitor cell populations.

Paper I

In order to find good targets for eradication of leukemic cells, we need to fully understand the normal hematopoiesis. Traditionally, the HSC compartment has been considered to be a rare and rather homogenous population. Due to the low numbers of these cells in BM, many studies in the past have rather focused at full BM. With this project, we set out to generate a differentiation map of stem and progenitor cells. Additionally, we investigated the effects of aging on HSC heterogeneity, physiology and function.

In the first section of the paper, we utilized 62 000 $CD34^+$ high quality cells from 15 healthy donors, from three age groups (young: 20-23, midage: 52-65 and old: 70-84 years of age). Clustering analysis provided a comprehensive map of early steps in human HSC differentiation across life. Our results showed a hierarchical, stepwise commitment, an early separation of megakaryocytic-erythroid (MkE) lineage from HSC/MPP cells, and a later separation of myeloid and lymphoid progenitors. These results are in line with

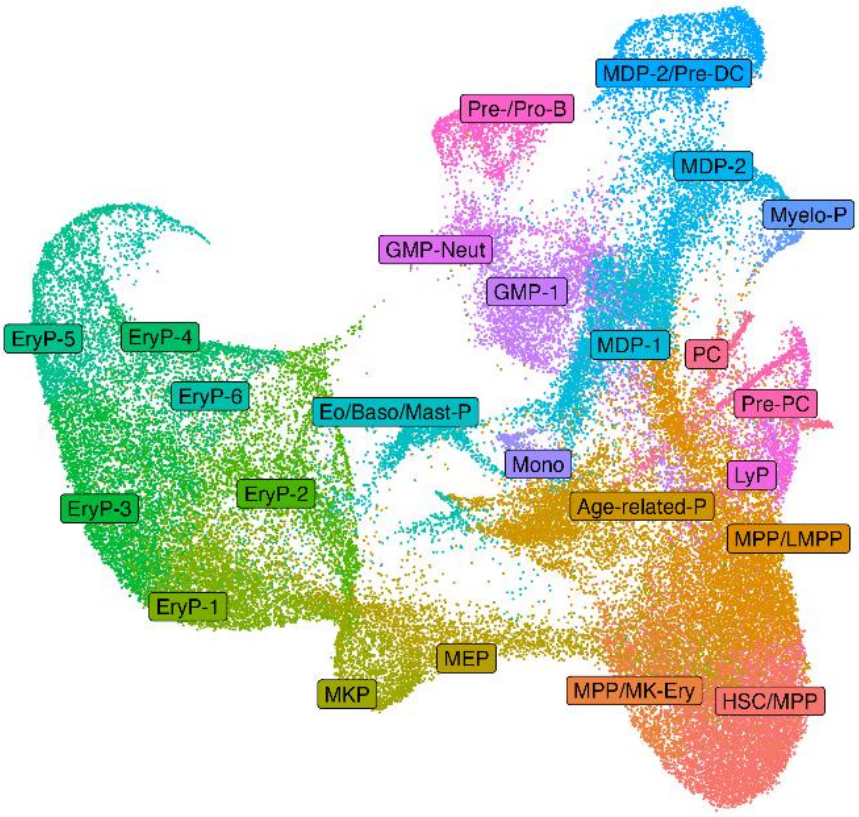

Figure 6. Refined CD34+ HSPC differentiation map. MK-Ery = megakaryocytic-erythroid progenitor, MEP = megakaryocytic-erythroid progenitor, MKP = megakaryocytic progenitor, EryP = erythroid progenitor, LMPP = lympho/myelo primed progenitor, LyP = lymphoid progenitor, Pre-PC = pre-plasma cells, PC = plasma cells, Mono = monocytes, MDP = myeloid progenitor, pre-DC = pre-dendritic cells, GMP = granulocyte-macrophage progenitor.

many recently published studies (43,51,241,242). Multiomic profiling allowed detailed characterization and annotation of all cell subtypes, even though we used targeted gene and protein panels with myeloid bias (Figure 6).

To get more insight into the earliest transitions from HSC to lineage committed states, we shifted our focus to clusters harboring the majority of pre-sorted $CD34^{+}CD38^{-}$ cells. Refined clustering of >20 000 immature cells revealed clear separation of nine cell subsets, with HSC-1 representing the apex of the hematopoiesis (Figure 7A). HSC-1′ cells were defined as quiescent/slowly cycling cells, with upregulation of well-described HSC markers, such as *CRHBP, MLLT3, MPL* and *HLF*. Furthermore, we detected an upregulation of few previously unreported genes, including *DLK1* and *ADGRG6* (GPR126).

Huang et al. recently reported that *DLK1*, Delta Like Non-Canonical Notch Ligand 1, was upregulated on murine long term HSC (LT-HSC) and was necessary for HSC homeostasis (243). Upon *DLK1* knock out (KO), LT-HSC entered cell cycle and increased metabolic activity. These results suggest that *DLK1* expression is confined to the most immature non-cycling cells, and that it is needed to keep the cells in a quiescent state. On the other hand, not much is known about GPR126 expression on immature cells.

To shed light on the lineage reconstruction and inference of pseudotime, SlingShot trajectory analysis was utilized. Psuedotime of one cell represents its distance from the lineage origin, and can be considered as an increasing function of time (Figure 7B). Briefly, four trajectory lineages were observed (MEP, LYP, GMP, MDP) without significant differences between age groups. While cell distribution analyzes showed increased frequency of the most immature cells in aged donors, HSC from young donors had higher differentiation activity and early lineage commitment. These results are mostly in line with previous studies, except that our results do not validate previously reported myeloid-bias in aged donors (58).

The pseudotime information was further used for trajectory-based differential expression (TradeSeq) to corroborate and comprehensively define the most primitive cell profiles and lineage-commitment patterns. Furthermore, the analysis allowed to detect the switch on/off patterns for over 200 genes along the pseudotime. Hence, for the first time, we report lineage-specific gene expression profiles. Potential novel stemness markers *DLK1* and *ADGRG6* showed early downregulation along pseudotime, perfectly matching the expression pattern of know HSC markers, such as *CRHBP* (Figure 7C).

Besides molecular profiling, our analyses included expression data of 50 surface proteins. While many of them were used as an additional layer of information or for flow-cytometry like gating, CD273/PD-L2 surprisingly stood out. To the best of our knowledge, we are the first to report that CD273 is significantly more expressed in cells of the HSC-1´ cluster as compared to HSC-2´ (Figure 7D). This, to the best of our knowledge, has not been reported before. CD273/PD-L2 and CD274/PD-L1 are ligands to a Programmed death 1 receptor (PD-1). PD-1 interaction with either of the ligands influences activation, proliferation and cytotoxic function of T cells (244). In our study, CD274/PD-L1 was not upregulated on HSC, in contrast to previous results from murine studies (245,246) Functional experiments with CD273/PD-L2 confirmed our multiomic findings. Flow cytometry analyses of mobilized PB (mPB) of the most immature HSC ($CD34^+CD38^-CD45RA^-CD90^+CD49f^+$) showed significant upregulation of CD273 compared to $CD34^+$ cells and highly significant in comparison to $CD34^+CD38^-CD45RA^-$ and $CD34^+CD38^-$

(Figure 7E). To further validate these findings, we isolated $CD273^{hi}$ and $CD273^{low}$ cells and performed *in vitro* assays. $CD273^{hi}$ cells showed increased quiescence and delayed differentiation, as well as increased protein expression of stem cell markers (*HLF, ATP1B1*) and decreased expression of cycling marker *CDK6*. These results together provide evidence for the quiescent nature of $CD273^{+/hi}$ HSC.

According to the current definition, HSC reside within $CD34^{+}CD38^{-}Lin^{-}CD45RA^{-}CD49f^{+}CD90^{+}$ cell compartment. However, a combination of these markers does not yield satisfactory outcomes in HSC isolation. Thus, novel HSC markers delineated in this paper (*ADGRG6, DLK1* and CD273) hold significant promise and warrant further investigation.

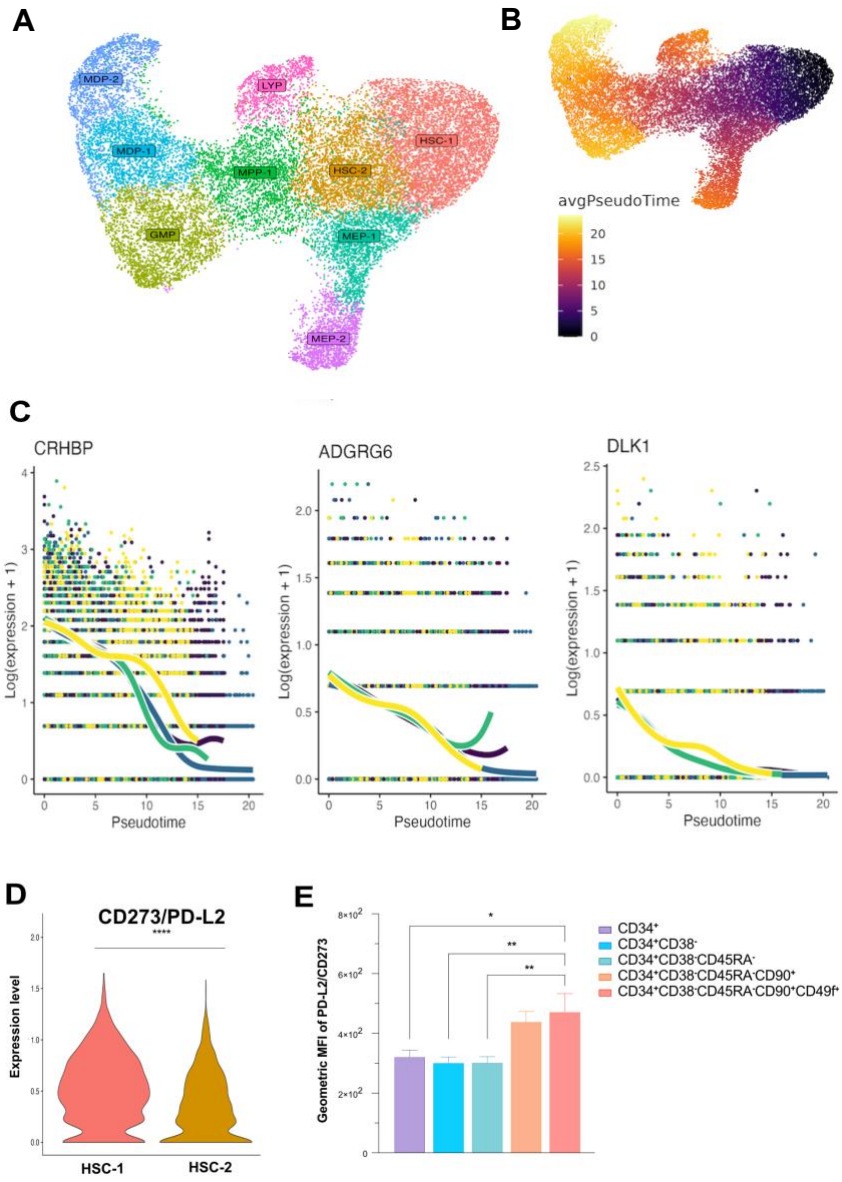

Figure 7. Human BM immature cells show upregulation of novel markers. A) Immature cell clustering, B) Pseudotime analysis, C) Early downregulation of expression of known and novel HSC markers along pseudotime (yellow: MEP, green: LYP, purple: GMP, blue: MDP). D-E) Significant upregulation of CD273 in most immature cells D) in multiomic experiments, E) in flow-cytometry experiments

Papers II and III

While CML has demonstrated a relatively favorable prognosis since the introduction of TKIs, the benefits remain unclear for certain patients. The principal challenge lies in the persistence of TKI-resistant clones, which are responsible for disease relapses or progression towards the more critical blast crisis phase. Hence, the ultimate challenge to obtain the goal of treatment-free remission for A main hurdle for a successful targeted treatment is the CML LSC phenotype. Both HSC and LSC have high expression of CD34 and low or absent expression of CD38, with *BCR-ABL1* fusion gene as the only definitive LSC feature. Even though multiple additional LSC surface markers have been reported so far, including CD26, CD25, IL-1RAP, the field mostly refers to $CD34^{+}CD38^{-/low}$ when referring to LSC. This urges the need for further LSC characterization that would allow easier separation from HSC and targeted eradication that would not harm HSC in close proximity.

To achieve this, we performed detailed characterization of the stem and progenitor cell (SPC) compartments in CML in **Paper II**, while **paper III** focused on assessing the effects of short-term HU treatment on the most immature population of cells.

Although there are many scRNAseq studies that have focused on CML hematopoiesis, to our knowledge none of them succeeded in combining the *BCR-ABL1* fusion point detection as an additional layer to the proteo-genomic profiling. Currently available high-throughput scRNA-seq-based methods rely on 3' end capture of transcripts, thus provide sequence information relatively close to the 3' end. Since the fusion point is located >5000 b from poly-A tail, the transcript captured by BD Rhapsody is identical to a normal *ABL1* transcript. Thus, we decided to bring the fusion point closer to the poly-A tail by adapting a method published by Nam *et. al* (247). The approach consisted of multiple PCR amplifications and circularizations in order to remove the majority of the long *ABL1* sequence and bring the *BCR-ABL1* fusion point close to the poly-A tail. Subsequently, this allowed *BCR-ABL1* sequencing in combination to cell labeling and merging with multiomic dataset.

The initial analyses of **paper II** focus on multiomic (CITEseq) profiling of 70 000 $CD14^{-}CD34^{+}$ cells from 16 CP CML patients and five healthy donors (HD). Both CML and HD cells were used for generation of cell-annotation references by employing transcriptome-based clustering. Using gene and protein expression data, we were able to identify stem and progenitor cell subsets in both cohorts. The analysis confirmed findings from **paper I**, with an early separation of MkE lineage from HSC and later separation of myeloid and lymphoid progenitors in HD. Of note, cells from different HD were used in

these two papers. Hematopoiesis in CML at diagnosis, on the other hand, varied a lot between patients, nevertheless had a significant enrichment of erythroid and myeloid cells, while few stem and almost no lymphoid progenitor cells were observed. Analyses of TKI-treated cells showed normalization of cell subset frequencies, enrichment of HSC and decrease of LSC subsets, thus suggesting the efficacy of TKI in majority of patients from our cohort.

We next zoomed in on the most immature cells by taking advantage of the protein expression data for flow cytometry-like gating of $CD38^{-/low}$ cells. Cells from CML and HD were merged and reclustered. Similar to **paper I,** this population that was previously assumed to be homogenous, revealed a significant heterogeneity. Among 15 clearly separated clusters, 4 showed features of immature cells, with high expression of CD34 and CD90; and low/ absent expression of maturation markers CD38 and CD45RA. However, only two of these clusters showed upregulation of CD25 and CD26 suggesting their leukemic nature. As stated above, *BCR-ABL1* is the most reliable LSC feature, and its presence overlapped with CD25 and CD26 expressing clusters. The remaining two immature clusters were *BCR-ABL1*$^{-}$ and probably comprise healthy HSC. This was additionally confirmed by plotting samples from different cohorts. Cells from HD were observed to be overlapping with HSC clusters, while cells from CML patients were found in both LSC and only one HSC cluster.

More thorough characterization of LSC clusters revealed the quiescent nature of LSC-I with G0/G1-related gene expression and downregulation of cKIT – characteristics that were previously reported to be associated with a TKI-resistant population (85). This was contrary to LSC-II cluster, which expressed cKIT and S/G2/M-phase-related genes, indicating its cycling capacity.

The most striking findings of **paper II** involve upregulated expression of protein TIM3 and the gene *VWF* (von Willebrand factor) on the LSC-I. TIM3 is an immune checkpoint with a role in immune suppression. It has been reported as an AML LSC marker, and allowed successful separation of LSC and HSC. Furthermore, it has been shown that a vast majority of AML patients express TIM3 on LSC at diagnosis. Hence, targeting TIM3 might not cause serious effects on normal hematopoiesis as it is not expressed on HSC or progenitor populations. This finding has paved the way for many preclinical and clinical trials aiming at AML LSC eradication by targeting TIM3. Upregulation of TIM3 gene in CML LSC has been recently reported (248), further strengthening the idea of TIM3 targeting in order to eradicate the relapse driving LSC.

VWF is one of the main megakaryocytic markers and its encoded protein is involved in platelet aggregation (249). It has been shown that *VWF*$^{+}$ HSC have platelet and myeloid bias and are believed to sit at the top of the hematopoietic hierarchy, given their self-renewal capacity (250,251). This fits very well with an early separation model in normal hematopoiesis discussed earlier. It could also explain the significant accumulation of myeloid-primed cells in our CML cohort which goes in line with the classical hematopoiesis model where myeloid CMP give rise to megakaryocytes, myeloid and erythroid cells.

In summary, **paper II** provides evidence for two distinct LSC populations at the time of CML diagnosis, one highly quiescent and TKI-resistant population and another population with cKIT expression that seems to be proliferative. The quiescent/TKI resistant cell phenotype is refined to CD34^{+} CD38$^{-/low}$CD4RA^{-}CD90^{+}CD25^{+}CD26^{+}CD35^{-}cKIT^{-}TIM3^{+} together with *BCR-ABL1* presence and *VWF* upregulation. Although the TIM3 finding needs further confirmation, it shows potential to be explored as an immunotherapeutic target for the relapse prevention (Figure 9).

Hydroxyurea (HU) rapidly inhibits ribonucleotide reductase without serious adverse side effects. Prior to the introduction of TKI, HU was a common therapy for CML patients. Today, it is mainly used in cases of hyperleukocytosis or high platelet counts, mostly while patients are waiting for the diagnosis confirmation (252). However, no studies have focused on understanding the effects of HU on the stem cells in CML.

The initial flow cytometry analyses in **paper III** suggested HU-induced changes in the proportion of CD34^{+} cells, with significant reduction observed in BM CD38^{-} cells among CD34^{+} and resembling trend in blood. These results were further confirmed by analysis of three paired pre-/post-HU blood samples.

The scCITEseq analysis of 26 000 CD34^{+} cells from two patients (blood pre-/post-HU and BM post-HU) showed no patient-specific cell subsets. However, when looking at the cell distribution, post-HU samples showed increased frequency in clusters of more mature erythroid progenitors (EP-II and EP-Cy) and consequent reduction in EP-I. The highest enrichment of EP-II cells was observed in BM samples.

Cell-cycle analyses showed that EP-I cells were mostly in G0/G1 phase, in contrast to S/G2/M phase cells in EP-Cy and EP-II clusters. To further validate these findings, we used results from **paper II** (253) and looked into cell subsets between patients who underwent short HU treatment prior to sampling to those who did not. SPC from HU-treated patients showed a significantly higher

fraction of cells in the mature and cycling erythroid clusters. Of note, in both of these studies, EP-cycling clusters showed upregulation of checkpoint-related genes, including *CHEK2*, while mature erythroid clusters showed upregulation of *HBA1*, *HBA2*, and *HBB* (hemoglobin subunits). Hence, we propose that HU-induces a shift towards S/G2/M phase and erythroid maturation (Figure 8).

We next shifted our focus to 4000 $CD34^{+}CD38^{-}$ cells where similar effects were observed, suggesting that HU affects cells regardless of the differentiation stages and lineages. As discussed in **paper II**, we identified two different LSC populations, LSC-I with a quiescent profile and LSC-II with cycling features. To elucidate HU effects on the cells of the main interest, we performed cell-label transfer from **paper II** dataset. The results revealed a proportional shift from the LSC-I in G0/G1 phase to the LSC-II subpopulation in S/G2/M phase. Hence, we speculate that HU induces a cell-cycle arrest in S phase or eventually slows the progression of the cell cycle leading to an accumulation of cells in S/G2/M phases. Furthermore, when looking at the patient responses, two patients did not achieve a complete cytogenetic response (CCyR, 0% Ph^{+} cells) after three months of TKI treatment. Interestingly, both of them were treated with HU prior to TKI, showed the highest proportion of LSC-II and required the third generation TKI, ponatinib, to reach CCyR.

In summary, we speculate that HU hinders the cell progression through cell cycle and alters cells of all differentiation stages. Among the most immature cells, this effect leads to a relative reduction of quiescent LSC-I frequency in favor of the cycling LSC-II subset (Figure 8). Our study highlights the need to take pre-HU treatment into account when studying LSCs in CML. More clinical studies of the benefit/harm of HU are also warranted as some studies suggest that patients treated with HU show inferior TKI response and lower MMR rate (144,254).

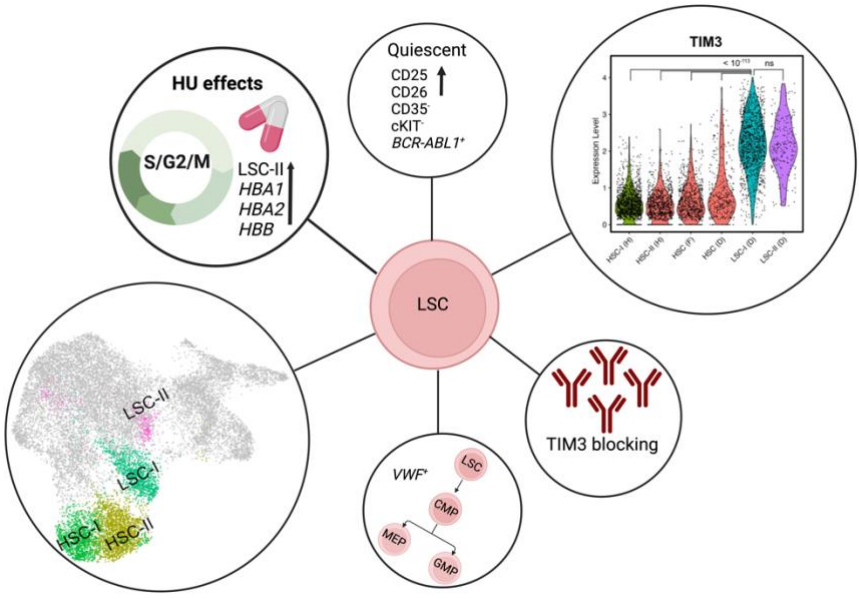

Figure 8. Summary of findings from papers II and III. Two LSC populations were observed, with quiescent LSC expressing previously reported markers in combination with *BCR-ABL1*, TIM3 and *VWF*. HU induces *S* phase arrest and increases frequency of EP with upregulation of hemoglobin subunit genes

Paper IV

Given the heterogeneity and complexity of AML, many patients relapse in the post-CR phase despite repeated courses of consolidation chemotherapy (172). The main reason for disease persistence is the residual LSC clone. While allo-SCT is a curable option for a fraction of fit patients, aged AML patients undergo a limited selection of remission maintenance treatments, without warranted success.

A phase III randomized AML trial investigated the effects of histamine dihydrochloride and interleukin-2 (HDC/IL-2) in CR1. The results showed significantly improved LFS in HDC/IL-2 treated patients <60 years of age compared to the standard of care (232). A follow-up single-armed phase IV trial (Re:Mission) included 84 AML patients with *de novo* or secondary AML in CR1 who received HDC/IL-2 as a relapse-prevention treatment. The treatment was administered by subcutaneous injection twice a day (0.5mg HDC, 16,400 IU/kg IL-2) in ten three-week cycles over a period of 18 months. In **paper IV**, we set out to investigate the correlation between HLA-B genotypes and response to the HDC/IL-2 immunotherapy. The main effector pathway discussed is NK cells cytotoxicity towards cells with absent or reduced HLA class I expression, known as missing self-recognition. Certain HLA-B alleles contain a motif that makes it a ligand to the inhibitory NK cell receptor KIR3DL1 and can shape its function.

It is important to distinguish between HLA allele and supertype, with supertypes consisting of alleles with similar peptide biding and presentation features (255). Many HLA-B alleles and/or supertypes have previously been associated with the treatment outcome in different malignancies, including melanoma, uveal melanoma, lung cancer and diffuse large B-cell lymphoma (256–259) .

The most interesting finding of our study was the finding that the HLA-B*44 allele was associated with significantly worse LFS (Figure 9A) and OS. HLA-B supertype analysis showed that patients who did not carry any alleles belonging to B44 supertype had a significantly better LFS. However, the effect was driven exclusively by HLA-B*44 allele. HLA-B molecules can have either Bw4 or Bw6 motif, with only Bw4 being a ligand for an inhibitory KIR3DL1 receptor (260) To our surprise, there was a clear difference within HLA-B44 supertype in regards to Bw4/Bw6 motif. The majority of alleles encode the Bw6 motif, HLA-B*49 allele harbors Bw4-with isoleucine at position 80 (Bw4-80I), while HLA-B*37, **HLA-B*44**, and HLA-B*47 alleles contain threonine at position 80 of Bw4 (Bw4-80T). This latter Bw4 variant was previously shown to have a lower affinity for KIR3DL1. Since B*44 allele was

driving the supertype effect, we hypothesized that the interaction with NK cell receptors might be the key player. To understand the role of NK cells, we next investigated if relapse incidence was associated with motif presence. The results showed that patients carrying the Bw4-80I allele or Bw6/Bw6 had a significantly superior LFS ($p < 0.0001$) compared to patients with at least one 80T allele and no 80I allele (Figure 9B). The analysis of KIR3DL1/HLA-B interaction strength proposed that weak interacting pairs were associated with significantly reduced LFS (Figure 9C) and OS compared to the strong interacting pair (details for classification can be found **paper IV**).

At first glance, these results may seem counter-intuitive as AML cells with weak inhibitory ligands should be cleared more easily via NK cell cytotoxicity. However, ligands to inhibitory NK cell receptors have dual functions. In addition to their role in negatively regulating NK cell cytotoxicity, they also participate in the process of NK cell education, which in short means that the most responsive and active NK cells are the ones that are fed with the strongest inhibitory signaling at steady state (261). Thus, in individuals with the strong Bw4-80I HLA-B variant, NK cells would be highly functional and may be more efficient in targeting the residual leukemic cells.

Accordingly, *in vitro* co-cultures of IL-2-stimulated healthy donor NK cells and K562 cells showed significantly higher degranulation and cytokine producing response in 80I donors, suggesting impaired NK education in Bw4-80T donors. These effects were not observed in KIR2DL1^{sp+} NK cells, confirming the Bw4/KIR3DL1 education axis (Figure 9D).

In summary, we report that HLA-B*44 allele presents a weak ligand to KIR3DL1 and is incapable of inducing strong anti-leukemic effects, due to relatively unresponsive NK cells. Hence, immunotherapeutic regimen consisting of HDC and IL-2 seems to be especially efficacious in this subgroup of AML patients and could be involved in the eradication of the LSC in AML.

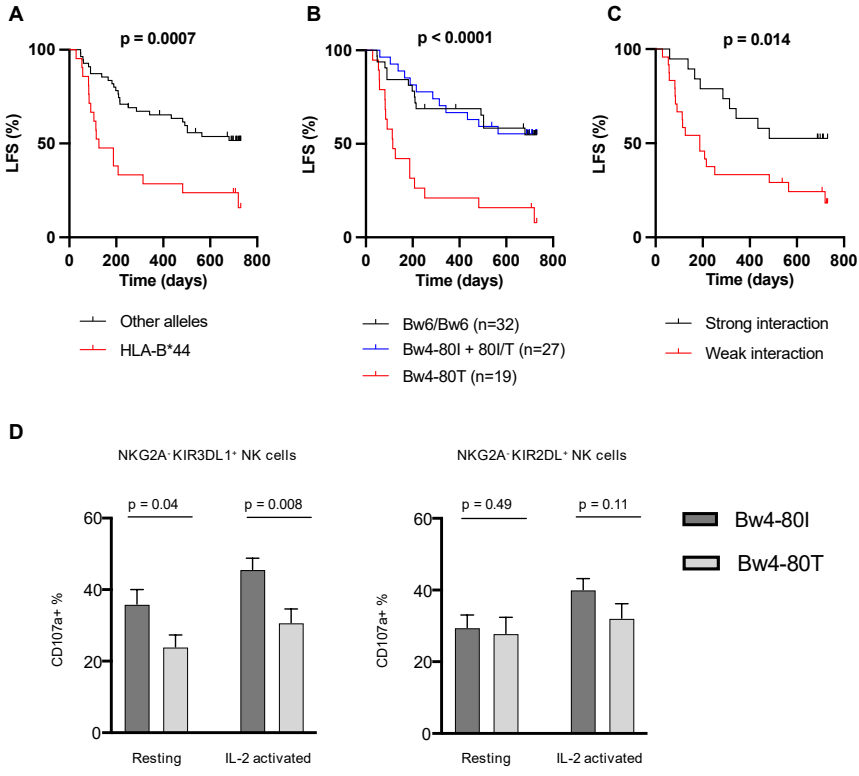

Figure 9. HDC/IL-2 immunotherapy effects on HLA-B genotype A) HLA-B*44 allele is associated with significantly inferior LFS. B) AML patients with no Bw4-80 T allele show superior clinical outcome. C) Weak interaction between KIR3DL1 and Bw4 drives the inferior survival, D) NK cells from Bw4-80I donors show significantly higher degranulation

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The main aim of this thesis was to provide further insights into the heterogeneity of leukemic stem cells (LSC) in myeloid leukemia and pave the way for development of novel immunotherapeutic approaches that would lead to desired outcomes.

In order to understand the aberrant hematopoiesis in malignancies, one has to start with characterization of the normal HSC. As many cell-surface proteins are upregulated on both HSC and LSC, additional markers for their distinction are of utmost importance. One of the limitations of our multiomic studies is the chosen targeted approach with a limited number of markers. Whole transcriptome analysis (WTA) is bias-independent and provides wide-screening, however, it is not very efficient in picking up low-expressed genes in quiescent cells.

In **Paper I** we show that selection of markers in combination with deep sequencing was sufficient to confidently define cell entities and provide a comprehensive map of human bone marrow stem and progenitor cell differentiation stages. The results support hierarchical hematopoiesis with early separation of MEP progenitors. The study design allowed enrichment of immature cells, making our study stand out from many other studies that mostly focused on the whole BM or the $CD34^+$ compartment. To the best of our knowledge, we were the first to perform such an extensive analysis of the immature compartment with a special focus on early commitment by multiomic profiling and functional validation. The major findings of the study are upregulation of CD273/PD-L2, *ADGRG6*, and *DLK1* on immature HSC, as well as trajectory mapping.

Taken together, **papers II** and **III** provided detailed profiling of stem and progenitor populations in CML, by simultaneous measurements of gene and protein expression, further complemented with detection of *BCR-ABL1* expression in the same cells. **Paper II** focused on defining targetable markers on residual leukemic cells in chronic myeloid leukemia (CML). Even though the disease prognosis significantly improved with introduction of TKI and has allowed a subset of patients to stop treatment and remain in TFR, a fraction of patients relapses and requires life-long TKI therapy. The advances in scRNAseq technology during the past few years, contributed to better understanding of the disease progression and/or relapse. However, there are no

approved immunotherapies that would allow direct eradication of the persistent and treatment-resistant LSC. We report upregulation of TIM3 and *VWF* on LSC. TIM3 represents an interesting finding as it is expressed on cell surface and has been explored in preclinical and clinical AML studies, as a target for immunotherapeutic LSC elimination. As CD26 protein seemed like a very reliable marker for distinguishing LSC from HSC in our multiomic study, we employed it in subsequent flow cytometry analysis. It was observed that LiveCD14$^-$CD34$^+$CD38$^-$CD45RA$^-$CD26$^+$ LSC had significantly increased TIM3 expression in comparison to LiveCD14$^-$CD34$^+$CD38$^-$CD45RA$^-$CD26$^-$ cells. Additionally, TIM3 was significantly lower in HSC, clearly indicating its potential to be targeted. We are currently in the process of setting up a protocol to trigger TIM3 antibody-dependent cytotoxicity (ADCC) by NK cells resulting with LSC killing. Results in **paper III** suggest that HU treatment does affect cells of all stages of differentiation and inhibits cell cycle, by either arresting cells in S phase or making them progress rather slowly. Clinical parameters suggest a need to take HU treatment into consideration, as it might be related to the poor response.

One possible strategy for targeting the residual LSC in TKI-treated CML is NK cell-stimulatory immunotherapy with HDC/IL-2. An ongoing Phase I/II trial lead by our group is testing the feasibility to combine this immunotherapeutic regimen with TKI. High NK cell numbers have been reported to be a biomarker for successful TKI discontinuation (262), and our previous studies in AML have shown that HDC/IL-2 increase NK cell counts and NK cell function (234). Results in **paper IV** show other aspects of the importance of NK cells in HDC/IL-2-treated AML. The presented results suggest that the capacity of the patient's HLA genotype to generate strong NK cell education impacts on treatment outcome in AML. This study further underscores the importance of NK cells for the outcome of relapse-preventive HDC/IL-2 immunotherapy in AML.

ACKNOWLEDGEMENT

First of all, I would like to thank my main supervisor, **Fredrik Bergh Thorén**. You gave me a chance for a better life and I am truly thankful for that. I learnt tremendously much from you: your creativity and amazing scientific reasoning are truly inspiring. You helped me when I struggled the most, finding a way to keep me motivated. Thanks for believing in me and for being such a great role model.

My co-supervisor, **Lovisa Wennström**, you have been an amazing support and asset to our team throughout all these years. Thank you for the hard work within CML projects, but mostly, thank you for being such a great person. Thank you to my other co-supervisor, **Anders Ståhlberg**. Even though my PhD project changed the direction, I always felt I could count on your help.

I am so happy I got to spend a lot of time learning from you, **Anna Martner**. Our CML meetings were always so inspiring and I really enjoyed being part of the CML project. **Kristoffer Hellstrand**, thanks for the scientific input and for being a great leader. I always felt safe and protected at work. **Ka-Wei Tang,** thanks for making my PhD days **fantastic** and memorable.

Malin, we have been through a lot since 2018, but always there for each other. All the issues, super long evenings, thousands of vials and UMAPs....Thanks for supporting me and being the best work partner I could ask for. **Brwa,** you are one of the nicest people I have ever met and I am sure our friendship will last no matter where life takes us. **Linnea,** we started our PhD journey around the same time and it has been a blast going through it all with you. **Rob**, positive energy and calm nature bring a lot to the lab. I am happy to call you a friend. **Elin**, thanks for your support during my studies, but mostly thank you for encouraging me to stand up for myself. It's been great learning from you. **Hanna GW**, lab is not the same without you. Thanks for all your help throughout the years. **Yarong** thanks for being a great friend and for skipping yoga. **Alan,** my favorite IT guy, time to teach you some Jokic/Balkan dance moves.

TIMM Lab has been a fun place all these years, and it is because of many amazing people on the floor. My sincerest thanks to all current and previous lab members. Keep shining, but don't forget to take care of yourselves! Thank you for your support and Friday fika over the years.

Many thanks to **SCCR people**! I really enjoyed talking to many of you on the daily basis. **Kompis Azbija**, hvala na svemu! **Gulay** abla, sagol!

It's been a pleasure collaborating with **Michael Rieger** (Frankfurt) and **Bruno Paiva** (Pamplona) and their group members. I am very grateful to the amazing **BD Multi-omics alliance**.

Sincerest thanks to all patients, donors and their families, as well as hard-working and dedicated physicians and nurses at Hematology and Orthopedics department at Sahlgrenska University Hospital. Many thanks to all funding agencies that supported our research.

I have been very privileged to come across some outstanding people who quickly became my second family: **Ebru,** you were my first friend here and I think you are one of the reasons I didn't run back home after two weeks. I hope you know how much you and Sofi mean to me. **Ahmed** you made my time here sooooo much better. All the silly shopping runs, food discussions and laughing sessions. Thanks for being my rock when skies were falling. My dear Pink Cat ladies, **Junkoooo** and **Beatrice**. The three of us couldn't be more different, but we make a great team and I love spending time with you. **Silvia,** you are a big part of my life and even though we don't see each other often, I am always just a phone call away. I miss you! **Ezgi**, thanks for many lahmacun dates and all the great fun we have had. You are the only person who buys more useless things than me, and I love how silly we are. **Mukul** and **Vasu**, thanks for all the support and great moments we shared. **Zerina** and **Ulf**, tack för er kärlek och stöd.

Some of my favorite people from Uni days in Sarajevo stayed in my life even after leaving Bosnia. **Erna**, I am so happy you joined Thorén group. You deserve sooooo much. Thanks for being by my side, I am always here for you. **Prof. Amina** thank you for believing in me and pushing me.

None of this would be possible without people who have supported me for years. I have been surrounded by **AMAZING FRIENDS** and even though bad situation in Bosnia made many of us leave home, we have always been there for each others. I love you and miss you guys so much.

Over the past few years, my family and me have been through a lot, but it made us closer than ever. It is a blessing to have a family like mine, whose love, support and understanding make me stronger. Thank you from the bottom of my heart. Sreca i blagodat je biti okruzen porodicom poput vas. Iako sam davno otisla od kuce, vasa ljubav i podrska su mi dale krila. Hvala sto ste uvijek uz mene, volim vas! **Majka Timka**, tvoja snaga mi je motivacija. **Majka Zahida**, **deda**, **dido**, nedostajete mi!

Shortly after I started writing this thesis, my cousin **Adnan** passed away, changing our lives forever. **Ado**, otisao si iznenada, tiho kako si i zivio. Ostala sam te zeljna, ali ces zauvijek biti u mom srcu.

Finally, I would like to thank those who never left my side. **Mom Aida**, **dad Adem**, you got me when your lives were in danger every single second, you had so little, almost nothing, but nevertheless always put Sabina and me first. You've kept following us along the way, giving us enourmous amounts of love, trust, support and sacrifice. You never stood in the way of following our dreams, but instead did everything to get us there quicker.

Mama, **tata**, odakle da pocnem? Sve sto jesam, dugujem vama. Vasa neizmjerna ljubav i razumijevanje mi nisu dali da odustanem. Znala sam da ste uvijek uz mene, cak i onda kada smo jedni od drugih krili probleme. Ne postoje rijeci kojima vam se mogu zahvaliti na svemu, ali se nadam da cu ostatak zivota imati priliku da vam uzvratim bar malo.

Sabina, my favorite (and only) sister... You have been the best birthday gift ever and you make me proud every single day. Your support and love made me survive many bad days, and I know that no matter what happens, we can fight it together.

Mom, **dad**, **Sabina**, a big part of my PhD title belongs to you. Love you more than words can say and can't wait to celebrate with you. **Mama**, **tata**, **Sabina**, ovu tezu posvecujem vama. Volim vas!

References

1. Till JE, McCulloch EA. Hemopoietic stem cell differentiation. Biochim Biophys Acta. 1980 Nov 26;605(4):431–59.
2. Dunn CD. The differentiation of haemopoietic stem cells. Ser Haematol 1968. 1971;4(4):1–71.
3. Seita J, Weissman IL. Hematopoietic stem cell: self-renewal versus differentiation. Wiley Interdiscip Rev Syst Biol Med. 2010;2(6):640–53.
4. Weissman IL, Shizuru JA. The origins of the identification and isolation of hematopoietic stem cells, and their capability to induce donor-specific transplantation tolerance and treat autoimmune diseases. Blood. 2008 Nov 1;112(9):3543–53.
5. Deutsches Textarchiv – Haeckel, Ernst: Natürliche Schöpfungsgeschichte. Berlin, 1868. [Internet]. [cited 2023 Dec 25]. Available from: https://www.deutschestextarchiv.de/book/view/haeckel_schoepfungsgeschichte_1868?p=6
6. Laurenti E, Göttgens B. From haematopoietic stem cells to complex differentiation landscapes. Nature. 2018 Jan 24;553(7689):418–26.
7. Becker AJ, McCULLOCH EA, Till JE. Cytological Demonstration of the Clonal Nature of Spleen Colonies Derived from Transplanted Mouse Marrow Cells. Nature. 1963 Feb;197(4866):452–4.
8. Siminovitch L, Mcculloch EA, Till JE. THE DISTRIBUTION OF COLONY-FORMING CELLS AMONG SPLEEN COLONIES. J Cell Comp Physiol. 1963 Dec;62:327–36.
9. Till JE, McCULLOCH EA. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res. 1961 Feb;14:213–22.
10. Wu AM, Till JE, Siminovitch L, McCulloch EA. Cytological evidence for a relationship between normal hemotopoietic colony-forming cells and cells of the lymphoid system. J Exp Med. 1968 Mar 1;127(3):455–64.
11. Ogawa M. Differentiation and proliferation of hematopoietic stem cells. Blood. 1993 Jun 1;81(11):2844–53.
12. Rossi DJ, Seita J, Czechowicz A, Bhattacharya D, Bryder D, Weissman IL. Hematopoietic stem cell quiescence attenuates DNA damage response and permits DNA damage accumulation during aging. Cell Cycle Georget Tex. 2007 Oct 1;6(19):2371–6.
13. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature. 2001 Nov 1;414(6859):105–11.
14. Weissman I. Stem cell research: paths to cancer therapies and regenerative medicine. JAMA. 2005 Sep 21;294(11):1359–66.
15. Kondo M, Weissman IL, Akashi K. Identification of Clonogenic Common Lymphoid Progenitors in Mouse Bone Marrow. Cell. 1997 Nov 28;91(5):661–72.
16. Morrison SJ, Weissman IL. The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. Immunity. 1994 Nov;1(8):661–73.
17. Osawa M, Hanada K, Hamada H, Nakauchi H. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science. 1996 Jul 12;273(5272):242–5.
18. Kiel MJ, Yilmaz OH, Iwashita T, Yilmaz OH, Terhorst C, Morrison SJ. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell. 2005 Jul 1;121(7):1109–21.
19. Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature. 2000 Mar;404(6774):193–7.
20. Inlay MA, Bhattacharya D, Sahoo D, Serwold T, Seita J, Karsunky H, et al. Ly6d marks the earliest stage of B-cell specification and identifies the branchpoint between B-cell and T-cell development. Genes Dev. 2009 Oct 15;23(20):2376–81.
21. Rieger MA, Schroeder T. Hematopoiesis. Cold Spring Harb Perspect Biol. 2012 Dec;4(12):a008250.
22. Majeti R, Park CY, Weissman IL. Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. Cell Stem Cell. 2007 Dec 13;1(6):635–45.
23. Zhang CC, Lodish HF. Cytokines regulating hematopoietic stem cell function. Curr Opin Hematol. 2008 Jul;15(4):307–11.
24. Metcalf D. Hematopoietic cytokines. Blood. 2008 Jan 15;111(2):485–91.

25. Cabezas-Wallscheid N, Klimmeck D, Hansson J, Lipka DB, Reyes A, Wang Q, et al. Identification of regulatory networks in HSCs and their immediate progeny via integrated proteome, transcriptome, and DNA methylome analysis. Cell Stem Cell. 2014 Oct 2;15(4):507–22.
26. Chambers SM, Boles NC, Lin KYK, Tierney MP, Bowman TV, Bradfute SB, et al. Hematopoietic fingerprints: an expression database of stem cells and their progeny. Cell Stem Cell. 2007 Nov;1(5):578–91.
27. Novershtern N, Subramanian A, Lawton LN, Mak RH, Haining WN, McConkey ME, et al. Densely interconnected transcriptional circuits control cell states in human hematopoiesis. Cell. 2011 Jan 21;144(2):296–309.
28. Laurenti E, Doulatov S, Zandi S, Plumb I, Chen J, April C, et al. The transcriptional architecture of early human hematopoiesis identifies multilevel control of lymphoid commitment. Nat Immunol. 2013 Jul;14(7):756–63.
29. Chen L, Kostadima M, Martens JHA, Canu G, Garcia SP, Turro E, et al. Transcriptional diversity during lineage commitment of human blood progenitors. Science. 2014 Sep 26;345(6204):1251033.
30. Notta F, Doulatov S, Laurenti E, Poeppl A, Jurisica I, Dick JE. Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. Science. 2011 Jul 8;333(6039):218–21.
31. Laurenti E, Frelin C, Xie S, Ferrari R, Dunant CF, Zandi S, et al. CDK6 levels regulate quiescence exit in human hematopoietic stem cells. Cell Stem Cell. 2015 Mar 5;16(3):302–13.
32. Anjos-Afonso F, Currie E, Palmer HG, Foster KE, Taussig DC, Bonnet D. CD34(-) cells at the apex of the human hematopoietic stem cell hierarchy have distinctive cellular and molecular signatures. Cell Stem Cell. 2013 Aug 1;13(2):161–74.
33. Oguro H, Ding L, Morrison SJ. SLAM family markers resolve functionally distinct subpopulations of hematopoietic stem cells and multipotent progenitors. Cell Stem Cell. 2013 Jul 3;13(1):102–16.
34. Kent DG, Copley MR, Benz C, Wöhrer S, Dykstra BJ, Ma E, et al. Prospective isolation and molecular characterization of hematopoietic stem cells with durable self-renewal potential. Blood. 2009 Jun 18;113(25):6342–50.
35. Pietras EM, Reynaud D, Kang YA, Carlin D, Calero-Nieto FJ, Leavitt AD, et al. Functionally Distinct Subsets of Lineage-Biased Multipotent Progenitors Control Blood Production in Normal and Regenerative Conditions. Cell Stem Cell. 2015 Jul 2;17(1):35–46.
36. Milyavsky M, Gan OI, Trottier M, Komosa M, Tabach O, Notta F, et al. A Distinctive DNA Damage Response in Human Hematopoietic Stem Cells Reveals an Apoptosis-Independent Role for p53 in Self-Renewal. Cell Stem Cell. 2010 Aug 6;7(2):186–97.
37. van Galen P, Kreso A, Mbong N, Kent DG, Fitzmaurice T, Chambers JE, et al. The unfolded protein response governs integrity of the haematopoietic stem-cell pool during stress. Nature. 2014 Jun;510(7504):268–72.
38. Bryder D, Rossi DJ, Weissman IL. Hematopoietic Stem Cells. Am J Pathol. 2006 Aug;169(2):338–46.
39. Haas S, Hansson J, Klimmeck D, Loeffler D, Velten L, Uckelmann H, et al. Inflammation-Induced Emergency Megakaryopoiesis Driven by Hematopoietic Stem Cell-like Megakaryocyte Progenitors. Cell Stem Cell. 2015 Oct 1;17(4):422–34.
40. Notta F, Zandi S, Takayama N, Dobson S, Gan OI, Wilson G, et al. DISTINCT ROUTES OF LINEAGE DEVELOPMENT RESHAPE THE HUMAN BLOOD HIERARCHY ACROSS ONTOGENY. Science. 2016 Jan 8;351(6269):aab2116.
41. Yamamoto R, Morita Y, Ooehara J, Hamanaka S, Onodera M, Rudolph KL, et al. Clonal analysis unveils self-renewing lineage-restricted progenitors generated directly from hematopoietic stem cells. Cell. 2013 Aug 29;154(5):1112–26.
42. Haas S, Trumpp A, Milsom MD. Causes and Consequences of Hematopoietic Stem Cell Heterogeneity. Cell Stem Cell. 2018 May 3;22(5):627–38.
43. Velten L, Haas SF, Raffel S, Blaszkiewicz S, Islam S, Hennig BP, et al. Human haematopoietic stem cell lineage commitment is a continuous process. Nat Cell Biol. 2017 Apr;19(4):271–81.
44. Karamitros D, Stoilova B, Aboukhalil Z, Hamey F, Reinisch A, Samitsch M, et al. Heterogeneity of human lympho-myeloid progenitors at the single cell level. Nat Immunol. 2018 Jan;19(1):85–97.
45. Mercier FE, Scadden DT. Not All Created Equal: Lineage Hard-Wiring in the Production of Blood. Cell. 2015 Dec 17;163(7):1568–70.
46. Paul F, Arkin Y, Giladi A, Jaitin DA, Kenigsberg E, Keren-Shaul H, et al. Transcriptional Heterogeneity and Lineage Commitment in Myeloid Progenitors. Cell. 2015 Dec 17;163(7):1663–77.

47. Perié L, Duffy KR, Kok L, de Boer RJ, Schumacher TN. The Branching Point in Erythro-Myeloid Differentiation. Cell. 2015 Dec 17;163(7):1655–62.
48. Nestorowa S, Hamey FK, Pijuan Sala B, Diamanti E, Shepherd M, Laurenti E, et al. A single-cell resolution map of mouse hematopoietic stem and progenitor cell differentiation. Blood. 2016 Aug 25;128(8):e20–31.
49. Hamey FK, Göttgens B. Machine learning predicts putative hematopoietic stem cells within large single-cell transcriptomics data sets. Exp Hematol. 2019 Oct;78:11–20.
50. Mair F, Erickson JR, Voillet V, Simoni Y, Bi T, Tyznik AJ, et al. A Targeted Multi-omic Analysis Approach Measures Protein Expression and Low-Abundance Transcripts on the Single-Cell Level. Cell Rep. 2020 Apr 7;31(1):107499.
51. Triana S, Vonficht D, Jopp-Saile L, Raffel S, Lutz R, Leonce D, et al. Single-cell proteo-genomic reference maps of the hematopoietic system enable the purification and massive profiling of precisely defined cell states. Nat Immunol. 2021 Dec;22(12):1577–89.
52. Hanahan D, Weinberg RA. The Hallmarks of Cancer. Cell. 2000 Jan 7;100(1):57–70.
53. Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. Cell. 2011 Mar 4;144(5):646–74.
54. Hanahan D. Hallmarks of Cancer: New Dimensions. Cancer Discov. 2022 Jan 12;12(1):31–46.
55. de Haan G, Lazare SS. Aging of hematopoietic stem cells. Blood. 2018 Feb 1;131(5):479–87.
56. Mejia-Ramirez E, Florian MC. Understanding intrinsic hematopoietic stem cell aging. Haematologica. 2020 Jan;105(1):22–37.
57. López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The Hallmarks of Aging. Cell. 2013 Jun 6;153(6):1194–217.
58. Pang WW, Price EA, Sahoo D, Beerman I, Maloney WJ, Rossi DJ, et al. Human bone marrow hematopoietic stem cells are increased in frequency and myeloid-biased with age. Proc Natl Acad Sci U S A. 2011 Dec 13;108(50):20012–7.
59. Florian MC, Klose M, Sacma M, Jablanovic J, Knudson L, Nattamai KJ, et al. Aging alters the epigenetic asymmetry of HSC division. PLoS Biol. 2018 Sep 20;16(9):e2003389.
60. Signer RAJ, Montecino-Rodriguez E, Witte ON, McLaughlin J, Dorshkind K. Age-related defects in B lymphopoiesis underlie the myeloid dominance of adult leukemia. Blood. 2007 Sep 15;110(6):1831–9.
61. Jaiswal S, Ebert BL. Clonal hematopoiesis in human aging and disease. Science. 2019 Nov 1;366(6465):eaan4673.
62. von Bonin M, Jambor HK, Teipel R, Stölzel F, Thiede C, Damm F, et al. Clonal hematopoiesis and its emerging effects on cellular therapies. Leukemia. 2021 Oct;35(10):2752–8.
63. Miyamoto T, Weissman IL, Akashi K. AML1/ETO-expressing nonleukemic stem cells in acute myelogenous leukemia with 8;21 chromosomal translocation. Proc Natl Acad Sci U S A. 2000 Jun 20;97(13):7521–6.
64. Jan M, Snyder TM, Corces-Zimmerman MR, Vyas P, Weissman IL, Quake SR, et al. Clonal Evolution of Pre-Leukemic Hematopoietic Stem Cells Precedes Human Acute Myeloid Leukemia. Sci Transl Med. 2012 Aug 29;4(149):149ra118.
65. Shlush LI, Zandi S, Mitchell A, Chen WC, Brandwein JM, Gupta V, et al. Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. Nature. 2014 Feb;506(7488):328–33.
66. Corces-Zimmerman MR, Hong WJ, Weissman IL, Medeiros BC, Majeti R. Preleukemic mutations in human acute myeloid leukemia affect epigenetic regulators and persist in remission. Proc Natl Acad Sci U S A. 2014 Feb 18;111(7):2548–53.
67. Fabre MA, de Almeida JG, Fiorillo E, Mitchell E, Damaskou A, Rak J, et al. The longitudinal dynamics and natural history of clonal haematopoiesis. Nature. 2022 Jun;606(7913):335–42.
68. Steensma DP, Bejar R, Jaiswal S, Lindsley RC, Sekeres MA, Hasserjian RP, et al. Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. Blood. 2015 Jul 2;126(1):9–16.
69. Essers MAG, Trumpp A. Targeting leukemic stem cells by breaking their dormancy. Mol Oncol. 2010 Oct;4(5):443–50.
70. Misaghian N, Ligresti G, Steelman L, Bertrand F, Bäsecke J, Libra M, et al. Targeting the leukemic stem cell: the Holy Grail of leukemia therapy. Leuk Off J Leuk Soc Am Leuk Res Fund UK. 2009 Jan;23(1):25–42.
71. Soverini S, De Santis S, Monaldi C, Bruno S, Mancini M. Targeting Leukemic Stem Cells in Chronic Myeloid Leukemia: Is It Worth the Effort? Int J Mol Sci. 2021 Jan;22(13):7093.

72. Udomsakdi C, Eaves CJ, Swolin B, Reid DS, Barnett MJ, Eaves AC. Rapid decline of chronic myeloid leukemic cells in long-term culture due to a defect at the leukemic stem cell level. Proc Natl Acad Sci U S A. 1992 Jul 1;89(13):6192–6.
73. Holyoake T, Jiang X, Eaves C, Eaves A. Isolation of a Highly Quiescent Subpopulation of Primitive Leukemic Cells in Chronic Myeloid Leukemia. Blood. 1999 Sep 15;94(6):2056–64.
74. Graham SM, Jørgensen HG, Allan E, Pearson C, Alcorn MJ, Richmond L, et al. Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro. Blood. 2002 Jan 1;99(1):319–25.
75. Holtz MS, Slovak ML, Zhang F, Sawyers CL, Forman SJ, Bhatia R. Imatinib mesylate (STI571) inhibits growth of primitive malignant progenitors in chronic myelogenous leukemia through reversal of abnormally increased proliferation. Blood. 2002 May 15;99(10):3792–800.
76. Gerber JM, Qin L, Kowalski J, Smith BD, Griffin CA, Vala MS, et al. Characterization of chronic myeloid leukemia stem cells. Am J Hematol. 2011 Jan;86(1):31–7.
77. Copland M, Hamilton A, Elrick LJ, Baird JW, Allan EK, Jordanides N, et al. Dasatinib (BMS-354825) targets an earlier progenitor population than imatinib in primary CML but does not eliminate the quiescent fraction. Blood. 2006 Jun 1;107(11):4532–9.
78. Stoklosa T, Poplawski T, Koptyra M, Nieborowska-Skorska M, Basak G, Slupianek A, et al. BCR/ABL Inhibits Mismatch Repair to Protect from Apoptosis and Induce Point Mutations. Cancer Res. 2008 Apr 15;68(8):2576–80.
79. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med. 1997 Jul;3(7):730–7.
80. Eisterer W, Jiang X, Christ O, Glimm H, Lee KH, Pang E, et al. Different subsets of primary chronic myeloid leukemia stem cells engraft immunodeficient mice and produce a model of the human disease. Leukemia. 2005 Mar;19(3):435–41.
81. Järås M, Johnels P, Hansen N, Ågerstam H, Tsapogas P, Rissler M, et al. Isolation and killing of candidate chronic myeloid leukemia stem cells by antibody targeting of IL-1 receptor accessory protein. Proc Natl Acad Sci. 2010 Sep 14;107(37):16280–5.
82. Herrmann H, Sadovnik I, Cerny-Reiterer S, Rülicke T, Stefanzl G, Willmann M, et al. Dipeptidylpeptidase IV (CD26) defines leukemic stem cells (LSC) in chronic myeloid leukemia. Blood. 2014 Jun 19;123(25):3951–62.
83. Sadovnik I, Herrmann H, Blatt K, Eisenwort G, Mueller N, Stefanzl G, et al. Evaluation of Cell Surface Markers and Targets in Leukemic Stem Cells (LSC) Reveals Distinct Expression Profiles, Unique Drug Effects, and Specific Checkpoint Regulation in AML LSC and CML LSC. Blood. 2016 Dec 2;128(22):4234.
84. Kinstrie R, Horne GA, Morrison H, Moka HA, Cassels J, Dunn K, et al. CD93 Is a Novel Biomarker of Leukemia Stem Cells in Chronic Myeloid Leukemia. Blood. 2015 Dec 3;126(23):49.
85. Warfvinge R, Geironson L, Sommarin MNE, Lang S, Karlsson C, Roschupkina T, et al. Single-cell molecular analysis defines therapy response and immunophenotype of stem cell subpopulations in CML. Blood. 2017 Apr 27;129(17):2384–94.
86. Sommarin MNE, Dhapola P, Safi F, Warfvinge R, Ulfsson LG, Erlandsson E, et al. Single-Cell Multiomics Reveals Distinct Cell States at the Top of the Human Hematopoietic Hierarchy [Internet]. bioRxiv; 2021 [cited 2023 Dec 6]. p. 2021.04.01.437998. Available from: https://www.biorxiv.org/content/10.1101/2021.04.01.437998v2
87. Warfvinge R, Ulfsson LG, Dhapola P, Safi F, Sommarin MNE, Soneji S, et al. Single cell multi-omics analysis of chronic myeloid leukemia links cellular heterogeneity to therapy response. eLife [Internet]. 2023 Nov 30 [cited 2023 Dec 25];12. Available from: https://elifesciences.org/reviewed-preprints/92074
88. Goardon N, Marchi E, Atzberger A, Quek L, Schuh A, Soneji S, et al. Coexistence of LMPP-like and GMP-like Leukemia Stem Cells in Acute Myeloid Leukemia. Cancer Cell. 2011 Jan 18;19(1):138–52.
89. Jan M, Chao MP, Cha AC, Alizadeh AA, Gentles AJ, Weissman IL, et al. Prospective separation of normal and leukemic stem cells based on differential expression of TIM3, a human acute myeloid leukemia stem cell marker. Proc Natl Acad Sci U S A. 2011 Mar 22;108(12):5009–14.
90. Saito Y, Kitamura H, Hijikata A, Tomizawa-Murasawa M, Tanaka S, Takagi S, et al. Identification of Therapeutic Targets for Quiescent, Chemotherapy-Resistant Human Leukemia Stem Cells. Sci Transl Med. 2010 Feb 3;2(17):17ra9.
91. Touzet L, Dumezy F, Roumier C, Berthon C, Bories C, Quesnel B, et al. CD9 in acute myeloid leukemia: Prognostic role and usefulness to target leukemic stem cells. Cancer Med. 2019;8(3):1279–88.

92. Sadovnik I, Herrmann H, Eisenwort G, Blatt K, Hoermann G, Mueller N, et al. Expression of CD25 on leukemic stem cells in BCR-ABL1+ CML: Potential diagnostic value and functional implications. Exp Hematol. 2017 Jul;51:17–24.
93. Sadovnik I, Hoelbl-Kovacic A, Herrmann H, Eisenwort G, Cerny-Reiterer S, Warsch W, et al. Identification of CD25 as STAT5-Dependent Growth-Regulator of Leukemic Stem Cells in Ph+ CML. Clin Cancer Res Off J Am Assoc Cancer Res. 2016 Apr 15;22(8):2051–61.
94. Saito Y, Uchida N, Tanaka S, Suzuki N, Tomizawa-Murasawa M, Sone A, et al. Induction of cell cycle entry eliminates human leukemia stem cells in a mouse model of AML. Nat Biotechnol. 2010 Mar;28(3):10.1038/nbt.1607.
95. Valent P, Sadovnik I, Ráčil Z, Herrmann H, Blatt K, Cerny-Reiterer S, et al. DPPIV (CD26) as a novel stem cell marker in Ph+ chronic myeloid leukaemia. Eur J Clin Invest. 2014 Dec;44(12):1239–45.
96. Jilani I, Estey E, Huh Y, Joe Y, Manshouri T, Yared M, et al. Differences in CD33 Intensity Between Various Myeloid Neoplasms. Am J Clin Pathol. 2002 Oct 1;118(4):560–6.
97. Chao MP, Weissman IL, Majeti R. The CD47-SIRPα Pathway in Cancer Immune Evasion and Potential Therapeutic Implications. Curr Opin Immunol. 2012 Apr;24(2):225–32.
98. Jaiswal S, Jamieson CHM, Pang WW, Park CY, Chao MP, Majeti R, et al. CD47 is up-regulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis. Cell. 2009 Jul 23;138(2):271–85.
99. Oehler VG, Walter RB, Cummings C, Sala-Torra O, Stirewalt DL, Fang M, et al. CD52 Expression In Leukemic Stem/Progenitor Cells. Blood. 2010 Nov 19;116(21):2743.
100. van Rhenen A, van Dongen GAMS, Kelder A, Rombouts EJ, Feller N, Moshaver B, et al. The novel AML stem cell–associated antigen CLL-1 aids in discrimination between normal and leukemic stem cells. Blood. 2007 Oct 1;110(7):2659–66.
101. Askmyr M, Ågerstam H, Hansen N, Gordon S, Arvanitakis A, Rissler M, et al. Selective killing of candidate AML stem cells by antibody targeting of IL1RAP. Blood. 2013 May 2;121(18):3709–13.
102. Kikushige Y, Shima T, Takayanagi S ichiro, Urata S, Miyamoto T, Iwasaki H, et al. TIM-3 Is a Promising Target to Selectively Kill Acute Myeloid Leukemia Stem Cells. Cell Stem Cell. 2010 Dec 3;7(6):708–17.
103. Boer B de, Prick J, Pruis MG, Keane P, Imperato MR, Jaques J, et al. Prospective Isolation and Characterization of Genetically and Functionally Distinct AML Subclones. Cancer Cell. 2018 Oct 8;34(4):674-689.e8.
104. Hosen N, Park CY, Tatsumi N, Oji Y, Sugiyama H, Gramatzki M, et al. CD96 is a leukemic stem cell-specific marker in human acute myeloid leukemia. Proc Natl Acad Sci U S A. 2007 Jun 26;104(26):11008–13.
105. Vetrie D, Helgason GV, Copland M. The leukaemia stem cell: similarities, differences and clinical prospects in CML and AML. Nat Rev Cancer. 2020 Mar;20(3):158–73.
106. Giustacchini A, Thongjuea S, Barkas N, Woll PS, Povinelli BJ, Booth CAG, et al. Single-cell transcriptomics uncovers distinct molecular signatures of stem cells in chronic myeloid leukemia. Nat Med. 2017 Jun;23(6):692–702.
107. Lane SW, Scadden DT, Gilliland DG. The leukemic stem cell niche: current concepts and therapeutic opportunities. Blood. 2009 Aug 6;114(6):1150–7.
108. Christopherson KW II, Hangoc G, Broxmeyer HE. Cell Surface Peptidase CD26/Dipeptidylpeptidase IV Regulates CXCL12/Stromal Cell-Derived Factor-1α-Mediated Chemotaxis of Human Cord Blood CD34+ Progenitor Cells1. J Immunol. 2002 Dec 15;169(12):7000–8.
109. Jin L, Tabe Y, Konoplev S, Xu Y, Leysath CE, Lu H, et al. CXCR4 up-regulation by imatinib induces chronic myelogenous leukemia (CML) cell migration to bone marrow stroma and promotes survival of quiescent CML cells. Mol Cancer Ther. 2008 Jan 17;7(1):48–58.
110. Weisberg E, Azab AK, Manley PW, Kung AL, Christie AL, Bronson R, et al. Inhibition of CXCR4 in CML cells disrupts their interaction with the bone marrow microenvironment and sensitizes them to nilotinib. Leukemia. 2012 May;26(5):985–90.
111. Peled A, Hardan I, Trakhtenbrot L, Gur E, Magid M, Darash-Yahana M, et al. Immature Leukemic CD34+CXCR4+ Cells from CML Patients Have Lower Integrin-Dependent Migration and Adhesion in Response to the Chemokine SDF-1. Stem Cells. 2002 May 1;20(3):259–66.
112. Goldman JM, Melo JV. Targeting the BCR-ABL Tyrosine Kinase in Chronic Myeloid Leukemia. N Engl J Med. 2001 Apr 5;344(14):1084–6.
113. Holyoake TL, Vetrie D. The chronic myeloid leukemia stem cell: stemming the tide of persistence. Blood. 2017 Mar 23;129(12):1595–606.

114. Nowell PC, Hungerford DA. Chromosome Studies on Normal and Leukemic Human Leukocytes. JNCI J Natl Cancer Inst. 1960 Jul 1;25(1):85–109.
115. Rowley JD. A New Consistent Chromosomal Abnormality in Chronic Myelogenous Leukaemia identified by Quinacrine Fluorescence and Giemsa Staining. Nature. 1973 Jun;243(5405):290–3.
116. Jabbour E, Kantarjian H. Chronic myeloid leukemia: 2018 update on diagnosis, therapy and monitoring. Am J Hematol. 2018;93(3):442–59.
117. Gong Z, Medeiros LJ, Cortes JE, Zheng L, Khoury JD, Wang W, et al. Clinical and prognostic significance of e1a2 BCR-ABL1 transcript subtype in chronic myeloid leukemia. Blood Cancer J. 2017 Jul;7(7):e583.
118. Cancer Facts & Figures 2017 [Internet]. [cited 2024 Jan 2]. Available from: https://www.cancer.org/research/cancer-facts-statistics/all-cancer-facts-figures/cancer-facts-figures-2017.html
119. Höglund M, Sandin F, Simonsson B. Epidemiology of chronic myeloid leukaemia: an update. Ann Hematol. 2015 Apr 1;94(2):241–7.
120. Chang CS, Lee K, Yang YH, Lin MT, Hsu CN. Estimation of CML incidence: Disagreement between national cancer registry and health claims data system in Taiwan. Leuk Res. 2011 May 1;35(5):e53–4.
121. Chen Y, Wang H, Kantarjian H, Cortes J. Trends in chronic myeloid leukemia survival in the United States from 1975–2009. Leuk Lymphoma. 2013 Jul;54(7):1411–7.
122. de la Fuente J, Baruchel A, Biondi A, de Bont E, Dresse MF, Suttorp M, et al. Managing children with chronic myeloid leukaemia (CML). Br J Haematol. 2014;167(1):33–47.
123. Rohrbacher M, Hasford J. Epidemiology of chronic myeloid leukaemia (CML). Best Pract Res Clin Haematol. 2009 Sep 1;22(3):295–302.
124. Berger U, Maywald O, Pfirrmann M, Lahaye T, Hochhaus A, Reiter A, et al. Gender aspects in chronic myeloid leukemia: long-term results from randomized studies. Leukemia. 2005 Jun;19(6):984–9.
125. Lin Q, Mao L, Shao L, Zhu L, Han Q, Zhu H, et al. Global, Regional, and National Burden of Chronic Myeloid Leukemia, 1990–2017: A Systematic Analysis for the Global Burden of Disease Study 2017. Front Oncol. 2020 Dec 15;10:580759.
126. Huang X, Cortes J, Kantarjian H. Estimations of the Increasing Prevalence and Plateau Prevalence of Chronic Myeloid Leukemia in the Era of Tyrosine Kinase Inhibitors Therapy. Cancer. 2012 Jun 15;118(12):3123–7.
127. Gunnarsson N, Sandin F, Höglund M, Stenke L, Björkholm M, Lambe M, et al. Population-based assessment of chronic myeloid leukemia in Sweden: striking increase in survival and prevalence. Eur J Haematol. 2016;97(4):387–92.
128. Arber DA, Orazi A, Hasserjian R, Thiele J, Borowitz MJ, Le Beau MM, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood. 2016 May 19;127(20):2391–405.
129. Khoury JD, Solary E, Abla O, Akkari Y, Alaggio R, Apperley JF, et al. The 5th edition of the World Health Organization Classification of Haematolymphoid Tumours: Myeloid and Histiocytic/Dendritic Neoplasms. Leukemia. 2022 Jul;36(7):1703–19.
130. Kantarjian H, Schiffer C, Jones D, Cortes J. Monitoring the response and course of chronic myeloid leukemia in the modern era of BCR-ABL tyrosine kinase inhibitors: practical advice on the use and interpretation of monitoring methods. Blood. 2008 Feb 15;111(4):1774–80.
131. Schoch C, Schnittger S, Bursch S, Gerstner D, Hochhaus A, Berger U, et al. Comparison of chromosome banding analysis, interphase- and hypermetaphase-FISH, qualitative and quantitative PCR for diagnosis and for follow-up in chronic myeloid leukemia: a study on 350 cases. Leukemia. 2002 Jan;16(1):53–9.
132. Jabbour E, Cortes JE, Kantarjian HM. Molecular Monitoring in Chronic Myeloid Leukemia. Cancer. 2008 May 15;112(10):2112–8.
133. Hughes T, Deininger M, Hochhaus A, Branford S, Radich J, Kaeda J, et al. Monitoring CML patients responding to treatment with tyrosine kinase inhibitors: review and recommendations for harmonizing current methodology for detecting BCR-ABL transcripts and kinase domain mutations and for expressing results. Blood. 2006 Jul 1;108(1):28–37.
134. Cortes J, Quintás-Cardama A, Kantarjian HM. Monitoring Molecular Response in Chronic Myeloid Leukemia. Cancer. 2011 Mar 15;117(6):1113–22.
135. Molica M, Noguera NI, Trawinska MM, Martinelli G, Cerchione C, Abruzzese E. Treatment free remission in chronic myeloid leukemia: Lights and shadows. Hematol Rep. 2020 Sep 21;12(Suppl 1):8950.

136. Senapati J, Sasaki K, Issa GC, Lipton JH, Radich JP, Jabbour E, et al. Management of chronic myeloid leukemia in 2023 – common ground and common sense. Blood Cancer J. 2023 Apr 24;13(1):1–12.
137. Hehlmann R, Heimpel H, Hasford J, Kolb HJ, Pralle H, Hossfeld DK, et al. Randomized Comparison of Interferon-α With Busulfan and Hydroxyurea in Chronic Myelogenous Leukemia. Blood. 1994 Dec 15;84(12):4064–77.
138. Wolfe HR, Rein LAM. The Evolving Landscape of Frontline Therapy in Chronic Phase Chronic Myeloid Leukemia (CML). Curr Hematol Malig Rep. 2021 Oct 1;16(5):448–54.
139. Allan NC. Therapeutic options in chronic myeloid leukaemia. Blood Rev. 1989 Mar 1;3(1):45–52.
140. Speck B, Bortin MortimerM, Champlin R, Goldman JohnM, Herzig RogerH, Mcglave PhilipB, et al. ALLOGENEIC BONE-MARROW TRANSPLANTATION FOR CHRONIC MYELOGENOUS LEUKAEMIA. The Lancet. 1984 Mar 24;323(8378):665–8.
141. Chronic Myeloid Leukemia Trialists' Collaborative Group. Interferon Alfa Versus Chemotherapy for Chronic Myeloid Leukemia: A Meta-analysis of Seven Randomized Trials. JNCI J Natl Cancer Inst. 1997 Nov 5;89(21):1616–20.
142. Lange T, Niederwieser C, Gil A, Krahl R, von Grünhagen U, Al-Ali HK, et al. No advantage of Imatinib in combination with hydroxyurea over Imatinib monotherapy: a study of the East German Study Group (OSHO) and the German CML study group. Leuk Lymphoma. 2020 Oct 14;61(12):2821–30.
143. Thiesing JT, Ohno-Jones S, Kolibaba KS, Druker BJ. Efficacy of STI571, an Abl tyrosine kinase inhibitor, in conjunction with other antileukemic agents against Bcr-Abl–positive cells. Blood. 2000 Nov 1;96(9):3195–9.
144. Kockerols CCB, Geelen I, Levin MD, Janssen JJWM, Dinmohamed AG, Hoogendoorn M, et al. The use of hydroxyurea pretreatment in chronic myeloid leukemia in the current tyrosine kinase inhibitor era. Haematologica. 2022 Mar 31;107(8):1940–3.
145. Soverini S, Mancini M, Bavaro L, Cavo M, Martinelli G. Chronic myeloid leukemia: the paradigm of targeting oncogenic tyrosine kinase signaling and counteracting resistance for successful cancer therapy. Mol Cancer. 2018 Feb 19;17:49.
146. Xie Y, Davies SM, Xiang Y, Robison LL, Ross JA. Trends in leukemia incidence and survival in the United States (1973–1998). Cancer. 2003;97(9):2229–35.
147. Druker BJ, Guilhot F, O'Brien SG, Gathmann I, Kantarjian H, Gattermann N, et al. Five-Year Follow-up of Patients Receiving Imatinib for Chronic Myeloid Leukemia. N Engl J Med. 2006 Dec 7;355(23):2408–17.
148. Cortes JE, Saglio G, Kantarjian HM, Baccarani M, Mayer J, Boqué C, et al. Final 5-Year Study Results of DASISION: The Dasatinib Versus Imatinib Study in Treatment-Naïve Chronic Myeloid Leukemia Patients Trial. J Clin Oncol. 2016 Jul 10;34(20):2333–40.
149. Hochhaus A, Saglio G, Hughes TP, Larson RA, Kim DW, Issaragrisil S, et al. Long-term benefits and risks of frontline nilotinib vs imatinib for chronic myeloid leukemia in chronic phase: 5-year update of the randomized ENESTnd trial. Leukemia. 2016 May;30(5):1044–54.
150. Hehlmann R. How I treat CML blast crisis. Blood. 2012 Jul 26;120(4):737–47.
151. Cortes J, Goldman JM, Hughes T. Current Issues in Chronic Myeloid Leukemia: Monitoring, Resistance, and Functional Cure. J Natl Compr Canc Netw. 2012 Oct 1;10(Suppl_3):S-13.
152. Hughes TP, Ross DM. Moving treatment-free remission into mainstream clinical practice in CML. Blood. 2016 Jul 7;128(1):17–23.
153. Saußele S, Richter J, Hochhaus A, Mahon FX. The concept of treatment-free remission in chronic myeloid leukemia. Leukemia. 2016 Aug;30(8):1638–47.
154. Mahon FX. Treatment-free remission in CML: who, how, and why? Hematol Am Soc Hematol Educ Program. 2017 Dec 8;2017(1):102–9.
155. Druker BJ, Tamura S, Buchdunger E, Ohno S, Segal GM, Fanning S, et al. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr–Abl positive cells. Nat Med. 1996 May;2(5):561–6.
156. O'Brien SG, Guilhot F, Larson RA, Gathmann I, Baccarani M, Cervantes F, et al. Imatinib Compared with Interferon and Low-Dose Cytarabine for Newly Diagnosed Chronic-Phase Chronic Myeloid Leukemia. N Engl J Med. 2003 Mar 13;348(11):994–1004.
157. Lombardo LJ, Lee FY, Chen P, Norris D, Barrish JC, Behnia K, et al. Discovery of N-(2-Chloro-6-methyl- phenyl)-2-(6-(4-(2-hydroxyethyl)- piperazin-1-yl)-2-methylpyrimidin-4- ylamino)thiazole-5-carboxamide (BMS-354825), a Dual Src/Abl Kinase Inhibitor with Potent Antitumor Activity in Preclinical Assays. J Med Chem. 2004 Dec 1;47(27):6658–61.

158. O'Hare T, Walters DK, Stoffregen EP, Jia T, Manley PW, Mestan J, et al. In vitro Activity of Bcr-Abl Inhibitors AMN107 and BMS-354825 against Clinically Relevant Imatinib-Resistant Abl Kinase Domain Mutants. Cancer Res. 2005 Jun 1;65(11):4500–5.
159. Tokarski JS, Newitt JA, Chang CYJ, Cheng JD, Wittekind M, Kiefer SE, et al. The Structure of Dasatinib (BMS-354825) Bound to Activated ABL Kinase Domain Elucidates Its Inhibitory Activity against Imatinib-Resistant ABL Mutants. Cancer Res. 2006 Jun 1;66(11):5790–7.
160. Shah NP, Tran C, Lee FY, Chen P, Norris D, Sawyers CL. Overriding Imatinib Resistance with a Novel ABL Kinase Inhibitor. Science. 2004 Jul 16;305(5682):399–401.
161. Atallah E. Update on Treatment-Free Remission in CML. Clin Lymphoma Myeloma Leuk. 2021 Sep 1;21:S156–7.
162. Atallah E, Sweet K. Treatment-Free Remission: the New Goal in CML Therapy. Curr Hematol Malig Rep. 2021 Oct 1;16(5):433–9.
163. Stagno F, Breccia M, Di Raimondo F. On the road to treatment-free remission in chronic myeloid leukemia: what about 'the others'? Expert Rev Anticancer Ther. 2020 Dec 1;20(12):1075–81.
164. Annunziata M, Bonifacio M, Breccia M, Castagnetti F, Gozzini A, Iurlo A, et al. Current Strategies and Future Directions to Achieve Deep Molecular Response and Treatment-Free Remission in Chronic Myeloid Leukemia. Front Oncol. 2020;10.
165. Jabbour E, Cortes J, Santos FPS, Jones D, O'Brien S, Rondon G, et al. Results of allogeneic hematopoietic stem cell transplantation for chronic myelogenous leukemia patients who failed tyrosine kinase inhibitors after developing BCR-ABL1 kinase domain mutations. Blood. 2011 Mar 31;117(13):3641–7.
166. Lee SJ, Kukreja M, Wang T, Giralt SA, Szer J, Arora M, et al. Impact of prior imatinib mesylate on the outcome of hematopoietic cell transplantation for chronic myeloid leukemia. Blood. 2008 Oct 15;112(8):3500–7.
167. Mellqvist UH, Hansson M, Brune M, Dahlgren C, Hermodsson S, Hellstrand K. Natural killer cell dysfunction and apoptosis induced by chronic myelogenous leukemia cells: role of reactive oxygen species and regulation by histamine. Blood. 2000 Sep 1;96(5):1961–8.
168. Hughes A, Clarson J, Tang C, Vidovic L, White DL, Hughes TP, et al. CML patients with deep molecular responses to TKI have restored immune effectors and decreased PD-1 and immune suppressors. Blood. 2017 Mar 2;129(9):1166–76.
169. Hsieh YC, Kirschner K, Copland M. Improving outcomes in chronic myeloid leukemia through harnessing the immunological landscape. Leukemia. 2021 May;35(5):1229–42.
170. Nakajima H, Zhao R, Lund TC, Ward J, Dolan M, Hirsch B, et al. The BCR/ABL Transgene Causes Abnormal NK Cell Differentiation and Can Be Found in Circulating NK Cells of Advanced Phase Chronic Myelogenous Leukemia Patients1. J Immunol. 2002 Jan 15;168(2):643–50.
171. Matsushita M. Novel Treatment Strategies Utilizing Immune Reactions against Chronic Myelogenous Leukemia Stem Cells. Cancers. 2021 Oct 29;13(21):5435.
172. Döhner H, Weisdorf DJ, Bloomfield CD. Acute Myeloid Leukemia. N Engl J Med. 2015 Sep 17;373(12):1136–52.
173. Bennett JM, Catovsky D, Daniel M, Flandrin G, Galton DAG, Gralnick HR, et al. Proposals for the Classification of the Acute Leukaemias F RENCH -A MERICAN -B RITISH (FAB) C O-OPERATIVE G ROUP. Br J Haematol. 1976 Aug;33(4):451–8.
174. Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A, et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood. 2009 Jul 30;114(5):937–51.
175. Genomic and Epigenomic Landscapes of Adult De Novo Acute Myeloid Leukemia. N Engl J Med. 2013 May 30;368(22):2059–74.
176. Döhner H, Wei AH, Appelbaum FR, Craddock C, DiNardo CD, Dombret H, et al. Diagnosis and management of AML in adults: 2022 recommendations from an international expert panel on behalf of the ELN. Blood. 2022 Sep 22;140(12):1345–77.
177. Dong Y, Shi O, Zeng Q, Lu X, Wang W, Li Y, et al. Leukemia incidence trends at the global, regional, and national level between 1990 and 2017. Exp Hematol Oncol. 2020 Jun 19;9(1):14.
178. Shallis RM, Wang R, Davidoff A, Ma X, Zeidan AM. Epidemiology of acute myeloid leukemia: Recent progress and enduring challenges. Blood Rev. 2019 Jul 1;36:70–87.
179. Juliusson G, Antunovic P, Derolf Å, Lehmann S, Möllgård L, Stockelberg D, et al. Age and acute myeloid leukemia: real world data on decision to treat and outcomes from the Swedish Acute Leukemia Registry. Blood. 2009 Apr 30;113(18):4179–87.

180. Juliusson G, Abrahamsson J, Lazarevic V, Antunovic P, Derolf Å, Garelius H, et al. Prevalence and characteristics of survivors from acute myeloid leukemia in Sweden. Leukemia. 2017 Mar;31(3):728–31.
181. Döhner H, Estey EH, Amadori S, Appelbaum FR, Büchner T, Burnett AK, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. Blood. 2010 Jan 21;115(3):453–74.
182. Dick JE. Acute Myeloid Leukemia Stem Cells. Ann N Y Acad Sci. 2005;1044(1):1–5.
183. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature. 1994 Feb;367(6464):645–8.
184. Thomas D, Majeti R. Biology and relevance of human acute myeloid leukemia stem cells. Blood. 2017 Mar 23;129(12):1577–85.
185. Clarke MF, Dick JE, Dirks PB, Eaves CJ, Jamieson CHM, Jones DL, et al. Cancer Stem Cells—Perspectives on Current Status and Future Directions: AACR Workshop on Cancer Stem Cells. Cancer Res. 2006 Oct 3;66(19):9339–44.
186. Boddu P, Kantarjian HM, Garcia-Manero G, Ravandi F, Verstovsek S, Jabbour E, et al. Treated secondary acute myeloid leukemia: a distinct high-risk subset of AML with adverse prognosis. Blood Adv. 2017 Jul 19;1(17):1312–23.
187. Sekeres MA, Elson P, Kalaycio ME, Advani AS, Copelan EA, Faderl S, et al. Time from diagnosis to treatment initiation predicts survival in younger, but not older, acute myeloid leukemia patients. Blood. 2009 Jan 1;113(1):28–36.
188. Lichtman MA. A historical perspective on the development of the cytarabine (7days) and daunorubicin (3days) treatment regimen for acute myelogenous leukemia: 2013 the 40th anniversary of 7+3. Blood Cells Mol Dis. 2013 Feb 1;50(2):119–30.
189. Löwenberg B, Ossenkoppele GJ, van Putten W, Schouten HC, Graux C, Ferrant A, et al. High-Dose Daunorubicin in Older Patients with Acute Myeloid Leukemia. N Engl J Med. 2009 Sep 24;361(13):1235–48.
190. Fernandez HF, Sun Z, Yao X, Litzow MR, Luger SM, Paietta EM, et al. Anthracycline Dose Intensification in Acute Myeloid Leukemia. N Engl J Med. 2009 Sep 24;361(13):1249–59.
191. Döhner H, Estey E, Grimwade D, Amadori S, Appelbaum FR, Büchner T, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. Blood. 2017 Jan 26;129(4):424–47.
192. Dombret H, Gardin C. An update of current treatments for adult acute myeloid leukemia. Blood. 2016 Jan 7;127(1):53–61.
193. Castaigne S, Pautas C, Terré C, Raffoux E, Bordessoule D, Bastie JN, et al. Effect of gemtuzumab ozogamicin on survival of adult patients with de-novo acute myeloid leukaemia (ALFA-0701): a randomised, open-label, phase 3 study. The Lancet. 2012 Apr 21;379(9825):1508–16.
194. Lancet JE, Uy GL, Cortes JE, Newell LF, Lin TL, Ritchie EK, et al. CPX-351 (cytarabine and daunorubicin) Liposome for Injection Versus Conventional Cytarabine Plus Daunorubicin in Older Patients With Newly Diagnosed Secondary Acute Myeloid Leukemia. J Clin Oncol. 2018 Sep 10;36(26):2684–92.
195. Stone RM, Mandrekar SJ, Sanford BL, Laumann K, Geyer S, Bloomfield CD, et al. Midostaurin plus Chemotherapy for Acute Myeloid Leukemia with a FLT3 Mutation. N Engl J Med. 2017 Aug 3;377(5):454–64.
196. Stubbins RJ, Francis A, Kuchenbauer F, Sanford D. Management of Acute Myeloid Leukemia: A Review for General Practitioners in Oncology. Curr Oncol. 2022 Aug 30;29(9):6245–59.
197. Kantarjian H, Kadia T, DiNardo C, Daver N, Borthakur G, Jabbour E, et al. Acute myeloid leukemia: current progress and future directions. Blood Cancer J. 2021 Feb 22;11(2):1–25.
198. Löwenberg B. Sense and nonsense of high-dose cytarabine for acute myeloid leukemia. Blood. 2013 Jan 3;121(1):26–8.
199. Schaich M, Röllig C, Soucek S, Kramer M, Thiede C, Mohr B, et al. Cytarabine Dose of 36 g/m2 Compared With 12 g/m2 Within First Consolidation in Acute Myeloid Leukemia: Results of Patients Enrolled Onto the Prospective Randomized AML96 Study. J Clin Oncol. 2011 Jul;29(19):2696–702.
200. Löwenberg B, Pabst T, Vellenga E, van Putten W, Schouten HC, Graux C, et al. Cytarabine Dose for Acute Myeloid Leukemia. N Engl J Med. 2011 Mar 17;364(11):1027–36.
201. Burnett AK, Russell NH, Hills RK, Hunter AE, Kjeldsen L, Yin J, et al. Optimization of Chemotherapy for Younger Patients With Acute Myeloid Leukemia: Results of the Medical Research Council AML15 Trial. J Clin Oncol. 2013 Sep 20;31(27):3360–8.

202. Cornelissen JJ, Blaise D. Hematopoietic stem cell transplantation for patients with AML in first complete remission. Blood. 2016 Jan 7;127(1):62–70.
203. Barrett J. Why is a 3-year NRM following allogeneic transplantation still stuck at approximately 20%? Best Pract Res Clin Haematol. 2018 Dec 1;31(4):414–9.
204. Gratwohl A, Pasquini MC, Aljurf M, Atsuta Y, Baldomero H, Foeken L, et al. One million haemopoietic stem-cell transplants: a retrospective observational study. Lancet Haematol. 2015 Mar 1;2(3):e91–100.
205. Passweg JR, Baldomero H, Bader P, Bonini C, Cesaro S, Dreger P, et al. Hematopoietic SCT in Europe 2013: recent trends in the use of alternative donors showing more haploidentical donors but fewer cord blood transplants. Bone Marrow Transplant. 2015 Apr;50(4):476–82.
206. Niederwieser D, Baldomero H, Szer J, Gratwohl M, Aljurf M, Atsuta Y, et al. Hematopoietic stem cell transplantation activity worldwide in 2012 and a SWOT analysis of the Worldwide Network for Blood and Marrow Transplantation Group including the global survey. Bone Marrow Transplant. 2016 Jun;51(6):778–85.
207. Juliusson G, Karlsson K, Lazarevic VL, Wahlin A, Brune M, Antunovic P, et al. Hematopoietic stem cell transplantation rates and long-term survival in acute myeloid and lymphoblastic leukemia. Cancer. 2011;117(18):4238–46.
208. Ravandi F, Cortes J, Faderl S, O'Brien S, Garcia-Manero G, Verstovsek S, et al. Characteristics and outcome of patients with acute myeloid leukemia refractory to 1 cycle of high-dose cytarabine-based induction chemotherapy. Blood. 2010 Dec 23;116(26):5818–23.
209. de Lima M, Roboz GJ, Platzbecker U, Craddock C, Ossenkoppele G. AML and the art of remission maintenance. Blood Rev. 2021 Sep 1;49:100829.
210. Ossenkoppele GJ, Janssen JJWM, van de Loosdrecht AA. Risk factors for relapse after allogeneic transplantation in acute myeloid leukemia. Haematologica. 2016 Jan;101(1):20–5.
211. Wei AH, Döhner H, Pocock C, Montesinos P, Afanasyev B, Dombret H, et al. Oral Azacitidine Maintenance Therapy for Acute Myeloid Leukemia in First Remission. N Engl J Med. 2020 Dec 24;383(26):2526–37.
212. Larson RA, Mandrekar SJ, Huebner LJ, Sanford BL, Laumann K, Geyer S, et al. Midostaurin reduces relapse in FLT3-mutant acute myeloid leukemia: the Alliance CALGB 10603/RATIFY trial. Leukemia. 2021 Sep;35(9):2539–51.
213. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. Nat Immunol. 2008 May;9(5):503–10.
214. Vivier E, Nunès JA, Vély F. Natural Killer Cell Signaling Pathways. Science. 2004 Nov 26;306(5701):1517–9.
215. Carlsten M, Järås M. Natural Killer Cells in Myeloid Malignancies: Immune Surveillance, NK Cell Dysfunction, and Pharmacological Opportunities to Bolster the Endogenous NK Cells. Front Immunol. 2019 Oct 11;10:2357.
216. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol. 2001 Nov 1;22(11):633–40.
217. Martner A, Thorén FB, Aurelius J, Hellstrand K. Immunotherapeutic strategies for relapse control in acute myeloid leukemia. Blood Rev. 2013 Sep 1;27(5):209–16.
218. Fauriat C, Just-Landi S, Mallet F, Arnoulet C, Sainty D, Olive D, et al. Deficient expression of NCR in NK cells from acute myeloid leukemia: evolution during leukemia treatment and impact of leukemia cells in NCRdull phenotype induction. Blood. 2006 Aug 29;109(1):323–30.
219. Henney CS, Kuribayashi K, Kern DE, Gillis S. Interleukin-2 augments natural killer cell activity. Nature. 1981 May;291(5813):335–8.
220. Liao W, Lin JX, Leonard WJ. IL-2 Family Cytokines: New Insights into the Complex Roles of IL-2 as a Broad Regulator of T helper Cell Differentiation. Curr Opin Immunol. 2011 Oct;23(5):598–604.
221. Janice P. Dutcher MD. Current Status of Interleukin-2 Therapy for Metastatic Renal Cell Carcinoma and Metastatic Melanoma. 2002 Nov 1 [cited 2024 Jan 23];16. Available from: https://www.cancernetwork.com/view/current-status-interleukin-2-therapy-metastatic-renal-cell-carcinoma-and-metastatic-melanoma
222. Rosenberg SA. Raising the Bar: The Curative Potential of Human Cancer Immunotherapy. Sci Transl Med. 2012 Mar 28;4(127):127ps8.
223. Kolitz JE, Hars V, DeAngelo DJ, Allen SL, Shea TC, Vij R, et al. Phase III Trial of Immunotherapy with Recombinant Interleukin-2 (rIL-2) Versus Observation in Patients < 60 Years with Acute Myeloid Leukemia (AML) in First Remission (CR1): Preliminary Results from Cancer and Leukemia Group B (CALGB) 19808. Blood. 2007 Nov 16;110(11):157.

224. Baer MR, George SL, Caligiuri MA, Sanford BL, Bothun SM, Mrózek K, et al. Low-Dose Interleukin-2 Immunotherapy Does Not Improve Outcome of Patients Age 60 Years and Older With Acute Myeloid Leukemia in First Complete Remission: Cancer and Leukemia Group B Study 9720. J Clin Oncol. 2008 Oct 20;26(30):4934–9.
225. Pautas C, Merabet F, Thomas X, Raffoux E, Gardin C, Corm S, et al. Randomized Study of Intensified Anthracycline Doses for Induction and Recombinant Interleukin-2 for Maintenance in Patients With Acute Myeloid Leukemia Age 50 to 70 Years: Results of the ALFA-9801 Study. J Clin Oncol. 2010 Feb 10;28(5):808–14.
226. Berry SM, Broglio KR, Berry DA. Addressing the Incremental Benefit of Histamine Dihydrochloride When Added to Interleukin-2 in Treating Acute Myeloid Leukemia: A Bayesian Meta-Analysis. Cancer Invest. 2011 Apr 7;29(4):293–9.
227. Buyse M, Squifflet P, Lange BJ, Alonzo TA, Larson RA, Kolitz JE, et al. Individual patient data meta-analysis of randomized trials evaluating IL-2 monotherapy as remission maintenance therapy in acute myeloid leukemia. Blood. 2011 Jun 30;117(26):7007–13.
228. Hansson M, Asea A, Ersson U, Hermodsson S, Hellstrand K. Induction of apoptosis in NK cells by monocyte-derived reactive oxygen metabolites. J Immunol. 1996 Jan 1;156(1):42–7.
229. Hellstrand K, Asea A, Dahlgren C, Hermodsson S. Histaminergic regulation of NK cells. Role of monocyte-derived reactive oxygen metabolites. J Immunol. 1994 Dec 1;153(11):4940–7.
230. Brune M, Hansson M, Mellqvist UH, Hermodsson S, Hellstrand K. NK cell-mediated killing of AML blasts: role of histamine, monocytes and reactive oxygen metabolites. Eur J Haematol. 1996;57(4):312–9.
231. Brune M, Hellstrand K. Remission maintenance therapy with histamine and interleukin-2 in acute myelogenous leukaemia. Br J Haematol. 1996;92(3):620–6.
232. Brune M, Castaigne S, Catalano J, Gehlsen K, Ho AD, Hofmann WK, et al. Improved leukemia-free survival after postconsolidation immunotherapy with histamine dihydrochloride and interleukin-2 in acute myeloid leukemia: results of a randomized phase 3 trial. Blood. 2006 Jul 1;108(1):88–96.
233. Buyse M, Michiels S, Squifflet P, Lucchesi KJ, Hellstrand K, Brune ML, et al. Leukemia-free survival as a surrogate end point for overall survival in the evaluation of maintenance therapy for patients with acute myeloid leukemia in complete remission. Haematologica. 2011 Aug;96(8):1106–12.
234. Martner A, Rydström A, Riise RE, Aurelius J, Anderson H, Brune M, et al. Role of natural killer cell subsets and natural cytotoxicity receptors for the outcome of immunotherapy in acute myeloid leukemia. Oncoimmunology. 2015 May 5;5(1):e1041701.
235. 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):1473–84.
236. Boudreau JE, Le Luduec JB, Hsu KC. Development of a Novel Multiplex PCR Assay to Detect Functional Subtypes of KIR3DL1 Alleles. PLoS ONE. 2014 Jun 11;9(6):e99543.
237. Rieger MA, Schroeder T. Analyzing cell fate control by cytokines through continuous single cell biochemistry. J Cell Biochem. 2009;108(2):343–52.
238. Pellin D, Loperfido M, Baricordi C, Wolock SL, Montepeloso A, Weinberg OK, et al. A comprehensive single cell transcriptional landscape of human hematopoietic progenitors. Nat Commun. 2019 Jun 3;10(1):2395.
239. Buenrostro JD, Corces MR, Lareau CA, Wu B, Schep AN, Aryee MJ, et al. Integrated Single-Cell Analysis Maps the Continuous Regulatory Landscape of Human Hematopoietic Differentiation. Cell. 2018 May;173(6):1535-1548.e16.
240. Ranzoni AM, Tangherloni A, Berest I, Riva SG, Myers B, Strzelecka PM, et al. Integrative Single-Cell RNA-Seq and ATAC-Seq Analysis of Human Developmental Hematopoiesis. Cell Stem Cell. 2021 Mar 4;28(3):472-487.e7.
241. Anjos-Afonso F, Buettner F, Mian SA, Rhys H, Perez-Lloret J, Garcia-Albornoz M, et al. Single cell analyses identify a highly regenerative and homogenous human CD34+ hematopoietic stem cell population. Nat Commun. 2022 Apr 19;13(1):2048.
242. Weng C, Yu F, Yang D, Poeschla M, Liggett LA, Jones MG, et al. Deciphering cell states and genealogies of human hematopoiesis. Nature. 2024 Jan 22;1–3.
243. Huang D, Han Y, Tang T, Yang L, Jiang P, Qian W, et al. Dlk1 maintains adult mice long-term HSCs by activating Notch signaling to restrict mitochondrial metabolism. Exp Hematol Oncol. 2023 Jan 18;12(1):11.
244. Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. Nat Immunol. 2001 Mar;2(3):261–8.

245. Yamashita M, Passegué E. TNFα coordinates hematopoietic stem cell survival and myeloid regeneration. Cell Stem Cell. 2019 Sep 5;25(3):357-372.e7.
246. Zheng J, Umikawa M, Zhang S, Huynh H, Silvany R, Chen BPC, et al. Ex vivo expanded hematopoietic stem cells overcome the MHC barrier in allogeneic transplantation. Cell Stem Cell. 2011 Aug 5;9(2):119–30.
247. Nam AS, Kim KT, Chaligne R, Izzo F, Ang C, Taylor J, et al. Genotyping of Transcriptomes links somatic mutations and cell identity. Nature. 2019 Jul;571(7765):355–60.
248. Irani YD, Kok CH, Clarson J, Shanmuganathan N, Branford S, Yeung DT, et al. Association of TIM-3 checkpoint receptor expression on T cells with treatment-free remission in chronic myeloid leukemia. Blood Adv. 2023 Jun 6;7(11):2364.
249. Denis C, Methia N, Frenette PS, Rayburn H, Ullman-Culleré M, Hynes RO, et al. A mouse model of severe von Willebrand disease: Defects in hemostasis and thrombosis. Proc Natl Acad Sci U S A. 1998 Aug 4;95(16):9524–9.
250. Cheng H, Zheng Z, Cheng T. New paradigms on hematopoietic stem cell differentiation. Protein Cell. 2020 Jan 1;11(1):34–44.
251. Sanjuan-Pla A, Macaulay IC, Jensen CT, Woll PS, Luis TC, Mead A, et al. Platelet-biased stem cells reside at the apex of the haematopoietic stem-cell hierarchy. Nature. 2013 Oct;502(7470):232–6.
252. Hochhaus A, Baccarani M, Silver RT, Schiffer C, Apperley JF, Cervantes F, et al. European LeukemiaNet 2020 recommendations for treating chronic myeloid leukemia. Leukemia. 2020;34(4):966–84.
253. Nilsson MS, Komic H, Gustafsson J, Sheybani Z, Paul S, Rolfson O, et al. Multiomic single-cell analysis identifies von Willebrand factor and TIM3-expressing *BCR-ABL1* $^{+}$ CML stem cells [Internet]. Cancer Biology; 2023 Sep [cited 2024 Jan 31]. Available from: http://biorxiv.org/lookup/doi/10.1101/2023.09.14.557507
254. Rinaldi I, Reksodiputro AH, Jusman SW, Harahap A, Setiabudy R, Wanandi SI, et al. Longer Hydroxyurea Administration Prior to Imatinib Mesylate is Risk Factor for Unsuccessful Major Molecular Response in Chronic-Phase Chronic Myeloid Leukemia: Possibility of P-Glycoprotein Role. Asian Pac J Cancer Prev APJCP. 2019;20(12):3689–95.
255. Sidney J, Peters B, Frahm N, Brander C, Sette A. HLA class I supertypes: a revised and updated classification. BMC Immunol. 2008 Jan 22;9(1):1.
256. Chowell D, Morris LGT, Grigg CM, Weber JK, Samstein RM, Makarov V, et al. Patient HLA class I genotype influences cancer response to checkpoint blockade immunotherapy. Science. 2018 Feb 2;359(6375):582–7.
257. Cummings AL, Gukasyan J, Lu HY, Grogan T, Sunga G, Fares CM, et al. Mutational landscape influences immunotherapy outcomes among patients with non-small-cell lung cancer with human leukocyte antigen supertype B44. Nat Cancer. 2020 Dec;1(12):1167–75.
258. Alcoceba M, Sebastián E, Marín L, Balanzategui A, Sarasquete ME, Chillón MC, et al. HLA specificities are related to development and prognosis of diffuse large B-cell lymphoma. Blood. 2013 Aug 22;122(8):1448–54.
259. Maat W, Haasnoot GW, Claas FHJ, Schalij-Delfos NE, Schreuder GMT, Jager MJ. HLA Class I and II Genotype in Uveal Melanoma: Relation to Occurrence and Prognosis. Invest Ophthalmol Vis Sci. 2006 Jan 1;47(1):3–6.
260. Gumperz JE, Litwin V, Phillips JH, Lanier LL, Parham P. The Bw4 public epitope of HLA-B molecules confers reactivity with natural killer cell clones that express NKB1, a putative HLA receptor. J Exp Med. 1995 Mar 1;181(3):1133–44.
261. Brodin P, Lakshmikanth T, Johansson S, Kärre K, Höglund P. The strength of inhibitory input during education quantitatively tunes the functional responsiveness of individual natural killer cells. Blood. 2009 Mar 12;113(11):2434–41.
262. Ilander MM, Olsson-Strömberg U, Lähteenmäki H, Kasanen T, Koskenvesa P, Söderlund S, et al. Disease Relapse After TKI Discontinuation In CML Is Related Both To Low Number and Impaired Function Of NK-Cells:Data From Euro-SKI. Blood. 2013 Nov 15;122(21):379.