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# Cell Surface Markers as Therapeutic Targets in Myeloid Leukemia

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FACULTY OF MEDICINE | LUND UNIVERSITY



# Cell Surface Markers as Therapeutic Targets in Myeloid Leukemia

Niklas Landberg



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DOCTORAL DISSERTATION

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Abstract <p>Chronic myeloid leukemia (CML) and acute myeloid leukemia (AML) are two distinct hematological entities of myeloid cellular origin. CML is well-studied with a homogenous genetic background and effective treatment, whereas AML is a heterogenous disease lacking effective targeted therapies and with a poor prognosis. The relapse causing leukemic stem cells are not fully characterized and rarely eliminated by treatment in CML or AML, necessitating most CML patients to receive life long daily treatment and leaving AML patients with a significant risk of relapse. In this thesis, the overall aims have been to 1) study previously described cell surface markers as surrogate markers for leukemic stem cell burden in CML and their applicability for predicting therapeutic response, 2) identify new cell surface markers on primitive CML cells and evaluate their possible roles as targets for antibody-based therapies, 3) perform arrayed cytokine screens using primary CML cells to identify positive regulators of primitive CML cells and their corresponding receptors, and 4) discover new cell surface markers on primitive AML cells in <i>TP53</i> mutated AML for antibody-based therapeutic targeting.</p> <p>In <b>Article I</b> it is shown that IL1RAP can be used as a marker for leukemic stem cell burden at diagnosis of CML. The leukemia stem cell burden was subsequently used to predict response to tyrosine kinase inhibitor treatment for CML patients at diagnosis.</p> <p>In <b>Article II</b>, the immature CML cell phenotype was examined more broadly by RNA sequencing of FACS sorted CD34<sup>+</sup>CD38<sup>low</sup> cells from CML patients at diagnosis. Compared to corresponding immature cells from healthy donors, a specific upregulation of CD36 and LEPR was identified. The CD34<sup>+</sup>CD38<sup>low</sup>IL1RAP<sup>+</sup>CD36<sup>+</sup> CML cells were shown to be less sensitive to imatinib treatment, but could be killed by antibody-dependant cellular cytotoxicity using an anti-CD36 antibody.</p> <p>In <b>Article III</b>, immature CD34<sup>+</sup>CD38<sup>low</sup> CML cells were subjected to a cytokine screen with 313 arrayed cytokines. Myostatin propeptide (MSTNpp) was shown to expand immature CML cells and increase survival of immature healthy hematopoietic cells in suspension cultures and colony forming assays. This is suggested to be dependant on MSTNpp binding to the cell surface and inducing phosphorylation of STAT5 and SMAD2/3. MSTNpp was found to be produced by mesenchymal stromal cells.</p> <p>In <b>Article IV</b>, immature CD34<sup>+</sup>CD38<sup>low</sup> AML cells from <i>TP53</i> mutated AML samples were subjected to an antibody screen containing 362 antibodies. Compared to healthy controls, SLAMF6 was shown to be specifically upregulated and could be targeted for antibody-dependant cellular cytotoxicity using an anti-SLAMF6 antibody, suggesting clinical implication for a subgroup of AML with very poor prognosis.</p> <p>In summary, this thesis shows that IL1RAP<sup>+</sup>CD36<sup>+</sup> CML cells are treatment resistant and targetable cells in CML and that MSTNpp plays a role in maintaining immature CML cells as well as healthy hematopoietic cells. SLAMF6 is shown to be a marker of immature AML cells in <i>TP53</i> mutated AML that can be specifically targeted using an anti-SLAMF6 antibody. Collectively, the results from this thesis should hopefully facilitate the development of targeted therapies in myeloid leukemia.</p>		
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# Cell Surface Markers as Therapeutic Targets in Myeloid Leukemia

Niklas Landberg



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*There is little success where there is little laughter*  
Andrew Carnegie

# Table of Contents

<b>Original articles .....</b>	<b>8</b>
<b>Abbreviations.....</b>	<b>10</b>
<b>Hematopoiesis .....</b>	<b>12</b>
Blood lineages .....	12
Regulation of Hematopoiesis .....	14
<b>Myeloid leukemia.....</b>	<b>16</b>
Studying leukemia.....	16
Chronic myeloid leukemia .....	19
Clinical aspects.....	19
Biological aspects.....	21
Acute myeloid leukemia.....	22
Clinical aspects.....	22
Biological aspects.....	24
The concept of leukemic stem cells .....	27
<b>Leukemic cell surface makers .....</b>	<b>29</b>
Cell surface markers in CML .....	29
IL1RAP .....	30
CD25 .....	31
CD26 .....	32
CD36 .....	33
Other cell surface markers.....	34
Cell surface markers in AML.....	37
CD123 .....	39
CD47 .....	40
TIM-3 .....	41
IL1RAP .....	41
CLL-1 .....	42
SLAMF6.....	43
CD33 .....	44
Other cell surface markers.....	45
Therapeutic targeting of cell surface markers .....	49

Naked antibodies .....	49
Antibody drug conjugates.....	50
Bispecific antibodies .....	50
CAR T cells .....	51
<b>Present investigation .....</b>	<b>52</b>
Aims .....	52
Results in CML .....	53
Article I.....	53
Article II .....	53
Article III.....	54
Results in AML .....	54
Article IV.....	54
Discussion .....	55
Conclusions .....	57
<b>Concluding remarks .....</b>	<b>58</b>
<b>Populärvetenskaplig sammanfattning .....</b>	<b>60</b>
<b>Acknowledgements .....</b>	<b>62</b>
<b>References .....</b>	<b>64</b>

# Original articles

This thesis is based on the following articles:

## Article I

Landberg N, Hansen N, Askmyr M, Ågerstam H, Lassen C, Rissler M, Hjorth-Hansen H, Mustjoki S, Järås M, Richter J, Fioretos T. **IL1RAP expression as a measure of leukemic stem cell burden at diagnosis of chronic myeloid leukemia predicts therapy outcome.** *Leukemia*. 2016;30(1):253–257

## Article II

Landberg N, von Palffy S, Askmyr M, Lilljebjörn H, Sandén C, Rissler M, Mustjoki S, Hjorth-Hansen H, Richter J, Ågerstam H, Järås M, Fioretos T. **CD36 defines primitive chronic myeloid leukemia cells less responsive to imatinib but vulnerable to antibody based therapeutic targeting.** *Haematologica*. 2018;103(3):447-455

## Article III

von Palffy S, Landberg N, Sandén C, Zacharaki D, Shah M, Nakamichi N, Hansen N, Askmyr M, Lilljebjörn H, Rissler M, Karlsson C, Scheduling S, Richter J, Eaves C, Bhatia R, Järås M, Fioretos T. **A high-content cytokine screen identifies myostatin propeptide as a positive regulator of primitive chronic myeloid leukaemia cells.** *Haematologica* [Epub ahead of print]

## Article IV

Landberg N, von Palffy S, Sandén C, Puente-Moncada N, Lilljebjörn H, Orsmark Pietras C, Rissler M, Ågerstam H, Fioretos T. **The cell surface marker SLAMF6 is upregulated on immature TP53 mutated AML cells and can be targeted with anti-SLAMF6 antibodies.** *Manuscript*

Articles not included in this thesis:

Sandén C, Lilljebjörn H, Orsmark Pietras C, Henningsson R, Saba KH, Landberg N, Thorsson H, von Palffy S, Pena-Martinez P, Högberg C, Rissler M, Gisselsson D, Lazarevic V, Juliusson G, Ågerstam H, Fioretos T. **Clonal competition within complex evolutionary hierarchies shapes AML over time.** *Nature Communications* [Accepted for publication]

Landberg N, Dreimane A, Rissler M, Billström R, Ågerstam H. **Primary cells in BCR/FGFR1-positive 8p11 myeloproliferative syndrome are sensitive to dovitinib, ponatinib, and dasatinib.** *Eur J Haematol.* 2017;99(5):442–448

Askmyr M, von Palffy S, Hansen N, Landberg N, Högberg C, Rissler M, Ågerstam H, Fioretos T. **Transgenic expression of human cytokines in immunodeficient mice does not facilitate myeloid expansion of BCR-ABL1 transduced human cord blood cells.** *PLoS One.* 2017;12(10):e0186035

Ågerstam H, Hansen N, von Palffy S, Sandén C, Reckzeh K, Karlsson C, Lilljebjörn H, Landberg N, Askmyr M, Högberg C, Rissler M, Porkka K, Wadenvik H, Mustjoki S, Richter J, Järås M, Fioretos T. **IL1RAP antibodies block IL-1–induced expansion of candidate CML stem cells and mediate cell killing in xenograft models.** *Blood.* 2016;128(23):2683-2693

Askmyr M, Ågerstam H, Lilljebjörn H, Hansen N, Karlsson C, von Palffy S, Landberg N, Högberg C, Lassen C, Rissler M, Richter J, Ehinger M, Järås M, Fioretos T. **Modeling chronic myeloid leukemia in immunodeficient mice reveals expansion of aberrant mast cells and accumulation of pre-B cells.** *Blood Cancer J.* 2014;4(12): e269

Hansen N, Ågerstam H, Wahlestedt M, Landberg N, Askmyr M, Ehinger M, Rissler M, Lilljebjörn H, Johnels P, Ishiko J, Melo JV, Alexander WS, Bryder D, Järås M, Fioretos T. **SOCS2 is dispensable for BCR/ABL1-induced chronic myeloid leukemia-like disease and for normal hematopoietic stem cell function.** *Leukemia.* 2013;27(1):130–135

# Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
AML	Acute myeloid leukemia
APL	Acute promyelocytic leukemia
ALL	Acute lymphoblastic leukemia
BPDCN	Blastic plasmacytoid dendritic cell neoplasm
CAR T	Chimeric antigen receptor T
CD	Cluster of differentiation
CDC	Complement-dependent cytotoxicity
CHIP	Clonal hematopoiesis of indeterminate potential
CLL	Chronic lymphocytic leukemia
CLP	Common lymphoid progenitor
CML	Chronic myeloid leukemia
CMP	Common myeloid progenitor
EMP	Erythro-myeloid progenitor
EPO	Erythropoietin
FACS	Fluorescence activated cell sorting
GMP	Granulocyte-macrophage progenitor
HSC	Hematopoietic stem cell
IL	Interleukin
IL1RAP	Interleukin 1 receptor accessory protein
ITD	Internal tandem duplication
LMPP	Lymphoid-primed multipotent progenitor
LSC	Leukemic stem cell

MDS	Myelodysplastic syndrome
MEP	Megakaryocyte-erythroid progenitor
MPP	Multipotent progenitor
MRD	Measurable residual disease
MSTNpp	Myostatin propeptide
NK	Natural killer
NOD/SCID	Non-obese diabetic/severe combined immune-deficient
NGS	Next generation sequencing
NSG	NOD/SCID gamma or NOD.Cg-Prkdc <sup>scid</sup> -IL2Rgc <sup>-/-</sup>
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RTqPCR	Reverse transcriptase quantitative polymerase chain reaction
SLAM	Signaling lymphocytic activation molecule
TCGA	The cancer genome atlas
TKD	Tyrosine kinase domain
TKI	Tyrosine kinase inhibitor
TPO	Thrombopoietin
WHO	World health organization

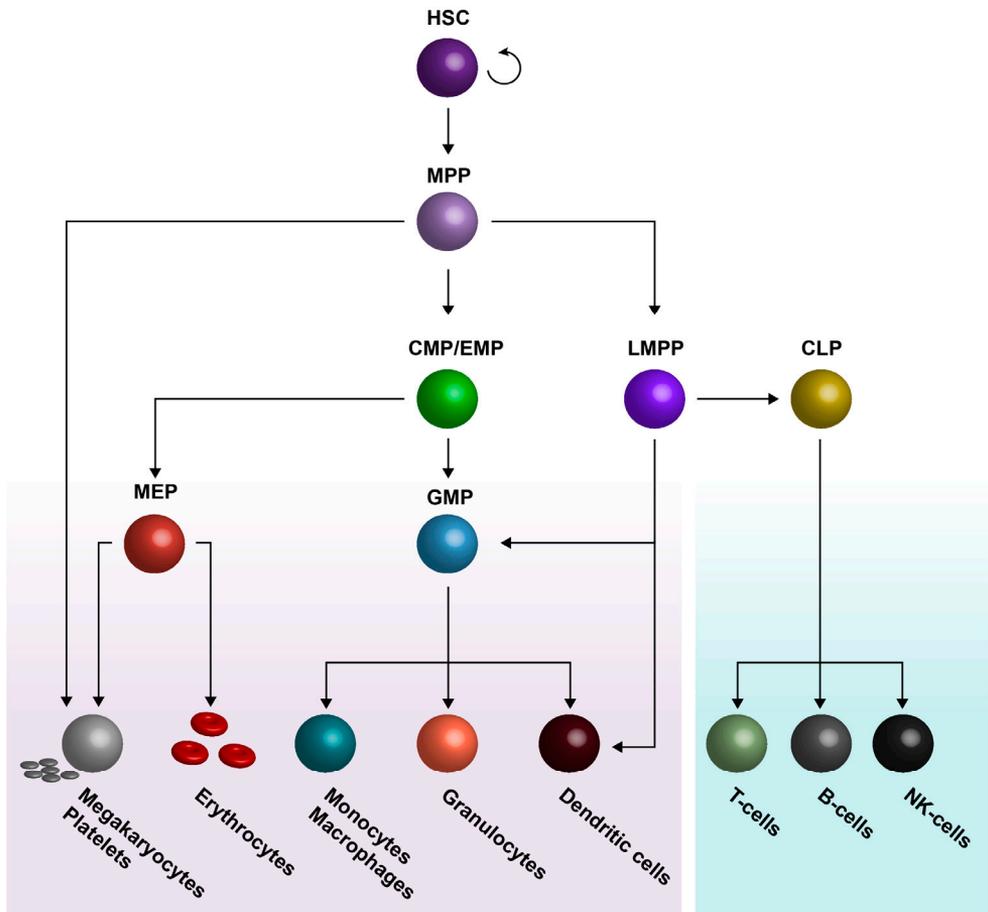
# Hematopoiesis

Hematopoiesis, the process of generating the cellular components of the blood, is built upon a hierarchical structure with the hematopoietic stem cell (HSC) at the very top [1]. The schematic illustration of the hierarchy as a branching tree with more committed progenitors further down the differentiation tracks is still used but a growing body of evidence suggests that this might be an over simplification of a not yet fully understood process [2]. Even though the exact relationship between lineage commitment and self-renewal potential is not fully understood, it is clear that the hematopoietic system needs to be tightly regulated to allow for a daily production of trillions of cells with minimal error [3]. It also allows for expanded and skewed production in order to meet demands during stress such as blood loss or infection [4]. If this balance is disturbed, the owner of the aberrant hematopoiesis might end up at the hematology clinic.

## Blood lineages

The major functions of the hematopoietic system are transportation of oxygen, carbon dioxide and nutrients by erythrocytes, hemostasis by thrombocytes and defense against infections by leukocytes. All cells originate from the HSCs, which are defined by their capacity to self-renew and retain multipotency after cell division [5]. Cells that have multipotency to produce all hematopoietic cell types but, as opposed to HSCs, lack self-renewability as defined by only transient engraftment in irradiated immunodeficient mice, are termed multipotent progenitors (MPP) [6]. The cells downstream of the HSCs and MPPs are divided into two main subgroups, the myeloid and the lymphoid system [7].

The mature cells of the myeloid system arise through different maturation pathways and originate from different progenitors (**Figure 1**). Erythrocytes and thrombocyte-producing megakaryocytes arise from the megakaryocyte-erythroid progenitor (MEP), megakaryocytes can also arise from MPPs [8]. MEPs in turn, are the progenies of a common myeloid progenitor (CMP). CMPs can also give rise to granulocyte-macrophage progenitors (GMP) which in turn, as the name suggests,



**Figure 1. The hematopoietic tree**

The hematopoietic tree shows the hierarchical arrangement from a self-renewing, multipotent hematopoietic stem cell (HSC) at the apex with subsequently more restricted progenitors downstream and finally the mature myeloid and lymphoid blood cells.

give rise to granulocytes, monocytes, macrophages and dendritic cells. CMPs were recently proposed to be more lineage restricted than previously believed and a cellular state with capacity to form erythroid cells and some granulocytes but not neutrophils termed erythro-myeloid progenitor (EMP) have instead been suggested [9]. The exact differentiation pathways and the oligopotency of these intermediate stages are still a matter of debate and recent evidence suggests that the progenitor groups might consist of many different unipotent progenitors that result in multipotency for the group as a whole [8,10].

The lymphoid system arises downstream of lymphoid-primed multipotent progenitors (LMPP) that, passing a cellular state denominated common lymphoid

progenitors (CLP), give rise to T-lymphocytes, B-lymphocytes, antibody producing plasma cells and natural killer cells (NK-cells) [11]. LMPPs can also produce dendritic cells [12]. The lymphoid system, excluding NK-cells, make up the adaptive immune system, whereas the leukocytes of the myeloid branch constitute the innate immune system.

## Regulation of Hematopoiesis

HSCs primarily reside in the bone marrow. As a consequence of disease, they can be displaced from their native environment with resulting extramedullary hematopoiesis, primarily in the spleen but also in the liver, lymph nodes and paravertebral regions [13]. Even in a normal physiological state, HSCs can be found circulating in the peripheral blood, a process with a circadian rhythm controlled by the sympathetic nervous system via CXCL12 release [14]. They can further be pharmacologically made to circulate to a higher degree using granulocyte colony-stimulating factor (G-CSF) or a CXCR4 antagonist, both used in a clinical setting for harvesting cells for hematopoietic stem cell transplantation [15,16].

### *HSC niche*

Within the bone marrow, the localization of HSCs has been an area of discussion and intense research. It is now believed that a majority of HSCs in adults reside within a specific microenvironment termed the perivascular niche [17-19]. The cells that make up this niche are important factors in the extrinsic control and maintenance of HSCs and the subsequent regulation of hematopoiesis. Suggested components of the niche are osteoblasts and osteoclasts [20,21], endothelial cells from sinusoids and arterioles [22], megakaryocytes [23], CXCL12-abundant reticular cells [24], non-myelinating Schwann cells [25], Nestin<sup>+</sup> mesenchymal stromal cells (MSCs) [26], as well as cellularly produced cytokines, adhesion molecules and extracellular matrix [27,28]. Well-studied cytokines with a regulatory role are CXCL12 that binds to CXCR4 (also known as CD184), stem cell factor (SCF) binding to KIT (also known as CD117), and thrombopoietin (TPO) binding to MPL (also known as CD110 or TPO-R) [29]. The interplay is complex and not yet fully understood, but remains an interesting aspect that could have direct clinical implications when closer delineated.

### *HSC phenotype*

HSCs are exceedingly rare with a vast majority of cells in the bone marrow being of more mature cellular states. The study of hematopoiesis and hematopoietic stem cells often rely on mouse models and the mouse HSC was studied in detailed before the human counterpart was. However, the phenotype of HSCs differs between

mouse and human, for example human HSCs are enriched in CD34<sup>+</sup> cells, which is not the case in mouse [30]. Today, the phenotype of human HSCs has been specified to be Lin<sup>-</sup>CD34<sup>+</sup>CD38<sup>low</sup>CD45RA<sup>-</sup>Thy1<sup>+</sup>Rho<sup>low</sup>CD49f<sup>+</sup> based on single cell transplantation assays in immunodeficient NOD.Cg-Prkdc<sup>scid</sup>-IL2Rgc<sup>-/-</sup> (NSG) mice [5]. In this setting, Lin<sup>-</sup> indicates that the cell population had been depleted by magnetic bead separation using antibodies targeting CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b, CD235a. The Rho<sup>low</sup> designation demarks cells with high efflux of a mitochondrial dye. Others have shown that HSCs defined as Lin<sup>-</sup>CD34<sup>+</sup>CD38<sup>low</sup> might express more mature myeloid markers such as CD13, CD33 and CD123, results with relevance in the development of targeted therapies where on-target off-tumor toxicity is of central importance [31].

### *Lineage commitment*

Differentiation from stem cell to mature cells requires a thorough change of gene expression and protein production. Two well-described transcription factors closely involved in this regulation are *GATA1* and *SP11* (also known as *PU.1*). When *GATA1* is preferentially expressed, an enforcement of differentiation towards megakaryocytic and erythroid cells ensues, whereas the *PU.1* transcription factor enforces a more granulocytic-monocytic differentiation. The previous conception that the actual lineage decision is dependent on the *GATA1* and *PU.1* balance has been revised and these transcription factors rather seem to enhance a lineage choice previously made [32].

Gene expression and cell differentiation are not only controlled by transcription factors. Epigenetic regulation is emerging as another important level of control and adjusting chromatin accessibility via DNA methylation and histone acetylation at promoters and enhancers affect when and in which cells genes are expressed [33]. An example of this is DNA Methyltransferase 3 Alpha (*DNMT3A*) that is crucial for differentiation and abrogation in a mouse model results in increased levels of HSCs at the expense of their differentiation [34].

Circulating or locally produced factors can also impact production and levels of mature cells. Erythropoietin (EPO) leads to proliferation of erythroid progenitors, TPO stimulates megakaryocyte progenitors, and G-CSF stimulate neutrophil production. These factors or agonists are currently used in clinical context to stimulate specific cell populations in patients [35,36].

# Myeloid leukemia

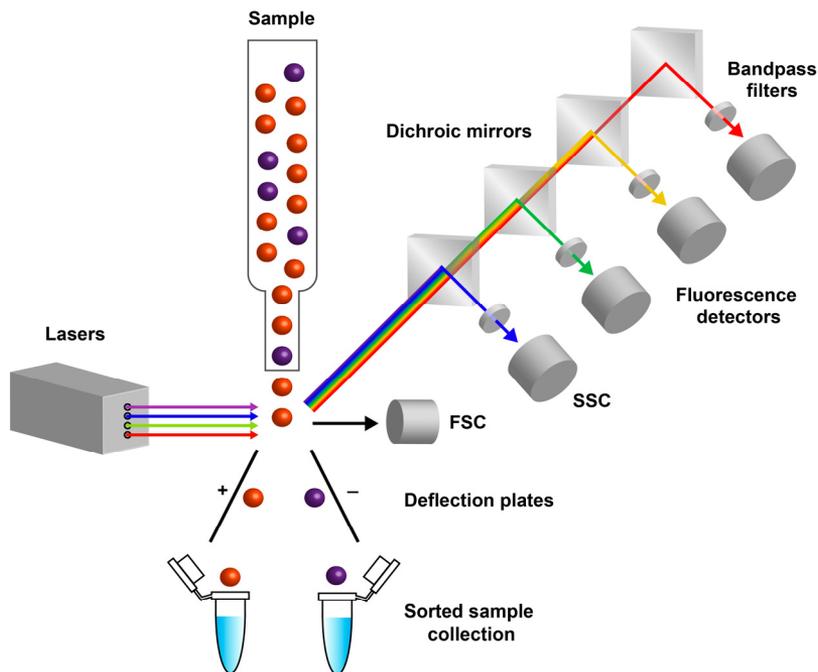
The high cell turnover needed for homeostasis requires the hematopoietic system to constantly produce vast amounts of mature cells of different lineages [35]. This process in turn has to be tightly regulated to avoid inappropriate expansion of inefficient or redundant cell types, making the hematopoietic system susceptible to somatic mutations and subsequent malignant transformation [37,38]. The two major types of myeloid leukemia, with different transforming events and most likely different cells of origin, are acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) [39]. Until recently, the outcome of both CML and AML were dismal [40]. However, the genetic dissection of the disease-causing genetic alteration defining CML cells made the development of targeted tyrosine kinase inhibitors (TKIs) possible. This has revolutionized the treatment of CML where life expectancies now are close to normal [41,42]. The treatment of AML, a disease entity comprised of a number of subtypes with different complex pathobiology, has not changed dramatically during the last six decades, although a number of targeted drugs have recently become available [43]. In order to better understand AML and to develop more efficient, less toxic treatment options, CML can be used as both a model system of leukemic disease and as a guiding example of successful targeted therapies.

## Studying leukemia

Historically, the diagnosis and classification of leukemia was based on morphology alone. In 1976, seven hematologists coined the French-American-British (FAB) classification to differentiate acute leukemia into lymphoid and myeloid acute leukemia with AML subdivided into M1-M6 based on the differentiation stage at which the bulk of leukemic cells were arrested [44]. Later, M0 was added for AML with an undifferentiated phenotype as well as M7 for acute megakaryoblastic leukemia. Today, the World Health Organization (WHO) describes how AML can be classified based on a combination of clinical characteristics, morphology, immunophenotype and genetics [39]. The tools needed for diagnosis and follow up of AML largely overlap with the techniques used in the research presented in this thesis. Below follows a short description of some of the main methods used in the

articles on which this thesis is based. For a more detailed description of the methods used, see **Articles I-IV**.

Flow cytometry uses a combination of lasers of specific wave lengths, antibodies specifically binding to predetermined epitopes, conjugated to excitable fluorochromes, detectors and filters to separate many fluorescent signals simultaneously (**Figure 2**). This is combined with a fluidic system to precisely align cells in a row for subsequent single cell analysis. This technique facilitates the immunophenotypic description of individual cells and multiple cell surface markers, generally with a distinct cluster of differentiation (CD) number, that can be assessed in parallel on all cells in a sample. With all single cells being suspended in individual droplets with a predetermined charge, cell sorting based on the immunophenotypic data from the flow cytometry analyses is possible with the help of deflection plates with adjustable charge and separate collection tubes downstream of the analyses. This is the main approach to assess cell surface marker expression and sort viable cells accordingly with fluorescence-activated cell sorting (FACS).



**Figure 2. Fluorescence activated cell sorting**

Fluorescence activated cell sorting (FACS) is based on spatially separated exciting lasers of defined wavelengths that excite different fluorochromes conjugated to antibodies that are in turn bound to cells based on their immunophenotype. The light emitted from these fluorochromes pass through dichroic mirrors and specific bandpass filters and is detected by photodiodes or photomultiplier tubes (PMT) and converted to electrical signals visualized in the flow cytometry software. The fluidics of the FACS separates the input sample into single cells encapsulated in droplets with a specific charge, allowing for sorting through adjusting the voltage on the deflection plates. The cells light scattering properties can also be measured and forward scatter (FSC) informs of the cell size and side scatter (SSC) informs of the cells internal complexity, often coinciding with its granularity.

Fluorescence in situ hybridization (FISH) is a relatively old method that can be used to determine large chromosomal aberrations in single cells and is based on DNA binding probes linked to fluorescent molecules, visualized by fluorescence microscopy. FISH is a rapid assay currently used both in research and clinical diagnostics to detect structural chromosomal aberrations. One example of use is the assessment of *BCR/ABL1* rearrangement at the single cell level.

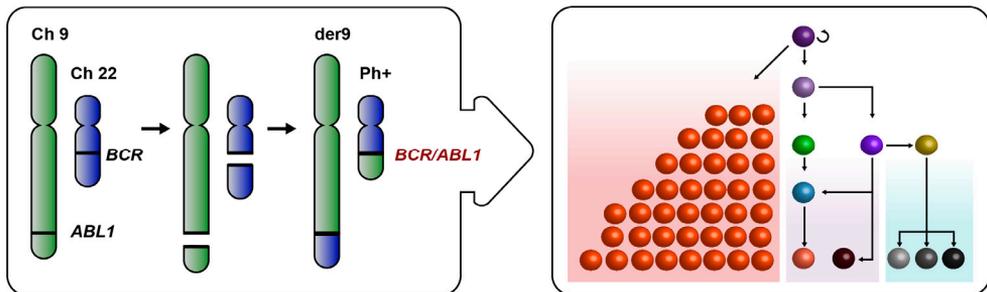
Breakthroughs in massive parallel sequencing have made RNA- and DNA-sequencing readily available, often referred to as next generation sequencing (NGS). It is now possible, in a fast and relatively inexpensive way, to sequence expressed RNA or whole genome DNA [45]. Today, clinical sequencing of a panel of genes commonly mutated in AML is part of everyday practice, often contributing to improved diagnostics, prognostication and disease follow-up of patients with hematological malignancies. These technologies are rapidly evolving, costs are decreasing, and in the near future hematological malignancies are likely to be analyzed by RNA- and whole genome sequencing at the time of diagnosis, as part of the routine clinical procedure. In addition, a new area of great promise to elucidate genetic heterogeneity and biology of disordered hematopoiesis, is single cell RNA- and DNA-sequencing [46-48].

To examine stem cell potential, the golden standard is to analyze engraftment potential in mouse models. The *in vivo* models for studying human hematopoiesis are mainly based on mice with varying degrees of abrogated immune system to allow the engraftment of human cells. In addition, *in vitro* surrogate assays are available that provide faster results from which stem cell potential often can be inferred. One of these rely on colony forming capacity of cells and is determined as potential to generate different colonies during culture in semi-solid medium.

One way of targeting cells for destruction with the use of an antibody is based on the recruitment of effector cells from the immune system. This can be experimentally tested by the addition of antibodies specifically binding to a cell surface receptor on the leukemic target cells and subsequently subject the cells to human effector cells, for example NK-cells. The antibody bound to the target cells then mediates antibody-dependent cellular cytotoxicity (ADCC) via interactions between the fragment crystallizable (Fc) region of the antibody and Fc-receptors on the NK-cells, for example CD16 [49]. The specific antibody-dependent cell killing can then be assessed using various methods including flow cytometry.

# Chronic myeloid leukemia

CML is the most well-studied leukemia and one of few neoplastic disorders where the genetic aspect of the pathobiology is well characterized. The balanced, reciprocal  $t(9;22)(q34;q11)$  is pathognomonic and gives rise to CML if it occurs in a hematopoietic stem cell (**Figure 3**) [50]. The resulting fusion gene subsequently translates into a constitutively activated tyrosine kinase, the BCR/ABL1 fusion protein, which elicits uncontrolled proliferation and accumulation of mature myeloid cells [51]. The disease defining event was first described, but not fully understood, in 1960 by Hungerford and Nowell. The observed aberrant chromosome was named the Philadelphia chromosome based on the city of discovery and still today CML cells are often referred to as  $Ph^+$  when containing the 9;22-translocation [52]. In 1973, Janet Rowley determined the structural basis of the Ph-chromosome and reported that it arises after a balanced translocation between chromosomes 9 and 22 [53]. Subsequently, the molecular basis for the 9;22-translocation generating a chimeric *BCR/ABL1* fusion gene was described in 1983 [54]. The therapeutic revolution came with the introduction of the TKI imatinib with Druker and colleagues pioneering the work [55].



**Figure 3. The 9;22-translocation generating the *BCR/ABL1* gene fusion leading to CML**

The chromosomal translocation  $t(9;22)(q34;q11)$  leads to the formation of the *BCR/ABL1* fusion gene. The resulting constitutively active tyrosine kinase leads to uncontrolled cell proliferation and the development of clinically manifest CML.

## Clinical aspects

At diagnosis, CML is often asymptomatic. Suspicion of disease is instead usually based on abnormal blood counts or clinical signs detected during routine physical examinations. The 50% of patients who do experience symptoms most commonly present with fatigue due to anemia, weight loss or symptoms related to splenomegaly such as early satiety or abdominal fullness. Less frequently, patients present with bleeding due to thrombocytopenia and thrombocyte dysfunction, thrombosis, or signs of leukostasis such as dyspnea [56]. At time of diagnosis, the

median white blood count in Swedish CML patients during 2014-2016 was  $101 \times 10^9/l$  with a hemoglobin level of 116g/l and thrombocytes at  $396 \times 10^9/l$ . The median age of diagnosis during this period was 59 years and the yearly incidence 1/100 000 people [57]. To confirm the CML diagnosis, bone marrow is aspirated and chromosomal analysis, FISH, or reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) are performed to confirm the presence of the 9;22-translocation or the corresponding *BCR/ABL1* gene rearrangement. If the white blood count is considered high and the patient is in need of treatment before the diagnosis is confirmed, hydroxyurea is sometimes used for cytoreduction [56,57].

CML is divided into three phases, 90-95% are diagnosed in chronic phase with approximately 5% in accelerated phase and 3% in blast crisis [56,57]. Accelerated phase CML is defined by WHO as either a blast count in peripheral blood or bone marrow of 10-19%, more than 20% basophiles in peripheral blood or other criteria based on therapy response, splenomegaly persistence, thrombocyte levels or additional genetic abnormalities. Blast crisis is defined as a blast count of  $\geq 20\%$  or extramedullary blast accumulation [39]. CML blast crisis is often myeloid resembling AML (70%) and less frequently lymphoid where the disease resembles ALL (30%). Almost all patients with CML in chronic phase are treated with a TKI, the majority receiving imatinib [58]. Patients in accelerated phase or blast crisis are often treated with later generation TKIs and considered for allogenic stem cell transplantation [59].

Progression from chronic to accelerated phase or blast crisis during imatinib treatment has in Sweden been shown at a rate of 2.4% during the first 2 years of treatment, with the majority occurring during the first year. The risk of progression was associated with higher European Treatment and Outcomes Study (EUTOS) score and although rare, it was coupled to a dismal prognosis with a median survival of 1.4 years [60].

RT-qPCR on peripheral blood for *BCR/ABL1* transcript levels converted to an international scale (*BCR/ABL1*<sup>IS</sup>) is the basis for monitoring measurable residual disease (MRD) in CML. Achieving specific levels at defined time points have been shown to aid in detecting patients at risk of treatment failure [61]. Close monitoring and adequate use of imatinib and the later generation TKIs dasatinib, nilotinib, bosutinib and ponatinib have resulted in a close to normal life expectancy for chronic phase CML patients [41,42].

The high survival rates achieved with the use of TKIs come at the price of some side effects, mainly myelosuppression, gastrointestinal problems, hepatotoxicity, hyperglycemia, pleural effusion or cardiovascular events [62,63]. To alleviate side effects and to relieve patients of daily medication, treatment discontinuation trials have been conducted. Approximately half of CML patients can stop their treatment after at least two years of TKI therapy if they have achieved and sustained a deep

molecular response (defined as  $BCR/ABL1^S \leq 0.0032\%$ , also referred to as MR<sup>4.5</sup>) and still not experience a relapse [64,65]. Some of these patients still harbour very low levels of *BCR/ABL1* positive cells that are believed to be held back by the restored immune system [66].

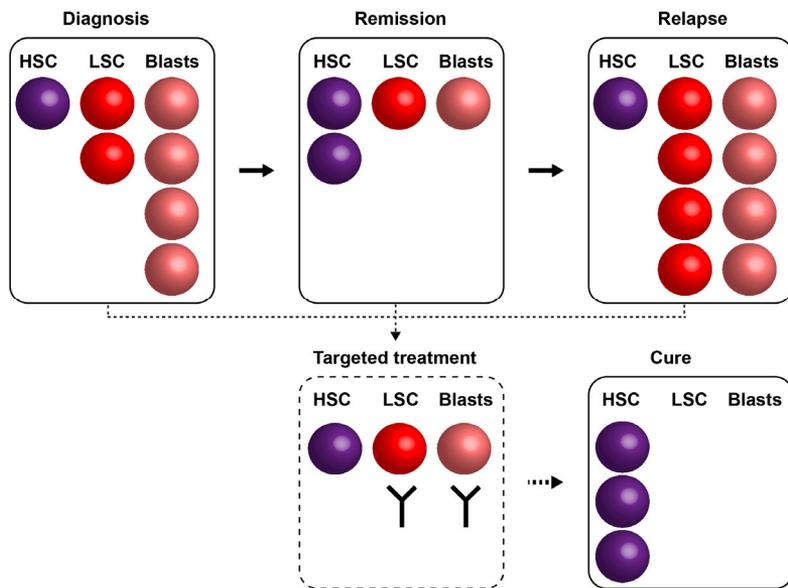
## Biological aspects

The cell of origin in CML, i.e. the cell that first acquires the *BCR/ABL1* rearrangement, is believed to be a hematopoietic stem cell as evidenced by their similar phenotypes and the presence of *BCR/ABL1* in both myeloid and lymphoid cells [67]. Low levels of *BCR/ABL1* transcripts can also be detected in healthy individuals, suggesting that a translocation has occurred in a cell lacking leukemia-initiating capacity, or that other factors also affect disease development [68-70]. In the context of CML, the constitutively active tyrosine kinase BCR/ABL1 activates a number of signaling pathways e.g. PI3K/AKT, JAK/STAT and WNT/ $\beta$ -catenin, affects gene expression, proliferation, and susceptibility to additional DNA damage [50]. Although the vast majority of CML cells respond to TKI treatment, the CD34<sup>+</sup>CD38<sup>low</sup> CML stem cells are less sensitive to TKI treatment and can remain quiescent and unharmed by treatment for long periods of time [71-73]. The primitive cells do, however, respond to cytokine stimulation such as IL-1B and MSTNpp as shown in **Article III**, suggesting alternative points for therapeutic targeting [74-76].

A suggested way of eradicating CML stem cells is by using the anti-diabetic drugs glitazons that via their agonistic effect on PPAR $\gamma$  and resulting decrease in STAT5 activity have a synergistic effect together with TKI [77]. This approach is currently being evaluated in a clinical study (NCT02767063). Use of the allosteric ABL1 inhibitor ABL001 (Asciminib) has also shown preclinical promise in combination with TKI and this combination is also currently being evaluated in clinical trials (e.g. NCT03906292). Alternatives to TKI treatment are needed both with regards to patients that develop TKI resistance and those that respond to treatment but have persisting MRD. One of the more well-described causes of resistance is mutations in the *ABL1* kinase domain but resistance is most likely often multifactorial including effects on drug metabolism and efflux from target cells, leaving patients unresponsive to TKI [78,79]. During progression to accelerated phase and blast crisis the cells acquire additional genetic aberrations including larger chromosomal alterations and point mutations [80]. Common mutations in blast crisis are found in *ABL1*, *IKZF1*, *RUNX1*, *ASXL1* and *TP53* with an overrepresentation of *ABL1* and *IKZF1* mutations in lymphoid blast crisis [81]. Currently, no antibody-based therapies targeting CML stem cells are in clinical trials for CML although several promising preclinical studies are available [74,82,83].

# Acute myeloid leukemia

AML still remains a great clinical challenge. It is a heterogenous disease with a homogenous treatment where the intensity of the given treatment is one of few factors that differ between patients. The intensity of the treatment in turn is often dependent on the patient's age and comorbidities rather than biological aspects of the disease. The outcome of patients with AML have remained poor for the last decades with the last incremental increase in survival coming from optimizing patient care in connection to allogeneic stem cell transplantations [84]. The understanding of AML pathobiology is however steadily increasing and new targeted therapies including FLT3, IDH1, IDH2, and BCL-2 inhibitors are now arriving, some showing potential to increase both survival and cure rates (**Figure 4**).



**Figure 4. The concept of LSC targeting therapy and effects on stages of disease**

Standard chemotherapy is generally effective in killing AML blasts but leukemic stem cells (LSCs) can persist in remission and cause relapse, sometimes with higher LSC frequencies. An LSC targeted treatment could potentially eradicate these LSCs and enhance the chance of cure.

## Clinical aspects

Symptoms at diagnosis of AML can vary from acute bleeding and severe infections to merely fatigue and cachexia. The incidence of AML is about 4/100 000 annually with a median age of 68 years in USA and 71 years in Sweden with a slight male predominance [85,86]. Diagnostic procedures include peripheral blood counts, bone

marrow morphology, flow cytometry, cytogenetics and mutational analyses. An AML diagnosis requires  $\geq 20\%$  myeloid blasts in peripheral blood or bone marrow or the presence of t(15;17), t(8;21), inv(16) or t(16;16) regardless of blast count. The 2016 revised WHO classification recognizes the following AML subgroups: AML with recurrent genetic abnormalities, AML with myelodysplasia-related changes, therapy-related myeloid neoplasm, AML not otherwise specified, myeloid sarcoma, myeloid proliferations related to Down's syndrome and acute leukemia of ambiguous lineage including mixed phenotype acute leukemia [39]. In 2017, the European Leukemia Net suggested that AML can be divided into three risk groups based on genetic aberrations, favourable risk including t(8;21), inv(16), t(16;16), biallelic *CEBPA* mutations and *NPM1* mutation without *FLT3*-internal tandem duplication (ITD) or with low allelic ratio, intermediate risk including mutated *NPM1* with *FLT3*-ITD with high allelic ratio and t(9;11), and high risk for patients with t(6;9), t(v;11), t(9;22), t(3;3), -5, -7, -17, inv(3), del(5q), del(17p), complex karyotype or mutations in *RUNX1*, *ASXL1* or *TP53* [87]. Additional suggestions of genomic classification of AML were proposed by Papaemmanuil in 2016 where eleven genetic subgroups were described [88].

Considering the many ways of classifying AML, the treatment approaches are remarkably similar for all patients except for those with acute promyelocytic leukemia where arsenic trioxide (ATO) and all-trans retinoic acid (ATRA) have shown great effect with high overall survival rates [89]. A majority of AML patients receive induction therapy with daunorubicin and cytarabine followed by consolidation therapy with similar regimens and allogeneic stem cell transplantation for intermediate- and high-risk patients. In patients not fit for intensive chemotherapy, hypomethylating agents such as azacitidine are alternative treatment regimens and for cytoreduction in palliative cases, hydroxyurea or low dose cytarabine can be used. Recently, the Swedish AML guidelines implemented the addition of gemtuzumab ozogamicin (an anti-CD33 drug-conjugated antibody) for favourable risk AML and midostaurin (a *FLT3* inhibitor) for AML with *FLT3* tyrosine kinase domain (TKD) mutation or *FLT3*-ITD, in combination with standard induction therapy [90]. Many patients also receive addition of new small molecules as part of clinical trials including BCL-2 inhibitors, IDH1 and IDH2 inhibitors, and later generation *FLT3* inhibitors.

The prognosis for patients with AML remains poor with the median overall survival being less than one year and a 5-year survival of 24% during 2016 in USA [86]. The survival is, however, highly age dependant and Swedish patients <60 years diagnosed between 2007-2014 showed a 5-year survival of close to 50% [85]. Outcome is also negatively affected by previous MDS or MPN disease indicating secondary AML, and prior exposure to cytotoxic therapy indicating therapy-related AML. Performance status and comorbidities affect the tolerance to chemotherapy and allogeneic stem cell transplantation, thereby affecting the prognosis [87].

Another prognostic factor at the time of diagnosis is the amount of CD34<sup>+</sup>CD38<sup>low</sup> cells, suggested to represent the leukemic stem cell (LSC) burden [91,92]. This has been further refined in a study where the LSCs were distinguished from HSCs and quantified using antibodies against CD2, CD7, CD11b, CD14, CD15, CD19, CD22, CD33, CD34, CD36, CD38, CD44, CD56, CD96, CD117, CD123, CD133, HLA-DR, CLL-1, and TIM-3. Using a cut-off of 0.03% to group CD34 positive AMLs into LSC<sup>high</sup> and LSC<sup>low</sup> showed a substantially longer overall survival in the LSC<sup>low</sup> patients [93]. Specific gene expression patterns at time of diagnosis have also been shown to predict outcome, initially using 42, 47 or 54 genes derived from microarray or mass spectrometry experiments [94-96]. A shorter LSC signature composed of 17 genes has since shown superiority in predicting outcome and the predictive value of LSC17 held true in multivariate analyses including age, de novo versus secondary disease, white blood count and cytogenetic risk including *FLT3*-ITD and *NPM1* status [97].

During follow up, complete remission (CR) is often defined as <5% blasts in the bone marrow and normalized peripheral blood counts. Substantial residual disease can still be present in CR and determining MRD with flow cytometry or detection of transcript levels of mutated *NPM1*, t(8;21), inv(16) or t(16;16) using RT-qPCR provides prognostic information. New methods for MRD detection are currently being explored and include digital droplet PCR and NGS-based monitoring of mutations detected at diagnosis or common mutations in AML [98,99].

## Biological aspects

### *Clonal hematopoiesis of indeterminate potential*

AML is often preceded by somatic mutations, sometimes many years before leukemia develops [100-102]. The concept of clonal hematopoiesis of indeterminate potential (CHIP) was introduced during the last decade and can be considered analogous to monoclonal gammopathy of undetermined significance (MGUS), which instead precedes the development of myeloma, both with risk of progression to overt hematological disease of about 1% annually [103,104]. The risk of developing AML in the setting of CHIP appears to increase with the number of mutations and their variant allele frequencies and depends on the genes affected [101,102]. The most commonly mutated genes in the context of CHIP are *DNMT3A*, *TET2* and *ASXL1*, sometimes referred to as DTA mutations, but can also involve *TP53*, *JAK2*, *SF3B1*, *SRSF2* as well as other genes [105]. The preleukemic stem cells retain multilineage potential as shown by their presence in additional cell lineages aside from the leukemic clone, such as T-cells [106]. These cells can gain further advantage by acquiring additional mutations, but the leukemic transformation is not fully understood [107]. The DTA CHIP mutations can usually

also be found during remission and their presence do not seem to hold prognostic value and cannot be used for MRD detection, although additional studies are needed to reach a firm conclusion [99]. In addition to the increased risk of leukemia, CHIP also confers an increased risk of atherosclerotic cardiovascular disease, in the case of *TET2* mutations possibly by defect macrophage function and increased inflammation in atherosclerotic plaques [108]. The frequency of CHIP increases with age but little is known of how patients with CHIP should be clinically handled.

### *Myelodysplastic syndrome*

Myelodysplastic syndrome (MDS) is both its own disease entity and a preleukemic state with a high risk of progression to AML. An arbitrary cut off for blast count at 20% in bone marrow is used to differ between MDS with excess blasts and AML. The field is, however, evolving and determining the underlying genetic aberrations causing the disease is becoming a part of the clinical diagnostic procedure to distinguish MDS and secondary AML from de novo AML [109]. Chromosomal aberrations in MDS include del(5q), -7/del(7q) and +8, whereas the most common mutations occur in genes related to splicing, DNA methylation and chromatin modification, including *TET2*, *SF3B1*, *ASXL1*, *SRSF2*, *DNMT3A*, *RUNX1*, *U2AF1*, *ZRSR2*, *STAG2*, *TP53* and *EZH2* [110,111]. Typically, MDS patients have cytopenia and dysplastic changes in the bone marrow and, as opposed to AML, only some patients with MDS develop the need for cytotoxic treatment such as daunorubicin with cytarabine, azacytidine or lenalidomide. Others have an indolent disease and may not need active treatment, these patients can instead be monitored for signs of progression and supported with EPO or erythrocyte transfusions.

### *Genetic alterations in AML*

In contrast to many solid tumors, relatively few mutations are required for AML development with an average of 13 mutations per patients and only 5 recurring mutations per patient [112]. Larger chromosomal alterations are common in AML and approximately 50% of patients have a normal karyotype. Acute promyelocytic leukemia (APL) is a specific genetic subgroup of AML that, similar to CML, is defined by a translocation. In APL the pathognomonic aberration is the 15;17-translocation, generating the fusion gene *PML-RARA*. The t(8;21) gives rise to a *RUNX1-RUNX1T1* gene fusion and together with inv(16) or t(16;16) with a resulting *CBFB-MYH11* gene fusion, are associated with a favorable prognosis. The *KMT2A*-rearrangement, previously known as *MLL*-rearrangement or t(v;11;q23) (apart from t(9;11)), is instead associated with a poor prognosis, as is t(6;9) resulting in *DEK-NUP214*, t(3;3), and the proposed de novo AML carrying a *BCR/ABL1* gene fusion, although the latter is hard to differentiate from a CML in myeloid blast crisis. Deletions of chromosome 5, 7, 17, inv(3), del(5q), del(17p), a monosomal karyotype or a complex karyotype defined as three or more chromosomal abnormalities, all indicate a poor prognosis [87].

The Cancer Genome Atlas (TCGA) Research Network published RNA sequencing data and whole genome or exome data on 200 de novo AML samples in 2013, shedding light on common recurring mutations and translocations. These can be stratified into the following nine functional groups; transcription-factor fusions (*PML-RARA*, *MYH1-CBFB*, *RUNX1-RUNX1T1*), myeloid-transcription-factor gene mutations (*RUNX1*, *CEBPA*), *NPM1* mutation, tumor suppressor gene mutations (*TP53*, *WT1*, *PHF6*), DNA methylation-related gene mutations (*DNMT3A*, *TET2*, *IDH1*, *IDH2*), activated signaling gene mutations (*FLT3-TKD*, *FLT3-ITD*, *KIT*, *KRAS*, *NRAS*), chromatin-modifying gene mutations (*ASXL1*, *EZH2*, *KMT2A*-fusions), cohesion-complex gene mutations (*STAG2*, *RAD21*), and spliceosome-complex gene mutations (*SRSF2*, *SF3B1*, *U2AF1*, *ZRSR2*) [112]. These alterations result in impaired differentiation, aberrant protein localization, resilience to apoptosis, altered methylation, chromatin modification, impaired chromosomal segregation, and deregulated RNA processing respectively.

De novo and secondary AML are genetically different and mutations in *NPM1*, core binding factor-rearrangements t(8;21), inv(16), and t(16;16), as well as *KMT2A*-rearrangements are highly specific for de novo AML, whereas mutations in splicing factors *SRSF2*, *SF3B1*, *U2AF1*, *ZRSR2*, epigenetic regulators *ASXL1*, *EZH2*, *BCOR* and cohesin complex member *STAG2* are associated with secondary AML developing after MDS [109]. Also worth noting is that familial versions of AML and MDS exist with germline mutations in *RUNX1*, *DDX41*, *GATA2*, *CEBPA*, *ETV6*, *TP53*, *SAMD9*, *SAMD9L* with implications in prognosis, use of familial donor for transplantation and potential risk for additional disease development in the family [113].

## The concept of leukemic stem cells

Healthy stem cells, including HSCs, are defined as rare, undifferentiated cells capable of symmetrical cell division with resulting production of additional identical stem cells as well as the capacity to produce progenitors and mature cells specialized for their intended tissue [114]. The definition of cancer stem cells, including LSCs, is more problematic. Conceptually, LSCs are defined as the cells exclusively capable of initiating and propagating leukemia indefinitely by self-renewal and by producing the other cell types of the leukemia [115]. This definition has shown to be difficult to fully implement in experimental settings, resulting in different surrogate assays to try to approximate the LSC capacity of cells and, in some cases, a skewed definition of LSC.

In CML, it is generally believed that a normal HSC acquires a *BCR/ABL1* rearrangement, thereby generating a CML stem cell. The BCR/ABL1 fusion protein activates a number of signaling pathways with resulting decreased apoptosis, increased self-renewal, and increased production of myeloid progenitors and mature cells, constituting the bulk of the leukemia [50]. With the massive expansion of mature cells, the leukemia as a whole contains only a small fraction of LSCs at the top of a hierarchy similar to that of HSCs in normal hematopoiesis [67]. However, this description of the CML stem cells is mainly conceptual, and how to functionally and phenotypically define and quantify CML stem cells remains controversial.

To functionally evaluate if a cell harbours LSC-potential, the golden standard is to assess engraftment in serial transplantations in immune deficient mice. When working with primary CML samples, mouse xenograft models can be problematic due to low or transient engraftment as well as engraftment of residual healthy cells [116]. Therefore, surrogate assays such as long-term culture-initiating cell (LTC-IC) assays or syngeneic mouse models are often used, making inter-study comparisons more difficult.

Studying AML stem cells in immunodeficient mouse models is associated with other problems. The extent to which the murine immune system is compromised will affect engraftment levels [117]. Engraftment can also be improved by the expression of human cytokines in the mouse [118]. Further, the genetic factors of a particular AML sample will also affect engraftment [119]. Stem cell properties can thus be context dependent. In a field with rapidly evolving humanized immunodeficient mouse models, this might skew the operational definition of an LSC with obvious implications in the readout of stem cell potential.

The cell of origin is a similar topic of discussion. CML has long been considered to originate in an HSC based on the fact that *BCR/ABL1* can be found in other lineages such as B-cells [120]. This, however, could potentially also be explained by a more mature cell gaining stem cell like features and experience a de-differentiation, much

like the process of generating inducible pluripotent stem cells (iPSC) [121]. Support for the HSC and not a more mature progenitor as the cell of origin in CML was strengthened when mouse CMPs and GMPs transduced with *BCR/ABL1* failed to show leukemic transformation [122].

In AML the cell of origin could potentially be, depending on the underlying molecular cause, a number of different cells. The HSC was early suggested to be the starting cell based on the shared  $CD34^+CD38^{low}$  phenotype between LSCs and HSCs [123,124]. However, in a mouse model of AML, using an *MLL-ELN* fusion gene, more mature progenitors but not HSCs could be transformed to produce leukemia [125]. It has also become evident that primary AML samples have LSC potential in different cellular subsets, including cells more similar to healthy GMPs and LMPPs than HSCs when assessing immunophenotype and gene expression data [126]. A recent example of mature leukemic cells going through de-differentiation and regaining leukemia initiating capacity was shown by suppressing PU.1 in an AML mouse model [127]. It thus seems possible that the LSCs in AML originate either from HSCs or from more mature progenitors that gain self-renewal capacity from their acquired genetic alterations.

# Leukemic cell surface makers

A fundamental cell property that allows studying and stratification of hematopoietic and leukemic cells are their immunophenotype. Assessable by immunohistochemistry and flow cytometry it has become the basis of hematological diagnosis, remission assessment and prediction of prognosis [87]. Further, cell surface markers make cell separation and purification possible by FACS and a majority of pivotal discoveries are based on FACS separation of cellular subpopulations that differ in their immunophenotype [123,128]. Cell surface markers are also the basis for many targeted therapies where the therapy utilizes the difference in cell surface expression between the leukemic target cells and their healthy counterparts to increase therapeutic effects and reduce toxicities.

## Cell surface markers in CML

The first description of quiescent CML cells expressing CD34 was published by Connie and Allen Eaves in 1999 [129]. The CML LSCs were later specified to be CD34<sup>+</sup>CD38<sup>low</sup> using FACS sorted cells from CML patients and xenotransplantation assays in immune deficient mice [130]. This indicated that LSCs in CML share many features with HSCs, but also sparked interest in trying to identify cell surface markers with the capacity to differentiate the two cell types. Below, cell surface markers suggested to be differentially expressed on CML stem cells are summarized (**Table 1**) and briefly discussed.

**Table 1. Cell surface markers in CML**

Name	Alias	Protein Name	Key Reference*
IL1RAP	IL1R3	Interleukin 1 Receptor Accessory Protein	Järås et al. 2010
CD25	IL2RA	Interleukin 2 Receptor Subunit Alpha	Sadovnik et al. 2016
CD26	DPPV	Dipeptidyl Peptidase 4	Herrmann et al. 2014
CD36	SCARB3	CD36 Antigen	Landberg et al. 2018
CD123	IL3RA	Interleukin 3 Receptor Subunit Alpha	Nievergall et al. 2014
LEPR	CD295, OB-R	Leptin Receptor	Landberg et al. 2018
CD56	NCAM1	Neural Cell Adhesion Molecule 1	Janssen et al. 2011
CD93	C1QR1	Complement Component C1q Receptor	Kinstrie et al. 2015
IL1R1	IL1RA	Interleukin 1 Receptor Type 1	Ågerstam et al. 2016
CD44	ECMR-III	CD44 Antigen	Krause et al. 2006
CD33	SIGLEC3	Myeloid Cell Surface Antigen CD33	Herrmann et al. 2012
CD117	SCFR, C-KIT	Mast/Stem Cell Growth Factor Receptor Kit	Florian et al. 2006

\* References provided in the table represent the first descriptions of expression in a relevant cell context and are complemented by additional references in the main text.

## IL1RAP

Interleukin 1 Receptor Accessory Protein 1 (IL1RAP) is a co-receptor to the interleukin 1 receptor (IL1R1) that, upon binding of IL-1A or IL-1B, signals through NF- $\kappa$ B and AKT phosphorylation in normal hematopoiesis [131-133]. In 2010, our group identified IL1RAP to be specifically expressed on immature CD34<sup>+</sup>CD38<sup>low</sup> CML cells, whereas CD34<sup>+</sup>CD38<sup>low</sup>IL1RAP<sup>-</sup> cells from the same patients were shown by flow-drop-FISH to be *BCR/ABL1* negative residual healthy HSCs [134]. Notably, it was also shown that antibodies directed towards IL1RAP could kill primitive CML cells *ex vivo* by ADCC [134]. In a follow-up study, newly generated IL1RAP antibodies were shown to mediate specific cell killing in a xenograft model of chronic phase and blast crisis CML, as well as to block IL-1B signaling [135]. As described in **Article I**, by assessing the leukemic stem cell burden defined as CD34<sup>+</sup>CD38<sup>low</sup>IL1RAP<sup>+</sup> cells at diagnosis, IL1RAP can be used as a maker to predict response to TKI treatment [136]. IL1RAP has also been shown by us [135,137], and others [138], to be expressed on primitive AML and MDS cells. The expression of IL1RAP on candidate CML, AML and MDS stem cells and the lack of expression on normal HSCs, makes it an interesting target for therapy in myeloid malignancies with potentially limited toxicity. IL1RAP has further been shown to be dispensable for normal hematopoiesis in mice [139]. Chimeric antigen receptor (CAR) T cells engineered to target IL1RAP have shown potent anti-leukemic effect against primary CML samples *in vitro* and in preclinical *in vivo* CML cell line models [140]. Whether CAR T cell therapy directed against IL1RAP will provide a basis for clinical studies remains to be determined.

The correlation between IL1RAP expression and other markers described in CML was further studied in **Article I**. Within the immature CD34<sup>+</sup>CD38<sup>low</sup> CML

compartment, IL1RAP is expressed on almost all *BCR/ABL1* positive cells, whereas CD25 is expressed only on a subpopulation of the CD34<sup>+</sup>CD38<sup>low</sup>IL1RAP<sup>+</sup> cells. Moreover, the percent positive IL1RAP cells better correlated to the percent *BCR/ABL1* positive cells as determined by FISH. In combination with the robust expression of IL1RAP on CD34<sup>+</sup>CD38<sup>+</sup> CML cells, this suggest that targeting IL1RAP would have a greater anti-leukemic effect than targeting CD25 [136].

So far, no clinical trials have been initiated in myeloid leukemia using anti-IL1RAP antibodies. However, a first-in-man clinical phase I/II trial using an anti-IL1RAP antibody (nidanimab, CAN04) is conducted in non-small cell lung cancer, pancreatic ductal adenocarcinoma, breast cancer and colorectal cancer (NCT03267316). Initial results suggest a manageable safety profile and an expansion phase is currently ongoing in non-small cell lung cancer and pancreatic cancer [141].

## CD25

Interleukin 2 Receptor Subunit Alpha (IL2RA), commonly known as CD25 is a part of the IL-2 receptor complex and is normally expressed on lymphocytes, including B-cells and regulatory T-cells [142,143]. CD25 has been shown to be upregulated on CD34<sup>+</sup>CD38<sup>low</sup> CML cells as compared to corresponding cells from healthy bone marrow [144]. It was later shown that in CML patients, only the LSCs and basophiles express CD25, with the majority of more differentiated CML cells lacking CD25 expression [145]. CD25 expression was also shown to be dependent on STAT5 activity and lower CD25 expression was seen during treatment with the TKIs nilotinib or ponatinib. Upon shRNA mediated downregulation of CD25, the CML cell line KU812 showed higher proliferation and engraftment in NSG mice [145]. CD25 was therefore suggested to be a marker of CML stem cells and a negative regulator of growth. Somewhat contradicting data from a murine model of CML with *BCR/ABL1* retrovirally transduced into LSK cells instead showed that there are both CD25<sup>+</sup> and CD25<sup>-</sup> LSCs and that *in vivo* treatment with anti-CD25 antibodies improved survival, as did anti-IL-2 antibodies, whereas IL-2 treatment instead shortened survival [146]. This was further supported by the increased colony forming capacity observed in human CML samples upon IL-2 stimulation, concluding that the IL-2-CD25 axis is important to maintain a subset of CML stem cells. CD25 was however again shown to be upregulated on both human and murine immature CML cells as well as blast crisis CML cells, consolidating CD25 as an LSC marker in CML although the exact function remains uncertain. The CD25 expression data are consistent with the results presented in **Article I** and **Article II**. CD25 is also expressed in AML, MDS and *BCR/ABL1* positive ALL [147-150].

Clinical trials with an anti-CD25 antibody conjugate to pyrrolobenzodiazepine (camidanlumab tesirine, ADCT-301) are currently conducted in AML and ALL

(NCT02588092), as well as lymphomas (NCT04052997 and NCT02432235), and other solid tumors (NCT03621982). Immunotoxins linked to anti-CD25 antibodies fragments (IMTOX25 and LMB-2) are also being evaluated but not for patients with CML or AML.

## CD26

Dipeptidyl Peptidase 4 (DPP4) is commonly referred to as CD26. It is normally expressed on a variety of cells including subsets of both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells and can deliver T-cell activating signals and display peptidase activity in cleaving dipeptides from polypeptides [151,152]. It is involved in many different biological functions among which the regulatory role of incretin hormones such as GLP-1 and subsequent glucose levels are one of the best studied [153]. This effect has led to anti-diabetic drugs such as sitagliptin which, by inhibiting CD26 mediated cleaving of GLP-1, results in higher insulin release and lower glucose levels [154]. CD26 can also cleave and inactivate CXCL12 with resulting effects on chemotaxis and reduced homing to the bone marrow [155]. Inhibition or deletion of CD26 in mouse HSCs results in higher bone marrow engraftment [156]. CD26 expression in a subgroup of CD34<sup>+</sup> cells from umbilical cord blood has also been described [157]. This spurred clinical trials where sitagliptin inhibition of CD26 activity showed a tendency towards enhance engraftment in patients receiving umbilical cord blood transplantation, however the relevance of this effect has yet to be determined [158,159].

Specific CD26 expression on CD34<sup>+</sup>CD38<sup>low</sup> CML cells compared to corresponding healthy cells has been described by others and was confirmed by us in **Article I** and **Article II** [144]. FACS sorting of primary lineage negative CML cells into CD26<sup>+</sup> and CD26<sup>-</sup> fractions and transplantation into NSG mice revealed *BCR/ABL1* positive CML engraftment only from the CD26<sup>+</sup> fraction [144]. In the same study, CML stem cells showed CD26 enzymatic activity and the authors propose that the cleaving of CXCL12 by CML cells can lead to reduced chemotaxis, increased niche escape, and spread of disease. This could be a reversible phenomenon as gliptins were shown to inhibit CD26 and CML cells treated with gliptins displayed reduced LTC-IC capacity and reduced engraftment, although no direct effect was observed in assays of colony forming capacity [144]. Two case reports were also provided where CML patients, upon addition of gliptin treatment to their nilotinib treatment, achieved marked reduction of *BCR/ABL1* mRNA levels.

In a large cross-sectional study in Italy, peripheral blood from 468 CML patients were assessed for CD26 expression in their immature CD34<sup>+</sup>CD38<sup>low</sup> compartment at diagnosis, during TKI treatment, and during treatment-free remission. The relative frequency of CD34<sup>+</sup>CD38<sup>low</sup>CD26<sup>+</sup> cells was higher in blood than in bone marrow, although the absolute counts were comparable, consistent with previously

described data suggesting that CD26 activity makes CML cells less prone to bone marrow sequestering. At diagnosis, 100% of CML patients displayed CD26 expression on CD34<sup>+</sup>CD38<sup>low</sup> cells, during TKI treatment about 70% retained CD26 expression and during treatment-free remission 66% expressed CD26. However, no correlation between CD34<sup>+</sup>CD38<sup>low</sup>CD26<sup>+</sup> cells and *BCR/ABL1* transcript levels was observed, possibly explained by CML stem cells being quiescent and transcriptionally silent [160]. These results would be strengthened by actually investigating the *BCR/ABL1* status at the DNA level in the different cell populations during treatment and after TKI secession. Further support for CD26 as a CML stem cell marker during TKI treatment came from a study where FACS index sorting combined with single cell qPCR analyses of 95 genes identified Lin<sup>-</sup>CD34<sup>+</sup>CD38<sup>low</sup>CD45RA<sup>-</sup>cKIT<sup>-</sup>CD26<sup>+</sup> cells to persist during TKI treatment and to have a primitive and quiescent expression profile, [161].

An anti-CD26 CAR T cell therapy has been proposed as CML treatment but showed early problems with fratricide *in vitro*, precluding the relevance of those particular CAR T cells and possibly CD26 as a target for future CAR T cell therapies [162]. CD26 has also been suggested to be upregulated in other malignancies, including CLL, mesothelioma and renal clear cell carcinoma [163-165].

## CD36

CD36 (also known as SCARB3) is a scavenger receptor and a receptor for thrombospondin-1 and is normally expressed on monocytes, macrophages, thrombocytes, adipocytes and some endothelial cells [166]. In **Article II** we define a specific CD36 expressing subset of CD34<sup>+</sup>CD38<sup>low</sup> CML cells not present in normal bone marrow. The differential expression of CD36 was detected by RNA sequencing of FACS sorted CD34<sup>+</sup>CD38<sup>low</sup> CML and normal bone marrow cells and confirmed by flow cytometry. The CD36 expressing cells were shown to be less sensitive to TKI treatment and targetable for ADCC mediated killing using an anti-CD36 antibody [83]. CD36 also appears to be downregulated during TKI treatment, however, residual CD34<sup>+</sup>CD38<sup>low</sup>CD36<sup>+</sup> cells still contain *BCR/ABL1* to a higher degree than CD34<sup>+</sup>CD38<sup>low</sup>CD36<sup>-</sup> cells from the same patient, suggesting that CD36 is a marker of immature CML cells also during TKI treatment. CD36 is further expressed in approximately 50% of AML and some blast crisis CML and has been suggested to have a role in the metastatic spread of solid tumors [167-169]. In AML and blast crisis CML, CD36 has been shown to mediate fatty acid uptake and to confer distinct metabolic properties. CD36 expressing AML cells are also more quiescent, reside in fatty tissue and are less sensitive to chemotherapy [168]. These properties have not yet been studied in detail in chronic phase CML.

## Other cell surface markers

### *CD123*

CD123 (IL3RA) is overexpressed in both chronic phase and blast crisis CML, AML, MDS and other neoplasms [170]. In CML, CD123 is expressed in both CD34<sup>+</sup> and CD34<sup>+</sup>CD38<sup>low</sup> cells, findings confirmed in **Article I** [82,171]. However, both CD34<sup>+</sup>CD38<sup>low</sup>CD123<sup>+</sup> and CD34<sup>+</sup>CD38<sup>low</sup>CD123<sup>-</sup> show LTC-IC capacity with resulting *BCR/ABL1* positive colonies, allowing ADCC mediated killing of only a subset of LSCs in CML [82]. An anti-CD123 antibody was also shown to reduce the growth promoting effects of IL-3 on CML cells. Moreover, an anti-CD123 targeting fusion protein conjugated to diphtheria toxin could also induce apoptosis in primary CML samples carrying a T315I mutation in *ABL1* [172]. CD123 is also expressed on normal hematopoietic cells albeit at lower levels, which has halted therapeutic endeavours in CML. However, CD123 still remains an interesting target in AML were toxicity is more acceptable due to the worse prognosis, as discussed further below.

### *LEPR*

The leptin receptor (LEPR), also known as Ob-R or CD295, has been shown to be specifically upregulated on primary CD34<sup>+</sup>CD38<sup>low</sup> CML cells (**Article II**). The biological effect of this upregulation is not clear as stimulation with leptin did not confer a growth advantage to CML cells [173]. Leptin has, however, been shown to have a role in obesity-induced tumor growth, regulating adipogenesis and osteogenesis in the bone marrow, and to promote AML cell growth [174-176]. Further studies are therefore needed to establish whether LEPR has a functional role in CML and if it could provide a target for directed therapies.

### *Putative MSTNpp receptor*

To identify positive regulators of primitive CML cells, a cytokine screen using 313 different cytokines, primary CD34<sup>+</sup>CD38<sup>low</sup> CML cells, and healthy controls was performed in **Article III**. Based on this screen and subsequent experiments, myostatin propeptide (MSTNpp) was identified to expand both immature CML cells and corresponding cells from normal bone marrow by increased phosphorylation of SMAD2/3 and STAT5. Using the CML cell line KU812 and an anti-MSTNpp antibody, MSTNpp could be shown to bind to the cell surface. This suggests that there is a receptor for MSTNpp expressed on the surface of CML cells. This putative receptor of MSTNpp is most likely also expressed on healthy cells, based on the observation of similar growth promoting effects of MSTNpp in this cell context. Further studies are needed to elucidate the presumed MSTNpp receptor and its role in CML.

### *CD56*

NCAM1 commonly referred to as CD56, is normally expressed on NK-cells and has been shown to be upregulated in both CML and AML [177,178]. In CML, it is expressed on CD34<sup>+</sup> cells but only weakly expressed in CD34<sup>+</sup>CD38<sup>low</sup> cells, the expression however appears to be higher than on corresponding healthy cells [177,179]. The expression of CD56 was confirmed in **Article II**. The anti-CD56 drug-conjugated antibody lorvotuzumab mertansine (IMGN901) was tested in clinical trials for hematological malignancies including AML (NCT02420873), as well as solid tumors, but have since been discontinued [180]. An ongoing clinical trial in AML will evaluate CAR T cells targeting multiple proteins including CD56 (NCT03222674).

### *CD93*

CD93 (C1QR1) has been shown to be upregulated on immature Lin<sup>-</sup>CD34<sup>+</sup>CD38<sup>low</sup>CD45RA<sup>-</sup>CD90<sup>+</sup> CML cells as well as on AML cells [181,182]. In CML, CD93 confers signals for self-renewal and proliferation and this does not appear to be dependent on ligand binding [183]. Further, these effects of CD93 could potentially be mitigated by the use of the clinically available antiemetic metoclopramide, results in need of further validation. In **Article II** we could confirm the expression of CD93 in CD34<sup>+</sup>CD38<sup>low</sup> CML cells.

### *IL1R1*

IL1R1, which mediates IL-1 signaling together with its co-receptor IL1RAP, is also expressed on both CD34<sup>+</sup> and CD34<sup>+</sup>CD38<sup>low</sup> CML cells. However, the expression is not high and healthy cells also express IL1R1, primarily in CD34<sup>+</sup> cells but also at low levels in CD34<sup>+</sup>CD38<sup>low</sup> cells [74,184].

### *CD44*

Some markers are expressed on CML cells but with simultaneous high expression on healthy hematopoietic cells and in other tissue. CD44 is one such example. CD44 is expressed on CML cells and regulate homing to the bone marrow as shown in a transgenic mouse model of CML [170,185]. CD44 binds to E-selectin, hyaluronic acid and other components of the extracellular matrix, mediating adhesion and homing, and an anti-CD44 antibody has been shown to reduce LSC burden in a xenograft mouse model of human blast crisis CML [186,187]. The feasibility of using an anti-CD44 approach for treating CML patients remains to be determined and caution is advisable considering the CD44 expression on HSCs.

### *CD33*

CD33, also known as SIGLEC3, is highly expressed on CD34<sup>+</sup>CD38<sup>low</sup> CML cells as well as AML cells and the expression in CML is higher than in normal bone marrow [188-190]. These CD33 expressing CML cells can be targeted by an anti-CD33 drug conjugated antibody [189]. The relevance of high CD33 expression in CML is, however, limited due to expression on healthy hematopoietic stem cells [31]. CD33 targeting therapies are used in AML despite the HSC expression and possible on-target off-tumor effects.

### *CD117*

Early reports showed that CML cells express CD117 (KIT), the receptor for SCF [170]. CD117 has also been shown to be expressed on CD34<sup>+</sup>CD38<sup>low</sup> CML cells, but the expression appears higher on healthy cells [184]. Further, CD117 negative CML cells could potentially be less sensitive to TKI treatment, suggesting a reduced importance of CD117 expression in CML [161]. The expression of CD117 was confirmed in **Article I**.

### *ST2*

ST2 (IL1RL1) also known as IL33R, is another receptor suggested to have a role in CML. It was first described to be expressed on CD34<sup>+</sup> CML cells [191]. More recent research could not confirm the expression of ST2 on immature CML cells [184].

## Cell surface markers in AML

Many markers have been shown to be upregulated on AML cells. Some are specifically expressed on AML cells or AML stem cells and others simply have a higher expression on AML cells compared to normal cells. Some are also the focus of clinical therapeutic endeavours.

LSCs in AML were first believed to reside exclusively within the CD34<sup>+</sup>CD38<sup>low</sup> compartment [123,124,192]. It was later shown that the anti-CD38 antibodies used for the LSC separation could induce cell killing of CD38<sup>+</sup> cells through NK-cell activity as well as other mechanisms [193]. This most likely skewed the results, masking LSC potential in the CD38 expressing population. The genetic changes defining a particular AML sample will affect the LSC immunophenotype of a sample. For example, *NPM1* mutated AML that generally have low or no CD34 expression have been shown to have LSC potential in both CD34<sup>+</sup>CD38<sup>low</sup> and CD34<sup>+</sup>CD38<sup>+</sup> cells as well as in some cases only within the CD34<sup>-</sup> compartment [194,195]. This has further been demonstrated in other subgroups of AML, where both CD34<sup>+</sup>CD38<sup>low</sup> and CD34<sup>+</sup>CD38<sup>+</sup>, as well as AML cells expressing lineage markers, have been shown to harbor LSC potential [126,196]. Therefore, the LSCs in AML cannot be said to always reside within a specific CD34/CD38 compartment. However, in a majority of AML samples, the LSCs still appear to be enriched in the CD34<sup>+</sup>CD38<sup>low</sup> cell fraction, making it a preferred population for studying LSCs in AML [95,115,126,196].

Few studies have evaluated multiple cell surface markers and their co-expression, with some notable exceptions [197-199]. Ongoing work in our group is using a new technique based on cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq), to further elucidate the co-expression and immunophenotype of LSCs in AML.

Cell surface markers that have been described to be upregulated in AML are summarized in **Table 2** and short descriptions of the most well-studied markers are provided below.

**Table 2. Cell surface markers in AML**

Name	Alias	Protein Name	Targeted Drugs*	Key Reference#
CD123	IL3RA	Interleukin 3 Receptor Subunit Alpha	mab, adc, dab, CAR T	Jordan et al. 2000, Jin et al. 2009
CD47	MER6	CD47 Antigen	mab, other	Majeti et al. 2009, Jaiswal et al. 2009
TIM-3	HAVCR2, CD366	Hepatitis A Virus Cellular Receptor 2	mab	Kikushige et al. 2010, Jan et al. 2011
IL1RAP	IL1R3	Interleukin 1 Receptor Accessory Protein	(mab in solid tumors)	Barreyro et al. 2012, Askmyr et al. 2013
CLL-1	CLEC12A, CD371	C-Type Lectin Domain Family 12 Member A	dab, CAR T	Bakker et al. 2004, Van Rhenen et al. 2007
SLAMF6	CD352, NTB-A	SLAM Family Member 6	-	Landberg et al. 2019
CD33	SIGLEC3	Myeloid Cell Surface Antigen CD33	mab, adc, dab, rab, CAR T	Hauswirth et al. 2007
CD7	GP40	T-cell Antigen CD7	CAR T	Lo Coco et al. 1989
CD25	IL2RA	Interleukin 2 Receptor Subunit Alpha	adc	Saito et al. 2010
CD32	FCGR2A	Fc Fragment Of IgG Receptor IIa	-	Saito et al. 2010
CD36	SCARB3	CD36 Antigen	-	Ye et al. 2016
CD37	TSPAN26	Leukocyte Antigen CD37	mab (terminated), CAR T	Pereria et al. 2015
CD43	SPN, Sialoporphin	Leukosialin	-	Gillissen et al. 2017
CD44	ECMR-III	CD44 Antigen	mab, CAR T	Jin et al. 2006
CD45	PTPRC, LCA	Protein Tyrosine Phosphatase Rec. Type C	rab	Mawad et al. 2014
CD52	EDDM5	CAMPATH-1 Antigen	mab	Blatt et al. 2014
CD56	NCAM1	Neural Cell Adhesion Molecule 1	adc (discontinued), CAR T	Sasca et al. 2019
CD70	CD27L, TNFSF7	CD70 Antigen	mab	Riether et al. 2016
CD73	NT5E	5'-Nucleotidase	-	Jakobsen et al. 2019
CD82	TSPAN27	CD82 Antigen	-	Nishioka et al. 2013
CD89	FCAR	Fc Fragment Of IgA Receptor	-	Mladenov et al. 2015
CD90	THY1	Thy-1 Cell Surface Antigen	-	Buccisano et al. 2004
CD93	C1QR1	Complement Component C1q Receptor	-	Iwasaki et al. 2015
CD96	TACTILE	T-cell Surface Protein Tactile	-	Hosen et al. 2007
CD97	ADGRE5	CD97 Antigen	-	Martin et al. 2019
CD98	SLC3A2	4F2 Cell-surface Antigen Heavy Chain	mab	Bajaj et al. 2016
CD99	MIC2	CD99 Antigen	-	Chung et al. 2017
CD105	ENG	Endoglin	(mab in solid tumors)	Dourado et al. 2017
CD133	PROM1	Prominin 1	CAR T	Wuchter et al. 2001
CD135	FLT3	Fms Related Tyrosine Kinase 3	mAb	Kuchenbauer et al. 2005
CD157	BST1	Bone Marrow Stromal Cell Antigen 1	mab	Krupka et al. 2017
CD200	OX-2	Cell Surface Glycoprotein CD200 Rec. 1	mab	Coles et al. 2011
CD244	SLAMF4, NKR2B4	Natural killer cell receptor 2B4	-	Zhang et al. 2017
CD300LF	IREM-1, CLM1	CMRF35-like Molecule 1	-	Korver et al. 2009
CD302	CLEC13A	CD302 Antigen	-	Lo et al. 2019
CXCR4	CD184	C-X-C Motif Chemokine Receptor 4	mab, other	Zhang Yanyan et al. 2017
JAM3	JAMC	Junctional Adhesion Molecule 3	-	Zhang Yaping et al. 2018
GPR56	ADGRG1	Adhesion G-protein Coupled Receptor G1	-	Pabst et al. 2016
OX40	TNFRSF4, CD134	TNF Receptor Superfamily Member 4	mab	Neubling et al. 2018

\* Types of targeted drugs in in clinical trials for AML. Abbreviations; mab, naked monoclonal antibody; adc, antibody-drug conjugate; dab, dual-affinity antibody; rab, radiolabeled antibody, CAR T, chimeric antigen receptor T cells; other, other types of targeted compounds.

# References provided in the table represent the first descriptions of expression in a relevant cell context and are complemented by additional references in the main text.

## CD123

Interleukin 3 Receptor Subunit Alpha (IL3RA) is commonly known as CD123. In 2000, CD123 was described to be upregulated on immature CD34<sup>+</sup>CD38<sup>low</sup> AML cells compared to their cellular counterpart in healthy bone marrow [200]. IL-3 signals through the heterodimer of CD123 and CD131 and the IL-3 effects are species specific [201]. IL-3 has long been known to give a potent proliferative effect on AML cells [202]. It has also been shown that an anti-CD123 antibody both inhibits the proliferative effect of IL-3, reduces homing of AML cells to the bone marrow, and marks cells for killing by the innate immune system in non-obese diabetic-severe combined immune-deficient (NOD/SCID) mice [203]. Further, the level of CD34<sup>+</sup>CD38<sup>low</sup>CD123<sup>+</sup> cells at diagnosis, as assessed by flow cytometry, has been shown to correlate to overall survival and patients with <1% of these cells have a particularly good prognosis [204]. A link between CD123 and *FLT3*-ITD has also been suggested since it was shown that only CD34<sup>+</sup>CD38<sup>low</sup>CD123<sup>+</sup> and not CD34<sup>+</sup>CD38<sup>low</sup>CD123<sup>-</sup> cells carried a *FLT3*-ITD in a small group of primary AML samples [205].

CD123 is, however, expressed in normal hematopoiesis and can be used to differentiate CD123<sup>low</sup> CMPs and GMPs from CD123<sup>-</sup> MEPs [206]. It also appears to be expressed on HSCs from cord blood and, albeit at low levels, on some HSCs from normal bone marrow [31]. This low expression is relevant since it suggests that an anti-CD123 therapy might also affect residual normal hematopoiesis. This has been confirmed in preclinical *in vivo* experiments where a CD123 directed CAR T cell therapy for AML resulted in myeloablation [207]. Nevertheless, ongoing trials are exploring the use of both autologous anti-CD123 CAR T cells (NCT02159495) and allogeneic UCART123 (NCT04106076) in AML. CD123 is also expressed in some cases of B-ALL, blastic plasmacytoid dendritic cell neoplasms (BPDCN) and hairy cell leukemia [208].

Many compounds targeting CD123 are currently, or have recently been, studied in clinical trials for AML, including bispecific or dual affinity CD123-CD3 antibodies (XmAb14045 in NCT02730312, JNJ-63709178 in NCT02715011 and flotetuzumab, formerly MGD006 or S80880 in NCT02152956), drug conjugated anti-CD123 antibodies (IMGN632, NCT03386513), and anti-CD123 CAR T cells (UCART123 in NCT03190278 and MB-102 in NCT04109482). Tagraxofusp, formerly SL-401, is a CD123 directed recombinant protein combining IL-3 and truncated diphtheria toxin that has shown striking effects in BPDCN with overall response rates of 90% in previously untreated patients, although 19% also experienced capillary leak syndrome [209]. Tagraxofusp is currently also in clinical trials for AML in combination with azacitidine and venetoclax (NCT03113643), as well as in the form of consolidation treatment in complete remission (NCT02270463). Other compounds have been withdrawn or clinical trials

terminated due to lack of effect or unfavourable risk/benefit profiles, including naked CD123 antibody talacotuzumab (derived from CSL362, NCT02472145) and KHK2823 (NCT02181699), as well as drug conjugated antibody SGN-CD123A (NCT02848248) [180,210]. The efficacy and safety of targeting CD123 in AML have therefore yet to be conclusively decided.

## CD47

CD47 was first described by the Weissman group to be overexpressed on AML cells including immature Lin<sup>-</sup>CD34<sup>+</sup>CD38<sup>low</sup>CD90<sup>-</sup> AML cells, compared to normal HSC and MPP populations [211]. They further showed macrophage dependant phagocytosis and specific killing of AML cells with an anti-CD47 antibody both *in vitro* and in preclinical *in vivo* experiments, as well as a shorter survival for AML patients with high CD47 expression. In a separate study, they also showed CD47 to be more highly expressed in blast crisis than chronic phase CML and that mobilized mouse HSCs made to circulate transiently upregulate CD47 to avoid phagocytosis [212]. This ‘don’t eat me’ signal conferred by CD47 is a result of interaction with SIRP $\alpha$  on the surface of macrophages [213]. CD47 is, however, also expressed on a variety of normal tissue, albeit at lower levels than many malignancies [214]. Indeed, CD47 has been shown to be upregulated in a variety of cancers including breast, colon, prostate and ovarian tumors [215].

There are currently many clinical trials for therapies targeting CD47. Recent reports show promising effects of the recombinant human monoclonal IgG4 antibody Hu5F9-G4 in diffuse large B-cell lymphoma as well as more indolent follicular lymphomas [216]. Phase I trials for Hu5F9-G4 in AML and MDS are currently ongoing but no results are available (NCT02678338 and NCT03248479). SRF231 is another anti-CD47 antibody in clinical trials for different hematologic malignancies (NCT03512340). The phase I trial of Celgene’s antibody CC-90002 in AML was terminated due to unfavourable preliminary data (NCT02641002), the antibody is now evaluated in solid tumors, myeloma and lymphoma (NCT02367196). ALX148 is instead a high-affinity variant of SIRP $\alpha$  fused to an inactive Fc-domain that upon binding to CD47 blocks signaling, acting as an antagonist and potentially enhancing the phagocytic effect of other anti-tumor antibodies [217]. ALX148 is currently evaluated in a clinical trial for solid tumors and lymphomas (NCT03013218). A similar compound, TTI-621 is also evaluated in a clinical trial for hematological malignancies (NCT02663518). Therefore, CD47 is an interesting target with potential in AML as well as many other malignancies and further results from these clinical trials are eagerly awaited.

## TIM-3

Hepatitis A Virus Cellular Receptor 2 (HAVCR2) is commonly known as T-cell Immunoglobulin Mucin Family Member 3 (TIM-3) or CD366. TIM-3 is specifically upregulated on bulk AML cells and LSCs in most AML samples compared to hematopoietic stem cells and can be used to separate LSCs from residual HSCs in an AML sample [218]. An anti-TIM-3 antibody has been shown to induce ADCC and inhibit AML but not normal HSC engraftment in NOD/SCID mice [219]. Galectin-9 (Gal-9) is a ligand for TIM-3, also produced by AML stem cells, constituting an autocrine stimulatory loop with activation of NF- $\kappa$ B and  $\beta$ -catenin upon binding to TIM-3, stimulating leukemia progression [220]. TIM-3 can facilitate the secretion of Gal-9 from AML cells, which has been suggested to inhibit cytotoxic T and NK-cells, possibly contributing to AML immune evasion [221]. TIM-3 can also be secreted in a soluble form, possibly further preventing NK-cell activation via inhibition of IL-2 production [222]. TIM-3 plays a similar role as PD-1 in negatively regulating immune response and TIM-3 is expressed on normal regulatory T, B and NK-cells, as well as on macrophages, dendritic cells, and mast cells [223]. Furthermore, it is expressed together with PD-1 on exhausted T-cells in cancer and it has been suggested that blocking both PD-1 and TIM-3 might give synergistic therapeutic effects [224].

Two clinical phase I trials in AML are currently investigating MGB453, a humanized monoclonal anti-TIM-3 IgG4 antibody, one of which combines MGB453 with a PD-1-inhibiting antibody and decitabine (NCT03066648). It is also being tested in combination with hypomethylating agents in patients with MDS (NCT03946670). TIM-3 is thus currently being studied more as a target for immune check-point inhibition than a specific AML cell surface target.

## IL1RAP

As described above, the role of IL1RAP in leukemia was first studied in CML [134]. In parallel, work by Steidl's group demonstrated IL1RAP overexpression in immature cells from AML and MDS patients [138]. By performing FISH on FACS sorted IL1RAP<sup>+</sup> and IL1RAP<sup>-</sup> cells from an AML samples with del(7), it was shown that only the IL1RAP expressing cells were part of the leukemic clone. The functional relevance of IL1RAP was studied by shRNA mediated IL1RAP knock-down in AML cell lines, which resulted in reduced clonogenic potential. IL1RAP is expressed in a majority of AML samples and can be targeted with an anti-IL1RAP antibody, inducing ADCC mediated killing of primary AML cells *in vitro* [137]. It was later also shown that, in addition to NK-cell mediated ADCC effects in preclinical *in vivo* experiments, an anti-IL1RAP antibody can reduce proliferation of primary AML cells by blocking IL-1 signaling, providing a dual mode of action

[135]. IL1RAP is further strengthened as a target for therapy due to it being dispensable for normal hematopoiesis in mice as well as involved in FLT3-ligand and SCF signaling by physical interactions with FLT3 and KIT in AML cells [139]. This could possibly also indicate that targeting IL1RAP might have a less pronounced effect of blocking IL-1B signaling in AML patients with constitutively activated FLT3 signaling as conferred by *FLT3*-TKD or *FLT3*-ITD, although the ADCC effect would presumably still be present. Some AML samples have also been shown to express IL1RAP on subclones with a parallel subclone in the same patient lacking IL1RAP expression [225]. As mentioned above, a clinical phase I-II trial evaluating an anti-IL1RAP antibody is currently conducted in solid tumors (NCT03267316), but so far, no clinical trials have been initiated in AML or high-risk MDS.

## CLL-1

C-type Lectin Domain Family 12 Member A (CLEC12A) is often referred to as C-type Lectin-like Molecule-1 (CLL-1) or CD371. CLL-1 was first described to be expressed on AML cells in phage display experiments published in 2004 [226]. This study showed that CLL-1 is expressed in more than 90% of AML patients and that the CD34<sup>+</sup>CD38<sup>low</sup> LSCs express CLL-1. CLL-1 is also expressed on healthy granulocytes and monocytes, as well as on some CD34 expressing progenitors, but not in the CD34<sup>+</sup>CD38<sup>low</sup> HSC compartment or in other types of tissue [226]. CLL-1 expression on healthy progenitors vary but appears to be higher on GMPs (on average 80% cells expressed CLL-1) than CMPs (40%) and MEPs (10%) [227]. Further evidence of CLL-1 as a specific marker for LSCs was provided when a larger cohort of AML samples were shown to express CLL-1 in their CD34<sup>+</sup>CD38<sup>low</sup> compartment and that FACS sorted primary CD34<sup>+</sup>CLL1<sup>+</sup> AML cells could engraft with a leukemic phenotype in NOD/SCID mice [228]. CLL-1 expression is also retained upon relapse and is not aberrantly expressed in healthy cells in bone marrow from patients in remission after chemotherapy – a central aspect often overlooked in studying other therapeutic targets [228,229].

The functional role of and ligands for CLL-1 in AML is not fully discerned but CLL-1 has been suggested to be involved in inhibiting granulocyte and monocyte function, thereby limiting inflammation [230]. It is however known that CLL-1 internalizes upon antibody binding, an aspect similar to CD33 that facilitates drug delivery via drug conjugated anti-CLL-1 antibodies [231]. Preclinical experiments have shown that an anti-CLL-1 antibody conjugated to the DNA-binding D211 (isoquinolidinobenzodiazepine) induces cell killing of AML blasts and LSCs with minimal effects in healthy progenitors both *in vitro* and in an NSG mouse system [231]. The effects on healthy hematopoietic cells were less pronounced than the control antibody-drug conjugate targeting CD33. Another approach of targeting

CLL-1 is using a bispecific, full-length antibody directed towards CLL-1 and CD3. These experiments have shown promising anti-leukemic effect and acceptable levels of adverse events in preclinical models [232].

So far only one clinical trial using an anti-CLL-1 antibody in AML has been initiated (NCT03038230). This phase I first-in-man study evaluates MCLA-117 also known as tepoditamab, a bispecific full-length human IgG antibody targeting CLL-1 and CD3, engineered to lack Fc-mediated functions through abrogated binding to the Fc-receptors CD16, CD32, C1q and CD64 for increased specificity. No results from this clinical trial are currently available, but preclinical data suggests that tepoditamab can redirect autologous T-cells from AML patients for specific CLL-1 targeted cell lysis *in vitro* [233]. Clinical trials are currently conducted to assess the feasibility of CLL-1 directed CAR T cell systems, including a compound CAR T cell system targeting both CLL-1 and CD33 (NCT03795779).

## SLAMF6

Signaling Lymphocytic Activation Molecule Family 6 (SLAMF6), also known as CD352, Ly108, and NTB-A, is a homophilic self-ligand cell surface receptor in the immunoglobulin superfamily [234]. Commonly expressed on NK, B, and T-cells, SLAMF6 has immunomodulatory functions including NK-cell activation through SAP and EAT-2 signaling [235,236]. In addition, inhibition of T-cell and B-cell adhesion and interaction through SHP-1 recruitment [237], and a role in T-cell development through SAP and FYN signaling have been described [238]. The different effects of SLAMF6 signaling depends on the cellular context, tyrosine phosphorylation of one of the two ITSM on SLAMF6 cytoplasmic tail either recruits SAP and signal through FYN, or recruit SHP1/2 with activating or inhibitory outcomes respectively [239]. SLAMF6 is also expressed on normal eosinophils but not basophiles and neutrophils [240]. SLAMF6 expression has been proposed to be restricted to the hematopoietic system [239].

In **Article IV** we show that SLAMF6 is specifically expressed on CD34<sup>+</sup>CD38<sup>low</sup> AML cells compared to corresponding healthy cells. The expression is most pronounced in *TP53* mutated AML samples. We further show that an anti-SLAMF6 antibody induces ADCC mediated killing of KG1 cells.

Recent data suggests that an anti-SLAMF6 antibody can also re-activate exhausted CD8<sup>+</sup> T lymphocytes, providing a possible dual mode of action for an anti-SLAMF6 antibody-based therapy [241]. SLAMF6 has also been shown to be aberrantly expressed in CLL and myeloma [242-244]. An anti-SLAMF6 antibody conjugated to pyrrolbenzodiazepine has been evaluated in a clinical trial for myeloma (NCT02954796), but no results have been published and no other trial in hematological malignancies has been registered at ClinicalTrials.gov.

## CD33

CD33, also known as SIGLEC3, is a myeloid differentiation marker commonly expressed on many types of healthy myeloid cells including myeloid progenitors, monocytes, macrophages, granulocyte precursors and mast cells [245]. CD33 expression has been shown to be significantly higher on AML blasts (CD45<sup>dim</sup>) compared to healthy progenitor cells and CD33 expression can be detected in almost 90% of AML samples [190]. Importantly, CD33 has also been suggested to be expressed on LSC, increasing its potential relevance as a target for therapy [188].

Whether or not CD33 is expressed on healthy HSCs is still under discussion with conflicting data reported. The CD34<sup>+</sup>CD38<sup>low</sup> compartment from healthy bone marrow was shown to express high levels of CD33 [31]. The same study further showed that healthy Lin<sup>-</sup>CD34<sup>+</sup>CD38<sup>low</sup> cord blood and bone marrow cells expressing CD33 could induce multilineage engraftment after serial transplantations into NOD/SCID mice. Older clinical studies, however, show that CD33 depleted grafts give rise to full bone marrow engraftment, but with a longer latency than non-depleted grafts [246]. More recently, a bispecific antibody targeting CD33 and CD3 was shown to inhibited AML growth without effect on healthy hematopoietic cells, as determined by colony forming capacity and engraftment potential in NSG mice [247].

Initial clinical experiments using a CD33 antibody showed no effect on disease progression in AML, however, a high internalization upon binding of the antibody to CD33 was observed [248]. This led to the development of gemtuzumab ozogamicin, a CD33 targeting antibody conjugated to a DNA-damaging antibiotic derived from calicheamicin. The linker between antibody and drug is stable in peripheral circulation, but upon binding to CD33 the receptor-antibody complex is internalized and the low pH in the lysosome induces release of the toxic payload with subsequent DNA damage and cell apoptosis. However, large variations of the susceptibility to this toxic effect on leukemic cells are seen, suggested to be dependent on slow internalization, drug efflux, other pro- or anti-apoptotic signals in the cell as well as varying expression of the target molecule CD33 [249]. The antibody was retracted from market but have now been re-introduced following a meta analyses of multiple clinical trials showing a significant improvement in overall survival for AML patients of favourable and intermediate risk groups when added to standard induction therapy [250].

Several other approaches of targeting CD33 have also been suggested. One of these is the now withdrawn CD33 antibody conjugated to pyrrolbenzodiazepin lintuzumab, also known as SGN-CD33A or vadastuximab talirine. Initial positive results were followed by a phase III trial (NCT02785900) in older AML patients that was discontinued due to increased fatal infections in the treatment group. Other compounds in clinical trials include the anti-CD33 antibody BI836858

(NCT02632721 and NCT03207191), the radioimmunoconjugate HuM195 also known as Lintuzumab Ac-225 or Actimab-A (NCT03867682 and NCT03932318), bispecific T-cell engaging antibodies AMG330 (NCT02520427), GEM333 (NCT03516760) and JNJ-67571244 (NCT03915379), other T-cell engagers such as AMV564 (NCT03144245), and the tri-specific fusion protein OXS-3550 also known as GTB-3550 that targets CD33 and CD16 and is linked to a modified form of IL-15 intended to engage and activate NK-cells (NCT03214666). Ongoing trials also evaluate the effects of CAR T cells targeting CD33 (NCT02958397), as well as a bispecific compound CAR T cell therapy targeting both CD33 and CLL-1 (NCT03795779). The effects on residual healthy hematopoietic cells from these treatments and the potential need for subsequent allogeneic stem cell transplantation remains to be determined.

## Other cell surface markers

At least 32 additional markers have been described as possible targets in AML. In the following section, a few of these markers that have been studied more extensively are briefly discussed. Key references for the remaining markers are provided in **Table 2**.

### CD7

The known T-cell and NK-cell marker CD7 has long been recognized to be aberrantly expressed in 10-35% of AML cases with a slight predominance in normal karyotype AML, less differentiated M0 and M1 AML and younger patients [199,251-254]. CD7 expression is associated with poor prognosis and it is inversely correlated with *CEBPA* mutation although there does not seem to be a causative link between the mutation and CD7 expression in AML [255]. CD7 is also expressed in primitive CML cells albeit at low levels [173,256]. Targeting CD7 with CAR T cells have been shown to have potent anti-leukemic effect *in vitro* and in xenograft models of AML but require the CAR T cells to be genetically edited to not express CD7 in order to avoid fratricide [257]. A clinical trial is currently testing the feasibility of using CAR T cells targeting CD7 expressing neoplasia including AML (NCT04033302), it is unclear if these studies are conducted using T-cells with silenced CD7.

### CD25

CD25 is expressed in CML and described in more detail above. In AML 10-30% of patients have been shown to express CD25 and expression is associated with a poor prognosis [147,258,259]. Leukemic engraftment in NSG mice has been shown for both CD25 positive and negative cells, indicating that CD25 is expressed on some but not all LSCs [260]. CD25 is expressed on quiescent, chemotherapy refractory

LSCs and can initiate AML upon transplantation into immunodeficient mice [261]. In addition, CD25 is not expressed on healthy HSCs, increasing its potential relevance as a target for therapy. Anti-CD25 toxin conjugated antibody camidanlumab tesirine (ADCT-301) has been tested in a clinical trial for AML (NCT02588092) but no results have been reported. The radiolabelled anti-CD25 antibody daclizumab have been studied in Hodgkin's lymphoma and multiple sclerosis but not in AML and is currently withdrawn due to safety concerns. The recombinant immunotoxin LMB-2 targeting CD25 is being evaluated in T-cell lymphoma but not AML.

### *CD32*

CD32 (FCGR2A) is an activating Fc-receptor commonly expressed on T-cells, monocytes, macrophages and neutrophils [262]. CD32 here should not be confused with the structurally similar but inhibitory FCGR2B and the activating FCGR2C receptors. CD32 is expressed on quiescent, chemotherapy refractory LSCs that can initiate AML upon transplantation into immunodeficient mice, CD32 is, however, not expressed on healthy HSCs [261]. There are no ongoing clinical studies targeting CD32 in AML.

### *CD36*

As shown in **Article II**, CD36 is expressed on immature CML cells and is described in detail above. CD36 is also expressed in about 50% of AML patients where it marks a subgroup of LSCs that are quiescent and rely on fatty acid oxidation for their metabolism [168]. These CD36 expressing LSCs are suggested to reside in a fatty rich niche that confers resistance to chemotherapy. CD36 is also highly expressed on acute myelomonocytic leukemia (M4) and acute monoblastic/monocytic leukemia (M5), presumably based on the CD36 expression on normal monocytes [263].

### *CD37*

CD37 is part of the tetraspanin superfamily, it is expressed on B-cells and B-cell malignancies such as CLL and anti-CD37 antibodies and antibody-drug conjugates are being developed for the use in CLL [264,265]. It was recently shown to also be expressed on AML cells and potentially on LSCs [266]. However, the clinical trial evaluating the effect of the anti-CD37 antibody-drug conjugate AGS67E in AML (NCT02610062) was recently terminated by the sponsor and no results have been published. A clinical trial evaluating anti-CD37 CAR T cells in CD37 expressing hematological malignancies is planned for next year, potentially enrolling AML patients (NCT04136275).

### CD43

CD43, also known as SPN or Sialophorin, is expressed in a sialylated form on all WHO 2008 defined subgroups of AML and MDS [267]. The discovery of CD43 expression was based on experiments analysing antibodies produced from the allogeneic stem cell graft in a patient in complete remission two years after treatment for AML. Mass spectrometry revealed that the graft-produced antibodies specifically bound to sialylated CD43 on AML cells and these antibodies induced ADCC and complement-dependent cytotoxicity (CDC) in AML cell lines *in vitro* and *in vivo* as well as in primary AML cells. Later work using a bispecific antibody targeting CD43 and CD3 has shown potent anti-leukemic effect with minimal effect on healthy hematopoietic cells in mice models, even though CD43 can be detected at low levels on hematopoietic progenitors, monocytes and granulocytes [268].

### CD44

CD44 is expressed in AML, CML, ALL, and healthy tissue, and it binds to E-selectin, hyaluronic acid and other components of the extracellular matrix, mediating adhesion and homing [185,269,270]. Anti-CD44 antibodies have been shown to both inhibit AML proliferation, homing, and engraftment, as well as induce differentiation, with a less pronounced effect on healthy hematopoietic cells [269,271]. Post transcriptional splicing of CD44 results in different variants (CD44v) and expression of some of these distinct variants have been shown to be AML specific and to correlate to overall survival [272]. CD44v6 is highly expressed on AML cells and LSCs but only on few normal CD34<sup>+</sup> cells, allowing anti-CD44v6 CAR T cells to mediate potent anti-leukemic effects while sparing normal hematopoiesis *in vitro* [273]. The clinical utility of targeting CD44 is still not determined. The humanized anti-CD44 antibody RG7356 showed poor clinical efficacy in an AML trial with only one of 44 patients achieving CR [274]. An ongoing clinical trial in AML and myeloma is investigating CD44v6 targeting CAR T cells but no results are yet available (NCT04097301).

### CD45

CD45 (PTPRC), a receptor-like protein tyrosine phosphatase, is ubiquitously expressed on all nucleated hematopoietic cells [275]. Hence, it is not a good target for therapy in AML. It is, however, also expressed on AML blasts and therefore currently explored as a target for radiolabeled antibodies intended for myeloablation in conditioning regimens preceding allogeneic stem cell transplantation [276]. Ongoing clinical trials in AML are evaluating anti-CD45 antibodies conjugated to radioisotopes include Iomab-B (NCT02665065) and <sup>211</sup>Astaine-BC8-B10 (NCT03670966). CD45 is not pursued as a specific AML cell surface target.

### *CD70*

CD70, also known as TNFSF7, is the only known ligand for CD27, and the CD70/CD27 interaction is involved in immune activation and lymphocyte expansion [277]. CD70 is normally expressed on activated lymphocytes and dendritic cells but has also been shown to be aberrantly expressed on AML cells and LSCs [278]. Soluble CD27 (CD27s) increase as a result of CD70/CD27 interaction and high levels of CD27s in AML patients at diagnosis have been shown to correlate to poor prognosis. Blocking this interaction with an anti-CD70 antibody resulted in reduced cell growth, increased differentiation, and prolonged survival in xenograft studies of AML in a murine NSG model without effects on HSCs [278]. Current clinical trials in AML and MDS are evaluating the effects of the anti-CD70 antibody Argx-110 also known as cusatuzumab (NCT03030612 and NCT04150887).

### *CD97*

CD97 (ADGRE5) is an adhesion molecule expressed on activated lymphocytes, granulocytes, monocytes, macrophages, and dendritic cells. It is frequently expressed on AML cells and LSCs where it promotes proliferation and maintenance of an undifferentiated state [279]. High CD97 expression has been shown to correlate to *NPM1* mutations and is associated with a poor prognosis [280]. Genetic downregulation of CD97 showed anti-leukemic effect but also affected normal hematopoiesis to some extent, indicating that further research is needed to determine if CD97 targeting would be clinically feasible in AML [279].

### *Additional cell surface markers in AML*

In addition to those described above, many cell surface markers have been suggested to be relevant in AML. These include CD52 [281], CD56/NCAM1 [178], CD73/NT5E [282], CD82 [283], CD89/FCAR [284], CD90/THY1 [285], CD93 [182], CD96 [286], CD98/SLC3A2 [287], CD99 [288], CD105/ENG [289], CD133/PROM1 [290], CD135/FLT3 [291], CD157/BST1 [292], CD200 [293], CD244/SLAMF4 [294], CD300LF [295], CD302/CLEC13A [296], CXCR4 [297], JAM3 [298], GPR56/ADGRG1 [299], and OX40/TNFRSF4/CD134 [300]. Expression of many of these markers were also confirmed in *TP53* mutated AML in **Article IV**. A full list of AML cell surface markers and key references are provided in **Table 2**.

## Therapeutic targeting of cell surface markers

Among the many suggested cell surface markers, most solid data have been provided for CLL-1, CD123, IL1RAP, CD47, TIM-3, and CD33 as targets for therapeutic intervention. The fact that LSCs can change phenotype and increase in frequency after relapse or due to chemotherapy should indicate that a treatment targeting the LSCs would be more effective if introduced early on in treatment schemes [301]. An opposing concern is that a treatment depending on residual healthy immune cells as effector cells might have a reduced or abolished effect if introduced at diagnosis or in close proximity to chemotherapeutic agents. Additionally, hypomethylating agents such as azacytidine has shown to enhance anti-leukemic immune response in AML, potentially affecting other simultaneous immune-based treatments [302,303]. Thus, the optimal timepoint of introducing a treatment targeting LSCs in AML is not known. There are however several ways to utilize the differential expression between leukemic and normal cells when designing therapies targeting cell surface markers [210].

### **Naked antibodies**

The first clinically used antibodies were polyclonal anti-thymocyte antibodies, introduced in 1984 to prevent graft rejection and later graft versus host disease [304]. The first monoclonal antibody in clinical use was an anti-CD3 antibody, introduced in 1986 and used to prevent acute transplant rejection [305]. Since then, monoclonal antibodies targeting a variety of protein have been introduced and are now an important part of clinical practice. Upon binding specific epitopes on target cells, antibodies can inhibit or enhance signaling, opsonize cells for immune mediated cell killing such as ADCC and CDC, and mobilize LSCs from their bone marrow niche, possibly sensitizing them to chemotherapy [180]. The antigen binding fragment (Fab) region of the antibody determines the specificity of the antibody and different binding epitopes in turn can confer different biological effects. The Fc-part of the antibody will affect interactions with effector cells and, for example, antibodies with low fucosylation will induce more potent ADCC killing compare to antibodies with high fucosylation [306]. One example of a dual mode of action antibody is the anti-IL1RAP antibody nidanimab that inhibits IL-1B signaling and induce ADCC in CML and AML [74]. Based on annual sales, the naked antibodies most commonly used in clinical care target TNF $\alpha$  (adalimumab/Humira, infliximab/Remicade, and etanercept/Enbrel), VEGF (bevacizumab/Avastin), or HER2 (trastuzumab/Herceptin), whereas one of the most commonly used antibodies in hematology is the anti-CD20 antibody rituximab/Mabthera [307].

## **Antibody drug conjugates**

Conjugating a cytotoxic drug to an antibody has attracted increased attention during the last years and many ongoing clinical trials are evaluating this approach. Drug conjugate antibodies target an antigen that, upon binding to its target receptor, triggers internalization of the antibody-drug-receptor complex [308]. The cytotoxic payload is often stably linked to the antibody rendering it inert in the blood stream but upon internalization it is cleaved off and activated. Cleaving of the linker can be mediated by the lower pH in lysosomes [249]. Examples of targets for drug conjugated antibodies in myeloid leukemia are CD33 and CD123, and examples of conjugated drugs are calicheamicin-derivates and truncated diphtheria toxins [249,309]. Radioimmunoconjugates are also explored, such as the anti-CD33 antibody conjugated to a radionuclide [310]. A different approach is to direct the antibody to an epitope in the vicinity of the target cells. This has been shown with the radiolabeled anti-CD25 antibody daclizumab in Hodgkin's lymphoma where the antibody is directed towards non-malignant T-cells surrounding the rare Reed-Sternberg cells with subsequent killing of cells in the vicinity regardless of their lack of CD25 expression [311].

## **Bispecific antibodies**

Bispecific antibodies are also gaining increased attention as treatment for hematological malignancies. Here, recombinant antibodies or fragments thereof, are engineered to target two different epitopes. For example, one Fab region may bind to a target-cell-specific epitope and the other to an effector-cell epitope, in hematological malignancies often CD3, resulting in the recruitment and activation of T-cells and subsequent cell lysis of the target cells [312]. Bispecific T-cell engagers (BiTEs) use tandem single chain variable fragments (scFc) to elicit this response, whereas dual-affinity re-targeting antibodies (DARTs) have a different type of linker between the two binding fragments [312]. Ongoing clinical trials are evaluating bispecific antibodies targeting CD3/CD33, CD3/CD123, and CD3/CLL-1 for the use in AML. A tri-specific killer engager (TriKE) fusion protein targeting CD33 and CD16, linked to a modified version of IL-15 intended to engage and activate NK-cells, will soon be tested in a clinical trial for AML [313]. There are also examples of full-length antibodies with dual affinity such as CLT030 targeting CLL-1 and CD3, the full-length construct contributing to a longer half-life and possibly more favorable pharmacokinetic properties [232]. With increased knowledge of cell surface targets on LSCs and immunological cells, combined with improved possibilities to engineer recombinant antibodies, we are likely to see several new drugs entering clinical trials for hematological malignancies in the near future.

## **CAR T cells**

The most recent and very promising approach of immunotherapies are the chimeric antigen receptor T-cells [314]. Initial experiments and clinical trials targeting CD19 in B-ALL show promise with high anti-leukemic effect in heavily pretreated patients [315]. The side effects can, however, be severe, including cytokine release syndrome and neurological toxicity [314]. The fact that CD19 expression is restricted to leukemic target cells and healthy B-cells allows for specific targeting. The lack of cell surface markers with similar specificity in myeloid leukemia has made development of CAR T cells for AML more challenging. Initial preclinical experiments with CAR T cells targeting CD123 showed myeloablative effects, indicating a need for both eradication of the CAR T cells as well as subsequent allogeneic stem cell transplantation [207]. Targeting CD33 would also target HSCs but a suggested way of circumventing this problem could be to subsequently transplant patients with allogeneic cells genetically engineered to not express CD33 [316]. The implementation of a safety switch in the CAR T cells is also being explored in order to increase safety and controllability [317]. Preclinical experiments of CAR T cells targeting CLL-1, CD44v6 and other markers show promise but further investigation is needed to determine their usability in AML [318]. A clinical trial in AML of CAR T cells targeting CD123 is ongoing (NCT03766126), as are trials using compound CAR T cells targeting CLL-1 and CD33 (NCT03795779), and Multi-CAR T cells targeting Muc-1/CLL-1/CD33/CD38/CD56/CD123 (NCT03222674). Many of these therapies will most likely be followed by allogeneic stem cell transplantation and the benefit in AML remains to be determined. Discovery of additional cell surface markers on AML cells with a restricted expression in healthy tissue would further improve the applicability of CAR T cell therapies in myeloid leukemia.

# Present investigation

## Aims

The overall aim for this work has been to identify and characterize the expression of cell surface markers on leukemic stem cells (LSCs) in myeloid leukemia. Through the identification of cell surface markers specifically expressed on LSCs, this thesis hopes to facilitate the development of novel targeted therapies with higher efficiency and lower toxicity in myeloid leukemia.

More specifically, the aims of this thesis were to:

- Study previously described cell surface markers as surrogate markers for leukemic stem cell burden in chronic myeloid leukemia (CML) and their use in predicting therapeutic response (**Article I**).
- Identify new cell surface markers on primitive CML cells by RNA sequencing of FACS sorted cells and evaluate their possible roles as targets for antibody-based therapies (**Article II**).
- Perform arrayed cytokine screens using primary CML cells to identify positive regulators of primitive CML cells and their corresponding receptors (**Article III**).
- Discover new cell surface markers on primitive acute myeloid leukemia (AML) cells in *TP53* mutated AML suitable for antibody-based therapeutic targeting (**Article IV**).

# Results in CML

## Article I

Tyrosine kinase inhibitors (TKIs) have greatly improved the outcome of patients with CML, but not all respond optimally to the initial treatment. The LSC-burden of CML patients at diagnosis has been shown to predict response to TKI therapy [319]. However, current protocols to detect LSC-burden in CML are not easily implemented in a clinical setting. In **Article I**, the aim was to establish a flow cytometry-based protocol to assess the LSC-burden at diagnosis of CML. First, it is shown that of seven cell surface markers previously reported to be upregulated on primitive CML CD34<sup>+</sup>CD38<sup>low</sup> cells, only IL1RAP, CD25 and CD26 were not expressed on corresponding normal hematopoietic stem cells. Furthermore, IL1RAP expression in the CML CD34<sup>+</sup>CD38<sup>low</sup> population at diagnosis was found to be superior in identifying *BCR/ABL1* positive cells. Most importantly, the proportion of CML CD34<sup>+</sup>CD38<sup>low</sup> cells expressing IL1RAP at diagnosis predicted response to TKI treatment. Patients with a low fraction of IL1RAP positive cells showed better molecular and cytogenetic response after 3 as well 6 months of treatment. It was concluded that flow cytometric analysis of IL1RAP on primitive cells from CML patients at diagnosis provides important prognostic information in predicting the response to TKI treatment.

## Article II

Despite TKIs being highly effective for the treatment of CML, few patients are cured. The major drawbacks regarding TKIs are their low efficacy in eradicating LSCs responsible for disease maintenance and relapse upon drug cessation [72]. In **Article II**, RNA sequencing performed on flow-sorted primitive (CD34<sup>+</sup>CD38<sup>low</sup>) and progenitor (CD34<sup>+</sup>CD38<sup>+</sup>) chronic phase CML cells, identified transcriptional upregulation of 32 cell surface molecules relative to corresponding normal bone marrow cells. Focusing on novel markers with increased expression on primitive CML cells, upregulation of the scavenger receptor CD36 and the leptin receptor (LEPR) was confirmed by flow cytometry. A subpopulation of primitive CML cells expressing CD36 was identified and shown to be less sensitive to imatinib treatment. Using anti-CD36 antibodies, CD36 positive cells were targeted and killed by antibody-dependent cellular cytotoxicity (ADCC). In summary, CD36 defines a subpopulation of primitive CML cells with decreased imatinib sensitivity that can be effectively targeted and killed using an anti-CD36 antibody.

## Article III

Aberrantly expressed cytokines in the bone marrow niche are increasingly recognized as critical mediators of survival and expansion of LSCs. To identify regulators of primitive CML cells, a high-content cytokine screen using primary CD34<sup>+</sup>CD38<sup>low</sup> chronic phase CML was performed. Out of the 313 unique human cytokines evaluated, 11 were found to expand cell numbers  $\geq 2$ -fold in a 7-day culture. Focusing on novel positive regulators of primitive CML cells, the myostatin antagonist myostatin propeptide (MSTNpp) was shown to give the largest increase in cell expansion and was chosen for further studies. It was demonstrated that MSTNpp expands primitive CML and normal bone marrow cells, as determined by increased colony-forming capacity. Retention of the immature cell surface marker CD34 was seen during culture of primary CML cells. Furthermore, it was shown that *MSTN* is expressed in CML mesenchymal stromal cells and that MSTNpp has a direct and instant effect on CML cells, independent of myostatin. This is suggested to depend on binding of MSTNpp to the cell surface, resulting in increased phosphorylation of STAT5 and SMAD2/3. In summary, MSTNpp was identified as a novel positive regulator of primitive CML cells and corresponding normal hematopoietic cells.

## Results in AML

### Article IV

AML is associated with an overall poor prognosis, particularly in patients with genetic high-risk disease such as *TP53* mutated AML that often relapse even if they respond to initial treatment. Relapse is thought to occur from residual LSCs unresponsive to standard cytostatic treatment. To date, no specific markers of LSCs in *TP53* mutated AML have been identified that would allow for specific therapeutic targeting of these cells. In **Article IV**, a flow cytometry-based arrayed screen was developed to evaluate the expression 362 different cell surface markers within the CD3<sup>-</sup>CD19<sup>-</sup>CD34<sup>+</sup>CD38<sup>low</sup> subpopulation of *TP53* mutated AML cells and corresponding normal bone marrow cells. SLAMF6 upregulation was identified and could be validated in independent experiments of additional samples. Recombinant anti-SLAMF6 antibodies were shown to induce ADCC mediated cell killing of an AML cell line. The results of this study show that SLAMF6 provides a possible target for antibody-based therapies in *TP53* mutated AML, a disease carrying a dismal prognosis.

## Discussion

The first work (**Article I**) was conducted to compare suggested markers for immature CML cells defined as CD34<sup>+</sup>CD38<sup>low</sup>. Research in general tends to skew towards novelty at the expense of utility. In this study, the aim was to evaluate the expression of previously described markers in CML and to put these findings into the context of our own findings. The conclusions drawn are of interest from a biological standpoint even though clinical implementation of determining LSC-burden via IL1RAP expression at the time of diagnosis might not change the clinical management of CML patients. The obvious limitations of this work are that not all cell surface markers were studied in detail. In retrospect, it would have been interesting to delineate the expression of CD26 as this marker since has been shown to be more relevant than CD25 and CD123 in CML [160,161]. Similar analyses of co-expression have been performed in AML [199], suggesting the relevance of evaluating multiple cell surface markers in myeloid leukemia. It is important to note that no proof of leukemic stem cell potential was provided in this work, the terminology was used on the basis of previous work in the field.

**Articles II** and **III** provide biological insight into CML pathogenesis. They also explore potential novel therapeutic approaches. These might or might not become clinically relevant in the context of CML, considering the extraordinary effects of TKI treatment, but the results further our insight into CML biology. Our use of RNA sequencing on FACS sorted CML cells advanced the field where previous similar efforts have relied on microarray gene expression data [320]. Following the conclusion of this work, acquiring single cell gene expression data in combination with *BCR/ABL1* status has become feasible, providing additional insight into CML subpopulations and biology [321].

The discovery of CD36 expression in CML would certainly have led us to also investigate its relevance in AML. However, Craig Jordan's group explored this in detail in parallel with our work [168]. Our work adds to the insights from Craig Jordan's research by showing the relevance in CML as well as suggesting a potential therapeutic approach by antibody targeting. Both the discovery of MSTNpp and CD36 in CML furthers our understanding of the function and phenotype of immature CML cells. We also show that MSTNpp is relevant in the setting of normal hematopoiesis. An unresolved aspect, not addressed in the present work, is how MSTNpp exerts its function. No receptor for MSTNpp has yet been described and to identify this putative receptor and to further delineate the role of MSTNpp in CML and normal hematopoiesis remain interesting objectives for future research. The methodological approaches used and the results obtained in **Articles I-III** may also have implications for other malignancies, highlighting the value of using a genetically simple disease such as CML to achieve knowledge to be implemented in genetically more complex disease such as AML.

The ongoing work in **Article IV** describes a novel cell surface marker in a genetic subtype of *TP53* mutated AML with very poor prognosis. Many cell surface markers have been suggested to be relevant targets in AML, including CD123, CLL-1 and IL1RAP, but few have been studied in the context of the underlying cause of the disease. There are also other promising therapeutic approaches emerging such as BCL-2 inhibitors [322], and some that targets specific molecular driver lesions such as inhibitors of FLT3 [323], IDH1 [324], and IDH2 [325]. The molecular and phenotypic heterogeneity of AML often provide obstacles in designing and interpreting experimental results. In this study, we focused on a clinically relevant genetic subtype of AML characterized by a *TP53* mutation, to test the hypothesis that specific markers may be upregulated on LSCs in specific subtypes of AML. The identification of SLAMF6 would not have been possible if examining all AML subtypes or bulk leukemia samples. Work is currently ongoing to explore the relation between SLAMF6 and *TP53* mutations, to further investigate the possible function of SLAMF6 in AML, and to study therapeutic targeting of SLAMF6 in preclinical xenograft models of AML. In addition, the expression of SLAMF6 on LSC-enriched AML cells in other subtypes of AML and in solid tumors will be studied.

# Conclusions

The main conclusions from the articles of which the thesis is based upon can be summarized as follows:

## *Article I*

- IL1RAP can be used as a marker for leukemic stem cell burden in CML
- IL1RAP expression at time of diagnosis predicts response to therapy

## *Article II*

- CD36 and LEPR are specifically expressed in immature CML cells compared to corresponding healthy cells
- CD36 expressing cells are less vulnerable to TKI treatment
- CD36 expressing cells can be specifically killed using an antibody targeting this cell surface marker

## *Article III*

- MSTNpp is a positive regulator of immature CML cells and healthy HSCs, possibly through phosphorylation of SMAD2/3 and STAT5
- MSTNpp is produced by stromal cells in CML patients

## *Article IV*

- SLAMF6 is specifically expressed on immature cells from *TP53* mutated AML compared to corresponding healthy cells
- SLAMF6 expressing cells can be targeted and killed using anti-SLAMF6 antibodies

# Concluding remarks

The clinical and biological understanding of myeloid leukemia is rapidly evolving. CML patients now have a life expectancy close to normal, but the prognosis for AML patients remains dismal. The genetically simple disease CML is a good and convenient model of leukemia and studies of this disease may provide important insight into more complex disorders such as AML. Many targeted therapies, including antibodies and small molecule inhibitors, are currently being explored for the treatment of AML [322,324-326]. Some show promising results but a common cure for all AML subgroups remains to be identified.

In this thesis, it is shown that IL1RAP and CD36 are specifically expressed on immature CML cells. These discoveries provide insight into the pathobiology of CML and facilitate future experiments by expanding our knowledge of the LSC immunophenotype. Both IL1RAP and CD36 can also be targeted for specific killing of LSCs in CML. The treatment effects of TKIs are truly remarkable but in order to provide an ultimate cure, additional therapeutic approaches should focus on targeting the residual LSCs. Considering the close to normal life expectancy of CML patients, new drugs targeting these LSCs need to be specific and associated with minimal toxicity. It remains to be determined if IL1RAP and CD36 might serve such a purpose in a clinical context. Both IL1RAP and CD36 have, however, also been found to provide targets in other diseases with more pressing needs for novel therapies, such as AML and solid tumors. The results of this thesis highlight the benefits of using a simpler disease model such as CML to achieve new knowledge with possible application in more complex disorders. The discovery of the role of MSTNpp as a positive regulator of both immature CML and normal healthy cells, also shows that our findings can perhaps be extrapolated and used in other settings, such as *in vitro* expansion of normal HSCs.

There are many suggested cell surface molecules that could be used as targets in treating AML. However, none have both solid evidence of eradicating AML LSCs and are clinically available. The CD33 targeting drug conjugate gemtuzumab ozogamicin is now available after a period of retraction but the clinical effect is restricted to favourable or intermediate risk patients [250]. Other antibodies targeting CD123, CLL-1, CD47, and TIM-3 are under investigation in clinical trials and the results of these trials will be of great interest.

Considering the molecular and phenotypic heterogeneity within AML, this thesis sought to define new cell surface markers in the specific genetic subgroup of *TP53* mutated AML. By focusing on the *TP53* mutated patients with a poor prognosis, we hypothesized that it would be possible to identify markers specifically expressed on LSCs of this molecular subtype of AML. By applying an antibody screen to immature  $CD34^+CD38^{low}CD3^-CD19^-$  cells, we detected high and specific expression of SLAMF6 on immature AML cells. This molecule has previously been suggested as a target in myeloma and CLL but we provide the first evidence of SLAMF6 as a suitable target for antibody-based therapy in AML, targeting the LSC-enriched cellular subpopulation.

Other approaches of directing treatment to specific subgroups of AML based on genetic lesions are being explored in ongoing trials of FLT3, IDH1 and IDH2 inhibitors. There are also ongoing studies of BCL-2 inhibitors, with promising effects across multiple genetic subtypes of AML. Exploring therapies with potential use in multiple subgroups of AML is particularly compelling because of the high costs associated with drug development and the relatively low incidence of AML in each subgroup. The ultimate efficacy of this approach has yet to be determined.

Going forward, we await the results from ongoing clinical trials with great enthusiasm. We also hope for the discovery of additional cell surface markers that may enhance the arsenal in the fight against myeloid leukemia.

# Populärvetenskaplig sammanfattning

Leukemi uppstår till följd av förvärvade genetiska förändringar såsom mutationer eller translokationer som leder till okontrollerad celledelning, minskad celldöd och i vissa fall utebliven cellmognad. Vissa typer av leukemi, så som kronisk myeloisk leukemi (KML) kan idag behandlas framgångsrikt men kräver livslång behandling. Andra former så som akut myeloisk leukemi (AML) kräver lång behandling med höga doser cellgift där dödligheten från både behandlingen och själva sjukdomen fortfarande är mycket hög.

Denna avhandling har undersökt cellytemarkörer, protein som sitter på KML- och AML-cellernas yta, och deras förmåga att särskilja sjuka från friska celler. Med hjälp av cellytemarkörer som uttrycks enbart på leukemiska celler är förhoppningen att kunna utveckla riktade behandlingar som båda minskar biverkningar och i bästa fall kan bota dessa sjukdomar.

I **Artikel I** utvärderades flera idag kända cellytemarkörer i KML och det kunde konstateras att markören IL1RAP kan användas för att kvantifiera den leukemiska stamcellsördan vid diagnos. Denna information kunde sedan användas för att förutsäga hur patienter svarar på behandling med tyrosinkinashämmare.

I **Artikel II** användes sorterade, omogna celler från KML-patienter och friska donatorer för att identifiera nya cellytemarkörer på de leukemiska cellerna. Genom RNA-sekvensering av dessa cellpopulationer genererades en lista över gener med specifikt uttryck i leukemiska celler jämfört med friska celler. Uttrycket av markörerna CD36 och LEPR bekräftades sedan även på proteinnivå. CD36 kunde därefter visas vara användbar som måltavla för antikroppsbaseerad behandling, en behandling som specifikt dödade celler som svarar sämre på gängse behandling.

I **Artikel III** användes en storskalig analys av cytokiner, små peptider som via receptorer på cellytan stimulerar eller hämmar cellers förmåga att dela sig och i vissa fall hjälper leukemin att växa. Denna analys visade att myostatin propeptid (MSTNpp) stimulerar celledelning av KML-celler men även friska blodceller. Därefter visades att MSTNpp bland annat bildas av bindvävs-celler i benmärgen och att effekten sannolikt beror på att MSTNpp aktiverar molekylerna SMAD2/3 och STAT5 som utgör viktiga signalvägar i cellen. Detta innebär att MSTNpp är relevant både inom KML men potentiellt även inom normal blodbildning.

I **Artikel IV** pågår arbetet fortfarande kring cellytemarkörer i den subgrupp av AML som kännetecknas av mutationer i *TP53*-genen, en förändring som medför en notoriskt dålig prognos. Här användes en storskalig antikroppspanel för att leta efter nya, specifikt uttryckta cellytemarkörer på AML-stamceller som ofta tros orsaka återfall i sjukdomen. Resultaten har visat att markören SLAMF6 är specifikt uttryckt på dessa celler och att detta protein kan användas som mål för en antikroppsbasead behandling som experimentellt har visats kunna döda AML celler.

Sammanfattningsvis har delarbetena i avhandlingen identifierat en rad olika markörer på ytan av sjuka celler från patienter med leukemi. Avhandlingen bidrar på så sätt till ökad förståelse för vad som skiljer sjuka leukemiceller från friska blodceller och underlättar vidare forskning inom området. Vidare visas även på experimentell väg att vissa av dessa markörer kan användas som måltavlor för behandling med antikroppar. Förhoppningsvis kan resultaten ligga till grund för utveckling av nya målinriktade behandlingar för patienter med leukemi.

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