Modeling normal and malignant hematopoiesis in vitro. To screen for extrinsic regulators and differentiation therapy.

Hultmark, Simon

2022

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

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To be defended on the 6th of October 2022 at 13:00 in Segerfalksalen, BMC A12, Sölvegatan 17, 223 62, LUND

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LUND UNIVERSITY
Faculty of Medicine
Department of Laboratory Medicine
Division of Molecular Medicine and Gene Therapy

Document name Doctoral dissertation

Date of issue 2022-10-06

Title
Modeling normal and malignant hematopoiesis in vitro to screen for extrinsic regulators and differentiation therapy

Abstract
The incredible thing with blood stem cells, also known as hematopoietic stem cells (HSC), is that they can restore normal hematopoiesis in patients that need a new blood system. Since a prerequisite for successful transplantation is immune compatibility, it requires large donor registries to find a suitable match for a recipient. Unfortunately, there is still a shortage of immune-compatible donors in these registries. Thus one potential approach to improve the quantity and quality of these registries is to expand HSC in umbilical cord blood units. However, robust in vitro expansion of human HSC is not possible yet. Thus, in vitro expansion of HSC is a high-value objective in hematological research.

A common reason patients need a new blood system is blood cancer. An added benefit of transferring someone's else blood system to a cancer patient is that the donor's immune cells can help eradicate the cancer cells. Because the transfer of a new blood system is not without risks, as the donor's immune cells also target normal tissues, physicians will only transplant when the cancer treatment is not potent enough to eradicate the cancer cells. Even though the cancer therapies of some AML subtypes are effective today, the prognosis of most cancer types would improve with new therapies. Thus, developing new therapies is another high-value objective in hematological research.

Here we addressed both of these objectives by using a mesenchymal stroma-based co-culture model for culturing primary acute myeloid leukemia (AML) cells and HSC to identify differentiation therapy of AML (**papers I and II**), improve culture conditions of HSC (**paper III**), and for investigating synthetic lethality in AML (**paper IV**). Thus, the common thread of the four papers included in this thesis is the use of OP9M2 cells to model normal and malignant hematopoiesis.

In **paper I**, we identified a natural product that induces differentiation in AML through activation of the PKC pathway. Moreover, we show that AML with FLT3-ITD or FLT3 mutations are resistant to differentiation, highlighting the importance of neutralizing the effect of mutated FLT3 in differentiation therapy. This study illustrates how small molecule screening and genetic profiling are powerful tools for developing personalized treatments.

Paper II is a small molecule screening protocol based on the OP9M2 co-culture model of primary AML cells. With a flow-cytometry readout, the protocol is highly adjustable to different study objectives, including screening for novel therapeutic agents, drug repurposing, drug synergism, patient selection, mechanism of action analysis, and drug resistance. Methods such as these will continue to be crucial for developing new therapies to improve outcomes for many patient groups.

In **Paper III**, we identified potential regulators of HSC, which we screened by shRNA knockdown in the OP9M2 model. However, it did not identify any candidates, likely due to a sub-optimal screening methodology. Still, the list of potential regulators could be helpful for similar studies. Improving in vitro culture conditions remains a high-value objective as cellular therapies will continue to be essential for treating hematological diseases.

Paper IV shows that STAG1 and STAG2 have a synthetic lethal interaction in primary AML cells. Thus, targeting STAG1 or STAG2 in STAG1- or STAG2-null AML is potentially a new precision medicine for molecular targeted therapy. This study shows how an in-depth understanding of disease heterogeneity and subtype-specific weaknesses is critical for developing precision medicine.

Supplementary bibliographical information	
ISSN 1652-8220	**Language** English
ISBN 978-91-8021-281-6	**Number of pages** 118

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ISBN 978-91-8021-281-6
ISSN 1652-8220
Doctoral Dissertation Series 2022:120

Printed in Sweden by Media-Tryck, Lund University
Lund 2019

"Where there is love there is life"
Mahatma Gandhi

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Preface

Writing the preface marks the end of my Ph.D. journey and the beginning of something new that I have yet to discover. I joined the research school in stem cell biology almost ten years ago due to my fascination for stem cells. I was especially fond of embryonic stem cells and the questions in vogue back then, such as how you explain the pluripotent stem cell state and how well epigenetic reprogramming works. Coming to Lund, I realized that Woods's group, which worked on obtaining hematopoietic stem cells from pluripotent stem cells, was already filled, and so was Flygare's group, which aimed to transdifferentiate fibroblasts to red blood cells. Luckily, Mattias, who would later become my Ph.D. supervisor, also had an exciting project about improving cell culture conditions for hematopoietic stem cells. Even though this study focused on an adult instead of embryonic stem cells, it still excited me due to its relatedness to the stem cell state.

We would later find out that the project was not working, which was a harsh realization for me as a young, naive, and ambitious student who thought hard work always pays off. I was devastated by the realization that my efforts were in vain and how this jeopardized my chances of fulfilling my dream of getting a Ph.D. in biomedical science. I was lucky, though, that Mattias assembled a small team with the addition of my colleagues Aurelie and Fredrik that would help me get started on a new study that aimed to identify new differentiation therapy for acute myeloid leukemia.

At that time, my passion for science was no longer there, not by choice, but my body had had enough, and I would later understand that I could put a checkmark on all the major indications for burnout. Therefore, I am very thankful to you, Mattias, who helped me get through that difficult time when I did not have the same energy as I used to. I am also very thankful to you, Aurelie, who I would say was my first mentor in the lab—working closely with someone with much more experience than I had greatly impacted me. I am also very thankful to you, Fredrik, for your support with chemicals and scientific input from project initiation to the publication of the paper. It is hard to believe that I will soon complete this major project that I started on January 1, 2013. Once again, thank you, team leukemia, i.e., Mattias, Aurelie, and Fredrik. You helped me get here to where I am today, soon ready to fulfill my dream of getting my Ph.D. in biomedical science.

Simon, Malmö, August 2022

Original papers

Paper I

Simon Hultmark, Aurélie Baudet, Ludwig Schmiderer, Pavan Prabhala, Sara Palma-Tortosa, Carl Sandén, Thoas Fioretos, Rajkumar Sasidharan, Christer Larsson, Sören Lehmann, Gunnar Juliusson, Fredrik Ek, and Mattias Magnusson. Combinatorial molecule screening identifies a novel diterpene and the BET inhibitor CPI-203 as differentiation inducers of primary acute monocytic leukemia cells. Haematologica, 2021.

Paper II

Aurélie Baudet, **Simon Hultmark**, Fredrik Ek, and Mattias Magnusson. Small Molecule Screening of Primary Human Acute Myeloid Leukemia Using Co-culture and Multiplexed FACS Analysis. Bio-protocol, 2022.

Paper III

Simon Hultmark, Rajkumar Sasidharan, David Yudovich, Ineke De Jong, Jonas Larsson, Hanna Mikkola, and Mattias Magnusson. Identification of extrinsic regulators of human hematopoietic stem cells using an shRNA screen in mesenchymal stromal cells. Manuscript.

Paper IV

Agatheeswaran Subramaniam, Carl Sandén, Larissa Moura-Castro, Kristijonas Žemaitis, Ludwig Schmiderer, Aurelie Baudet, Alexandra Bäckström, Elin Arvidsson, **Simon Hultmark**, Natsumi Miharada, Mattias Magnusson, Kajsa Paulsson, Thoas Fioretos, and Jonas Larsson. Inducing synthetic lethality for selective targeting of acute myeloid leukemia cells harboring STAG2 mutations. Haematologica, 2022.

Abbreviations

AHD-AML	Antecedent hematological disorder acute myeloid leukemia
ALL	Acute lymphocytic leukemia
AML	Acute myeloid leukemia
APL	Acute promyelocytic leukemia
Ara-C	Cytosine-arabinoside
ARCH	Age-related clonal hematopoiesis
ATO	Arsenic trioxide
ATP	Adenosine triphosphate
ATRA	All-trans-retinoic acid
RBC	Red blood cells
CCUS	Clonal cytopenia of unknown significance
CHIP	Clonal hematopoiesis with indeterminate potential
CHOP	Clonal hematopoiesis with oncogenic potential
CR	Complete remission
CRh	Complete remission with partial hematologic recovery
CR_{MRD-}	complete remission without detection of minimal residual disease
EMA	European Medicines Agency
FAB	French-American-British
FACS	Fluorescence-activated cell sorting
FDA	The United States Food and Drug Administration
GvHD	Graft versus host disease
GvT	Graft versus tumor
HCT	Hematopoietic cell transplantation
HLA	Human leukocyte antigens
HSC	Hematopoietic stem cells
HSCT	Hematopoietic stem cell transplantation
HSPC	Hematopoietic stem and progenitor cells
ICUS	Idiopathic cytopenia of undetermined significance
IDUS	Idiopathic dysplasia of undetermined significance
IFNα	Interferon-α
IPSS	The International Prognostic Scoring System
ITD	Internal tandem duplication
LAA	Leukemia-associated antigens
LSC	Leukemic stem cells
LT-HSC	Long-term hematopoietic stem cells
MDS	Myelodysplastic syndrome
MDS/MPN	Myelodysplastic/myeloproliferative neoplasms
MHC	Major histocompatibility complex
mIDH	mutated IDH

miHA	Minor histocompatibility antigens
MPN	Myeloproliferative neoplasms
MPP	Multipotent progenitors
MRD	Minimal residual disease
NSC	Neoplastic stem cells
OS	Overall survival
RARα	Retinoic acid receptor-α
PKC	Protein kinase C
PML	Promyelocytic leukemia protein
RIC	Reduced-intensity conditioning
s-AML	Secondary acute myeloid leukemia
ST-HSC	Short-term hematopoietic stem cells
t-AML	Therapy/toxin/radiation induced acute myeloid leukemia
TKD	Tyrosine kinase domain
TKI	Tyrosine kinase inhibitors
TLS	Tumor lysis syndrome
TLV	Tandvårds- och läkemedelsförmånsverket
UCB	Umbilical cord blood
VAF	Variant allele frequency
WHO	World Health Organization

Introduction of the hematopoietic system

Hematopoiesis

The human body is composed of trillions of cells that serve its multicellular community. The cells are spectacularly diverse in function and shape even though they share the same genome, and a long time ago, at least while not cancerous, they stopped serving their individual needs. Instead, they are part of a mission to serve the body as a whole as they have organized over evolutionary time into organs and tissues to sustain human life by optimizing for the survival and reproduction of the species[1].

This thesis focuses on one of the many sub-systems in the body, the hematopoietic system, composed of organs and liquid tissue, including the blood cells, bone marrow, spleen, thymus, and lymph nodes. Its role is to provide the body with all functionally distinct blood cells throughout life, in a process referred to as hematopoiesis (from the Greek hemato [blood] + poiesis [to make], or "to make blood"). In adults, hematopoiesis occurs mainly in the bone marrow (medullary) but may occur under pathological conditions in extramedullary sites such as the spleen, liver, and lymph nodes[2].

The formation of new blood cells is a unidirectional differentiation process by which the blood system's effector cells are formed from less specialized progenitors. As progenitors differentiate to more specialized cells, they become committed to specific lineages and cell types, involving dynamic changes in proliferation kinetics and loss of differentiation potentiality (Figure 1)[3]. The flux into each lineage is controlled by demand in a highly regulated process, so the right type of cells is produced at the right time. The estimated turnover is remarkable, with nearly one trillion (10^{12}) cells per day[4].

These specialized cells are the hematopoietic system's effector cells that serve the multicellular community by a remarkable width in function and dedication to specific roles. They all circulate in the cardiovascular system, but not all stay within the blood vessels as some cells use it to reach different tissues where they exert their different functions[5].

The different types of blood cells

Broadly, the blood cells are classified based on their color in white or red. The white cells, also known as leukocytes (from Greek leuko [white] + cytes [cells]), compose both myeloid (from the Greek myelo [marrow] + oid [form], or "from the marrow") and lymphoid (from the Greek lymph [water] + oid [form], or "from the water") cells that are responsible for protecting the body against bacteria, virus, cellular debris, and diseased cells. A critical function of these cells is to discriminate self from non-self and healthy from diseased cells. Depending on the mechanism in which they do that, the leukocytes are divided into innate immune cells that can launch a non-specific defense immediately and adaptive immune cells with an antigen-specific response, which takes time to develop, but, once developed, can act immediately on the foreign antigen[6].

Moreover, megakaryocytes and osteoclasts are myeloid cells but are not formally considered leukocytes. Megakaryocytes are responsible for producing thrombocytes (platelets) that prevent bleeding in the cardiovascular system and osteoclasts for degrading bone tissue that balances the bone remodeling process. The last but most abundant myeloid cell type is the red cells, also known as erythrocytes (from Greek erythro [red] + cytes [cells]) that circulate the cardiovascular system and transport O_2 and CO_2 between the lungs and the tissues[7]. See Figure 1 for an overview of the classification.

Stem cells: general principles

Stem cells today are defined as having the capacity to self-renew and give rise to differentiated cells. However, the meaning evolved since it first appeared in the late 19th and early 20th centuries. When the German biologist Ernest Haeckel first used the term in phylogenic tree diagrams called stem trees, the stem cell referred to a presumed ancestral unicellular organism from which all multicellular organisms evolved[8-10]. Later he used the term in embryology to refer to the fertilized egg that can give rise to all cells in the body[10,11]. Other early uses of the term described the primordial germ cells that give rise to the germ cells[10,12], and a common precursor postulated according to the unitarian theory to give rise to the red and white blood cells[10,13]. Of these, only the common precursor to the red and white blood cells has kept the term stem cell as the definition evolved.

Why stem cells are (unfortunately) called stem cells and not root cells

When the term stem cells started to appear in the literature, it lacked the functional component of today's definition. Instead, the focus was on the relational linkage between cells, apparent in the term stem, which usually refers to a plant's main body

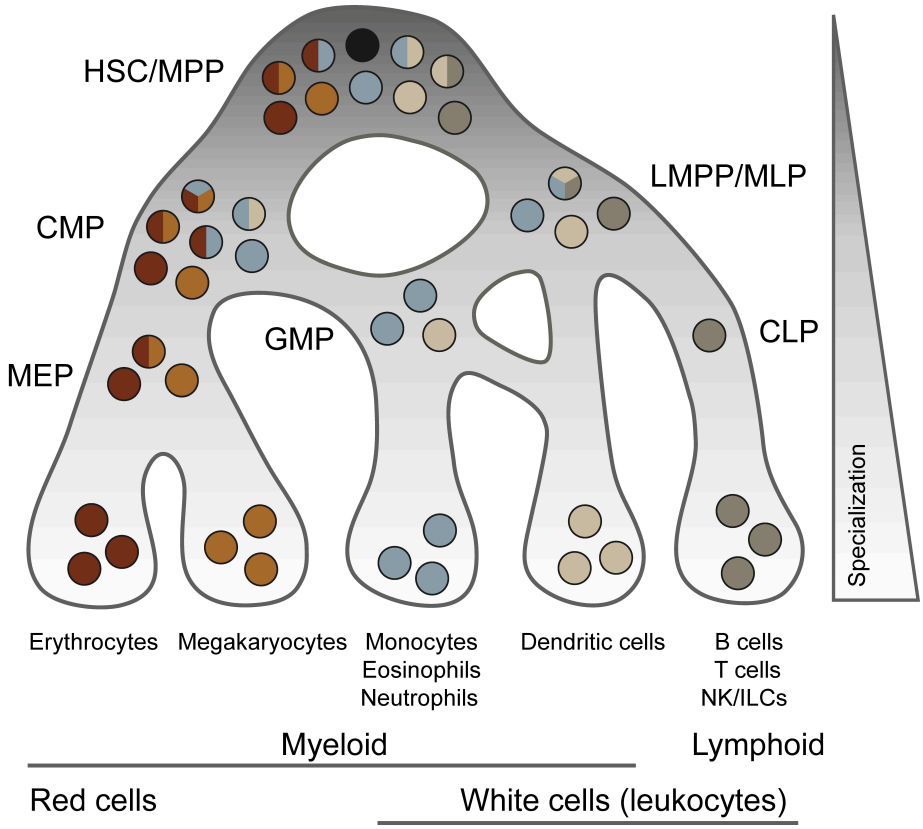

Figure 1: Model of steady-state hematopoiesis that visualizes heterogeneity in differentiation potential among stem and progenitor cells. In the traditional tree diagram, the circles or nodes are intuitively understood as a homogenous population of cells, which is in stark contrast to the more recent appreciation for the heterogeneity in differentiation potential within the hematopoietic stem and progenitor populations at the single-cell level. Also, since the differentiation potential in the traditional tree is based on transplantation and colony assays that measure differentiation during artificial stress and not in unperturbed hematopoiesis, it raises the question of the construction of a hematopoietic tree that visualizes differentiation in homeostasis conditions should also include multipotent progenitors (MPP) as the root of hematopoiesis. With inspiration from Elisa Laurenti and Berthold Göttgens, this Figure fuses their "cloud model" with their proposed alternative visualization of multiple parallel differentiation trajectories3. While the traditional tree diagram depicts a hierarchal tree of differentiation and self-renewal, it fails to convey the experimental observations of heterogeneity in differentiation potential within bulk populations. Therefore, new alternative trees need to emphasize the prevalence of early lineage choice and the cells' biological behavior in an unperturbed condition to depict the hematopoietic differentiation tree during steady-state conditions accurately. A cell's differentiation potential is indicated with colors in this Figure that allow for tracing possible differentiation trajectories by connecting the root cells in the HSC and MPP compartment through the commonly defined progenitor compartments to the specialized cells without visualized trajectories. The black circle represents a cell with multipotentiality, and the one, two, and three colored circles represent cells with uni-, bi-, and trilineage potential.

or stalk or, more generally, as the main section of something. In the stem trees proposed by Ernest Haeckel, the stem referred to the connection between the root node (e.g., the stem cell) and the downstream" children" nodes (e.g., progenitors or specialized cells). Only after Ernest McCulloch, James Till, and others in the early 1960s found

experimental methods to prove a common precursor to the red and white blood cells was a functional component added to the definition[14-16]. In retrospect, it would arguably be more intuitive to refer to stem cells as root cells, emphasizing their parent's root node location rather than the relational linkage between cells in the hierarchy.

The role of stem cells

The stem cells' location at the root of their hierarchies makes them fundamental to the integrity of the tissues they reside in as they supply new specialized cells as they are lost. Therefore, stem cells need to be stable and maintain themselves throughout life in a self-renewal process that sets them apart from progenitor cells that only transiently maintain themselves.

During embryonic and fetal development, cells with stem cell properties can arise and disappear or arise and stay as the body develops into an adult. Interestingly, it is possible to capture cells in development and keep them in a self-renewal state *in vitro*. Hence, these stem cells are maintained by the *in vitro* culture condition that stabilizes them in a self-renewal process without induction of differentiation. One example is the cells from the blastocyst's inner cell mass that only transiently exists during embryonic development but, when *in vitro* cultured, can be maintained as self-renewing embryonic stem cells with the ability to give rise to all cell types of the body if inserted back to the blastocyst[17]. Moreover, in relation to differentiation potential, a stem cell cannot exist without a stem structure; hence, stem cells need to give rise to at least one specialized cell type. With the current stem cell definition including a root node location, self-renewal capability, and differentiation potential, cells with stem cell properties have been identified in multiple tissues in the body, such as the central nervous system, intestine, skin, and blood[18,19].

The hematopoietic stem cell

This thesis focuses on one particular stem cell, the hematopoietic stem cell (HSC), which is the root cell of the hematopoietic system responsible for replenishing all blood cell types. They reside mainly in the bone marrow, but a small percentage can also be found in the spleen and the lungs, as well as circulating in the cardiovascular system in a pattern controlled by the circadian clock[20-22]. Since, by definition, a stem cell is self-renewing and has differentiation potential. Much research on HSC has focused on identifying the HSC population and defining the stem cell state's underlying cellular and molecular processes.

Introduction

After McCulloch, James Till, and others proved the existence of a common precursor of red and white blood cells[14-16], advancement in fluorescence-activated cell sorting (FACS) allowed for sorting out cells based on cell surface markers to test their functionality by transplantation. The transplantation assay gave rise to the concept of long-term HSC (LT-HSC), short-term HSC (ST-HSC), and downstream progenitors as defined by their sustained engraftment and differentiation potential after transplantation. Accordingly, LT-HSC has multilineage potential and long-term engraftment–measured 16 weeks after the transplantation–in both primary and secondary transplanted recipient mice[23-25]. They are thereby fulling the criteria of self-renewal by proving the existence of self-renewing HSC with multilineage potential in the primary recipient. While ST-HSC has multilineage potential, they do not engraft long-term in secondary recipients, thus lacking self-renewing capacity[23-25]. The progenitors have a restricted bi- or uni-lineage potential, and lack self-renewal capacity as their contribution to hematopoiesis is only transient after transplantation[23-25].

The HSC core defining qualities and progenitor boundaries

Following the FACS and the transplantation assay to test for HSC self-renewal, numerous studies have linked the self-renewal capacity to dormancy depth and a cell's time to exit quiescence[26-29]. With a variety in division rate between different HSC subsets from 2 to 12 divisions per year (in mice)[28,29], there is a dormancy gradient amongst the most primitive cells. This heterogeneity in quiescence correlates directly with the self-renewal capacities in transplantation assays[28,29]. Hence, FACS and transplantation provided an assay that could test self-renewal capacity in sorted populations and single cells and proved that the dept of quiescence correlates with greater self-renewal capacities defining quiescence as crucial for the HSC cellular identity.

In addition to quiescence, several additional cellular and molecular processes have been identified as key to the stem cell state. They include HSC dependency/reliance on autophagy[30,31], glycolysis[32,33], low mitochondria activity[34-36], and low protein translation rate[37]. However, some of the critical stem cell characteristics are not absolute since HSC occasionally during homeostasis, but especially by stress can exist quiescence and start dividing to return to the dormant state after a transient proliferation[28,38], although the number of times for this might be limited[39]. In contrast, and as a demarcation to HSC, progenitors are highly proliferative, metabolically active, and use oxidative phosphorylation[26,33,36]. In summary, unique quiescent and metabolic properties guard the HSC state's core defining self-renewal trait and differentiate HSC from downstream progenitors.

The role of HSC and progenitors in steady-state hematopoiesis

Until recently, the capacity for self-renewal and differentiation potential in transplantation assays defined a hematopoietic stem cell and a progenitor cell. However, distinguishing a stem cell from a progenitor with the transplantation assay imposes criteria to the definition that is not relevant for normal steady-state conditions. For instance, during the transplantation, the cells are exposed to *ex vivo* conditions and are tested for their homing ability to a radiation-damaged bone marrow, where they need to survive without losing their cellular properties until the bone marrow has healed. Consequently, the transplantation assay defines what a stem cell is on its ability to withstand an artificially high-stress level in addition to self-renewal and differentiation potential.

New studies using non-disruptive models have provided a new appreciation for the stem and progenitor cells' contribution to hematopoiesis during homeostasis. One of these studies used a sleeping beauty transposon that, when activated by doxycycline, integrates into unique sites that can be used for lineage tracing after a chaise period without inducing stress to the cells[40]. They found that the multipotent progenitors (MPP)–that lack self-renewing capacity in transplantation assays–and not HSC replenished, for the most part, the myeloid lineage with new cells during steady-state conditions.

Thus, this study put forward a paradigm where HSC still are the hematopoietic system's root cells but do not actively replenish the hematopoietic system with mature cells. Instead, their functional value is a cellular backup for progenitors that serve as the "root cell" of hematopoiesis in steady-state conditions throughout life (Figure 1). Thus, the importance of progenitors and the role of the HSC was clarified by studies using assays that removed artificial stress resistance in the definition of the cell types' functions.

The differentiation potential of HSC and progenitors

Given the apex position in the hematopoietic system hierarchy, HSC should have the ability to give rise to all downstream cell types. Hence, together with self-renewal, multipotentiality has intuitively been a key defining factor for the HSC identity. However, within the apex of the hematopoietic system, differentiation-restricted stem and early progenitor cells could, with a similar result to multipotent stem and progenitors, replenish lost cells and provide for the system's changing needs. This raises the question if the hematopoietic system requires stem cells that are all multipotent and, consequently, if multipotency is a strict HSC defining quality.

Since cell fate decisions are executed in individual cells, the study of a cell's differentiation potential is carried out at the single-cell level. These studies using single-cell transplantation and *in vitro* culture indicate that few single cells read out as multipotent within the stem and progenitor compartment[41-43]. However, some caution

to these results needs to be considered due to the artificial stressors transplantation assays expose single cells to and how a low contribution to some lineages can cause a misrepresentation of the single cell's true lineage potential. Similarly, the artificial environment of *in vitro* culture assays with unnatural instructive signals and cellular stress may misrepresent a cell's true differentiation potential. Finally, fate choices that are executed before the first cell division will hide the cell's true potential.

Nonetheless, in favor of a HSC population containing stem cells with restricted differentiation potential, transplantation of single HSC has shown consistent differentiation potential over serial transplantations, indicating an intrinsically determined differentiation bias[44-46]. Compatible with the idea that the HSC population–defined by self-renewing capacity–comprises a heterogeneous stem cell population with multi and restricted differentiation potentials. Taken together, differentiation potential analysis of single cells has shown that there is an abundance of unipotent progenitors and that cell fate choices occur high up in the hierarchy, possibly already in the HSC population (Figure 1). Furthermore, these studies suggest that the capacity for self-renewal alone, without the need for demonstrated multipotentiality, is enough to define a hematopoietic cell as a stem cell.

The clinical relevance of hematopoietic stem cell transplantation

At about the same time the current definition of stem cells was worked out in the 1960s–including self-renewal and multipotent differentiation potential–the practice of hematopoietic stem cell transplantation (HSCT) also started to take form. Highlighting its success, an estimated one and a half million transplants have since been performed to treat and sometimes cure patients with tumors and malignant and non-malignant blood diseases[47]. Noteworthy, HSCT is the only FDA-approved (The United States Food and Drug Administration) stem cell therapy to this date[48]. That is likely due to the close connection of HSC to the blood vessel vasculature and homing ability to their semi-solid bone marrow niches, qualities that make replacement of HSC relatively easy to perform compared to cellular systems situated within solid organs. Moreover, continued advancements in conditioning regimens, control of transplant-related toxicity, donor matching, and gene therapy access promise further improvement on current limitations. Therefore, as new technologies develop, the hematopoietic system is well-positioned to continue to lead the way in the cell and gene therapy arena.

Current indications for hematopoietic stem cell transplantation

The HSCT procedure is divided into two major categories depending on if the stem cell graft is from another person (allogeneic, from the Greek állos [other] + genḗs [kind], or "another person") or the patient's own cells (autologous, from the Greek autós [self] + logous [related], or "same person"). Which source to choose depends on whether the malignancy is blood-related and where the disease has its cellular origin in the hematopoietic hierarchy. A healthy allogeneic stem cell graft is needed when the disease has a known cellular origin in the $CD34^+$ hematopoietic stem and progenitor cells (HSPC). Whereas, if the disease originated further down the hierarchy or outside the blood system, the preferred approach is autologous transplantation, where the patient's healthy $CD34^+$ HSPC are reinfused back after the patient has gone through a therapy regimen[49].

Data from all patients

Data collected from mostly European transplantation centers in 2019 by the European Society of Blood and Marrow Transplantation (EBMT) show that 59% of the performed transplantations were autologous and 41% allogeneic[50]. The most common indications are lymphoid malignancies (64%), followed by myeloid malignancies (25%), and non-malignant diseases (7%). The major indications for allogeneic transplantation, and thus diseases with a cellular disease originating in the HSPC population, include acute myeloid leukemia (AML) (38%), acute lymphocytic leukemia (ALL) (16%), non-malignant disorders (14%), and myelodysplastic syndrome (MDS) or myelodysplastic/myeloproliferative neoplasms (MDS/MPN) (12%). The major indications for autologous transplantation where the patients' HSPC are healthy are plasma cell disorders (55%), non-Hodgkin lymphoma (27%), Hodgkin lymphoma (9%), and solid tumors (6%)[50].

Data from pediatric patients

In pediatric patients (<18 years at transplant) that make up every fifth transplantation, it is more common with allogenic (pediatric 78%; adults 38%) than autologous (pediatric 22%; adult 62%) transplantations, and indications differ from the overall population that includes all age group[50,51]. Non-malignant disorders such as PID, BMF, Thalassemia, IDM, and Sickle cell proportionally differ the most for pediatric allogeneic transplantations (43.9% compared to 12% in all ages), and indications for non-blood-related cancer differ the most for pediatric autologous transplantation (81% compared to 7.4% in all ages)[51]. In summary, it is more common in pediatric patients with allogeneic transplantations and treatment of non-malignant diseases than in adults (>18 years at transplant). As plasma cell disorders and lymphoma are rare in pediatric patients, autologous transplantations are mostly used for treating malignancies outside the blood system.

Conditioning

Before a patient can receive a stem cell transplantation, it is crucial to deplete the diseased cells and the patient's endogenous blood system to make room for a healthy stem cell graft. In the case of an allograft, it is also important to deplete the patient's immune cells to prevent graft rejection. The process of preparing for a successful graft is called conditioning. Depending on the disease, conditioning employs chemotherapeutics, radiotherapy, targeted therapy, and immunosuppressant to eradicate the diseased cells and facilitate the highest possible engraftment with the lowest amount of short- and long-term toxicity. However, there are often conflicts between the benefit and risks of many conditioning regimens. Therefore, when physicians choose a conditioning regimen, they must balance the risk-benefit ratio tailored to the patient's age, co-morbidities, and underlying disease specifics[52].

Myeloablative conditioning

The myeloablative regimen is the most intense protocol that facilitates effective engraftment but is associated with transplant-related complications, toxicity, and mortality. Partially due to the initial time without a well-functioning immune system and the damage it causes to the mucus membranes that can result in opportunistic bacterial, viral, and fungal infections[53,54]. The high transplant-related mortality has limited myeloablative regimens to younger and more fit patients under the age of 50 years[54].

Reduced-intensity conditioning

However, luckily older and unfit patients can be treated with reduced-intensity/toxicity conditioning (RIC) or non-myeloablative regimens combined with immunosuppressant drugs that decrease treatment-associated mortality and the risk of graft rejection by the endogenous immune system. While these less toxic regimens are not as effective in creating niche space and killing malignant cells, they have increased the three-year overall survival (OS) by reducing non-relapse mortality (NRM) in the fragile population. Together with improved supportive care, nonmyeloablative conditioning has allowed treating unfit and older patients up to 70 years, providing a much larger patient group access to HSCT[54-56].

In non-malignant diseases, RIC can be preferred over myeloablation in some cases because of reasons specific to the disease. For instance, RIC is recommended for patients with immunodeficiency diseases because the immunocompromised state prevents graft rejection and provides a proliferative advantage to the grafted lymphoid cells[57,58].

Another example is patients with Fanconi anemia who suffer from an impaired response to DNA damage, which makes them unfit for genotoxic conditioning and, due to increased apoptosis, extra vulnerable to graft versus host disease (GvHD), which happens with the grafted immune cells attacks the host tissues. The good thing is that

the condition provides a proliferative advantage to the grafted HSPC, making it possible to achieve desired engraftment levels with reduced non-genotoxic conditioning or even without conditioning at all. However, Fanconi anemia patients still run a risk of graft rejection and are sensitive to GvHD, making it essential to have an HLA-matched allogeneic donor or use autologous gene therapy[59,60].

Non-genotoxic myeloablation

However, RIC might not be enough for some non-malignant disorders such as lysosomal storage diseases and hemoglobinopathies to clear the necessary niche space needed to achieve the desired engraftment levels[61]. Since harsher myeloablative conditioning is not a good option in these patients, that are often very young, as it can lead to long-term complications, such as infertility, organ toxicity, impaired growth, and secondary malignancies, it shows the need for improved conditioning methods with fewer complications[62].

It is thus promising to see non-genotoxic myeloablation being developed based on antibodies targeting hematopoietic cell surface markers. One of these approaches uses anti-human CD117 (also known as c-kit and SCFR) that blocks the receptor-ligand interactions, thereby preventing the signaling transmission critical to HSC survival[63,64]. Another approach uses a hematopoietic cell-specific depletion strategy by targeting the pan-hematopoietic marker CD45[65].

Both approaches promise to condition patients without genotoxic side effects. If proven safe and efficient, these approaches are especially relevant for non-malignant diseases where safety and efficacy are the primary objectives without the need to deplete malignant cells in the process. Since conditioning-related toxicity is a significant burden for patients and treatment costs, developing nongenotoxic conditioning for non-malignant diseases would be a crucial advancement allowing more patients access to HSCT and improving their lives without adding an extra financial strain to the health care system.

Control of transplant-related toxicity in allogeneic transplantations

Historical overview

Humans never shared cells or organs successfully until the middle of the last century, when the modern era of organ and hematopoietic cell transplantation (HST) started. The first successful allogeneic HST was performed in 1959 to treat leukemia. Because the transplantation was between genetically identical twins, it was free from immunological complications with no risk for GvHD or graft rejection. Although the lethal dose of radiation was not enough to clear the disease that later relapsed, the transplantation proved that injection of bone marrow cells protected against radiation and highlighted the need for improved treatments[66]. However, it is noteworthy that in

one exceptional case from this time, a lymphoma patient who received chemotherapy followed by autologous transplantation spent the next 21 years in remission[67,68].

Initially, treating malignancies in conjunction with HSCT would prove challenging, particularly for allogeneic transplantations. Often, the host's immune system would reject the grafted cells if the conditioning regimen were not strong enough, or the immunocompromised state would lead to fatal infections, or fatal complications would also arise from the grafted cells attacking the host tissues. A review from 1970 concluded that none of about 200 allogenic transplantations had been successfull[68,69].

However, progress in the late 1960s and early 70s in HCT studies on dogs revealed the importance of histocompatibility testing and immune suppression to limit GvHD and prolong post-transplantation survival[70,71]. In addition, animal studies on mice had shown that HCT from unrelated donors creates an immune reaction that can eliminate the remaining leukemic cells after conditioning, although in conjunction with GvHD[72]. A report in 1979 proved this observation in humans by correlating GvHD with delayed relapse[73]. Hence, these early studies discovered the importance of matching the donor and recipient histocompatibility to prevent GvHD and the positive effect transplantation can have on eliminating malignant disease via the graft versus tumor (GvT) reaction.

The role of the major histocompatibility complex

The major histocompatibility complex (MHC), known in humans as the human leukocyte antigens (HLA), are the cell surface proteins that make histocompatibility matching essential. The MHC are central to the vertebrate immune system by their role in displaying intracellular and extracellular peptides to T cells. There are two major types of MHC, MHC class I which displays intracellular peptides to CD8 cytotoxic T-cells, and MHC class II, that display extracellular peptides to CD4 helper T cells. During the CD8 and CD4 T-cell receptors education in the thymus, thymic cells first positively select maturing T-cells with receptors that bind innate peptides. Then they eliminate the T cells with receptors that bind with too high affinity to innate peptides to prevent them from causing an autoimmune response. This procedure allows T cells to recognize foreign peptides displayed on MHC molecules as they bind them with a higher affinity than self-peptides. Upon binding, they either initiate a cell-killing process (MHC I – CD8 T-cell interaction) or an immune response (MHC II – CD4 T-cell interaction)[74].

If only a few alleles of each HLA type existed, matching donors and recipients would not be so challenging. However, the HLA loci contain six genes, and hence 12 alleles, with the most variable allele sets expressed in vertebrates. The three most variable genes, HLA-A, HLA-B, and HLA-DRB1, contain 243, 499, and 321 alleles, respectively[75]. This unusually high variability was favored during the vertebrate evolution since a high allele frequency in a population's gene pool made the detection of a larger number of pathogen antigens possible. A concept demonstrated by some alleles being better than others in detecting certain viruses and parasites[75,76].

However, major and minor immune compatibility issues stem from this system at transplantation. The major issue is due to the high allele frequency and the fact that when T cells are educated to recognize innate peptides displayed on one set of MHC molecules, they will reject cells expressing another MHC set, even if the peptides are the same. The minor immune compatibility issue stems from germline-encoded differences resulting in altered peptide sequences that the T cells may recognize as foreign.

These reasons explain why the recipient's T cells may reject the allografted cells and why the allografted T-cells would attack the host cells. Hence, to circumvent this, the recipient's T cells are sometimes depleted before an allogeneic graft to prevent graft rejection, and the grafted cells are sometimes depleted of T cells to prevent graft versus host disease. In conjunction with immunosuppressive medicine, these measures have increased the OS of patients receiving allogeneic transplantations[77].

The graft versus host and tumor response

The GvT effect is the immune cells' attack on cancer cells after the conditioning regimen. It involves T cells, B cells, NK cells, and dendritic cells and is present in both autologous and allogeneic transplantations[78]. In allogeneic transplantations, the HLA alleles between donor and recipient must be matched to avoid acute GvHD. However, GvHD and GvT still play a role even in a fully matched transplant setting. This histoincompatibility comes from antigens derived from germline differences in the donor and the recipient, resulting in peptides not recognized as innate by the allogeneic T cells. Since these antigens are expressed on both cancer cells and normal cells, it explains why it is impossible to decouple the GvHD and GvT responses from each other. However, there is a GvT-specific response in allogeneic and autologous transplantations due to mutations in the coding regions of the cancer cells, which results in cancer-specific peptides, and there can also be a GvT response to abnormally expressed innate proteins in the cancer cells. These minor histocompatibility components, which influence GvHD and the GvT response, will be introduced in more detail below.

The role of minor histocompatibility antigens

The antigens derived from germline-encoded differences between the donor and the recipient are known as minor histocompatibility antigens (miHA). Whole-exome sequencing comparing nine donor-recipient pairs found an average of 6445 nucleotide sites that changed the amino acid sequence and thus could present as a foreign peptide[79]. However, since only 1 in 200 of these sites is predicted to be expressed and displayed as a peptide by the HLA molecules[80], it would translate to about 32 miHA capable of inducing a T cell response. A broad expression pattern of miHA results in widespread GvHD, while a localized expression to the hematopoietic system results in

a GvT reaction. The GvHD and GvT reaction is not possible to decouple from each other since the expression pattern of miHA is not possible to control. However, if an HLA-matched sibling is available, it is possible to reduce the amount of miHA by about half compared to if an HLA-matched unrelated donor was used[81]. Moreover, matching gender when transplanting to males reduces the number of miHA since T-cells from a female donor do not recognize antigens expressed on the Y chromosome as innate. Hence, T cells from a female donor are more likely to launch an immune response when transplanted to male recipients than to female recipients. Furthermore, male donor cells transplanted to female recipients are more likely to be rejected because of this reason[82].

The role of neoantigens

Another category of antigens, referred to as neoantigens, is specific to the GvT response. Thus, they differ from the miHA, which results in an inseparable GvT and GvHD response in the allogeneic transplantation setting. Neoantigens are created from mutations in malignant cells and are thus cancer-specific peptides displayed by the HLA molecules and detected as foreign by T cells[77]. However, the mutational burden between cancer types differs, which means that some cancers generate more neoantigens than others. For instance, melanoma and lung cancer have a high mutational burden and will frequently generate neoantigens, whereas AML, ALL, and chronic lymphocytic leukemia (CLL) have one of the lowest mutational burdens of all cancers and will only occasionally generate neoantigens[83]. Hence, cancer types with a higher mutational burden and, thus, more neoantigens are more likely to evoke an immune response.

The role of leukemia-associated antigens

Leukemia-associated antigens (LAA) are GvT-associated antigens. These antigens are innate proteins expressed in tissues that are not surveyed by the immune system. That means that they are expressed in immune-privileged sites, such as the eyes, testis, and the central nervous system, in the placenta during fetal development or occasionally expressed in low levels in some adult cells. T cells may recognize LAA as foreign peptides because they lacked exposure to them during their maturation in the thymus. Since the abnormal gene expression in some cancer cells can result in overexpression of LAA, they may subject themselves to a T cell-mediated immune response[77]. A gene expression analysis of 116 AML samples found a significant correlation between improved survival and expression of at least one LAA, indicating their significance for disease control[84].

The role of the non-T-cell-mediated response

Although the GvT and GvHD are mostly T cells driven, B cells can also mount an immune response resulting in GvT and GvHD against miHA and an antileukemic effect towards LAA[85-87]. The dendritic cells, whose function is to present antigens and activate T cells, can contribute to GvT and GvHD depending on the antigen and type

of transplantion[88,89]. Natural killer cells, a type of innate lymphocytes, are the first cells to recover after transplantation and their promptness of engraftment correlates with a reduced risk of relapse without inducing GvHD[90].

The importance of conditioning

Conditioning is an integral part of the GvT response since the disease burden at diagnosis is already so high that the immature cells, also known as blasts, have quashed the immune system's capacity to suppress the disease. Hence, the elimination of cancer cells by conditioning helps the immune system regain control over the disease. Even with the GvT effect, it is imperative to achieve a deep remission since detecting minimal residual disease (MRD) correlates with a 4.51 times higher risk for AML relapse with a similar risk for both myeloablation and non-myeloablation regimens[56]. Thus, the conditioning aims to obtain as deep remission as possible, helping the immune system combat and hopefully eliminate the disease.

The more potent graft versus tumor response in allogeneic transplantation

The GvT effect is present in both autologous and allogeneic transplantations but is more robust in the latter due to major immune incompatibilities. Diseases such as lymphoma are normally transplanted with the patient's own cells since the HSPC compartment is not mutated. However, if the disease is aggressive, such as in relapsed lymphoma, allogeneic transplantation is an option due to the hope that the more robust GvT response from an HLA mismatch will result in a deeper remission, even though GvHD might be a problem. If the increased GvT response justifies the higher risk of NRM is not clear, however, as it has not been tested in a randomized study[91].

Finding a suitable donor for allogeneic transplantations

Since patient/donor matching is the major aspect that influences the outcome of allogeneic transplantation, much effort has focused on understanding how to select the best donor. It has led to the development of different criteria for what is considered an HLA match. For instance, in the US, an 8/8 match (eight HLA matched alleles) has been a standard, and in Europe, a 10/10 match. However, with larger patient registries, finding more than one match with the current standards is becoming more common, which means that improved matching criteria would benefit some patients. Therefore, new recommendations for patient registries and clinics are to use ultra-high-resolution HLA typing with 12/12 HLA allele matching, prioritize the lowest aged donor, and match the patient/donor on previous cytomegalovirus infection, gender, and ABO blood type[92,93].

The probability of finding a match

When searching for an HLA-matched donor, siblings are the first choice, which in Europe makes up 29% of the allogenic transplantions[50]. The probability of finding an HLA-matched sibling is 25% with one sibling, 44% chance with two siblings, 58% chance with three siblings, and 68% chance with four siblings[94]. For those patients that do not have an HLA-matched sibling, the second choice is to look for adult unrelated HLA-matched donors that make up 50% of the transplants. If that does not provide a match either, a haploidentical matched family member can be an option that makes up 18% of the transplants or an umbilical cord blood (UCB) unit that makes up 1.6% of the transplants[50]. The use of unrelated and haploidentical donors has steadily increased during the last decade, while cord blood decreases in popularity with a peak in use cases between 2008-2012.

Peripheral blood is the most common source of hematopoietic stem cells

What else is noteworthy is that peripheral blood as a stem cell source in transplantations has grown in popularity over the last 25 years. From being used equally to bone marrow extraction in the year 2000, peripheral blood is today used in >80% of the allogeneic transplantations and has been the primary choice for autologous transplantation since the middle of the 90s and is today essentially the only source used (99,8%)[50]. Contributing factors that made peripheral blood the preferred stem cell source are likely the convenience for the donors, the faster hematopoietic recovery, and the larger amounts of HSPC that can be collected from the blood thanks to mobilization agents like G-CSF and plerixafor (a CXCR-4 inhibitor) that promotes the egress of HSPC from the bone marrow to the blood circulation[68,95,96].

Ethnic origin determines the chance of finding a donor

It is also noteworthy that patient registries and cord blood banks do not provide all people an equal chance of finding a match. For example, in the US, the chance of finding an 8/8 match from adult donors for patients with white European ancestry is 75%, and a ≥7/8 match is 97%. In contrast, patients of black south or central African descent have the lowest probability, with only a 16% chance of an 8/8 match and a 66% chance for a ≥7/8 match[94]. Hence, there is an unequal distribution of probability for finding an HLA-matched donor depending on the patient's ethnic origin.

More donors are needed

Today there are 36 million donors registered worldwide. Tobiasregistret was initiated in Sweden in 1991 to meet the unmet and growing demand for unrelated HLA-matched donors. Volunteers between 18-35 years can register themselves at Tobiasregistret and have the chance to save someone's life. More donors are still needed since 30% of the patients do not find an HLA match in the registries and thus have to try their luck with inferior alternatives with more transplant-related complications[97].

Autologous gene therapy

Autologous HSPC gene therapy is a possible alternative to allogeneic transplantation for monogenic non-malignant blood disorders. These disorders are caused by single mutations, which result in altered proteins that affect normal cell maturation or cell function. Since there is a lack of optimally matched allogeneic donors, there is a need for gene therapy as a safer option for mismatched allogeneic transplantation as it removes the transplantation-related complications associated with GvHD. Hitherto, two products have secured regulatory approval for use in Europe, Strimvelis (Orchard Therapeutics) for ADA-SCID and Zynteglo (BlueBird Bio) for transfusion-dependent β-thalassemia patients over the age of 12 years without β0/β0-genotyp[61]. However, In Sweden, Strimvelis is currently evaluated by the council for new therapy (NT-rådet), and Zynteglo was denied approval due to the cost of 17 million SEK per patient, which was considered too high to justify the benefit[98]. Hence, gene therapy is still in an early phase, but with several additional products about to be launched in the coming years, gene therapy is expected to grow in importance.

The potential risk of neoplastic development

Lentiviral vectors are the primary vector choice for gene transfer due to the better safety profile than previously used retroviral vectors. The improved safety profile is mainly due to the genomic integration that predominantly occurs in gene bodies over promoters, resulting in a lower risk of inducing aberrant gene expression changes[61]. However, due to the unspecific nature of the genomic integration, there is still a risk that it can lead to deregulation of genes, as exemplified in a β-thalassemia study where one patient had a vector integration in the *HMGA2* gene resulting in its activation and as a result an expansion of a dominant but benign clone[99]. Hence, for gene therapy products like Zynteglo, it is recommended to do annual follow-ups for the first 15 years to monitor any sign of leukemia or lymphoma development[100].

Gene transfer optimization

Another unique aspect of the gene therapy treatment modality is the procedure where the patient's cells are harvested and put in a brief *in vitro* culture for genetic manipulation. Since the time in the cell culture can induce transcriptional and cellular changes that may reduce the number or comprise the functionality of the long-term repopulating cells. Research in this area has aimed to optimize cell culture conditions to minimize the loss of HSC function and improve viral transduction efficiency while keeping the FDA recommended of less than five integration copies per genome[101]. These efforts have included defining the ideal HSPC starting population for viral transduction, using viral vector entry enhancers, and optimizing cell culture conditions[102-105].

Increased possibilities with CRISPR-Cas9

Furthermore, the toolbox for genetic modification has recently increased with the opportunity to do site-specific genome editing and targeting with techniques such as CRISPR-Cas9. For instance, this technology opens up the possibility to inactivate harmful genes, disrupt the expression or binding of transcriptional repressors, perform site-specific corrections, and insert healthy genes in genomic safe harbors that provide predictable therapeutic gene expression without causing aberrant expression changes to nearby genes[61,106]. Currently, two clinical trials are evaluating the use of CRISPR-Cas9 to disrupt the expression of *BCL11A, a* transcriptional repressor of fetal hemoglobin, which would provide a treatment option for β-thalassemia patients[61]. In summary, if proven safe, efficient, and cost-effective, the HSPC gene therapy field has an exciting future ahead as it can provide a potential cure to more patients with non-malignant disorders that do not optimally match with an HLA donor.

Ex vivo expansion of hematopoietic cells

From a clinical standpoint, the driving forces behind the study of HSC self-renewal come from the benefit that larger amounts of transplanted donor cells would have for patients. Since the transplanted cell number has a linear correlation to the recovery time of the new blood system, the cell dose is a fundamental aspect of the transplantation's success[107]. Furthermore, without ex vivo expansion, at best, the same number of HSPC that was collected can be transplanted. Thus ex vivo expansion is a research area with high clinical potential. In line with this, in paper III of this thesis, we aimed to improve current culture conditions by identifying extracellular proteins that support HSC *ex vivo*.

The clinical benefits that improved *in vitro* culture conditions would bring are multifold, including expanding cells in cord blood units to an adequate cell dose for safe and efficient transplantation to adults. In addition, the increased availability of cord blood units for adult patients would improve HLA matching and potentially increase the demand for cord blood as a complementary source of unrelated HLA-matched donor cells. Further, the accelerated donor reconstitution by the transplantation of more cells reduces the risk of opportunistic infections and graft rejection and may also reduce the intensity of conditioning needed for certain non-malignant diseases. Finally, it would reconcile with advances in genetic manipulation techniques that may lead to more innovative autologous gene therapies. Moreover, research on both normal and malignant cells benefits from culture conditions that maintain cellular properties and expand cell numbers[108]. Hence improved culture conditions also contribute to discoveries in basic and preclinical research.

The relevance of ex vivo culture for umbilical cord blood transplantation

Even though patients could benefit from *in vitro* expanded cells from adult donors, it is not required since collecting enough cells for reasonably safe and efficient transplantations is possible. However, the number of cells obtained from cord blood units is often insufficient for transplantation to adults. A study that looked at the US banked cord blood inventory found that only 30% of the units met the criteria of acceptable total nucleated cell count and CD34+ content for transplantation to 30kg patients, and a mere 4% of the units to 70kg patients. With a double-unit cord blood transplantation, the units that met the minimum criteria for 30kg patients increased to 74% and for 70kg patients to 22%[107]. Hence, the current situation is that only a few units have enough cells to be an option for adult patients.

A known positive attribute to the UCB source is the lower number of mature T cells in the graft, which means that a less stringent HLA matching is required compared to the adult donor sources[109]. However, acute GVHD is still comparable to adult sources with the current HLA matching standard for cord blood. Thus anti-thymocyte globulin (ATG) that removes T cells from the UCB grafts is sometimes used to prevent severe GvHD[110]. However, its use is controversial since studies have shown that it increases overall mortality[111-113]. Furthermore, although chronic GVHD might be reduced compared to other donor sources, it remains a significant complication[110]. Hence, to optimize the outcome of cord blood transplantation, improved HLA matching is necessary, with higher HLA typing resolution required for cord blood banking in the future.

Another known attribute of the UCB source is its delayed reconstitution regardless of whether it is a single or a double unit graft, which increases the risk of opportunistic infections and graft failure[107]. These concerns have questioned the sustainability of cord blood banking, especially when haploidentical transplantations have faster reconstitution kinetics and acute and chronic GvHD is comparable to UCB. Consequently, haploidentical transplantations have significantly lower NRM and better OS and, thus, are often preferred over UCB when no adult HLA-matched unrelated donors are available[114-116].

However, transplanting *ex vivo* expanded grafts could make UCB a better source than haploidentical donors. First, the increased cell dose will translate to quicker reconstitution and better OS by reducing NRM. The shorter time to reconstitution that *ex vivo* expansion protocols lead to is evident in the clinical trials that have shown a median time of engraftment (neutrophil count ≥500 cells per µl of blood) of 13-21 days compared to 26-28 days with non-expanded UCB units, which is comparable to mobilized peripheral blood with the median time of 13-15 days to engraft[117]. Second, the increased cell dose will improve HLA matching by increasing the available units meeting the acceptable cell dose criteria for transplantations to adults[107,118].

Although initial results from clinical trials look promising, the realization of USB *ex vivo* expansion products will not come without hurdles to solve. One can anticipate

logistical challenges in shipping expanded cord blood units from the centralized production facilities to the final destinations. The *ex vivo* culture that can take 7 to 15 days, depending on the protocol, with the added risk of infections in the process, can make this approach impractical for patients in immediate need of a graft. Hence, shorter expansion protocols are desired or off-the-shelf solutions that can be thawed and infused without delay[119]. Although concerns for graft failure have so far not been realized in the current trials, long-term follow-up will be important to confirm that LT-HSC were not affected by the *in vitro* culture. Finally, a cost-benefit analysis has not been done for these emerging products where none has reached the market so far[120].

Introduction of myeloid neoplasms and leukemia

Malignant disease from a multicellular species perspective

How malignant disease develops from premalignant to late-stage cancer can be understood from a multicellular species perspective. Multicellular life forms evolved because of the survival benefits of the group of cells over some form of unicellular living. At this point, it created a new evolutionary pressure that suppressed cell-level fitness (i.e., the reproductive success of the individual cell) to promote organism-level fitness. This development incentivized cooperation between cells, promoting increased cell specialization and higher-level functioning of the multicellular body. Aktipis, C. A. et al. (2015) conceptualized these processes as the five foundations of multicellularity: proliferation inhibition, controlled cell death, creation and maintenance of the extracellular environment, division of labor, and resource allocation. In addition, tumor suppressors and immune cells evolved to protect the organism from cheating cells in more complex multicellular species. Cheating cells stop following one or more of the foundations of multicellularity, which leads to increased cell-level fitness that threatens the organism by uncontrolled proliferation, inappropriate cell survival, environment degradation, dysregulated differentiation, and resource monopolization, all features of neoplastic development to late-stage cancer[121]. Thus, cancer can be regarded as a manifestation of cells cheating the features of cooperation that evolved to promote organism-level fitness. Together, this viewpoint creates a philosophical and practical framework from which we can understand how premalignant and malignant diseases evolve.

Clonal evolution towards chronic and acute neoplasms

Introduction to hematological cancer

Approximately four of every ten people in the US will be diagnosed with cancer at some point during their lifetime[122]. That makes cancer the second most common cause of

death (21% of all deaths) after heart disease (23% of all deaths), and together, they make up the majority of all death causes with a wide margin[123]. Of all cancer types, blood cancer makes up approximately 4.6% of the diagnosed cancers and 1.9% of the cancer deaths after breast/prostate cancer and lung and bronchus cancer, which are both more common and more deadly[124].

Blood cancer is a neoplasm (from Greek "new creation") with an origin in blood cells with abnormal proliferation and differentiation. The stage when that threat becomes acute due to the overproduction of blasts is when the neoplasm is called malignant or cancerous. The three major types of malignant hematopoietic neoplasms are classified by disease location and solid or liquid manifestation, with lymphoma being the most common (48% in the US by 2020 statistics), followed by leukemia (34%) and myeloma (18%)[122]. However, the introduction of this thesis will go deeper into the disease evolution of leukemia with a specific focus on the myeloid branch and briefly mention lymphoma and myeloma.

Leukemia

Leukemia (from Greek "white blood") is a "liquid malignancy" that manifests as an accumulation of white blood cells in peripheral blood. The affected cells are mostly leukocytes (white blood cells) but can also be immature precursor cells of the megakaryocytic and red blood cell lineage in some cases of acute leukemia. Generally, the initial genetic lesion that results in the first clone, also known as the cell of origin, occurs in the HSPC population. However, in rare leukemias, the initial transforming event and driver lesion can occur in CD34- downstream progenitors as acute promyelocytic leukemia (APL) is an example of[125,126]. As the disease evolves, it spreads from the bone marrow to the blood and the extramedullary sites of hematopoiesis, such as the spleen, liver, and lymph nodes[127].

Leukemia is classified into slower-growing chronic leukemia and faster-growing acute leukemia. The defining feature is simply the percentage of blasts in the bone marrow or the blood where chronic leukemia has a cut-off of <20% blasts and acute leukemias ≥20% blasts, with the exception of some translocations that can directly qualify chronic leukemia as an acute form regardless of the blast count[128]. Furthermore, what matters for the subtype categorization of leukemia is the presence of specific molecular lesions, as well as the lineage and cell type of the affected cells that are either myeloid (granulocytes, monocytes, megakaryocytes, and erythrocytes), lymphoid (B, NK, and T cells), or of ambiguous lineage[129].

Lymphoma

Lymphoma (from Greek "water tumor") is a malignancy characterized by swollen masses, also known as tumors, in the lymphatic system that usually emerges in the lymph nodes. In most cases, lymphoma originates in lymphoid cells (B, NK, and T cells), but in rare cases can also originate from myeloid and mesenchymal cells, at which

point the diseases are confusingly referred to as sarcomas from the cell of origin perspective but grouped with lymphomas due to the location of the tumors in the lymphatic system[130]. Benign lymphoid neoplasms can look similar to malignant neoplasms but are slow-growing and do not readily spread to additional sites like aggressive lymphoma[131].

Unlike leukemia, which in most cases is a disease with an HSPC origin, lymphoma affects mostly late-stage differentiated B and T cells that are particularly susceptible to transformation events. Especially B cells risk accumulating genetic lesions during T cell-induced affinity maturation of the B cell receptor in the germinal centers. Also, B and T cells undergo extensive clonal expansion after antigen exposure and are long-lived as memory cells, which increases their susceptibility to transformation events relative to other immune cells[132]. However, the initial molecular lesions that lead to lymphoid neoplasia can still occur throughout the hematopoietic hierarchy. While the majority of the lymphoid neoplasms develop from molecular lesions in late-stage differentiated cells, some lesions occur in transitory precursor T cells in the thymus or precursor B cells in the bone marrow. It is also clear that the initiating lesions in some lymphomas can occur in the long-lived HSPC population, demonstrating the existence of premalignant lymphoid stem cells at the very top of the hierarchy[49,133].

The broadest classification divides lymphoma into Hodgkin's and non-Hodgkin's lymphoma. Hodgkin lymphoma (10% of all diagnosed lymphoma), named after the doctor who first described this distinctive type in 1832, contains two kinds of lymphoma characterized by large mature multinucleated B cells[122,130,134]. The remaining lymphomas labeled non-Hodgkin (90% of diagnosed lymphoma) include mature B-cell lymphomas, mature T- and NK-cell lymphomas, and soft-tissue and myeloid sarcomas referred to as histolytic and dendritic cell neoplasms. These more specific classifications have replaced the term non-Hodgkin's lymphoma, which is too heterogeneous to be diagnostically useful[122,130]. However, the term non-Hodgkin lymphoma is still used in contexts that do not require a precise classification.

Myeloma

Myeloma (from Greek "marrow tumor"), also known as multiple myeloma and plasma cell myeloma, is a "solid cancer" with tumors growing in the bone marrow. Myeloma originates from plasma cells that are non-dividing antibody-producing cells formed from differentiated B cells. The first molecular lesions leading to myeloma are acquired in the germinal center during the brief period of about three weeks when the B cell receptor undergoes affinity maturation to produce antibodies with higher antigen affinity. However, the first lesions are typically not enough to cause myeloma, as pre-malignant states characterized by uncontrolled proliferation and overproduction of monoclonal antibodies are often diagnosed before myeloma. It is first when the tumors multiply to the degree that leads to a defined set of symptoms that the disease is called myeloma[135]. Symptoms of myeloma include an increased bone breakdown by activated osteoclasts, low blood counts by competition of bone marrow space, and kidney

damage/failure by increased secretion of antibodies[136]. Myeloma is the only malignant disease in this category of blood cancers, but the World Health Organization (WHO) classification describes several pre-malignant conditions that may advance to myeloma[130].

The phases of cancer evolution

A one-hit lesion leading directly to a malignant state is rare; instead, almost all malignancies originate in a premalignant condition that progresses through an accumulation of genetic lesions to an acute and life-threatening disease. Therefore, as eloquently summarized by Valent P. et al. (2019) and briefly described below, cancer evolution often occurs in phases[137]. Some people are from the germline predisposed to developing a neoplasm from lacking a tumor suppressor or having a pro-oncogenic leasion[138,139]. Nevertheless, what marks the first phase is the initial genetic lesion or lesions that result in a persistent clone or clones with increased cell-level fitness. These clones are usually slow-cycling or dormant and consequently incapable or need time to create a premalignant neoplasm, thus representing a neoplastic precursor state that is only possible to detect by deep sequencing for the hematological-neoplasm-associated lesions[140,141]. In the next phase, the clones acquire driver lesions that lead to clonal expansion and an increased spatial dominance over normal cells making the organ gradually oligo or monoclonal with measurable clinical effects on normal hematopoiesis. With additional lesions, the clones may stay dormant, slow cycling, or continue expanding depending on the lesion and the cooperative lesions. From this premalignant stage, as soon as the additional driver lesions result in further sub-clonal expansion, usually with a high proliferative rate, the neoplasm advances to a life-threatening condition that will lead to death unless treated[137,142]. Consequently, cancer evolution usually requires cooperation between different pro-oncogenic lesions and tumor suppressors that accumulate over time, often years, resulting in sub-clones with a high proliferation rate and poor differentiation capacity that characterizes the malignant state.

CHIP and CHOP–clonal precursor states with indeterminate and oncogenic potential

As described above, the first stable clones that expand from the initial genetic lesions are not necessarily potent enough or require a latency period before they develop into a premalignant state. Indeed, with whole-exome sequencing, it was recently discovered that elderly individuals often carry clones with one or more lesions associated with hematological neoplasms despite being healthy. These healthy individuals have clones with a variant allele frequency (VAF) of > 2%– this cut-off is necessary because clones with hematological-neoplasms-associated lesions would otherwise be ubiquitous–and

have not had a persistent reduction of mature blood cells, also known as cytopenia (from Greek "cell poverty") that lasted for more than four months[140,141,143]. Thus, they are diagnostically not qualified for a premalignant condition even though they carry lesions associated with neoplastic development. With the above criteria, studies show that about 1% of healthy individuals below 50 carry clones with hematological-neoplasm-associated lesions, which increases to about 6% at age 60 and 18% at age 90[141,144-146].

Because of the increased frequency with age, the finding was referred to as age-related clonal hematopoiesis (ARCH) but later named clonal hematopoiesis with indeterminate potential (CHIP) to distinguish it from premalignant conditions that have a clinical presentation[141,144]. Although the risk of developing hematological cancer increases with CHIP, the risk is modest, with an absolute conversion rate of 1.0% per year, which means that few individuals will develop a disease[146]. However, clones with known disease-related or disease-specific genetic lesions or clones with multiple low-risk lesions increase the risk to a degree where most individuals will develop a disease at some point in their lives. Thus, these high-risk clonal precursor states have been suggested the name clonal hematopoiesis with oncogenic potential (CHOP)[137,142]. Consequently, as single defects, CHIP-associated mutations in genes such as *DNMT3A, TET2, ASXL1, SF3B1, GNB1, SRSF2, IDH1, IDH2,* and *GNAS* represent background lesions that may progress into a neoplasm with the aid of other cooperative lesions or loss of tumor suppressors. While multiple CHIP lesions or CHOP lesions such as *BCR-ABL p210, JAK V617F, KITD816V, RUNX1-RUNX1T1, CBFβ-MYH11, FIP1L1-PDGFRA, and FLT3-ITD, TP53, KRAS, and NPM1 mutations* indicate a high certainty that the affected individuals will develop a malignancy sometime in their lives[142,147]. Because of the involvement of CHOP-associated lesions in disease progression and relapse, they are attractive therapeutic targets for pharmaceutical companies, which is evident by the already approved therapies against *BCR-ABL, FIP1L1-PDGFRA, JAK2 V617F,* and *FLT3*-ITD mutations[142,147].

Even if early detection of CHIP and especially CHOP lesions certainly is desired, the current consensus seems skeptical that an NGS-based screening of the general population would be worthwhile given its costs. However, as NGS-sequencing is becoming cheaper and more widely used, it is anticipated to lead to more frequent detection of CHIP and CHOP lesions. Therefore, treatment strategies need to be developed for health care practitioners to ensure adequate monitoring and early treatment and, inversely, prevent the risk of neglect and potentially harmful overtreatment of these clonal conditions[141,148,149].

CCUS–clonal cytopenia of unknown significance

As CHIP and CHOP progress to a stage where it is diagnostically recognizable in peripheral blood tests, occasionally, the clinical manifestation is not enough to meet the minimal criteria for a myeloid neoplasm even though there is a detectable clone and a persistent cytopenia that has lasted for more than four months. This intermediate stage is called clonal cytopenia of unknown significance (CCUS)[150]. CCUS resembles MDS from the manifestation of cytopenia but lacks other MDS-related characteristics and has no or only mild <10% cell dysplasia (from Greek "abnormal development"; i.e., cells with abnormal morphology and function) of a given lineage[140]. Similar to the division of CHIP and CHOP, individuals with CCUS can be divided into low and high-risk disease progression based on the type of lesions, the number of lesions, and the clone sizes measured by VAF analysis[151].

However, the exact classification of CCUS and even if this classification should remain is up for debate. From the beginning, the cut-off for CCUS was set at a VAF>2%, similar to CHIP and CHOP. However, this cut-off seems too low because clones with low penetrance are unlikely to cause cytopenia, which at a low VAF is more likely due to secondary reasons–see idiopathic cytopenia ("cytopenia with unknown cause") of undetermined significance (ICUS) below. Accordingly, a group of researchers has proposed increasing the cut-off to a VAF≥20% instead[149]. However, at a VAF ≥20%, the disease progression rate to a myeloid neoplasm is >95% measured over a 10-year interval. Such a high conversion rate begs the question if these patients should be considered to have an early pre-leukemic myeloid neoplasm instead of CCUS with a necessary revision of the WHO classification of myeloid neoplasms[152]. Because the awareness of clonal precursor states is relatively new, classifying these premalignant sub-states may need some reiterations before practitioners in the field reach a consensus. Thus, more discussions and data will determine whether CCUS is removed or stays with some alteration, possibly with an increased VAF cut-off and a more precise risk stratification.

ICUS and IDUS–cytopenia or dysplasia of unknown cause and significance

Sometimes individuals are diagnosed with cytopenia or dysplasia without having underlying genetic lesions and other characteristics of hematological neoplasms. Thus, if neoplastic cells detected by sequencing cannot explain why the patient has cytopenia or dysplasia, the condition goes under the term ICUS or idiopathic dysplasia of undetermined significance (IDUS)[150]. Therefore, other causes of cytopenia or dysplasia need to be considered, such as inherited bone marrow failure, pure red cell aplasia, viral or bacterial infections, vitamin B12 or copper deficiencies, and chronic kidney or liver failure[140]. However, as soon as hematological-neoplasm-associated lesions are detected, the diagnosis changes to CCUS or a chronic neoplasm[150].

Chronic myeloid neoplasms

Chronic neoplasms are heterogeneous lymphoid and myeloid conditions with dysregulated differentiation, uncontrolled proliferation, or both. Thus, at a minimum, these conditions cheat the multicellular community by not following the rules of division of labor or proliferation inhibition. However, other types of cheating may also occur, especially when the diseases progress. These include avoiding the rules of controlled cell death, not contributing to resource sharing, and maintaining the local environment[121,129,130].

If blast cells in the chronic myeloid neoplasm increase to ≥20% in the blood or the bone marrow, it is called an acute phase, blast crisis, secondary AML, or blast- or leukemia transformation, which essentially means that the uncontrolled proliferation of blasts reached an acute phase analog to acute leukemia. However, in non-leukemic chronic neoplasms, i.e., conditions that do not cause abnormal proliferation in granulocytes and monocytes, fatal complications can occur without leukemia transformation from issues stemming from bone marrow failure, cytopenia, infections, severe blood clots, bleedings, or organ damage. Thus, the disease presentation varies considerably between the conditions, with median survival ranging from >25 to 2.2-0.6 years[153-159]. That means that the chronic conditions with the worst prognosis are similar or just slightly better than AML, with a median survival of six months in cases where transplantation is not an option[160].

WHO divides chronic neoplasms into four major groups: MDS, myeloproliferative neoplasms (MPN), MDS/MPN, and myeloid/lymphoid neoplasms with eosinophilia and rearrangement of PDGFRA, PDGFRB, or FGFR1, or with PCM1-JAK2. Arguably, mastocytosis, which consists of several subtypes, could be included as an additional group as it did prior to the 2008 update, from which it moved to MPN. However, in 2016 it was removed from MPN without being re-established as an independent group. Therefore, without creating a group of its own and not being listed under an existing group, it appears that WHO made mastocytosis go rogue, which should not be possible in a classification scheme[129,161,162]. The section below will introduce MDS, a group of conditions with cytopenia and usually dysplasia, MPN, disorders with abnormal proliferation without dysplasia, and MDS/MPN, which include MDS and MPN hybrid conditions with both abnormal proliferation and dysplasia.

Myelodysplastic syndromes

MDS are a collection of diseases with dysregulated differentiation as a hallmark feature, thereby cheating the multicellular cooperation by not contributing to the division of labor[121,163]. As a result, patients with MDS suffer from cytopenia in one or more lineages that usually also coincide with dysplasia in precursor and mature blood cells[129]. Depending on the affected lineage or lineages, common symptoms include frequent infections and fever from leukopenia, unusual paleness, fatigue, and poor concentration

from anemia, or easily bleeding and bruising from thrombocytopenia that can have a substantial impact on everyday life and be fatal even in cases where the MDS is not progressing to an acute leukemia[164].

MDS mainly affects older people with a median age of 71 years, where 77% of the patients are >60 years[155]. The older age for MDS is also naturally reflected in the incidence rate with 5.3-13.1 cases per 100,000 people per year in the population as a whole compared to 75-162 cases per 100,000 people aged ≥65 per year[165].

What is common for all MDS is cytopenia in at least one lineage. Thus, if there is persistent cytopenia in at least one lineage and not high enough blast counts for an AML diagnosis, there is a good chance the patient is suffering from MDS. MDS classification separates cases into single lineage dysplasia (MDS-SLD) and multilineage dysplasia (MDS-MLD). In cases with a del(5q) abnormality, the classification changes to MDS with isolated del(5q), the only genetic lesion defining a specific MDS subtype. In cases when physicians discover ring sideroblasts (RS)–a type of nucleated red blood cells (RBC) that cannot incorporate iron into hemoglobulin and thus transport oxygen–in the bone marrow to a level that exceeds ≥15%, that is mentioned such as MDS-RS-SLD or MDS-RS-MLD. Moreover, if there are high blast numbers in the bone marrow or the peripheral blood, this is mentioned in the classification as MDS with excess blasts (MDS-EB). In rare cases that do not fit any of these categories, the MDS is called MDS unclassifiable (MDS-U) with the added notion of how they are different[129].

The International Prognostic Scoring System (IPSS) for MDS divides patients into five risk groups based on the level of cytopenia, marrow blasts, and cytogenic profile. The clinical outcome for patients varies widely, with the lowest risk group having a median of 5.4 years survival to the highest risk group with 0.7 years survival. As expected, the higher the risk group is, the higher the risk is that the MDS progresses to acute leukemia[155,156].

The only curative treatment for any chronic myeloid neoplasm is allogeneic HSCT owing to the origin of these diseases in the HSPC population. Therefore, if there is an available immunocompatible donor, MDS patients up to the age of 70-75 who are fit for HSCT and at high risk for disease progression are directly given HSCT. Others with lower risk can initially be monitored and transplanted as the disease progresses. The most fragile patient group that cannot tolerate the conditioning regimen and those waiting for a donor are offered supportive care in the form of transfusions or growth factors to compensate for the lack of functional effector cells in the affected lineage or lineages. Other treatment options are Lenalidomide for low-risk del(5q) MDS patients, immunosuppressive treatment for certain patients, and hypomethylation agents for high-risk groups. Investigational therapy involves targeted therapy such as IDH1 and IDH2 mutation inhibitors (ivosidenib and enasidenib, respectively), a BCL2 mutation inhibitor (Venetoclax), modifiers of P53 signaling (ARP-246 and ALRN-6924), and a TGFβ inhibitor (Luspatercept) for MDS-RS[156].

Myeloproliferative neoplasms

MPN are a heterogeneous group of diseases with uncontrolled proliferation as a central feature. This leads to environmental degradation in some subtypes, which could be considered a secondary feature. Therefore, MPN are cheating the multicellular community by not following the rule of proliferation inhibition and, in some subtypes, by not contributing to a normal turnover of the extracellular environment[121,166]. However, the overproduction of blood cells is the root problem of MPN, leading to an increased number of blasts in the bone marrow, the peripheral blood, and eventually in extramedullary sites such as the spleen.

Symptoms derived from increased blasts can take time until they manifest and are vague at the beginning, such as headache, fatigue, weakness, dizziness, and excessive night sweats. As the disease progresses, affected people with MPN develop abdominal discomfort from an enlargement of the spleen, which puts pressure on the stomach and leads to reduced appetite and weight loss[166]. Other symptoms of disease progression are disease-specific and depend on the lineage that is affected by uncontrolled proliferation.

As chronic myeloid leukemia (CML) and chronic neutrophilic leukemia (CNL) progress–with root cause granulocytosis–, it leads to symptoms related to pancytopenia (see MDS above) from expanding blasts outcompeting normal hematopoiesis and bone pain from pressure on the nerves inside the bones[167,168]. Pancytopenia can also be caused by primary myelofibrosis (PMF; root cause megakaryocytosis and myelodysplasia[169]) and secondary myelofibrosis. Secondary myelofibrosis is a disease evolution of polycythemia vera (PV; root cause erythrocytosis and frequently also leukocytosis and thrombocytosis[170]) or essential thrombocythemia (ET; root cause thrombocytosis) with a conversion rate of 6-14% and 4-11% at 15 years, respectively[171], which means that secondary myelofibrosis makes up a substantial number of patients given that ET and PV patients have a relatively long median survival and an about three times higher incidence rate than PMF, see statistics below. In cases when PV and ET have not converted to secondary myelofibrosis, the excess RBC in PV thickens the blood and increases the risk for blood clots that lead to tissue and organ damage. In ET, the overproduction of platelets results in a malfunctioning of the blood clotting process with increased risk for blood clots and bleedings[153]. Moreover, uncontrolled proliferation of eosinophils in chronic eosinophilic leukemia not otherwise specified (CEL-NOS; root cause eosinophilia) leads to tissue and organ damage through tissue infiltration, fibrosis, blood clots, and inflammation, where the thickening and scarring of the heart is particular damning[172]. The last category of MPN is MPN unclassifiable (MPN-U), which contains cases where the WHO classification criteria cannot delineate a specific subtype. There are many reasons why the classification system fails in these cases, including early diagnosed cases that later will fall into one of the other categories, incomplete clinical and genetic data, cases with two concurrent neoplasms, and cases that for various reasons have altered clinical features due to advanced disease, concurrent inflammation, or prior cytoreductive treatment[161,166]. Thus, although

MPN-U represents a classification, it does not represent a specific condition with specific symptoms and disease progression.

Like MDS, MPN affects mainly people of older age, with a median age of 55 for *BCR-ABL1* positive CML[173], 62-63 for CEL-NOS[157,158], 65 for PV, 68 for ET, 70 for PMF, 73 for MPN-U[174], and 70-73 for CNL[159], resulting in a median of the medians of 68 years. The most common MPN is ET, with an incidence rate of 1.6 cases per 100,000 people per year, followed by PV at 1.48, MPN-U and CML at 0.85[175,176], PMF at 0.52[176], CEL-NOS at 0.04[177], and finally CNL, which is the least common MPN with 0.01 cases per 100,000 people per year[159]. Combining the data for MPN, the cumulative incidence rate is roughly 5.3 cases per 100,000 people per year, which is similar to MDS with 5.3-13.1 cases per 100,000 people per year[165].

The classification of MPN uses a combination of clinical, morphological, and genetic data. For instance, a morphological analysis of the bone marrow looking for the absence of dysplasia and presence of bone marrow hypercellularity, or abnormal megakaryocytic proliferation accompanied by reticulin or collagen fibers, or both, is a valuable approach for distinguishing MPN from most MDS and MDS/MPN[161]. Clinically, high peripheral blood counts, platelet counts, and different blood parameters such as hemoglobin, hematocrit, increased red cell mass, and serum erythropoietin levels are crucial for delineating the MPN type. Moreover, clinical genetics is also essential in the MPN-subtype classification as the detection of *BCR-ABL1* is a sign of CML, *CSF3R* mutations of CNL, *JAK2V617F* or *JAK2* exon 12 mutations of PV, and *JAK2*, *CALR*, or *MPL* mutations of ET[129].

Over the years, BCR/ABL1 positive CML has been the subtype that has improved OS the most due to tyrosine kinase inhibitors (TKI) targeting BCR/ABL1 signaling. First-line treatment includes a short course of the chemotherapeutic drug hydroxyurea if myelosuppression is necessary, followed by the first generation of TKI. If resistance develops, the second line of treatment includes the second generation of TKI; if that fails, allogeneic HSCT is considered[178]. The 8-year survival for CML patients in the chronic phase has improved from ≤15% survival before 1983 to 42-65% between 1983-2000, and 87% between 2001-2012[179]. The most recent statistics using the European Treatment Outcome Study (EUTOS) long-term survival (ELTS) score show an even greater improvement with 93%, 84%, and 81% OS after eight years for the low-, intermediate-, and high-risk group, respectively[180]. However, this treatment success is unique to CML as the other chronic leukemias CNL and CEL-NOS still have a poor prognosis with a median survival of 2.2-1.8 and 1.85-1.33 years, respectively[157-159]. In these cases, eligible patients are directly considered for transplantation. Otherwise, hydroxyurea is commonly used in CNL and corticosteroids, hydroxyurea, and interferon-α (IFNα) in CEL-NOS[167,181]. For the other MPN subtypes, ET has the best prognosis based on the IPSS system, with the low-risk group lacking a median survival because more than half the patients were still alive at the end of the study, and the median survival for the intermediate- and high-risk groups are 24.5 and 13.8 years, respectively. For PV, the median survival for the lowest to the highest risk group

assigned at diagnosis is 28, 19, and 11 years, and for PMF, 11.3, 7.9, 4, and 2.3 years[153]. PV and ET treatment aims to reduce the risk of fatal blood clots and bleedings. In low-risk groups are PV and ET treated with aspirin, and in PV blood removal, while PMF patients are monitored for disease progression. When these diseases progress to a higher risk group, the first-line treatment is myelosuppression using hydroxyurea or IFNα. If that fails, the second-line treatment for PV is a JAK1/2 inhibitor (ruxolitinib) and, in the case of ET and PMF, an alternative chemotherapeutic drug (busulfan). If blast transformation or accelerated bone marrow fibrosis occurs, allogeneic transplantation is considered the last therapeutic option[170,182,183].

Myelodysplastic/myeloproliferative neoplasms

MDS/MPN have overlapping features between MDS and MPN neoplasms resulting in both dysregulated differentiation and uncontrolled proliferation. Thus, akin to acute leukemias, MDS/MPN neoplasms are cheating the multicellular community by not contributing to the division of labor and proliferation inhibition but with less potency and thus blasts in the peripheral blood and the bone marrow[121,163]. Because of the overproduction of cells and cellular dysplasia, patients with MDS/MPN have both symptoms related to leukocytosis and cytopenia in different lineages and severity depending on the subtype and stage of the disease. Thus, symptoms are related to both MDS and MPN, such as frequent infections, fatigue, and bleedings caused by anemia, thrombocytopenia, and bone marrow fibrosis, as well as pain and fullness below the ribs from blasts cells infiltrating and enlarging the spleen and the liver[184].

Similar to MDS and MPN, most MDS/MPN neoplasms affect older people, with a median age of 75-76 years for chronic myelomonocytic leukemia (CMML), 62-73 years for *BCR-ABL1*–negative atypical chronic myeloid leukemia (aCML), 65-71 years for MDS/MPN-unclassifiable (MDS/MPN-U), and 63-75 years for MDS/MPN-ring sideroblasts and thrombocytosis (MDS/MPN-RS-T). The exception is juvenile myelomonocytic leukemia (JMML) which mainly affects infants and toddlers with a median age of <1 to 2 years, according to incidence data compiled by Kuendgen, A. et al. (2021)[185]. The most common MDS/MPN is CMML, which makes up 0.3-0.4 cases per 100,000 people per year, followed by JMML, with 0.1 cases per 100,000 people under the age of 14. The other three subtypes of MDS/MPN are rare, with an estimation that aCML makes up 1-2% of all BCR-ABL1-positive CML, and MDS-RS-T and MDS/MPN-U 1.45% and 0.05% of all MDS, respectively[185]. Calculated on the incidence data for CML and MDS (0.85 and 5.3-13.1 cases per 100,000 people per year, respectively), it would translate to 0.5-2, 8-19 and 0.3-0.7 cases per 10,000,000 people per year for aCML, MDS-RS-T and MDS/MPN-U, respectively[165,175,176]. Compiling the incidence data for all MDS/MPN subtypes, there are only about 0.4 cases per 100,000 people per year diagnosed with MDS/MPN, with CMML making up the majority of those cases. Thus, MDS/MPN neoplasms are rare, with at least one order of magnitude fewer cases than MDS and MPN.

Diagnosis of MDS/MPN subtypes based on molecular lesions alone is challenging. Thus a first step to narrowing down the diagnosis is to rule out molecularly defined neoplasms (BCR/ABL, PDGFRA, PDGFRB, FGFR1, PCM1-JAK2) with overlapping features and acute leukemia with peripheral blood or bone marrow blast counts >20%129,185. The next step is to make a combined assessment based on collected clinical, morphological, and genetic data. For instance, CMML requires detection of monocytosis that often coincides with TET2 and SRSF2 mutations (60% and 50% of cases, respectively) that skew hematopoiesis towards the monocytic lineage. Subsequent mutations are often ASXL1 (40% of cases) and RAS pathway mutations (30% of cases) that drive disease progression. The importance of disease progression is illustrated by the enrichment of RAS pathway mutations in the MPN-CMML proliferative subtype compared to the less aggressive MDS-CMML subtype categorized by fewer blasts in the blood186,187. The RAS pathway is also commonly mutated in the monocytic proliferative disease JMML, which often has germline or somatic mutations that activate the RAS pathway (up to 85% of the cases)188. For aCML characterized by dysplastic neutrophilia, no disease-specific mutations exist. However, the WHO 2016 classification included SETBP1 or ETNK1 mutations (10-30% and 10% of cases, respectively) as a supportive criteria129,154. For the rare MDS/MPN-RS-T disease that results in ring sideroblasts accumulation and thrombocytosis, frequent mutations are the spliceosome gene SF3B1 (90% of cases) and the JAK2V617F mutation (50% of cases), which made this subtype a full entity in the WHO classification in 2016129,154. MDS/MPN-U is a category used for rare cases where the WHO criteria cannot delineate a specific subtype with poorly defined overlapping features of both MDS and MPN185.

Similar to MDS and MPN, the severity of MDS/MPN neoplasms depends on the particular subtype and the disease stage. For instance, akin to the aggressive MPN neoplasms such as CNL, CEL-NOS, and CML prior to TKI-therapy, as well as high-risk MDS, the MDS/MPN neoplasm aCML have a poor prognosis with a median survival of about 1.5 to 0.6 years (according to the Mayo Prognostic Model)[154]. Thus, with the poor prognosis that coincides with a high risk of leukemia transformation (30-40%), HSCT is the preferred treatment that has increased the median survival to 70 months (5.8 years) for the cohort that can receive HSCT[154,189]. However, due to high age, many aCML patients are not fit for transplantation and are instead treated for their symptoms, including low dose cytoreductive treatment to reduce blast numbers[190]. Another subtype with a poor prognosis is JMML. Although the risk of leukemia transformation is low in JMML patients, this young patient group has a median survival of about 12 to 10 months without HSCT. Transplantation is thus an essential first-line treatment that has prolonged the five-year OS to 64%[154]. Moreover, patients diagnosed with the ill-defined and heterogeneous subgroup MDS/MPN-U also have a relatively short median survival of about 28 to 12 months[154]. Thus, fit patients are transplanted, but a consensus on optimal treatment for patients ineligible for HSCT is lacking[191].

However, two MDS/MPN subtypes have a relatively good prognosis if diagnosed early. For example, the Mayo Molecular Model divides CMML into four risk groups with a median survival of 97, 59, 31, and 16 months (8-1.3 years)[192]. Thus, early diagnosed patients can be monitored initially without treatment. First, when patients start displaying poor prognostic factors, including severe cytopenia or high-risk mutations, HSCT is considered, which coincides with an increased risk for leukemia transformation (15-30% for this subtype)[154,192]. During the time leading up to transplantation and for patients not eligible for HSCT, the treatment goal is to alleviate cytopenia with hematopoietic growth factors, red cell transfusions, and steroids and to reduce the number of blasts cells by hypomethylating agents or low-dose cytoreductive therapy without worsening the concurrent cytopenia[193]. Lastly, patients with MDS/MPN-RS-T have a relatively good prognosis with a low risk of leukemia transformation (<5%) and a median survival of 80, 42, and 11 months (6.6-0.9 years) dependent on the risk group from a recently developed prognostic system by the Maya Clinic[154,194]. The treatment of MDS/MPN-RS-T is similar to the related disease MDS-RS and ET, with ET having the best prognosis followed by MDS/MPN-RS-T and then MDS-RS-SLD with the least favorable prognosis[195,196].

The routes to acute leukemia

The routes to acute myeloid and lymphocytic leukemia follow the same principles[197]. However, this section will focus on the myeloid branch. As stated previously, a patient is in an acute phase when blasts reach the diagnostic threshold of ≥20% in the blood or the bone marrow. When this happens, it poses an immediate threat to patients with a median survival of two months if left untreated[160,198]. The acute phase is brought on by a combination of uncontrolled proliferation and an arrest of normal differentiation, leading to a quick accumulation of blasts that, in some cases, can be so abrupt that the condition goes undiagnosed before the blast phase occurs. Thus, as in MDS/MPN neoplasms, the accumulating blasts in acute leukemias are cheating the multicellular community by not following the rules of proliferation inhibition and division of labor and, in more problematic cases, controlled cell death. Moreover, the expanding blasts indirectly lead to issues in the hematopoietic organs and tissues, including adverse effects from the breakdown of waste product management, resource transport and sharing, and maintenance of a physical and reliable signaling environment. The result is blast cells that monopolize resources and destroy the bone marrow and extramedullary sites, which causes pancytopenia and a long list of life-threatening complications[121,199].

AML can be classified broadly into two groups at diagnoses that depend on whether prior known predispositions exist or not. For instance, secondary AML (s-AML) includes all cases with a known predisposition in the form of an antecedent hematological disorder (AHD-AML), such as an MDS, MPN, or MDS/MPN, a

known therapy-induced disease, or a disease caused by exposure to environmental toxins or radiation (t-AML), and AML with a known germline predisposition. In contrast, de novo (from Latin "anew") AML includes cases without a known predisposition, and this type is indeed the most common form. Data from Sweden collected between 1997-2013 show that de novo AML made up 78.3% of all cases compared to 21.7% for s-AML, of which t-AML made up 8.5% and AHD-AML 13.2%[200,201].

However, with a more in-depth understanding of disease evolution today, many leukemias classified as de novo likely evolved from undiagnosed neoplasms or clonal precursor states. Hence, some researchers have suggested that idiopathic ("of unknown cause") AML is a more suitable term than de novo[202]. From that perspective, the term de novo would mean that the overt neoplasm started from an initial potent driver lesion instead that directly transformed the cell into a malignant clone without clonal evolution. Such leukemias are rare but are known to arise from topoisomerase inhibitor-based chemotherapy that results in near single-hit leukemia transformations through the PML–RARα and MLL-containing fusion genes[202,203].

Neoplastic stem cells

Neoplastic stem cells (NSC) create hierarchal subsystems from their apex position in the clone. These subsystems might not affect hematopoiesis as seen in CHIP or CHOP, slightly skew differentiation as seen in low-risk MDS, or increase proliferation with maintained differentiation as seen in MPN. However, as the cell-level fitness (i.e., the reproductive success of the individual cell) increases with additional or more potent mutations, the hierarchal subsystems become increasingly more dysregulated in differentiation and uncontrolled in proliferation. Also, the hierarchal subsystems adopt a shallower hierarchy that is evident transcriptionally by increased expression of stemness genes that usually define HSC[204-206]. Thus, the hierarchal subsystems are enriched for neoplastic stem and progenitor cells during disease progression. This enrichment of NSC correlates clinically with a poor prognosis and increased risk of disease relapse after treatment[204]. Therefore, there are two hierarchies in a neoplasm with a clinical presentation, one present in each clone and another making up the clonal pedigree of acquired mutations. In the case of an AHD-AML, that tree diagram would show that the clones in proximity to the parent node contributed to the CHIP or CHOP (layer 1), clones at the intermediate nodes gave rise to the chronic neoplasm (layer 2), and finally, the clones at the leaf nodes caused the blast crisis (layer 3).

Hence, from the relatively recent acknowledgment of clonal precursor states and how WHO separates myeloid neoplasm into chronic and acute states, it is clear that there are three recognized layers in the hierarchy. The NSC in the clonal precursor state (layer 1) does not have a specific name other than NSC. In layer 2, the NSC are usually referred to as the condition followed by stem cells, e.g., MDS-stem cells (MDS-SC),

CML-SC, or MPN-SC[207-209]. In layer 3, which consists of de novo AML and s-AML, the NSC are referred to as leukemic stem cells (LSC). In addition, in schematic models and prospective and retrospective contexts, the NSC from layer one and layer two are sometimes referred to as pre-LSC[210]. However, the term pre-LSC can create confusion when used to indicate NSC from chronic leukemias that are not pre-leukemic but rather pre-acute-leukemic.

Therefore, to create some harmony in the classification, a suggestion is to add the terms chronic-LSC (c-LSC) and acute LSC (a-LSC) to the vocabulary. Furthermore, following the same principle, NSC from non-leukemic chronic neoplasms could be called chronic-NSC (c-NSC), and NSC from rare non-leukemic acute neoplasms (e.g., acute erythroid and megakaryocytic neoplasms) could be called acute neoplastic stem cells (a-NSC). Moreover, for consistency, NSC from the clonal precursor states (CHIP and CHOP) could be called precursor-NSC (p-NSC).

As a side note, the same suggested principles for labeling NSC in liquid tumors could also be applied to solid tumors such as lymphomas and myeloid sarcomas. In tumors are terms benign and malignant used instead of chronic and acute for separating less acute cases from critical cases. The equivalent term to c-NSC in benign tumors is pre-cancerous stem cells (pCSC), and the equivalent term to a-NSC in malignant tumors is cancer stem cells (CSC)[211]. Although this nomenclature is not confusing because cancer equals the malignant state only, unlike leukemia, which could be both the chronic and the acute phase, there is still arguably a need for a neutral name for pCSC that does not sound forward-looking or retrospective. Therefore, the suggested nomenclature above solves this issue by referring to NSC from benign tumors as benign-NSC (b-NSC) and NSC from malignant tumors as malignant-NSC (m-NSC). In summary, the suggested classification stratifies NSC into three easily understood layers, including the pre-clinically relevant clonal states, the chronic or benign phase, and the acute or malignant phase. It also allows for broad-unspecific labeling of chronic NSC when it is unnecessary to mention the underlying disease.

Since AML and the blast crisis are rapid events that could kill a patient in a matter of weeks to a few months if left untreated, there is considerable interest in developing targeted therapy that efficiently eliminates the a-LSC in de novo AML and the a-LSC and c-LSC in ADH-AML[160,198]. In younger patients, the ideal is that the therapy also eliminated all traces of p-NSC due to their risk of neoplastic development over time. In summary, the priority in treatment is to deplete the a-LSC successfully and, secondly, to wipe out the c-LSC that otherwise may cause a relapse[142].

Introduction of acute myeloid leukemia

Incidence

AML is mainly a disease of the elderly with a median age at diagnosis of 63 to 72 years in developed countries, including the US, UK, Canada, Australia, and Sweden212-216. Thus the number of cases increases with age as Swedish incidence data from 1997-2013 show that people below 44 years have less than 3 cases per 100,000 people per year, which increases to about 10 cases at age 60-64 and 38 cases at age 80-84217. Similarly, data from the US shows 2 AML cases per 100,000 people per year in people under 65, which increases to 20.1 cases in people aged 65 years or older212. The incidence of AML in all age groups was 3.6 cases per 100,000 people per year in Europe between 2000-2002218. Statistics from other developed countries reflect this number with 3.3 to 4.3 cases per 100,000 people per year in the US, Canada, and Australia212,214,215. Moreover, in Sweden, with about 10 million people, 398 new AML cases were registered annually between 2007-2020, which aligns with the incidence data above219. Thus, AML is quite common, with slightly fewer cases annually than MDS (5.3-13.1) and MPN (5.3)159,165,175-177.

Classification

During the last half-century, the classification of AML improved in two waves due to technological advancements. The first wave utilized simple technology based on cellular stains to visualize cell morphology with light microscopy. This classification scheme helped recognize the morphological heterogeneity between leukemias and provided some prognostic information. The second wave came about from new technologies in immunophenotyping and genetic analysis, which allowed for a more precise delineation of lineages and differentiation stages and the possibility of classifying acute leukemias based on chromosomal and mutational abnormalities. Consequently, the second wave allowed for a much more in-depth analysis of myeloid and lymphoid neoplasms heterogeneity. As a result, these advancements improved prognostication and

optimization of therapeutic interventions for specific subtypes. Below is the pioneering morphological-based FAB classification system and the more recent and advanced WHO classification of acute leukemia introduced.

French-American-British (FAB) classification

The FAB classification was created in 1976 to establish a uniform classification of acute leukemias based on cell morphology. At the time, it was known that ALL responded better to chemotherapy than AML. Thus, further subtype stratification of ALL and AML was believed to identify subgroups with superior therapy response and prognosis, especially since improved therapies were anticipated. In addition, further subgroups would improve confidence in results and conclusions between different clinical trials and preclinical studies that were plagued with discrepancies because of the clinical heterogeneity between acute leukemia cases[212,213].

Diagnosing acute leukemia with the FAB classification requires ≥30% blasts in the blood or the bone marrow. An advantage of the classification is that it does not require expensive equipment, is relatively easy to perform, and gives quick results. The classification can therefore be done all over the world as its procedure follows a simple sample staining to visualize the internal cell structures under a light microscope. The subsequent stratification of subgroups is then based on the blasts' lineage and degree of maturation. In the initial publication, the hematologist behind the proposal suggested three subgroups of the lymphoid lineage (L1-L3 with small, large, and variable blast sizes) and six subgroups of the myeloid lineage (M1-M3, granulocytic lineage; M4, both granulocytic and monocytic lineage; M5, monocytic lineage; M6 erythrocytic lineage)[212]. Later, M0, M4eo, and M7 (M0, minimal differentiation; M4eo and M4 with abnormal eosinophils; M7, megakaryocytic lineage), were added to the myeloid branch to a total of nine subgroups, and the M5 group was divided into M5a (poorly differentiated monoblasts) and M5b (differentiated monoblasts)[214]. During the following two decades after its first proposal, the FAB classification was adopted by hematologists around the world and the classification succeeded in recognizing the morphological heterogeneity in acute leukemias, especially in the myeloid branch[128].

However, technological advances in cytogenetic and genetic methods would later show that genetic lesions are superior to morphology for predicting treatment response and prognosis. Only in some cases can morphology have a predictive value of the genetic lesion that determines therapy, such as in the case of the FAB-M3 subgroup that strongly indicates APL with PML-RARA, even though APL in rare cases can be PML-RARA negative[128,215]. Despite these shortcomings, the FAB system can help set a preliminary diagnosis in some circumstances because it is quick and can complement classification and prognostication based on immunophenotypic and genetic features[214]. In addition, cell morphology can be a valuable aspect in preclinical studies where the differentiation block can be of biological importance, as shown in paper I of this thesis,

where FAB-M5 responds better to treatment. Therefore, the FAB system is still helpful for determining prognosis and best treatment next to the more recent technologies in immunophenotyping and genetic analysis.

World Health Organization (WHO) classification

In the 90s, advancements in genetic analysis and immunophenotyping reached a level of maturity that allowed for new innovative classification schemes. Hence, hematologists from the Society for Hematopathology in the United States and the European Association for Hematopathology were asked to modernize the current WHO classification of hematological neoplasms. Since the first proposal in the mid-90s, the WHO classification of myeloid and lymphoid neoplasms was updated in 2008 and 2016 to keep up with new information. In the latest iteration, the WHO divides hematological neoplasms into three major groups: lymphoid neoplasms, myeloid neoplasms, and acute leukemia. These major groups are subdivided further based on genetic, immunophenotypic, clinical, and morphological features. The idea of the WHO classification is that the disease entities are categorized based on the feature or features that best defines them as individual entities of prognostic and therapeutic significance or with the potential to have it in the feature. Therefore, in some cases, a single genetic lesion can be enough to define specific subtypes, whereas other subtypes require multiple features. Hence, the WHO classification is flexible in what features and how many it needs in classifying subgroups[128,129,162].

Compared to the Fab classification, the WHO lowered the threshold for acute leukemias from ≥30% to ≥20% blasts in the blood or the bone marrow because a lower threshold did not change the prognosis. In addition, genetic information made it possible to diagnose leukemia from recurrent genetic lesions, which made a diagnosis possible with lower blasts counts than ≥20% in some cases[162]. In the latest update from 2016, acute leukemias are categorized into four main groups: (1) AML and related neoplasms, (2) leukemias of ambiguous lineage, (3) B-lymphoblastic leukemia/lymphoma, and (4) T-lymphoblastic leukemia/lymphoma[129]. Below, the classification of AML and related neoplasms and leukemias of ambiguous lineage will be introduced further.

Acute myeloid leukemia and related neoplasms

The WHO classifies AML into four main groups, (1) AML with recurrent genetic abnormalities, (2) AML with myelodysplasia-related changes, (3) therapy-related AML (t-AML), and (4) AML not otherwise specified (AML-NOS). It also includes an AML subgroup related to Down syndrome and myeloid sarcoma. The latter is not a subgroup but an alternative presentation of AML that should be classified according to the other AML categories[129]. Each of these categories will be discussed in more detail.

AML with recurrent genetic abnormalities

In their first issue of AML classification, the WHO defined four AML subgroups with recurrent genetic abnormalities, including rearrangements of the genes *RUNX1-RUNX1T1*, *CBFB-MYH11*, *PML-RARA*, and *MLLT3-KMT2A*[161]. Moreover, they correctly predicted that the number of disease entities would increase over time. The latest iteration from 2016 includes nine subgroups defined by both chromosomal rearrangements and gene mutations, which now also include *DEK-NUP214*, *RBM15-MKL1,* and *GATA2* and *MECOM* rearrangements, and *NPM1*, and biallelic *CEBPA* mutations. In addition, AML with the *BCR-ABL1* rearrangement and mutated *RUNX1* were introduced in the 2016 iteration as provisional subgroups, which means further studies are required before they can be fully acknowledged as verified disease entities in the next iteration[129].

Furthermore, what is important to recognize is that only the most frequent rearrangement of a given gene is mentioned as a defining feature of a disease entity in the WHO classification scheme. For example, *KMT2A* (formerly *MLL*) can rearrange with 135 partner genes, of which 35 occur recurrently, and nine make up 90% of all cases[216]. Hence, despite the wide variety of possible combinations with *KMT2A*, the WHO classification only mentions the *MLLT3-KMT2A* translocation to indicate this subgroup. However, in the clinical investigation of a patient, the actual translocation partner to *KMT2A* should be specified in the diagnosis[128].

Moreover, even if a known recurrent genetic abnormality is associated with prognostic and therapeutic significance, the WHO classification will not include the genetic abnormality as a defining feature of a subgroup if the abnormality is present within multiple subgroups[162]. One such example is *FLT3* mutations, which do not make up a disease entity by the WHO classification even though knowledge of the *FLT3* mutation status provides prognostic and therapeutic value, and 30% of all AML is *FLT3* mutated[216]. However, even if the *FLT3* status does not help classify unique disease entities, WHO recommends examining the *FLT3* gene in all AML cases that are cytogenetically normal[128]. The inability of WHO to incorporate *FLT3* mutation status in their classification scheme illustrates their focus on identifying subgroups rather than providing recommendations for diagnosis and management of the various AML subtypes. Instead, the latter aim is the goal of the European LeukemiaNet (ELN) panel that complements the WHO classification scheme by providing recommendations to physicians to help set a prognosis and determine which treatment is best suited for each given patient case[217].

Furthermore, it is also noteworthy that topoisomerase inhibitor-based chemotherapy can cause rearrangements between the same genes as the recurrent genetic abnormalities. Meaning that if the rearrangement is due to previous chemotherapy, the AML should instead be classified as a t-AML[129]. Therefore, previous chemotherapy can influence if the AML is categorized as an AML with a recurrent genetic abnormality or a t-AML; see more about the therapy-induced classification below.

AML with myelodysplasia-related changes

AML cases assigned to this category have myelodysplasia-related changes as the most salient feature. Three independent criteria based on clinical history, cellular dysplasia, or cytogenetics are sufficient to diagnose AML with myelodysplasia-related changes. For instance, some cases of AML with myelodysplasia-related changes evolve from MDS or MDS/MPN, thus representing the most advanced stages of these neoplasms. Also, de novo AML can be assigned to this category if clinical data show dysplasia in ≥50% of cells in ≥2 myeloid lineages; alternatively, if cytogenetic data shows a complex karyotype with ≥3 abnormalities or identifies one of 16 chromosomal rearrangements considered associated with dysplasia to the degree that it is sufficient to give the diagnosis[128,129].

However, dysplasia has a weak predictive power in forecasting prognosis and is thus a weak feature in subgroup specification. Hence, if an AML with myelodysplasia-related changes is diagnosed with the mentioned recurrent genetic abnormalities or caused by prior therapy, these features are prioritized when deciding on the classification[129]. Hence, similar to the mentioned example with *FLT3* mutations, dysplasia can exist in multiple subgroups but, unlike *FLT3* mutations, is still helpful in setting a diagnosis due to the lower-ranked prognostic significance.

Therapy-related myeloid neoplasms

In the classification of myeloid neoplasms, the WHO has given therapy-induced disease the highest priority of all features. This means that if prior chemotherapy or radiation is determined to be the causative agent of overt disease, it will change the classification to therapy-related myeloid neoplasm (t-MN). The t-MN is further stratified into chronic and acute forms, for example, therapy-related MDS (t-MDS) or t-AML, followed by the identified cytogenetic abnormality mentioned as a secondary feature[129].

One implication of this categorization is that patients with identical genetic abnormalities can be classified into entirely different categories based on whether their genetic lesions occurred from a spontaneous cause or therapy-related reason. Moreover, unlike genetic abnormalities that cannot be used for classification when they exist in multiple subgroups, prior therapy as a feature for classification does not follow that limitation as it overpowers any other feature. This priority is not because the indirect effects of the therapy-induced disease have a more substantial prognostic and therapeutic significance than, for example, recurrent genetic abnormalities, but because it is a weak feature akin to myelodysplasia. Below is an attempt to explain why the current classification scheme based on prior therapy is confusing.

The WHO gives two reasons for using prior therapy in their classification scheme; First, the WHO indicated that, on average, therapy-induced neoplasms have a more severe prognosis than spontaneously evolved neoplasms diagnosed with the same recurrent abnormalities[128,162]. This conclusion implies that therapy-induced disease must acquire more cooperative genetic lesions than spontaneously evolved disease and,

thus, on average, be associated with a more severe prognosis. It is reasonable but not proven by the literature the WHO uses to back up its claim with[128,162]. Second, in the first two iterations, WHO justified the inclusion of this category by emphasizing that researchers were searching for a reason why some patients develop therapy-induced neoplasms while others do not and that this category could help understand the pathogenesis of leukemia evolving independently of preceding therapy[128,162]. Those are important points to consider but not solid reasons why t-MN should be used in classification and with higher priority than features with more substantial and proven prognostic and therapeutic significance.

Incidentally, why some people are prone to developing t-MN was later found to be because of germline mutations that make a certain subgroup of patients more vulnerable to therapy-induced disease[218-221]. For instance, in a 2016 study, it was found that breast cancer survivors who developed therapy-related leukemia were frequently carriers of mutated cancer susceptibility genes. Accordingly, they found that approximately 20% of patients with t-MN had mutations in these genes[218]. Thus, it is warranted that t-MN patients are screened for these mutations to uncover cancer predisposition in additional family members[129].

To summarize, arguably, the category t-MN should not remain in its current form as it does not provide any meaning to the individual patient case and has an unclear prognostic significance. However, if conclusively proven that, on average, therapy-induced diseases have a worse prognosis than spontaneously evolved diseases with the same clinical presentation and cytogenetic abnormalities, it would make a case for including therapy-induced disease as a secondary feature for additional subgroup stratification.

AML, NOS

AML, NOS, includes all cases that do not meet the criteria of other categories, such as AML with recurrent genetic abnormalities, AML with myelodysplasia-related changes, or t-AML. Hence, before assigning an AML case to the category AML, NOS, a clinical and genetic investigation must first exclude all other possibilities.

Moreover, it is noteworthy that the WHO classification includes nine subgroups of AML, NOS, even though the lack of prognostic or therapeutic features defines this category[129]. This is possible because the subcategorization is based on cell morphology following the FAB classification scheme, which is a weak feature that the WHO intended to replace. Because of this, it has been debated if not all subgroups should be grouped into one category with heterogeneous cell morphology instead[128,222]. Despite these concerns, the WHO still decided to keep the subcategories in the 2008 and 2016 iterations. The reasoning was that the subclassification could facilitate the identification of genetic abnormalities and that some of the subgroups could still be relevant for prognostic purposes[128,129]. However, it also highlights that the assumption that the WHO classification system only includes subgroups of proven prognostic and therapeutic significance is invalid as it contains subgroups that are not of current value

but could help identify genetic abnormalities or therapeutic vulnerabilities in future studies.

Myeloid sarcoma

Myeloid sarcoma (MS) is an extramedullary tumor of at least one myeloid lineage that appears in about 2-8% of all AML cases. MS is often found in proximity to the skin, soft tissues, lymph nodes, and bones but can essentially grow in any site of the body[223]. Because MS is considered a clinical presentation rather than an AML subtype, the appearance of MS does not change the AML subtype classification of an already diagnosed AML. In most cases, patients are first diagnosed with AML and later found to have MS. However, de novo identification also occurs, making the appearance of MS qualify for an AML diagnosis even in cases with less than 20% blasts in the bone marrow or the peripheral blood. Alternatively, it appears in patients with chronic neoplasms, which in those cases is considered evolution to AML or a blast cell transformation. Notably, the tumor should be investigated in the two former cases to classify it into an AML subgroup since no prior diagnosis of AML exists[128,129]. In summary, the appearance of MS justifies the AML diagnosis even in cases with less than 20% blasts in the bone marrow or the peripheral blood. Moreover, it is not a subgroup but a solid manifestation of an acute myeloid disease that follows the same rules for classification as all other subgroups.

Myeloid proliferations related to Down syndrome

WHO added the category myeloid proliferations related to Down syndrome in the 2008 iteration because of their unique properties. The category includes two subgroups: transient abnormal myelopoiesis (TAM) and myeloid leukemia associated with Down syndrome[128,129]. Like all features associated with Down syndrome, the root cause of the unusual properties of these neoplasms is due to the additional part of- or the entire extra copy of chromosome 21 that people with Down syndrome are born with. The additional genetic material on chromosomal 21 leads to aberrant hematopoiesis during fetal and early child development. In addition, when this genetic defect is combined with a specific mutation of *GATA1*, it results in TAM, which is associated with a transient wave of an abnormal proliferation of megakaryocytic precursors with, amongst other things, a heightened sensitivity towards chemotherapy. Although TAM spontaneously disappears within three months after birth, it can still result in life-threatening complications[224,225].

Furthermore, additional mutations during this early stage of life increase the risk of MDS and AML associated with Down syndrome. These neoplasms have similar attributes to TAM but are not transient and usually occur during the first three years of life. Noteworthy, Down-related MDS and AML neoplasms are biologically identical; the only difference is the blast count. Therefore, both presentations are included in the subgroup of myeloid leukemia associated with Down syndrome[129].

Acute leukemias of ambiguous lineage

The category of acute leukemia of ambiguous lineage (ALAL) includes cases that cannot be assigned to either the myeloid or the B or T cell lineage of the lymphoid differentiation branch. There are two major subcategories of ALAL, acute undifferentiated leukemia (AUL), which lacks lineage commitment to any differentiation branch, and mixed phenotype acute leukemia (MPAL), which displays lineage commitment to more than one lineage[129].

The identification of AUL and MPAL requires an extensive immunophenotypic analysis in order to determine the correct subgroup. For instance, leukemia cases classified as AUL cannot express any classical myeloid or lymphoid markers or dendritic, basophilic, mast cell, or natural killer cell markers to get assigned to this category. However, AUL typically expresses a limited set of markers associated with immature hematopoietic cells, such as HLA-DR, CD34, CD38, and Terminal deoxynucleotidyl transferase. Leukemia cases that fulfill these criteria are rare, so the prevalence of this subgroup is not known[226].

The second type of ALAL is the MPAL category, which includes four subtypes. The mixed-lineage phenotype in these leukemias can occur from either a single clone or multiple clones affiliated to at least two different lineages. These lineages include the myeloid and the lymphoid branch's B and T cell lineage. About 2-3% of all acute leukemias are categorized as MPAL, and the most common to the least common subtype is the B + myeloid subtype, followed by T + myeloid, B + T, and the trilineage subtype with a prevalence of 59%, 35%, 4%, and 2%, respectively[227,228]. Recurrent genetic abnormalities have priority over the MPAL classification, with two exceptions, including *BCR-ABL1* and rearrangement involving the *KMT2A* gene[128]. These two genetic abnormalities make up the MPAL subgroups: MPAL with *BCR-ABL1* and MPAL with *KMT2A rearranged*[129]. In summary, ALAL includes cases that cannot be assigned to a single lineage due to the lack of lineage commitment or involvement of more than a single lineage of the myeloid, T-, or B-cell branches.

Treatment of AML

This section summarizes the most important therapies for AML, including intensive chemotherapy, low-intensive therapy for AML patients ineligible for intensive chemotherapy, complementary therapy against specific subgroups of AML, and differentiation therapy against APL, which deserves its independent subcategory.

Intensive cytotoxic chemotherapy for fit patients with responding AML

Intensive cytotoxic chemotherapy has been the mainstay therapy for AML for about fifty years[229]. Cytotoxic agents interfere with DNA, which triggers apoptosis in fast-growing cells. Therefore, it is targeted since it selectively kills dividing cells present in cancer rather than healthy cells that are relatively quiescent. Cytotoxic chemotherapy will likely continue to be an essential treatment for AML also in the future. In part because of its effectiveness on diseases with favorable cytogenetics but also because it can be implemented quickly without the need for a complete molecular analysis and risk stratification in cases with rapidly advancing diseases[230].

Initial treatment

The chemotherapeutic regimen combines Cytosine-arabinoside (Ara-C) with an agent from the anthracycline class. The therapy is given in a short period over the first week and causes a profound suppression of the hematopoietic system[231]. Patients are therefore given blood transfusions and antibiotics to maintain adequate levels of mature blood cells in the peripheral circulation and to prevent opportunistic infections during the first weeks when the normal hematopoietic system is suppressed. Only after three to four weeks is hematopoiesis recovering from the bone marrow suppression[232].

Intensive cytotoxic chemotherapy aims to achieve complete remission (CR) without detecting MRD (CR_{MRD-}). The remission status is assessed after the patient's blood system has recovered after about four weeks. Achievement of CR means that the patient is transfusion independent, has recovered a certain cell number of circulating neutrophils and platelets with no detection of circulating blasts or extramedullary disease, and has <5% blasts in the bone marrow without any Auer rods[217]. Patients reaching CR should undergo consolidation treatment, which means that an additional one to three treatment cycles is initiated shortly after the recovery of normal blood parameters[233]. Complete remission occurs after one to two rounds of these treatments in about 75% of younger adults below 60 years and about 50% of older patients[217].

MRD is assessed with either flow cytometry or molecular techniques targeting a driver mutation, including real-time quantitative PCR, digital PCR, or methods based on next-generation sequencing. A patient classified as CR_{MRD-} has no blasts detected above the threshold determined by the sensitivity of the chosen method, which in the case of flow cytometry is below 0,1% and molecular techniques 0,001%[234]. Achieving CR has a great prognostic value since it means that the patient is symptom-free and transfusion free and that infections have a greater chance of healing[235]. The assessment of MRD is also important in patients with CR as CR_{MRD-} indicates a better prognosis[217].

Moreover, these two categories are relevant to the definition of hematological and molecular relapse. The hematological relapse occurs in patients in CR or CR_{MRD-} when bone marrow blasts reach ≥5% or when blasts in the circulation or extramedullary sites reappear, and a molecular relapse occurs in CR_{MRD-} patients when MRD reappears.

Knowledge of a molecular relapse allows early interventions to avoid an overt relapse and may inform what conditioning strategy to use for HSCT[217].

Continues treatment

If residual disease is detected, HSCT should be considered[236]. The chance of reaching a second CR after relapse is about 30%, which is lower than after the initial treatment, and the time spent in remission is often shorter[237]. If the patient is eligible for HSCT, it should be given shortly after the second remission, especially for adverse and intermediate-risk groups. In contrast, favorable cytogenetics benefit better from salvage therapy, i.e., alternative treatment to standard therapy, without HSCT due to the adverse effects associated with HSCT[236]. Another thing to consider is that repeated use of anthracyclines can cause heart toxicity. The initial treatment can be used a second time if the relapse occurs long after the initial treatment. Otherwise, should an alternative chemotherapeutic combination be used that usually consists of FA-IDA (fludarabine, cytarabine, and idarubicin) or ACE (amsacrine, cytarabine, and etoposide)[233,238].

Cytotoxic chemotherapy has been explored in clinical trials for its benefits as a longer-term maintenance therapy to prevent relapse after the initial treatment, but this has not led to any improvements in OS[239]. Therefore, it is better suited as an intense short-term therapy. In summary, cytotoxic chemotherapy is targeted as it selectively kills dividing cells, which explains its efficacy. Despite its limitations, it is still the optimal choice to achieve CR for most patients. The future use of cytotoxic therapy will likely involve the BCL-2 inhibitor venetoclax (VENCLEXTA, AbbVie Inc., and Genentech Inc.) and other targeted therapies, which will be discussed more below[230,240].

BCL-2 inhibition with intensive chemotherapy–a potential new standard of care

As mentioned above, for about fifty years, intensive induction therapy has been made up of a combination of cytotoxic drugs[229]. However, this might change soon if ongoing clinical trials conclude that the BCL-2 inhibitor venetoclax combined with intensive induction and consolidation therapy is superior to intensive chemotherapy alone. Indeed, preliminary data from the ongoing clinical trials indicates that the combination is an improvement to the current standard of care for younger fit AML patients[240,241]. Indeed, venetoclax has already changed the standard of care for low-intensity therapy, which is used to treat patients ineligible for intensive chemotherapy[240,242]. If the standard of care also changes intensive induction and consolidation therapy, it will have several implications for the field.

Venetoclax is an inhibitor that specifically targets the antiapoptotic protein BCL-2. Importantly, venetoclax is not a chemotherapeutic drug since it does not directly kill cells. Instead, cancer cells with upregulated BCL-2 expression are eliminated through the restoration of the apoptotic process. This finding, combined with the development of an effective inhibitor, is important since cancers with elevated BCL-2 expression are

more resistant to apoptosis and chemotherapy. Dependency of BCL-2 for survival can occur in some cancer types, which explains why these cancers are susceptible to apoptosis with BCL-2 inhibition. However, other cancer types, such as AML, require combination treatment with chemotherapy, hypomethylating agents, or targeted molecular therapy for venetoclax to be effective[240,243,244].

The preliminary results from the ongoing clinical trials of venetoclax show that the standard of care for intensive induction chemotherapy of AML is likely to change[241,245]. For instance, the addition of venetoclax to standard induction therapy led to CRMRD- in 74 out of 85 patients compared to 86 out of 140 patients in the intensive chemotherapy cohort. The higher frequency of CRMRD- means that the combination therapy leads to a deeper remission that correlates with improved long-term outcomes. Consequently, there was an improved event-free survival, and the deeper remission enabled patients to transition safely to consolidative HSCT already in the first remission, which is beneficial for intermediate to high-risk groups with a matched donor. Moreover, preliminary data from the trial indicate an improved OS compared to historical outcomes[240,241]. Future studies will clarify which AML subgroups benefit the most from the addition of venetoclax. In addition, future studies should also explore if the deeper remission could lead to a reduced need for consolidative HSCT in certain intermediate-risk groups, which is desired since consolidative HSCT should only be used when needed since it is also associated with adverse effects[240,241].

If the standard of care changes for intensive induction therapy, the implications will be the same as what is currently happening for low-intensity treatment based on hypomethylating agents. Phase III trials of novel therapy will need to be compared to the new standard of care with venetoclax instead of the previous benchmark treatment. The value of recently approved therapies will also need to be reconsidered and compared to the improved standard care[242,244,246]. Moreover, triplet therapy will become more relevant to explore in clinical trials. As an example, the experimental arm of a novel therapy in a triplet study will include the chemotherapy or the hypomethylating drug (component 1), the BCL-2 inhibitor (component 2), and the experimental therapy (component 3) that will be tested against the control arm including component 1, 2, and the placebo[244,247] More of this will be discussed below in the introduction of complementary therapy against specific subgroups of AML.

Low-intensive therapy for AML patients with intolerance or unresponsiveness to intensive chemotherapy

There are two main reasons why patients may not be eligible for intensive chemotherapy, including *intolerance* from older age (>80 years) or comorbidities, or *unresponsiveness* from refractory or relapsed disease or unfavorable cytogenetics such as *TP53* mutation, del(5q), or complex karyotype[248]. When patients are intolerant or unresponsive to intensive chemotherapy, is low-intensive therapy based on

hypomethylation agents recommended[248]. Monotherapy with a hypomethylation agent used to be the standard of care for this patient group. However, this is changing, and the emerging standard of care is now a combination therapy consisting of a hypomethylation agent combined with the BCL-2 inhibitor venetoclax[240,242,247,249].

Hypomethylating agents

Hypomethylating agents are the mainstay therapy for low-intensity AML therapy. It is based on a different mechanism of action from intensive cytotoxic chemotherapy. Instead of causing cell death, hypomethylating agents change the cancer cells' epigenome by inhibiting DNA methylation, which leads to gene transcription changes. The effect on the neoplastic cells is often slow, and the chance of remission is lower than with intensive chemotherapy[250].

Azacitidine (Vidaza, Celgene Corp.) and decitabine (Dacogen, MGI Pharma Inc., and SuperGen Inc.) are the most commonly used hypomethylating agents that are given in a monthly treatment cycle over a few consecutive days. To reach CR is usually 2-3 cycles needed[250]. Since hypomethylation agents are less intensive than cytotoxic chemotherapy, they can be used as maintenance therapy[251,252]. In summary, hypomethylating agents are used when the patient does not tolerate the harsher chemotherapy regimen or when the AML is not responding to chemotherapy. Similar to chemotherapy, the future use of hypomethylation agents will increasingly involve combination therapy with venetoclax and other targeted therapies to increase the treatment's efficacy.

BCL-2 inhibition with low intensive therapy–a new standard of care

Venetoclax has gained FDA and EMA regulatory approval as a combination therapy with hypomethylating agents and in the US market for low-dose cytarabine in newly diagnosed AML patients ≥75 years or with comorbidities that preclude them from intensive induction chemotherapy. Based on promising results in phase II trials, is the combination also used off-label–i.e., use for an unapproved indication–in refractory or relapsed disease with unfavorable cytogenetics or intolerance to the first cycle of cytotoxic induction treatment[233,253,254].

Venetoclax is given in a monthly treatment cycle with a hypomethylating agent for the first few days and then as a single agent until day 28. One treatment cycle is usually enough to reach CR. The rapid cell death can lead to tumor lysis syndrome (TLS) (about 18% of patients), which is a sudden release of intracellular components into the bloodstream resulting in an imbalance of electrolytes and metabolites that can lead to life-threatening complications such as kidney failure, epileptic seizures, and cardiac arrhythmias. Another adverse effect is neutropenia which can cause opportunistic infections (25% develop a new infection in the first cycle). In those cases, the hematopoietic system needs to recover before the next treatment cycle. Therefore, this treatment requires experienced physicians, especially when using it outside indication

in refractory and relapsed patients. Protective measures to minimize adverse effects include TLS prophylaxis, dose adjustments, and shortening of the treatment cycle[233,255].

The regulatory approval of venetoclax with azacitidine was based on clinical trial data that showed that the combination compared to azacitidine alone led to an improved median OS of 14.7 vs. 9.6 months, with more patients achieving composite CR (complete remission or complete remission with incomplete hematologic recovery) 66.4% vs. 28.3%[242]. Another trial investigated the combination of venetoclax with low-dose cytarabine and showed an improved median OS of 8.4 vs. 4.1 months and a composite CR of 48.3% vs. 13.2%[256]. These data led to regulatory approval from FDA in 2020, and the EMA followed that in 2021. The same year the combination was also included as a subsidized treatment in Sweden by tandvårds- och läkemedelsförmånsverket (TLV)[257-259].

Monotherapy with the BCL2 inhibitor venetoclax can be enough to restore the apoptotic process and induce cell death in cancer dependent on BCL-2 for their survival[260]. However, many cancer types depend on more than one antiapoptotic protein, with MCL-1 being an additional key protein that promotes survival in addition to BCL-2. Hence, it is necessary to neutralize both MCL-1 and BCL-2 to restore the apoptotic process in cancer cells with the overexpression of these antiapoptotic proteins. When cancer cells are exposed to a hypomethylating agent, it activates the integrated stress response pathway that upregulates the proapoptotic protein NOXA that sensitizes cells to apoptosis by inhibiting MCL-1 through sequestration. Thus, by neutralizing MCL-1, hypomethylating agents increase the cancer cells' dependency on BCL-2 for survival. Consequently, combining hypomethylating agents with the BCL-2 inhibitor venetoclax neutralizes both of the antiapoptotic proteins, which leads to a restored apoptotic process and induced cell death in cancer cells with survival dependency on these proteins. The broader inhibition of two antiapoptotic proteins explains the synergistic effect and efficacy of combining a hypomethylating agent and venetoclax[261,262].

Notably, AML with specific mutations, such as *FLT*-TKD, *SRSF2*, *ZRSR2*, *NPM1*, *IDH1*, *IDH2*, and *RUNX1*, have been reported to be more responsive to the combination treatment with venetoclax than low-intensity chemotherapy or hypomethylating agents alone[249,263-266]. In contrast, resistance to the combination has been associated with *FLT3-ITD* and mutated *PTPN11*, *KRAS*, *and NRAS*[263,265-267]. Interestingly, the increased responsiveness and resistance from a subset of these mutations were further clarified by two recent publications that looked at survival outcomes in patients with mutated *FLT3*-TKD, *IDH1*, *IDH2*, and *FLT-ITD*. Patients treated with the venetoclax combination with *FLT3* WT or *IDH1* and *IDH2* WT had a median OS of 14.7 months and 12.3 months, respectively, vs. 10.1 months with azacytidine alone[266,268]. What is interesting, however, is that *FLT3*-TKD patients had a clear survival benefit from the combination with a median OS of 19.2 vs. 10.0 months with azacytidine alone[266]. Similarly, there was a survival benefit in *IDH1* and *IDH2* mutated patients with a median OS of 24.5 months vs. 6.2 with

azacitidine alone[268]. As expected, patients with *FLT3-ITD* did not benefit from the combination, with a median OS of 9.9 vs. 8.5 months for the respective treatments[266]. Mutations correlated with improved response or resistance are highly relevant as they may affect prognosis and impact future treatment strategies and combination approaches[244]. Some of these implications will be addressed more below in the introduction of complementary therapy against specific subgroups of AML.

In conclusion, low-intensity AML therapy with azacitidine and venetoclax is associated with manageable side effects, a higher rate of CR, and improved OS compared to azacitidine monotherapy. Therefore, the combination has emerged as the current standard of care for low-intensity treatment of older and unfit AML patients[240,242,247,249].

Complementary therapy against specific subgroups of AML

Below is a summary of the most crucial precision-based therapies for AML. This field is called precision- or personalized medicine which is medical care that uses genetic profiling to maximize the therapeutic benefit in specific subgroups of patients. Consequently, genetic profiling is the critical difference between personalized medicine summarized here and the previous sub-section that included therapies that are agnostic of disease subtypes.

FLT3 inhibitors

The therapeutic tool kit is larger for patients below 70 years with *FLT3-TKD* mutation or *FLT3*-ITD (about 5% and 25% of all AML patients, respectively) than for many other AML subtypes[269,270]. The discovery of FLT3 inhibitors is important since especially the *FLT3*-ITD subgroup is relatively large and associated with an intermediate to adverse prognosis in cases with a high allele frequency[217]. In contrast, there is not a strong correlation between *FLT3*-TKD mutations and prognosis[271].

Two FLT3 inhibitors that have recently received regulatory approval are midostaurin (RYDAPT, Novartis Pharmaceuticals Corp.) and gilteritinib (XOSPATA, Astellas Pharma US Inc.). Midostaurin and gilteritinib are both type I inhibitors. It means that they inhibit FLT3 signaling with the same mechanism of action by competing with adenosine triphosphate (ATP) for binding to the ATP binding site of the intercellular TKD (tyrosine kinase domain), thereby blocking its ability to phosphorylate other proteins. They are effective on both *FLT3*-TKD mutated AML, which forces the TKD into an active conformation, and FLT3-ITD (internal tandem duplication), which increases FLT3 signaling from the loss of an inhibitory domain. However, midostaurin and gilteritinib differ in their selectivity and potency. Midostaurin is a first-generation FLT inhibitor, which means that it lacks specificity to FLT3 and targets multiple kinases located downstream and in parallel pathways to FLT3 signaling. Gilteritinib, on the other hand, is a second-generation inhibitor that has been purposely developed

to be a more specific and potent FLT3 inhibitor. Despite these differences, they are both effective in treating AML with *FLT3*-TKD mutation and FLT-ITD. The broader activity against many other kinases of midostaurin adds to the effectiveness of this inhibitor, although with more extensive on-target cytotoxic effects compared to gilteritinib[270].

Midostaurin is registered for use with cytotoxic chemotherapy during the initial therapy and later consolidation treatment rounds. It is not intended for use in refractory disease, nor in combination with azacitidine in older patients, and its benefit in maintenance treatment has been questioned[272,273]. It is given between and after chemotherapy infusions in tablet form between days 8 to 21. The FDA and EMA approved Midostaurin in 2017, and shortly after, it was included as a subsidized treatment in Sweden by TLV[274-276].

Gilteritinib is approved as a monotherapy in younger patients with relapsed and refractory *FLT3* mutated AML and is a treatment option for consolidation therapy in cases when the AML is unresponsive to chemotherapy and midostaurin[277]. The approval of gilteritinib was based on the phase III ADMIRAL trial that showed that single agent treatment with gilteritinib resulted in a higher response rate and OS than salvage chemotherapy regimens[278]. Gilteritinib is given in daily doses in tablet form as long as it remains effective without unacceptable toxicity and can thus be used as maintenance therapy as well[279]. The positive clinical trial data led to the approval of gilteritinib in 2018 by the FDA, followed by the EMA in 2019[280,281]. Shortly after, in 2021, it was included as a subsidized treatment in Sweden by the TLV[282]. However, gilteritinib is not recommended in older patients unfit for chemotherapy, and a recent phase III study concluded that the combination with azacitidine did not improve the OS compared to azacitidine alone[283].

Two more FLT3 inhibitors that are currently in use or previously believed to have potential are sorafenib (Nexavar, Bayer HealthCare Pharmaceuticals Inc.) and quizartinib (Vanflyta, Daiichi Sankyo), respectively. Sorafenib is a multi-targeted TKI of the first generation, but unlike midostaurin and gilteritinib, it is a type II inhibitor with a different mechanism of action. Instead of interacting with the ATP binding site as type I inhibitors do, Type II inhibitors bind to a hydrophobic region next to the ATP binding site, thereby preventing activation of the TKD. However, the hydrophobic region is only exposed in the inactive TKD confirmation. Thus, type II inhibitors can only be used to treat FLT-ITD patients as the hydrophobic region is hidden in point mutated FLT3 AML due to the forced activated confirmation of the TKD[270].

Sorafenib is not approved for treating AML but is registered for other non-blood-related cancer types. However, studies on AML have shown that it is effective as a monotherapy to prevent relapse and in combination with cytotoxic chemotherapy against refractory disease after HSCT[284-286]. Hence, the proven effectiveness is in a different therapeutic context to midostaurin. These positive results with sorafenib have led to off-label use in many countries, including Sweden[286,287]. However, similar to

midostaurin and gilteritinib, sorafenib is not recommended for older patients unfit for chemotherapy since the combination with azacitidine did not improve the OS compared to azacitidine alone[288].

Quizartinib was a competitive molecule to gilteritinib as they are both second-generation FLT3 inhibitors with the difference that quizartinib is a type II inhibitor that only affects FLT3-ITD AML. However, a much-anticipated phase III trial published in 2019 did not convince the FDA and EMA to approve quizartinib for the EU and the US markets. The reason was that the benefits were determined to be too small to justify the risks, and there were also reported issues in the study design[289,290]. The only market that approved quizartinib is Japan, although the decision has been criticized after its approval[291].

Moreover, FLT3 inhibitors are now tested in combination with venetoclax and azacitidine. The addition of a FLT3 inhibitor is especially relevant for AML with FLT3-ITD since this patient group is resistant to the venetoclax and azacytidine combination[265,266]. So far, a finished phase Ib trial has evaluated the efficacy and safety of venetoclax with gilteritinib in patients with relapsed or refractory *FLT3* mutated AML[292]. Further, an ongoing Phase II clinical study explores triple therapy of venetoclax, azacytidine, and FLT3 inhibitors in *FLT3* mutated AML in older patients or those unfit for intensive chemotherapy[247,293]. Further randomized controlled trials will determine if FLT3 inhibitors in combination with venetoclax or the venetoclax and azacytidine combination improve the survival outcome of patients with FLT3-ITD AML[244].

In summary, FLT3-ITD is an important target in AML due to its high prevalence and association with a relatively poor prognosis in cases with a high allele frequency. Even with the inhibitors mentioned above, the prognosis for this patient group is still relatively poor[278,294]. Future studies will determine if FLT3 inhibitors with venetoclax or venetoclax and azacytidine improve treatment outcomes, especially for FLT3-ITD AML[266]. Therefore, there is still a need for further optimization of therapies targeting the *FLT3* mutated AML[277].

Mutated IDH1 and IDH2 inhibitors

Patients in the US that are ineligible for intensive chemotherapy and diagnosed with *IDH1* or *IDH2* mutations (approximately 20% of all AML) can receive targeted molecular therapy. The FDA has approved two inhibitors for mutated *IDH* (m*IDH*), ivosidenib (Tibsovo, Servier Pharmaceuticals LLC), which targets m*IDH1* (6-16% of all AML), and enasidenib (IDHIFA, Celgene Corp.), which targets mIDH2 (8-19% of all AML)[295].

The IDH enzymes are part of the tricarboxylic acid cycle (TCA) that generates ATP from metabolizing acetyl-CoA–a metabolite derived from glucose, fatty acids, and amino acids. Normally the IDH enzymes convert isocitrate to alpha-ketoglutarate (α-KG). However, specific *IDH* point mutations result in a loss of normal enzymatic function that changes the conversion of isocitrate from α-KG to 2-hydroxyglutarate (2-

HG), which in turn leads to 2-HG mediated inhibition of α-KG-dependent enzymes. Two enzymes affected by this inhibition are TET2 and histone lysine demethylases, which are involved in DNA and histone demethylation. Consequently, *IDH* mutated AML has elevated DNA and histone methylation levels that contribute to the neoplastic state by impairing cellular differentiation[296].

Mutations in *IDH* occur at different stages of the clonal evolution to a more aggressive neoplasm. For instance, *IDH* mutations have been identified in the CHIP stage and appear to be early events in the clonal evolution to MDS and AML. *IDH* mutations can also be a later mutational event and have been associated with MPN transformation to AML in these cases[142,147]. Moreover, mutations in *IDH1* or *IDH2* are mutually exclusive at diagnosis, but double mutated clones can evolve in relapsed disease due to resistance to either ivosidenib (targets mIDH1) or enasidenib (targets mIDH2)[297].

FDA approved enasidenib in 2017 and ivosidenib in 2018 as monotherapies for relapsed or refractory AML with m*IDH2* or m*IDH1*, respectively[298,299]. In 2019, the FDA expanded the use case for ivosidenib to include newly diagnosed *IDH1* mutated patients who are ineligible for intensive chemotherapy[300]. The latest FDA approval from May 2022 is for a combination therapy of ivosidenib and azacitidine that outperformed azacitidine alone (median OS of 24.0 vs. 7.9 months) and ivosidenib as single-agent therapy (median OS 12.6 months)[246,301,302]. However, the EMA took another stance from the FDA and rejected the initial applications of enasidenib and ivosidenib as they determined that the data was insufficient to conclude that the treatments have a positive benefit-risk balance. The response from the EMA led to both companies withdrawing their applications in 2019 and 2020[303,304]. However, the combination therapy with ivosidenib and azacitidine is submitted to the EMA. If they grant it regulatory approval, it will be the first *IDH1* mutation-specific therapy in the European market[305].

When the phase III IDHentify trial of enasidenib monotherapy presented its results in mid-2022, it did not show a meaningful improvement in median OS compared to conventional care regimens (6.5 vs. 6.2 months) in older patients with relapsed or refractory AML with mutated *IDH2*[306]. However, an analysis of patients subgroups from this trial revealed that enasidenib is effective on patients with mutated *IDH2*-R172 (25% of cases) with a median OS of 14.6 months compared to 7.8 months with conventional care regiments but not on patients with mutated *IDH2*-R140 (75% of cases) with a median OS of 5.7 months in both treatment arms (data from a recent poster abstract)[307]. Thus, enasidenib is only effective on one of every fourth patient with *IDH2* mutated AML, which means further improvements to IDH2 targeted therapy is warranted. Moreover, enasidenib and ivosidenib are also being evaluated in combination with intensive induction therapy. A phase I trial for newly diagnosed *IDH1* and *IDH2* mutated AML concluded that the combination is well tolerated. However, the potential benefit is believed to come first after follow-up with

maintenance therapy which is currently evaluated in a phase III trial conducted in Swedish hospitals[308,309].

In the current drug pipeline of potential novel inhibitors of mIDH1 and mIDH2, a few candidates are evaluated for efficacy and safety, while others have recently had their development end. For instance, the development halted for the inhibitor IDH_{305} against mu*IDH1* after a phase I clinical study concluded that the therapeutic window was too narrow[310]. Similarly, the development stopped for the inhibitor BAY1436032 against mIDH1 due to modest efficacy[311]. However, olutasidenib, an inhibitor against m*IDH1,* is more promising and is currently evaluated as a monotherapy and combination therapy with azacytidine[312-314]. Similar to the venetoclax and azacitidine combination, the relationship between mutations and the clinical response showed that mutations *in FLT3*, *NRAS, KRAS*, and *PTPN11* correlated with a lower rate of CR and CR with partial hematologic recovery (CRh) (data from a recent poster abstract)[315]. Future studies will reveal how olutasidenib compares to the already approved ivosidenib. Moreover, vorasidenib and HMPL-306 are two more potential inhibitors with dual inhibition of both mIDH1 and mIDH2 currently in phase I trials. Vorasidenib also differentiates itself from the other inhibitors by an improved blood-brain barrier penetrance, which is why it is under development for glioma[316,317]. The dual inhibition of mIDH1 and mIDH2 is also advantageous as it would avoid relapse due to an acquired mutation in the other *IDH* gene[318].

When the combination of azacytidine and venetoclax was proven to be a more effective frontline therapy than azacytidine alone, it also revealed that patients with specific mutated genes such as *FLT3*-TKD, or *IDH1* and *IDH2* had a survival benefit from the combination[242,266,268]. When comparing the median OS of the venetoclax and azacytidine combination/azacytidine alone (15.2/2.2 months) with the ivosidenib and azacytidine combination/azacytidine alone (24.0/7.9 months), it appears as the ivosidenib combination is the better performing combination in AML patients with m*IHD1*[246,268]. However, such a conclusion is premature, as one should be cautious about comparisons between trials. Moreover, a standing question is whether combination therapy with ivosidenib or enasidenib with venetoclax or triple therapy with ivosidenib or enasidenib with venetoclax and azacytidine will improve the survival outcome even further for AML patients with m*IDH1* or m*IDH2*[244]. Furthermore, if venetoclax and intensive chemotherapy would become a standard of care, the current phase III trial evaluating IDH1 and IDH2 inhibitors with frontline induction therapy followed by maintenance therapy will need to be tested against the venetoclax-based induction regimen[240,308].

In summary, the development of inhibitors for mIDH1 and mIDH2 is an achievement in molecularly targeted precision medicine. Future clinical trials should evaluate different combinations of mIDH1/mIDH2 inhibitors with hypomethylating agents and venetoclax to determine the optimal therapy for the *IDH1* and *IDH2* mutated AML subgroups[244].

An anti-CD33 antibody with chemotherapy

For patients diagnosed with de novo AML with core binding factor (CBF) translocation (12- 15% of all AML), the recommended treatment is a combination of intensive chemotherapy and gemtuzumab ozogamicin (GO) (Mylotarg, Pfizer Inc.)[319].

GO is a toxin conjugated antibody that selectively kills CD33 positive cells– expressed on about 90% of all AML and normal myeloid progenitors, but not on cells outside the hematopoietic system[320]. The function of the antibody is to target and facilitate the uptake of the toxin, which, once released intercellularly, is metabolized to its active form that induces DNA strand breaks. In dividing cells, this results in cell cycle arrest as the cell tries to repair the damaged DNA. If apoptosis is triggered depends on several factors such as the CD33 expression level, the cell's ability to metabolize or extrude the toxin, the capacity for DNA repair, and the presence of pro and antiapoptotic proteins[321]. Clinical experience of GO has shown that the best responding AML patients are those with favorable cytogenetics that are diagnosed with de novo CBF AML, which is a subtype characterized by a fusion protein containing one of the subunits of the CBF transcription factor (RUNX1 or CBFB) with another fusion partner, e.g., RUNX1-RUNX1T1 or CBFB-MYH11[319,322].

GO should be given on day one at the start of the induction or consolidation treatment. Therefore, a fast diagnostic response time is necessary by FISH or PCR that detects if the patient has translocation of one of the CBF subunits[233]. Most side effects from GO combination therapy are the same as when chemotherapy is used alone, including bleeding and opportunistic infections from myelosuppression, TLS, and veno-occlusive disease (VOD) that is caused by damage to the endothelium by toxic metabolites[323]. However, clinical trials have detected an increased risk, albeit low, in the GO-treated arm for extended thrombocytopenia and risk of VOD compared to chemotherapy alone[324]. Moreover, due to the intravenous injection route, complications from infusion reactions are also associated to GO, which involves a response of the host's immune system that can lead to side effects such as fever, chills, hypotension, tachycardia, hypoxia, and respiratory failure[323,325].

GO was approved by the FDA in 2017 as a monotherapy and combination therapy with chemotherapy for the treatment of newly diagnosed and relapsed and refractory CD33-positive AML in adult patients and relapsed and refractory CD33-positive AML in pediatric patients[326]. In 2020, the approval was extended to include newly diagnosed pediatric patients from 1 month of age[327]. In Europe, the EMA approved GO in 2018, but only for newly diagnosed patients aged 15 or above that has not received any other treatment. Moreover, in the EU, approval was only given to the combination treatment with chemotherapy[328].

Many clinical trials have evaluated the efficacy and safety of adding GO to intensive chemotherapy regimens. Individual patient data from five of these studies were later analyzed in a meta-analysis that included 3300 patients[329]. In this study, the five-year survival rate was used to evaluate treatment efficacy, which indicates a treatment's long-term benefit, as most AML that will relapse does so during the first two years. Thus at

the five-year mark, one can determine how many patients the treatment saved from the most critical time in the patient's journey to recovery.

Without any subcategorization of patients, the meta-analysis estimated that 35,6% were still alive in the GO arm at the five-year mark compared to 32.2% in the control arm. However, when patients were divided into cytogenetic risk groups, the same analysis showed an estimated five-year survival of 77.5% vs. 55.0% in the favorable risk group, 40.7% vs. 35.5% in the intermediate risk group, and 9.1% vs. 7.9% (non-significant) in the adverse risk group. Thus, when put in clear proportions, out of 20 patients, there were 15 instead of 11 patients still alive in the favorable risk group and 8 instead of 7 patients still alive in the intermediate risk group at the five-year mark. Moreover, it was shown that the combination treatment reduced the risk of relapse but did not increase the proportion of patients reaching CR or CRh[329].

Consequently, the clinical data support the use of GO with chemotherapy for patients with favorable cytogenetics as four more patients out of 20 were still alive five years after diagnosis compared to chemotherapy alone. The benefit is considerably lower in the intermediate subgroup as only one more patient out of 20 was still alive five years after diagnosis[329]. In Sweden, it is not recommended to use GO for the intermediate risk group because of the uncertainty of the benefit-risk balance from the increased side effects and the differences in the chemotherapy regimen between Swedish hospitals and the clinical trials[287].

Additional combinations with GO that are currently or have recently been tested include an ongoing phase Ib trial with GO and venetoclax in refractory and relapsed AML and a phase I study evaluating triplet therapy consisting of GO, the FLT3 inhibitor midostaurin, and chemotherapy[330,331]. The maximum tolerated dose from this phase I study is now used in phase II trials to test the efficacy in CBF AML and *FLT3* mutated AML[331]. Moreover, a phase II clinical study from 2016 concluded that the hypomethylating agent decitabine and GO did not improve median OS compared to historical outcomes[332]. Thus, GO with a hypomethylating agent does not seem to have potential, especially now when venetoclax and hypomethylating agents have emerged as the new standard of care for low intensive treatment[242]. Therefore, at least in the near future, the use case for GO will not be extended to additional combinations or patient subgroups.

In summary, combination therapy with GO and chemotherapy is considered the standard of care for de novo CBF AML with favorable cytogenetic risk[319]. In contrast, AML with intermediate cytogenetic risk has only a low benefit from the combination, and patients with adverse cytogenetic risk do not benefit from the addition of GO[329].

Differentiation therapy against APL

APL (about 5% of all AML) deserves a separate subsection of AML treatment because of the dramatically improved prognosis in this subtype[333]. This improvement is due to

the exceptionally simple pathophysiology consisting of one driver that can be molecularly targeted by Tretinoin, also known as all-trans-retinoic acid (ATRA) (Vesanoid), and arsenic trioxide (ATO) (TRISENOX, Teva Pharmaceutical Industries Ltd.). These unique attributes have led to a high response rate and few relapses, which changed the APL prognosis from a long-term OS of about 60% to a reported OS above 95% in clinical trials for standard risk APL[334-339]. However, the clinical trials exclude patients with a high risk of early death due to poor performance status, major hemorrhage, or life-threatening coagulopathy. This reduces early death to around 5-8%, but since real-world data indicate the risk of early death to be around 19%, the actual OS is about 80% after the introduction of ATRA and ATO, which still makes APL the most curable AML subtype today[340,341].

PML-RARα–the main driver of the disease

Most APL cases are caused by the translocation between chromosome 15 and 17 *t*(15;17) (q22;q12-21), resulting in a fusion of the coiled-coil domain of the promyelocytic leukemia protein (PML) with the retinoic acid receptor-α (RARα) (PML-RARα). In those rare instances when this is not the case (2% of all APL), is RARα fused to another fusion partner, such as PLZF or NPM (1% of the cases), or is APL diagnosed morphologically without a RARα fusion protein (1% of the cases)[342]. The risk of being diagnosed with APL is almost constant over life, which highlights the pathophysiological simplicity of this disease as the PML-RARα translocation is the rate-limiting event and the main driver of the disease. Thus, other genetic lesions sometimes found in APL, such as mutated FLT3 and trisomy MYC, facilitate disease progression rather than driving it[335].

APL pathophysiology and therapy

RARα and other retinoic acid-responsive transcription factors are normally involved in inducing cell differentiation and PML in organizing nuclear- domains or bodies, which regulate apoptosis, cell cycle progression, senescence, and DNA damage responses[343,344]. However, in the event of the t(15;17), the resulting PML-RARα fusion protein causes an arrested differentiation at the promyelocytic stage of granulocytic differentiation by attracting co-suppressors that silence the RARα target genes. In addition, the PML-RARα fusion protein disrupts the formation of nuclear bodies, which results in dysfunctional regulation of apoptosis and cellular senescence that contributes to the uncontrolled proliferation and inappropriate cell survival seen in the APL phenotype[335].

The therapeutic efficacy of ATRA is explained by its direct binding to RARα, which results in the recruitment of co-activators that removes the transcriptional block and induces transcription of RARα target genes. High ATRA concentration also leads to degradation of the PML-RARα fusion protein through autophagy, allowing WT PML to organize nuclear bodies that restore normal regulation of proliferation and cell survival[335,345].

In contrast, ATO directly binds to the PML domain and is more effective than ATRA in degrading the PML-RARα fusion protein but does not activate RARα-bound genes. The efficacy of ATO is explained by the elimination of the fusion protein by proteasome-dependent degradation, leading to the re-organization of PML-nuclear bodies and clearance of PML-RARα fusion protein from RARα promotors, which restores normal differentiation[335,346].

Interestingly, monotherapy with ATO and ATRA results in a similar long-term OS of about 70%, which should be compared >95% (or ~80% if early deaths are included in the analysis) with the combination[336-338,340,341,347-349]. Moreover, clinical data from the 1% of APL cases with RARα fused to an alternative fusion partner have shown that these subtypes terminally differentiate upon ATRA treatment but are not cleared from leukemia, indicating the critical role of the combination treatment for complete clearance of the leukemic cells[350]. Hence, these data demonstrate the synergistic relationship between ATRA and ATO and how vital the combination is for optimizing the cure rate–i.e., the five-year survival rate that presumably indicates a cure–in PML-RARα driven APL.

In summary, the efficacy of this combination treatment is explained by the uncommon pathophysiological simplicity in APL paired with the molecular on-target effects of ATRA and ATO on the PML–RARα fusion protein[335].

Diagnosis and initial treatment

At diagnosis, APL is divided into three risk groups (low-, intermediate-, and high-risk) that stratify patients into different prognostic groups and determines which therapy they will receive. Patients in low- and intermediate-risk groups differ by platelet count, and high blast count defines patients in the high-risk group. The low- and intermediate-risk groups, also known as standard risk APL, are treated the same with a combination of ATO and ATRA as frontline treatment. In contrast, high-risk APL requires an initial treatment with chemotherapy (idarubicin) to reduce the blast count before initiating the ATO and ATRA combination therapy[125]. Reduction of the blast count is critical in these patients because they would otherwise have a high risk of developing severe differentiation syndrome. Differentiation syndrome is caused by the large amount of maturing granulocytes that suddenly appear with ATRA and ATO treatment. Complications occur when the granulocytes move from the circulation to the tissues in response to ongoing inflammation, leading to complications that can be life-threatening[351].

Because of the high efficacy of the combination therapy, death from treatment failure is no longer common. Rather most patients die of early death (about 19%) caused by bleeding and blood clots, followed by infection and differentiation syndrome during the first 30 days after diagnosis[341,352]. Thus, because of the high risk of fatal bleeding and blood clots, it is necessary to start with ATRA and supportive care to mitigate bleedings at the first suspicion of standard risk APL followed by ATO when t(15;17) is verified. If the analysis shows that APL is not the case, ATRA treatment can be

stopped and more suitable treatment initiated instead. However, if the patient has high blast counts and thus suspected high-risk APL, ATRA cannot be given since it could lead to severe differentiation syndrome. Instead, the t(15;17) must be verified before chemotherapy treatment is initiated[353].

In frontline treatment, ATRA is given daily until CR or day 60. ATO is given daily for the first five days and then twice weekly for seven weeks, even in cases when CR is reached earlier. Patients are checked for CR after four weeks of treatment. If CR is not achieved after four weeks, are weekly tests for CR recommended until day 60. If CR is not achieved after 60 days, it is necessary to evaluate the situation since the therapy is not working satisfactorily. However, CR is usually achieved between day 30 and day 60. CR_{MRD-} is first checked when the treatment is done. Patients with standard risk APL that achieved CR_{MRD-} do not need further MRD monitoring due to the low risk of relapse. However, it is recommended that standard risk APL that did not achieve CR_{MRD-} and high-risk patients, regardless of the depth of remission, is followed up for an additional two years to catch a relapse early[353,354].

Continuous treatment

The FDA and EMA approved the combination of ATO and ATRA in 2008 and 2010, respectively[355,356]. Since then, relapse is not common anymore. One study estimated the 4-year hematological and molecular relapse risk is 1% and 0%, respectively, which should be compared to 18% and 27% risk with the ATRA and chemotherapy combination[337]. To illustrate how rare relapse is, in Sweden, where only 19 patients per year are diagnosed with APL (according to data collected between 2008-2020), the estimated number of patients that will relapse from the combination treatment is only two patients per 10 years[357].

Thus, with such a low relapse rate, it is understandable that the experience of treating patients with relapse from ATRA and ATO frontline treatment is limited. However, the first consideration in Sweden is post-remission therapy with ATRA and idarubicin. If the treatment achieves CR with MRD, the recommendation is that two rounds of conditioning (ATRA and chemotherapy–idarubicin + Ara-C) are used to prepare for allogeneic HSCT in cases where a donor is available and autologous HSCT if no donor is available. However, autologous HSCT is always preferred for patients where post-remission treatment achieved CR_{MRD-} (i.e., no detection of PML-RARα by qPCR). Moreover, for patients that do not tolerate intensive chemotherapy and are thus ineligible for HSCT, the recommendation is to continue with ATRA and idarubicin or ATO monotherapy[353]. The estimated three-year OS after autologous and allogenic HSCT is about 80%, and for patients that are not transplanted, about 60%[358].

Summary and future directions

In summary, the combination therapy of ATRA and ATO for APL patients has changed this disease from a relatively poor prognosis to the AML subtype with the

highest cure rate. Since the treatment is effective and safe, the highest risk of death is due to early deaths, primarily from bleeding and blood clots unrelated to the treatment itself. Therefore, new drugs are unlikely to improve the frontline treatment of APL. Instead, an early start with ATRA and supportive care for bleeding at the first suspicion of APL is vital for improving OS further in this patient group[125,340].

Present investigation

Central to the four projects presented in this thesis is the use of OP9M2 stroma for supporting human HSPC or primary AML *in vitro*. Studies on using the OP9M2 stroma in co-culture and screening small molecules have previously been published and are thus the foundation of the work presented in this thesis[359,360]. The majority of the work in this thesis has addressed two main objectives: to identify new differentiation therapy of AML (Paper 1) and to identify new extrinsic regulators of HSC (Paper 3). We also show a detailed step-by-step protocol that includes all the accumulated experience of using the co-culture model for screening small molecules on primary AML cells (Paper 2). In addition, this thesis contains a collaboration project that utilized the stroma to support the viability of primary AML during genetic manipulation *in vitro* (Paper 4).

Results and discussion

Paper I

Combinatorial molecule screening identified a novel diterpene and the BET inhibitor CPI-203 as differentiation inducers of primary acute myeloid leukemia cells

Most patients with acute leukemia have a poor prognosis. The poor prognosis can partially be attributed to the high variance of recurrent genetic abnormalities that pose both a challenge and an opportunity for the future development of acute leukemia subtype-specific drugs. Also, multiple genetic abnormalities often cooperate, meaning that a single drug might not be sufficient to eliminate the leukemic cells effectively[361]. Recent studies have demonstrated that *ex-vivo* small molecule screening combined with genetic and clinical data can effectively identify patient-specific drug susceptibilities[362-364]. For that reason, in this study, we utilized a screening platform based on co-culture and multiplexed flow cytometry to screen a natural product library on primary AML samples. First, to identify differentiation-inducing compounds, and second, to conduct a combinatorial screen with a selected hit compound and a small molecule library with known drug targets for analyzing the mechanism of action and potential drug synergies[365]. Here we report on an identified natural product of jatrophane type that induces a Protein kinase C (PKC) mediated differentiation response in a subfraction of FLT3-WT AML samples that appears further bolstered with bromo- and extra-terminal domain (BET) inhibition. Although PKC agonists are previously known to induce AML differentiation and jatrophanes are known PKC agonists, jatrophanes have not been studied before in the context of AML differentiation[366,367]. In addition, our study shows that FLT-mutated AML are resistant to PKC-induced differentiation, confirming previous understanding that activating FLT mutations suppresses myeloid differentiation[368]. We also show that BET inhibition, which triggered much interest for its therapeutic potential in various cancer types, potentially boosts the differentiation response from the PKC agonist identified in this study[369]. These findings illustrate the value of unbiased multiplex screening platforms for identifying new compounds and developing combination therapies for myeloid neoplasms. *Ex-vivo* small molecule screening combined with genetic data will likely continue to be crucial for discovering new therapies. This will ultimately lead to improved precision-based treatment for myeloid neoplasms such as acute leukemia, resulting in improved prognosis for many patient groups.

Results in short

- We screened 512 natural products on primary AML samples and identified 44 compounds with differentiation or cytotoxic effect.
- Of these, we picked a promising molecule with a macrocyclic structure of jatrophane type that we refer to as molecule H4.
- We demonstrate that H4 induces differentiation in a monoclonal primary AML sample with P53-null genotype and monocytic FAB-M5 differentiation block by inducing immunophenotypic and genetic changes associated with differentiation towards the monocytic lineage, which translated to a drastic reduction in NSG-S engraftment.
- We show that differentiation occurs through the PKC and MAPK pathways, which leads to the activation of gene signatures of MYC targets and inflammatory signaling, including TNF signaling via NF-κB, and INFα and IFNγ response.
- The primary samples that responded to H4 were FLT-WT, with the most robust responders belonging to the FAB-M5 type, indicating that mutated FLT3 results in resistance to differentiation via the PKC pathway.
- Combinatorial screening identified a synergistic effect between H4 and a BET inhibitor that boosted immunophenotypic differentiation, with the most evident effect in FLT3 WT and FAB-M5 AML samples.

The starting point

At the outset of this study, our main objective was to identify promising molecules and therapeutic targets to develop new differentiation therapy for acute myeloid leukemia. To increase the potential of discovering a novel lead compound, we hand-picked 512 molecules optimized for diversity and potential for further drug refinement from a larger library of natural products. Another consideration we took was to screen the library in two concentrations on several primary samples, some of which were not included in the manuscript due to a lack of genetic data and general responsiveness. Below I will discuss some results in more detail that was beyond the scope of the manuscript with a focus on future directions.

The prospects of PKC agonists in therapy

This part of the discussion focuses on the clinical potential of PKC agonists in cancer therapy. Here I will try to answer whether it is realistic that forced activation of PKC can lead to a safe and efficacious therapy.

To start with, PKC is fundamentally an ambivalent drug target. Unlike some kinases with one or a few substrates it phosphorylates, PKC activates a large number of substrates that regulate many cellular responses that can be somewhat unpredictable as a result. The first effects of PKC activation are increased transcription and translation and altered regulation of ion channels, receptors, and secretion. These profound

alterations affect cells differently depending on cell type and context, including changes that induce proliferation, cell death, differentiation, migration, inflammatory processes, and altered cell-cell contact and cell morphology[370]. Hence, the effect can sometimes be opposing, which can depend on the cell type, as in the case where one cell type starts proliferating and another goes through cell death, or on context, where the same cell type responds in an opposing manner depending on the context of the stimulation[371,372]. Therefore, the on-target effects of forced PKC activation are not always the same, negatively affecting the predictability and promise of PKC as a druggable target.

Moreover, PKC is essential for normal cellular function and expressed but not necessarily active in all cell types in the body. When activated, the physiological PKC response is modulatory in its action on downstream pathways as many feedback mechanisms govern PKC signaling[370,373]. Interestingly, situations with sustained elevated activation of PKC are associated with abnormal cell function observed in many diseases, such as diabetes, cancer, ischemic heart disease, heart failure, autoimmune diseases, Parkinson's disease, Alzheimer's disease, bipolar, and psoriasis[370]. Thus, increased activation of PKC is naturally occurring. However, the level and duration of PKC activation are still within a relatively modest level compared to the activation that occurs from foreign PKC agonists. These PKC agonists, usually obtained from plants, induce an irreversible kinase activation due to the cells' inability to metabolize these molecules, which leads to an on-off switch response that is not physiologically normal or present in disease states[373]. Since PKC isoforms have a slightly different mechanism of action, it has been suggested that a possible approach to circumvent the toxicity would be to target specific PKC isoforms. However, it has been challenging to identify isoform-specific PKC agonists that would be efficacious without the undesired side effects[370]. Thus, given the vast amount of cellular responses and the widespread expression of PKC, it is not surprising that forced irreversible PKC activation is associated with unacceptable off-target effects. Some of these will be addressed more below.

An essential aspect of successful molecule-based therapy is the therapeutic window. A *therapeutic window* can be defined as the range of a drug or its concentration in the body that provides safe and effective therapy[374]. In the case of PKC agonists, this range is narrow or arguably even non-existent, meaning that the range between the lowest effective concentration and the toxic or even deadly concentration is exceedingly close. For example, the identified compound in our *in vitro* assays indicated a marked difference in response between 5μM to 10μM, indicating that 10μM is the concentration where the on-off switch effect kicks in. When we increased the concentration of our compound *in vitro*, the positive effect on differentiation did not substantially improve. However, had the same titer experiment been done *in vivo*, we would have likely seen unacceptable adverse effects. In support of this assumption, we treated mice with MLL-AF9 leukemia with the PKC agonist prostratin in an experiment not included in our study. Peritoneal injection of 0.5mg/kg prostratin was tolerable for the mice, although with signs of a relatively minor adverse reaction at the

injection site as the tissue hardened after repeated injections. However, increasing the dose to 1.0mg/kg led to an immediate reaction that killed one of the mice a few seconds after the injection, which meant we had to abort the experiment. The acute toxicity we observed is in line with a patent application that reported the maximum tolerable dose of prostratin to be 0.76mg/kg in mice[375]. Thus, the safety profile of prostratin is abysmal, and this is likely a general feature of PKC agonists due to the irreversible on-off switch activation they induce.

Hence, there should not be a surprise that the only PKC agonist that has reached medical approval is in the form of a topical cream formulation that was developed by the Danish company Leo Pharmaceuticals. Their product, Picato, was recommended for treating actinic keratosis, a skin neoplasm with a relatively high risk (0,15% to 80% annual risk) of progressing to invasive squamous cell carcinoma without treatment[376,377]. The active reagent in Picato is the natural product ingenol mebutate that, similar to the natural product identified in our study, activates PKC and the MAPK/ERK pathway[378]. Since Picato is a topical treatment applied externally on the skin, it acts locally by inducing cell death in the neoplastic cells at the application site, thereby avoiding the side effects it would have with a systemic administration. Picato was launched in 2012, but in 2020, the European Medicines Agency (EMA) decided to withdraw the market authorization due to a discovered increased cancer incidence rate in patients using the product, which prompted Leo Pharmaceuticals to remove the product worldwide[379]. So, unfortunately, what looked like a good application for a PKC-agonist-based treatment would prove unreliable as the benefits were smaller than the risks that even the cutaneous application design could not correct for.

In summary, efficacious PKC agonists without toxic side effects and a narrow therapeutic window have not been convincingly proven to exist. It is hard to see how novel agents would decouple the toxic effects from the positive effects of PKC activation, which is necessary for PKC to become a legitimate target. Therefore, from what is known today, the future promise of PKC agonists looks rather bleak.

Alternatives to PKC agonists

An alternative approach to the challenging task of identifying efficacious and safe PKC agonists is to target similar signaling pathways as the once PKC induces. Some of the most robust gene signatures upregulated by H4 identified in this study were the IFNα and IFNγ responses. Both IFNα and IFNγ are potential therapeutic candidates for AML. Of these two is IFNα better studied as a treatment of hematological neoplasms, but IFNγ is also compelling as a PKC-agonist alternative due to its involvement in monocytic differentiation[380,381].

Interestingly, lots of data demonstrate that IFNα is an efficacious and safe treatment of hematological neoplasms. The experience of using IFNα in the clinic goes back to the 80s[382]. More recently, pegylated-IFNα (peg-IFNα)–i.e., IFNα conjugated to a polyethylene glycol moiety for improved pharmacokinetics that gives it a longer-lasting effect *in vivo*–was recently approved as a monotherapy for the treatment of PV by both

EMA (2019) and FDA (2021)[383]. Clinical trials showed that it reduced the blasts and the LSC population (*JAK2*V617F allele burden) with acceptable toxicity compared to the control arm, which gave it the green light as a therapeutic option. Notably, the relative improvement with peg-IFNα is first seen after longer-term treatment, which is possible due to its low toxicity, where year one matched the control (hydroxyurea), and year three and five showed superior results compared to the control (best available treatment)[383,384]. Although the approval of peg-IFNα was for PV, based on previous clinical experience, clinicians are using IFNα for the treatment of other hematological neoplasms, including CML (before TKI revolutionized the treatment of CML and later in combination with TKI), aCML, ET, and systemic mast cell disease to name a few[382]. It has also been tested in AML with good results. For instance, a patient with transformed primary myelofibrosis with a prognosis of 6 months reached remission and continued to stay in peg-IFNα-dependent remission with continuous administration for >4 years[385,386]. Still, and a bit surprisingly, the place for peg-IFNα in the treatment of AML is unknown and has thus been pointed out as an area that warrants further study[381,386].

In contrast to IFNα, IFNγ is less studied but appears to be an exciting candidate for therapy. Its use as a potential differentiation inducer of the monocytic lineage seems especially promising since IFNγ is involved in the differentiation of monocytes to both dendritic cells and macrophages. For instance, the addition of IFNγ alone is enough to differentiate monocytes into macrophages *in vitro*[387]. Moreover, including IFNγ in the reprogramming protocol of fibroblasts to dendritic cells increased the reprogramming efficiency by 20-fold[388]. Hence, given the central role of IFNγ in the differentiation and activation of dendritic cells and macrophages, it would be interesting to explore its potential as a differentiation therapy for neoplasms with a differentiation block in the monocytic lineage. For *in vivo* experiments, peg-IFNγ has been developed with a 20-32 fold longer *in-vivo* half-life that was efficacious on a xenograft of ovarian cancer cells[389]. Interestingly, there is currently a phase I clinical trial investigating the effect of IFNγ as a monotherapy and in conjunction with donor leukocyte infusion for HSCT-relapsed AML and MDS[390]. Indeed, as others have alluded to, it will be interesting to see if IFN will have a renaissance in the treatment of hematological neoplasms that expands its use case beyond the recent market authorization for the treatment of PV[382,386].

Combinatorial treatment of FLT3-mutated AML and the implications of mutational heterogeneity in screening

During the revision of this study, the reviewers asked us to explore if FLT3 mutated AML were resistant to differentiation. By increasing the sample size, we could see a correlation between the FLT3 mutation status and the response to our molecule in primary AML samples but not in cell lines. However, in the cell lines, the three strongest responders had a differentiation block in the monocytic lineage–which is in line with increased sensitivity in the monocytic lineage for PKC agonists–with the most

potent response from the FLT3-WT THP1 cell line followed by the FLT3-mutated MM6 and MOLM-13 cell lines[391]. Thus, it appears as the FLT3 mutations also reduced differentiation in the cell lines, although the sample size is too small to draw a conclusion. Thereby, our study confirms the knowledge from other studies that FLT3 mutations suppress myeloid differentiation in addition to inducing uncontrolled proliferation and boosting cell survival[368,392].

Thus it would have been interesting to test if combination treatment with an FLT3 inhibitor unlocks or boosts the differentiation response in the FLT3-mutated samples. In line with this notion, although very preliminary, an earlier combination screen on the FLT3-mutated MM6 cell line showed that this might be the case. The screen demonstrated that FLT3 inhibition is highly toxic to MM6 cells and that the combination with molecule H4 boosted the differentiation response–albeit with few recorded events (data not shown).

A critical implication for screening for differentiation therapy is that FLT3 mutated samples should be considered to be screened in combination with FLT3 inhibitors to avoid false negatives. If not performed in the primary screen, it would be recommended to do it at least during follow-up experiments once the initial candidates have been identified.

Moreover, the FLT3-mutated differentiation block we highlight in this study is an excellent example of how the heterogeneity in underlying mutations results in different types of diseases requiring different treatment strategies. This mutational heterogeneity has implications for screening. For example, a small molecule screen on only a few samples could miss candidates due to the low sample size. Similarly, it should be expected that a screen conducted on a larger sample size may result in a response in one or only a few samples, with reasons that would be unknown at first but may lead to an important discovery if the reason why the sample is responding is found out. However, most small-molecule screenings are conducted on a small sample size due to the considerable resources it requires, and with that comes the risk that some potential patient-specific therapies might not be identified in the screening of a small molecule library.

Screening for molecules that upregulates therapeutically relevant cell surface markers

An unexpected result from the screen was that treatment with molecule H4 upregulates CD123 with a further boost in expression in combination with BET inhibition in a subfraction of the samples (data not shown). Since we included CD123 as a primitive marker, this upregulation was unanticipated as we expected it to downregulate during a differentiation response. Therefore the upregulation of CD123 is not related to differentiation, which was further strengthened by the potent upregulation of CD123 in the AML-2 sample (FAB-M2: neutrophil lineage) that was resistant to H4-induced differentiation (data not shown). However, the boosted expression of CD123 is interesting since CD123 is evaluated as a therapeutic target for hematological malignancies. Indeed, much research in the last decade has been concentrating on this

antigen, which has led to the development of multiple strategies to target it, including recombinant diphtheria toxin IL-3 fusion protein, antibody-drug conjugates, CAR T cells, and monoclonal and bispecific antibodies that utilizes the host's immune system to kill the CD123 expressing cells. Although some of these strategies are inefficient or have led to unacceptable bone marrow toxicities, ongoing research aims to minimize on-target cytotoxicity while maintaining therapeutic efficiency[393].

Targeted therapy of this type is, for understandable reasons, limited to malignancies that express high levels of the targeted antigen. Even then, relapse and treatment inefficiencies can arise from therapeutic selection pressure, which allows for the growth of cells that lack the targeted antigen or have acquired resistance to the therapy, i.e., following the mechanisms of natural selection or survival of the fittest originally laid out by Charles Darwin in his legendary book on the origin of species[394,395].

Thus, molecules like H4 that upregulate these druggable antigens are interesting as they could make these targeted therapies more efficient. Our screening platform, based on the detection of cell surface markers, is therefore well suited for screening molecules that upregulate druggable targets, such as the example with CD123. Thus when deciding on the objectives of a screen, such as in our case to identify differentiation therapy, it can also include the identification of molecules that enhance the treatment efficacy of targeted therapies directed towards cell surface markers.

General conclusion

The heterogeneity in hematological disease introduces complexity and an opportunity for the preclinical research community that works towards bringing safer and more efficient precision-based treatment to patients. In this study, we performed a screen to identify novel molecules that could lead to new differentiation therapy for subgroups of AML patients. Even though our lead compound that activates PKC may not be clinically applicable, our study still highlights important aspects for continuous research on precision-based treatment. First, AML of the monocytic lineage is sensitive to PKC-induced differentiation and, by reason, other molecules that induce similar pathways, for which IFN is a strong candidate with less toxicity. Second, FLT3 mutations introduce a differentiation block with implications for differentiation therapy, where combination therapy with FLT3 inhibitors should be considered. This should concern everyone working on differentiation therapy, from preclinical research to clinical trials.

From a personal perspective, I would like to summarize that even though the prospect of PKC as a therapeutic target is highly uncertain, our study illustrates that the incentives in academia to publish rather than pursue commercial outcomes (which allowed us to pursue this study) has its place as it can lead to unexpected discoveries that ultimately brings value. Thus, academia and the industry counterbalance each other's strengths and weaknesses and are together a net positive for our society. Since incentives influence motivations at all levels of a system's hierarchy, an essential question for people interested in pursuing science should be which incentive structure

aligns best with them, i.e., what motivates them more, the pressure to publish, or the pressure for commercial outcomes.

Paper II

Small Molecule Screening of Primary Human Acute Myeloid Leukemia Using Co-culture and Multiplexed FACS Analysis

Methods for screening and investigating drug responses in primary AML samples are more relevant than ever as targeted therapy is becoming more common[396]. However, primary AML cells are notoriously difficult to culture due to their propensity to go through cell death and differentiation when placed in inadequate cell culture conditions[397]. The advantage of using primary cells over leukemic cell lines is that they represent the full mutational heterogeneity of AML in contrast to cell lines that only represent a small number of AML subtypes that also lacks mutational authenticity from prolonged *in vitro* culture[397]. Thus an appropriate cell culture strategy of primary AML cells must be selected to get reliable results during drug screening and mechanistic studies. The various cell culture strategies that support primary AML cells *ex vivo* are designed to recreate the supportive conditions from the bone marrow or compensate for the lack of support in the cell culture dish by using supportive feeder cells, cytokines, or small molecules[360,398]. Our approach to creating a supportive *in vitro* condition is based on co-culture with a subclone of OP9 stromal cells (OP9M2) and myeloid cytokines that promote survival and hinder culture-induced differentiation of primary AML cells[360,399]. Here we describe how to use this co-culture system for small molecule screening and mechanistic studies. Although sample heterogeneity still impacts the extent of what the co-culture and screening platform is capable of, in general, the benefit of this cell culture method includes preservation of cell surface markers, support of cellular growth, and prevention of cell death in most samples[360,399]. Because flow cytometry is used in the readout, physical and chemical characteristics can be detected on a single-cell level, which allows for the analysis of subpopulations in individual samples. Moreover, the readout using flow cytometry is highly adjustable to the study objective, and multiple variables can be assayed simultaneously, such as cell survival, apoptosis, cell growth, cell cycle, cell differentiation, stemness (LSC subpopulations), and the upregulation of druggable cell surface markers. Hence, this cell culture and screening method can identify novel therapeutic agents or explore drug repurposing, drug synergism, patient selection, mechanism of action analysis, and drug resistance. As targeted therapies for AML are becoming more common, methods for screening and investigating drug responses in primary AML samples are more relevant than ever[396,397]. Methods for small molecule screening and mechanistic studies of primary AML samples will continue to be crucial for developing new therapies, ultimately resulting in improved outcomes for many patient groups.

Results in short

- We describe a step-by-step protocol for how to use OP9M2 stromal cells for small molecule screening of primary adult and pediatric AML cells, which include the procedures:
    - How to thaw OP9M2 stromal cells and primary AML cells.
    - How to culture OP9M2 cells and plate them with primary AML cells in 96 half-area well plates.
    - How to add small molecules.
    - How to perform the readout using a flow cytometer equipped with a high throughput sampler (HTS).
    - How to analyze the data using FlowJo.

Paper III

Identification of extrinsic regulators of human hematopoietic stem cells using an shRNA screen in mesenchymal stromal cells

A driving force behind the study of HSC self-renewal stems from the fact that the transplanted cell number has a linear correlation to the recovery time of the new blood system. Thus, an adequate amount of cells must be transplanted as it otherwise can lead to opportunistic infections and graft failure[107]. Since the cell number is a limiting factor in some applications, such as for UCB transplantations to adults and in some cases of autologous gene therapies, a robust approach for expanding HSPC would unlock the untapped potential of many applications in need of higher cell numbers before transplantation[108]. The expansion of cells *in vitro* requires an artificial environment that bolsters the natural processes of symmetric cell division and cell survival. One cell type closely associated with HSPC *in vivo* is the MSC, which expresses many of the known HSC niche factors, such as SCF, the ANGPTL family, and CXCL12[400-404]. In *ex vivo* co-culture, MSC supports the engraftment potential of HSC, but the mechanisms are still largely unknown, suggesting that MSC is a good screening model for extrinsic regulators of HSC function[405]. Here we generated a list of potential HSC regulators by comparing the gene expression of a non-supportive (BFC012) vs. supportive MSC-derived feeder cell line (OP9M2)[405,406]. We also show the results from an shRNA knock-down screen for proteins expressed in the OP9M2-MSC extracellular environment that we assay for HSPC supportive capacity by co-culture of UCB derived HSPC with flow cytometry as readout. Unfortunately, the shRNA screen failed to identify leads for further exploration, which likely had to do with a suboptimal screening approach based on RNA interference. Regardless, our results demonstrate that factors in the vicinity of the MSC microenvironment are more critical than secreted factors, and we present a list of potential regulators that should still be useful for identifying new regulators of HSC. Thus, we anticipate that our candidate list can

be a resource for other studies aiming to identify HSC niche factors. Even though our screen failed to identify any leads, improving the screening methodology with CRISPR/Cas9 could make these screens valuable. Improvements in ex *vivo* culture conditions are needed as they will contribute to the development of genetic engineering-based applications and potentially make UCB a trusted and reliable source for HSCT[108]. Thus, this will ultimately lead to better therapy for both non-malignant and malignant diseases of the hematopoietic system.

Results in short

- We demonstrate that the *ex vivo* MSC support of HSPC relies on cell-cell contact by showing that transwell inserts and OP9M2 conditioned media is inferior to co-culture.
- We provide a candidate list of proteins expressed by the MSC cells in the plasma membrane or the extracellular matrix with potential HSC regulatory functions.
- Of these candidates, we screened 74 genes for their supportive effect of HSPC using a screening methodology based on RNA interference, MSC-HSPC co-culture, and flow cytometry.
- The screen failed to identify any leads, which reasons we elaborated on and suggested possible improvements for in the discussion.

Paper IV

Inducing synthetic lethality for selective targeting of acute myeloid leukemia cells harboring STAG2 mutations

The improved recognition of disease heterogeneity in AML has opened up the floodgates for developing precision medicine. Precision medicine is tailored treatment optimized for therapeutic benefit in individual patient groups. Thus, precision medicine represents the next phase of development from the standardized cytotoxic chemotherapy that follows the one-drug-fits-all model that, notably after its introduction about 50 years ago, still is the mainstay therapy for most AML subtypes[229,407]. One mechanism that precision-based therapy can be designed to exploit is known as synthetic lethal interaction, which occurs in situations where the expression of two genes must be intact for the cell to stay viable. This phenomenon is exploitable in cancer cells with one of the genes mutated, which makes them compared to healthy cells disproportionally vulnerable to perturbation of the second gene. An example of gene pair with a synthetic lethal interaction is the paralogue genes *STAG1* and *STAG2*, which are part of the cohesin complex. *STAG1* and *STAG2* partially overlap functionally with redundancy in mediating cohesion between sister chromatids during cell division[408,409]. Mutations in subunits of the cohesin complex are mutually exclusive

and found in 5.9% of all AML with a frequency of 1,8% and 1,3% for *STAG1* and *STAG2*, respectively, which makes them the two most commonly mutated subunits in the cohesin complex[410]. However, although the synthetic lethal interaction between STAG1 and STAG2 genes is known earlier, the possibility of exploiting this vulnerability in primary AML cells has not been demonstrated. Here we show that knocking down STAG1 in STAG2-null primary AML cells results in a 2-5 fold reduction in cell expansion *in vitro* and a nearly complete depletion of engrafted cells at week 16 in NSG-S mice. Moreover, using UCB-HSPC cells, we demonstrate that STAG1 knockdown is well tolerated by HSPC, while it negatively affects proliferation in STAG2-KO HSPC, demonstrating the synthetic lethal interaction in healthy cells. These results demonstrate a proof-of-concept for the value of selectively targeting STAG1 within the hematopoietic system, further strengthening the rationale for developing targeted therapies toward STAG1 in hematological disease. Strategies suggested for targeting STAG1 include induction of protein degradation via PROteolysis TArgeting Chimera (PROTAC) technology and small molecule blocking of the STAG-RAD21 protein interaction[411]. Noteworthy, these potential therapies must have specific ligandability to STAG1 to be successful since unspecific affinity would create synthetic lethality in normal cells[412]. Looking ahead, improvements in AML treatment will utilize an in-depth understanding of disease heterogeneity to target AML subtype-specific weaknesses with the broad arsenal of tools developed for precision medicine[407]. This exciting development will ultimately lead to improved prognosis for many patient groups, which is a source of motivation for the continued effort in bringing precision-based therapy to the clinic.

Results in short

- We demonstrate that knockdown of STAG1 in STAG2-null HSPC and primary AML cells leads to reduced cell viability through synthetic lethality both *in vitro* and *in vivo*.
- In contrast, the knockdown of STAG1 in STAG2-WT HSPC is well tolerated both *in vitro* and *in vivo*.
- Importantly, this study demonstrates the first proof-of-concept of STAG1 and STAG2 mediated synthetic lethality in primary AML cells.

References

1 West, S. A., Fisher, R. M., Gardner, A. & Kiers, E. T. Major evolutionary transitions in individuality. *Proc Natl Acad Sci U S A* **112**, 10112-10119 (2015). https://doi.org:10.1073/pnas.1421402112

2 Yamamoto, K. *et al.* Extramedullary hematopoiesis: Elucidating the function of the hematopoietic stem cell niche (Review). *Mol Med Rep* **13**, 587-591 (2016). https://doi.org:10.3892/mmr.2015.4621

3 Laurenti, E. & Göttgens, B. From haematopoietic stem cells to complex differentiation landscapes. *Nature* **553**, 418-426 (2018). https://doi.org:10.1038/nature25022

4 Ogawa, M. Differentiation and proliferation of hematopoietic stem cells. *Blood* **81**, 2844-2853 (1993).

5 Nourshargh, S. & Alon, R. Leukocyte migration into inflamed tissues. *Immunity* **41**, 694-707 (2014). https://doi.org:10.1016/j.immuni.2014.10.008

6 Marshall, J. S., Warrington, R., Watson, W. & Kim, H. L. An introduction to immunology and immunopathology. *Allergy Asthma Clin Immunol* **14**, 49 (2018). https://doi.org:10.1186/s13223-018-0278-1

7 Rieger, M. A. & Schroeder, T. Hematopoiesis. *Cold Spring Harb Perspect Biol* **4** (2012). https://doi.org:10.1101/cshperspect.a008250

8 Haeckel, E. *Anthropogenie oder entwickelungsgeschichte des menschen*. (W. Engelmann, 1874).

9 Haeckel, E. *Natürliche Schöpfungsgeschichte. Gemeinverständliche wissenschaftliche Vorträge über die Entwickelungslehre im Allgemeinen und diejenige von Darwin, Goethe und Lamarck im Besonderen... Mit Tafeln, Holzschnitten, etc*. (1868).

10 Ramalho-Santos, M. & Willenbring, H. On the origin of the term "stem cell". *Cell Stem Cell* **1**, 35-38 (2007). https://doi.org:10.1016/j.stem.2007.05.013

11 Haeckel, E. *Anthropogenie: oder, Entwickelungsgeschichte des menschen, Keimes-und stammesgeschichte*. (W. Engelmann, 1877).

12 Häcker, V. *Die Kerntheilungsvorgänge bei der Mesoderm- und Entodermbildung von Cyclops, von Dr. V. Haecker*. (1892).

13 Kaushansky, K. *Williams hematology*, Univerza v Ljubljani, Medicinska fakulteta, (2010).

14 Becker, A. J., McCulloch, E. A. & Till, J. E. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. *Nature* **197**, 452-454 (1963).

15 Till, J. E. & Mc, C. E. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res* **14**, 213-222 (1961).

16 Till, J. E., McCulloch, E. A. & Siminovitch, L. A STOCHASTIC MODEL OF STEM CELL PROLIFERATION, BASED ON THE GROWTH OF SPLEEN COLONY-FORMING CELLS. *Proc Natl Acad Sci U S A* **51**, 29-36 (1964). https://doi.org:10.1073/pnas.51.1.29

17 Nichols, J. & Smith, A. The origin and identity of embryonic stem cells. *Development* **138**, 3-8 (2011). https://doi.org:10.1242/dev.050831

18 Maldonado-Soto, A. R. *et al.* Stem cells in the nervous system. *Am J Phys Med Rehabil* **93**, S132-144 (2014). https://doi.org:10.1097/phm.0000000000000111

19 Goodell, M. A., Nguyen, H. & Shroyer, N. Somatic stem cell heterogeneity: diversity in the blood, skin and intestinal stem cell compartments. *Nat Rev Mol Cell Biol* **16**, 299-309 (2015). https://doi.org:10.1038/nrm3980

20 Inra, C. N. *et al.* A perisinusoidal niche for extramedullary haematopoiesis in the spleen. *Nature* **527**, 466-471 (2015).

21 Lefrançais, E. *et al.* The lung is a site of platelet biogenesis and a reservoir for haematopoietic progenitors. *Nature* **544**, 105-109 (2017).

22 Méndez-Ferrer, S., Lucas, D., Battista, M. & Frenette, P. S. Haematopoietic stem cell release is regulated by circadian oscillations. *Nature* **452**, 442-447 (2008).

23 Oguro, H., Ding, L. & Morrison, S. J. SLAM family markers resolve functionally distinct subpopulations of hematopoietic stem cells and multipotent progenitors. *Cell Stem Cell* **13**, 102-116 (2013).

24 Majeti, R., Park, C. Y. & Weissman, I. L. Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. *Cell Stem Cell* **1**, 635-645 (2007).

25 Notta, F. *et al.* Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science* **333**, 218-221 (2011).

26 Laurenti, E. *et al.* CDK6 levels regulate quiescence exit in human hematopoietic stem cells. *Cell Stem Cell* **16**, 302-313 (2015).

27 Benveniste, P. *et al.* Intermediate-term hematopoietic stem cells with extended but time-limited reconstitution potential. *Cell Stem Cell* **6**, 48-58 (2010).

28 Wilson, A. *et al.* Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. *Cell* **135**, 1118-1129 (2008). https://doi.org:10.1016/j.cell.2008.10.048

29 Foudi, A. *et al.* Analysis of histone 2B-GFP retention reveals slowly cycling hematopoietic stem cells. *Nat Biotechnol* **27**, 84-90 (2009). https://doi.org:10.1038/nbt.1517

30 Warr, M. R. *et al.* FOXO3A directs a protective autophagy program in haematopoietic stem cells. *Nature* **494**, 323-327 (2013). https://doi.org:10.1038/nature11895

31 Ho, T. T. *et al.* Autophagy maintains the metabolism and function of young and old stem cells. *Nature* **543**, 205-210 (2017). https://doi.org:10.1038/nature21388

32 Simsek, T. *et al.* The distinct metabolic profile of hematopoietic stem cells reflects their location in a hypoxic niche. *Cell Stem Cell* **7**, 380-390 (2010).

33 Takubo, K. *et al.* Regulation of glycolysis by Pdk functions as a metabolic checkpoint for cell cycle quiescence in hematopoietic stem cells. *Cell Stem Cell* **12**, 49-61 (2013).

34 Vannini, N. *et al.* (2016).

35 Ito, K. *et al.* Self-renewal of a purified Tie2+ hematopoietic stem cell population relies on mitochondrial clearance. *Science* **354**, 1156-1160 (2016).

36 Mansell, E. *et al.* Mitochondrial Potentiation Ameliorates Age-Related Heterogeneity in Hematopoietic Stem Cell Function. *Cell Stem Cell* (2020). https://doi.org:10.1016/j.stem.2020.09.018

37 Signer, R. A., Magee, J. A., Salic, A. & Morrison, S. J. Haematopoietic stem cells require a highly regulated protein synthesis rate. *Nature* **509**, 49-54 (2014). https://doi.org:10.1038/nature13035

38 Takizawa, H., Regoes, R. R., Boddupalli, C. S., Bonhoeffer, S. & Manz, M. G. Dynamic variation in cycling of hematopoietic stem cells in steady state and inflammation. *J Exp Med* **208**, 273-284 (2011). https://doi.org:10.1084/jem.20101643

39 Bernitz, J. M., Kim, H. S., MacArthur, B., Sieburg, H. & Moore, K. Hematopoietic Stem Cells Count and Remember Self-Renewal Divisions. *Cell* **167**, 1296-1309.e1210 (2016). https://doi.org:10.1016/j.cell.2016.10.022

40 Sun, J. *et al.* Clonal dynamics of native haematopoiesis. *Nature* **514**, 322-327 (2014). https://doi.org:10.1038/nature13824

41 Notta, F. *et al.* Distinct routes of lineage development reshape the human blood hierarchy across ontogeny. *Science* **351**, aab2116 (2016). https://doi.org:10.1126/science.aab2116

42 Miyawaki, K. *et al.* Identification of unipotent megakaryocyte progenitors in human hematopoiesis. *Blood* **129**, 3332-3343 (2017). https://doi.org:10.1182/blood-2016-09-741611

43 Belluschi, S. *et al.* Myelo-lymphoid lineage restriction occurs in the human haematopoietic stem cell compartment before lymphoid-primed multipotent progenitors. *Nat Commun* **9**, 4100 (2018). https://doi.org:10.1038/s41467-018-06442-4

44 Yu, V. W. C. *et al.* Epigenetic Memory Underlies Cell-Autonomous Heterogeneous Behavior of Hematopoietic Stem Cells. *Cell* **168**, 944-945 (2017). https://doi.org:10.1016/j.cell.2017.02.010

45 Challen, G. A., Boles, N. C., Chambers, S. M. & Goodell, M. A. Distinct hematopoietic stem cell subtypes are differentially regulated by TGF-beta1. *Cell Stem Cell* **6**, 265-278 (2010). https://doi.org:10.1016/j.stem.2010.02.002

46 Müller-Sieburg, C. E., Cho, R. H., Thoman, M., Adkins, B. & Sieburg, H. B. Deterministic regulation of hematopoietic stem cell self-renewal and differentiation. *Blood* **100**, 1302-1309 (2002).

47 Niederwieser, D. *et al.* One and Half Million Hematopoietic Stem Cell Transplants (HSCT). Dissemination, Trends and Potential to Improve Activity By Telemedicine from the Worldwide Network for Blood and Marrow Transplantation (WBMT). *Blood* **134**, 2035-2035 (2019). https://doi.org:10.1182/blood-2019-125232

48 Reisman, M. & Adams, K. T. Stem cell therapy: a look at current research, regulations, and remaining hurdles. *P t* **39**, 846-857 (2014).

49 Husby, S. & Grønbæk, K. Mature lymphoid malignancies: origin, stem cells, and chronicity. *Blood Adv* **1**, 2444-2455 (2017). https://doi.org:10.1182/bloodadvances.2017008854

50 Passweg, J. R. *et al.* Hematopoietic cell transplantation and cellular therapy survey of the EBMT: monitoring of activities and trends over 30 years. *Bone Marrow Transplant* (2021). https://doi.org:10.1038/s41409-021-01227-8

51 Passweg, J. R. *et al.* Hematopoietic SCT in Europe: data and trends in 2012 with special consideration of pediatric transplantation. *Bone Marrow Transplant* **49**, 744-750 (2014). https://doi.org:10.1038/bmt.2014.55

52 Gyurkocza, B. & Sandmaier, B. M. Conditioning regimens for hematopoietic cell transplantation: one size does not fit all. *Blood* **124**, 344-353 (2014). https://doi.org:10.1182/blood-2014-02-514778

53 Hierlmeier, S., Eyrich, M., Wölfl, M., Schlegel, P. G. & Wiegering, V. Early and late complications following hematopoietic stem cell transplantation in pediatric patients - A retrospective analysis over 11 years. *PLoS One* **13**, e0204914 (2018). https://doi.org:10.1371/journal.pone.0204914

54 Henig, I. & Zuckerman, T. Hematopoietic stem cell transplantation-50 years of evolution and future perspectives. *Rambam Maimonides Med J* **5**, e0028 (2014). https://doi.org:10.5041/rmmj.10162

55 Lipof, J. J., Loh, K. P., O'Dwyer, K. & Liesveld, J. L. Allogeneic Hematopoietic Cell Transplantation for Older Adults with Acute Myeloid Leukemia. *Cancers (Basel)* **10** (2018). https://doi.org:10.3390/cancers10060179

56 Walter, R. B. *et al.* Comparison of minimal residual disease as outcome predictor for AML patients in first complete remission undergoing myeloablative or nonmyeloablative allogeneic hematopoietic cell transplantation. *Leukemia* **29**, 137-144 (2015). https://doi.org:10.1038/leu.2014.173

57 Lawitschka, A. *et al.* Paediatric reduced intensity conditioning: analysis of centre strategies on regimens and definitions by the EBMT Paediatric Diseases and

Complications and Quality of Life WP. *Bone Marrow Transplant* **50**, 592-597 (2015). https://doi.org:10.1038/bmt.2014.306

58 Hayashi, R. J. Considerations in Preparative Regimen Selection to Minimize Rejection in Pediatric Hematopoietic Transplantation in Non-Malignant Diseases. *Front Immunol* **11**, 567423 (2020). https://doi.org:10.3389/fimmu.2020.567423

59 Río, P. *et al.* Successful engraftment of gene-corrected hematopoietic stem cells in non-conditioned patients with Fanconi anemia. *Nat Med* **25**, 1396-1401 (2019). https://doi.org:10.1038/s41591-019-0550-z

60 Dufour, C. How I manage patients with Fanconi anaemia. *Br J Haematol* **178**, 32-47 (2017). https://doi.org:10.1111/bjh.14615

61 Ferrari, G., Thrasher, A. J. & Aiuti, A. Gene therapy using haematopoietic stem and progenitor cells. *Nat Rev Genet* (2020). https://doi.org:10.1038/s41576-020-00298-5

62 Bresters, D. *et al.* High burden of late effects after haematopoietic stem cell transplantation in childhood: a single-centre study. *Bone Marrow Transplant* **45**, 79-85 (2010). https://doi.org:10.1038/bmt.2009.92

63 Kwon, H. S. *et al.* Anti-human CD117 antibody-mediated bone marrow niche clearance in nonhuman primates and humanized NSG mice. *Blood* **133**, 2104-2108 (2019). https://doi.org:10.1182/blood-2018-06-853879

64 Agarwal, R. *et al.* Toxicity-Free Hematopoietic Stem Cell Engraftment Achieved with Anti-CD117 Monoclonal Antibody Conditioning. *Biology of Blood and Marrow Transplantation* **25**, S92 (2019). https://doi.org:https://doi.org/10.1016/j.bbmt.2018.12.172

65 Palchaudhuri, R. *et al.* Non-genotoxic conditioning for hematopoietic stem cell transplantation using a hematopoietic-cell-specific internalizing immunotoxin. *Nat Biotechnol* **34**, 738-745 (2016). https://doi.org:10.1038/nbt.3584

66 Thomas, E. D., Lochte, H. L., Jr., Cannon, J. H., Sahler, O. D. & Ferrebee, J. W. Supralethal whole body irradiation and isologous marrow transplantation in man. *J Clin Invest* **38**, 1709-1716 (1959). https://doi.org:10.1172/jci103949

67 Haurani, F. I. Thirty-one-year survival following chemotherapy and autologous bone marrow in malignant lymphoma. *Am J Hematol* **55**, 35-38 (1997). https://doi.org:10.1002/(sici)1096-8652(199705)55:1<35::aid-ajh6>3.0.co;2-4

68 Thomas, E. D. A history of haemopoietic cell transplantation. *Br J Haematol* **105**, 330-339 (1999). https://doi.org:10.1111/j.1365-2141.1999.01337.x

69 Bortin, M. M. A compendium of reported human bone marrow transplants. *Transplantation* **9**, 571-587 (1970). https://doi.org:10.1097/00007890-197006000-00006

70 Storb, R., Epstein, R. B., Graham, T. C. & Thomas, E. D. Methotrexate regimens for control of graft-versus-host disease in dogs with allogeneic marrow grafts. *Transplantation* **9**, 240-246 (1970). https://doi.org:10.1097/00007890-197003000-00007

71 Epstein, R. B., Storb, R., Ragde, H. & Thomas, E. D. Cytotoxic typing antisera for marrow grafting in littermate dogs. *Transplantation* **6**, 45-58 (1968). https://doi.org:10.1097/00007890-196801000-00005

72 Barnes, D. W. & Loutit, J. F. Treatment of murine leukaemia with x-rays and homologous bone marrow. II. *Br J Haematol* **3**, 241-252 (1957). https://doi.org:10.1111/j.1365-2141.1957.tb05793.x

73 Weiden, P. L. *et al.* Antileukemic effect of graft-versus-host disease in human recipients of allogeneic-marrow grafts. *N Engl J Med* **300**, 1068-1073 (1979). https://doi.org:10.1056/nejm197905103001902

74 Spurgin, L. G. & Richardson, D. S. How pathogens drive genetic diversity: MHC, mechanisms and misunderstandings. *Proc Biol Sci* **277**, 979-988 (2010). https://doi.org:10.1098/rspb.2009.2084

75 Piertney, S. B. & Oliver, M. K. The evolutionary ecology of the major histocompatibility complex. *Heredity (Edinb)* **96**, 7-21 (2006). https://doi.org:10.1038/sj.hdy.6800724

76 Bentkowski, P. & Radwan, J. Evolution of major histocompatibility complex gene copy number. *PLoS Comput Biol* **15**, e1007015 (2019). https://doi.org:10.1371/journal.pcbi.1007015

77 Sweeney, C. & Vyas, P. The Graft-Versus-Leukemia Effect in AML. *Front Oncol* **9**, 1217 (2019). https://doi.org:10.3389/fonc.2019.01217

78 Porrata, L. F. Autologous Graft-versus-Tumor Effect: Reality or Fiction? *Adv Hematol* **2016**, 5385972 (2016). https://doi.org:10.1155/2016/5385972

79 Jameson-Lee, M. *et al.* In silico Derivation of HLA-Specific Alloreactivity Potential from Whole Exome Sequencing of Stem-Cell Transplant Donors and Recipients: Understanding the Quantitative Immunobiology of Allogeneic Transplantation. *Front Immunol* **5**, 529 (2014). https://doi.org:10.3389/fimmu.2014.00529

80 Granados, D. P. *et al.* Impact of genomic polymorphisms on the repertoire of human MHC class I-associated peptides. *Nat Commun* **5**, 3600 (2014). https://doi.org:10.1038/ncomms4600

81 Martin, P. J. *et al.* Genome-wide minor histocompatibility matching as related to the risk of graft-versus-host disease. *Blood* **129**, 791-798 (2017). https://doi.org:10.1182/blood-2016-09-737700

82 Stern, M. *et al.* Influence of donor/recipient sex matching on outcome of allogeneic hematopoietic stem cell transplantation for aplastic anemia. *Transplantation* **82**, 218-226 (2006). https://doi.org:10.1097/01.tp.0000226156.99206.d1

83 Schumacher, T. N. & Schreiber, R. D. Neoantigens in cancer immunotherapy. *Science* **348**, 69-74 (2015). https://doi.org:10.1126/science.aaa4971

84 Greiner, J. *et al.* Expression of tumor-associated antigens in acute myeloid leukemia: Implications for specific immunotherapeutic approaches. *Blood* **108**, 4109-4117 (2006). https://doi.org:10.1182/blood-2006-01-023127

85 Wadia, P. P. *et al.* Antibodies specifically target AML antigen NuSAP1 after allogeneic bone marrow transplantation. *Blood* **115**, 2077-2087 (2010). https://doi.org:10.1182/blood-2009-03-211375

86 Nakasone, H. *et al.* Allogeneic HY antibodies detected 3 months after female-to-male HCT predict chronic GVHD and nonrelapse mortality in humans. *Blood* **125**, 3193-3201 (2015). https://doi.org:10.1182/blood-2014-11-613323

87 Gillissen, M. A. *et al.* AML relapse after rituximab treatment for GvHD: crucial role for B cells in GvL responses. *Bone Marrow Transplant* **51**, 1245-1248 (2016). https://doi.org:10.1038/bmt.2016.90

88 Toubai, T., Mathewson, N. & Reddy, P. The role of dendritic cells in graft-versus-tumor effect. *Front Immunol* **5**, 66 (2014). https://doi.org:10.3389/fimmu.2014.00066

89 Dean, R. *et al.* Dendritic cells in autologous hematopoietic stem cell transplantation for diffuse large B-cell lymphoma: graft content and post transplant recovery predict survival. *Bone Marrow Transplant* **36**, 1049-1052 (2005). https://doi.org:10.1038/sj.bmt.1705183

90 Baron, F. *et al.* What is the role for donor natural killer cells after nonmyeloablative conditioning? *Biol Blood Marrow Transplant* **15**, 580-588 (2009). https://doi.org:10.1016/j.bbmt.2009.01.018

91 Bhatt, V. R. Allogeneic Stem Cell Transplantation for Non-Hodgkin Lymphoma. *Current Hematologic Malignancy Reports* **11**, 196-207 (2016). https://doi.org:10.1007/s11899-016-0319-0

92 Shaw, B. E. *et al.* Recipient/donor HLA and CMV matching in recipients of T-cell-depleted unrelated donor haematopoietic cell transplants. *Bone Marrow Transplant* **52**, 717-725 (2017). https://doi.org:10.1038/bmt.2016.352

93 Hurley, C. K. *et al.* Regarding "Recipients Receiving Better HLA-Matched Hematopoietic Cell Transplantation Grafts, Uncovered by a Novel HLA Typing Method, Have Superior Survival: A Retrospective Study". *Biol Blood Marrow Transplant* **25**, e268-e269 (2019). https://doi.org:10.1016/j.bbmt.2019.05.026

94 Tiercy, J. M. How to select the best available related or unrelated donor of hematopoietic stem cells? *Haematologica* **101**, 680-687 (2016). https://doi.org:10.3324/haematol.2015.141119

95 Chen, J., Lazarus, H. M., Dahi, P. B., Avecilla, S. & Giralt, S. A. Getting blood out of a stone: Identification and management of patients with poor hematopoietic cell mobilization. *Blood Rev*, 100771 (2020). https://doi.org:10.1016/j.blre.2020.100771

96 Gertz, M. A. Current status of stem cell mobilization. *Br J Haematol* **150**, 647-662 (2010). https://doi.org:10.1111/j.1365-2141.2010.08313.x

97 Tobiasregistret. *Om tobiasregistret*, <https://www.tobiasregistret.se/om/> (2021, April 26).

98 Janusinfo. *Zynteglo (autologa cd34-positiva celler, genetiskt modifierade till att utrycka βa-t87q-globingenen) vid β-thalassemi*, <https://janusinfo.se/download/18.722d04cf175c8613c9dbce33/1606732898283/Zynteglo-vid-betathalassemi-201130.pdf> (2020, November 30).

99 Lidonnici, M. R. & Ferrari, G. Gene therapy and gene editing strategies for hemoglobinopathies. *Blood Cells Mol Dis* **70**, 87-101 (2018). https://doi.org:10.1016/j.bcmd.2017.12.001

100 European Medicine Agency. *Zynteglo*, <https://www.ema.europa.eu/en/medicines/human/EPAR/zynteglo#product-information-section> (2021, January 28).

101 Zhao, Y., Stepto, H. & Schneider, C. K. Development of the First World Health Organization Lentiviral Vector Standard: Toward the Production Control and Standardization of Lentivirus-Based Gene Therapy Products. *Hum Gene Ther Methods* **28**, 205-214 (2017). https://doi.org:10.1089/hgtb.2017.078

102 Zonari, E. *et al.* Efficient Ex Vivo Engineering and Expansion of Highly Purified Human Hematopoietic Stem and Progenitor Cell Populations for Gene Therapy. *Stem Cell Reports* **8**, 977-990 (2017). https://doi.org:10.1016/j.stemcr.2017.02.010

103 Masiuk, K. E. *et al.* Improving Gene Therapy Efficiency through the Enrichment of Human Hematopoietic Stem Cells. *Mol Ther* **25**, 2163-2175 (2017). https://doi.org:10.1016/j.ymthe.2017.05.023

104 Schott, J. W. *et al.* Enhancing Lentiviral and Alpharetroviral Transduction of Human Hematopoietic Stem Cells for Clinical Application. *Mol Ther Methods Clin Dev* **14**, 134-147 (2019). https://doi.org:10.1016/j.omtm.2019.05.015

105 Petrillo, C. *et al.* Cyclosporine H Overcomes Innate Immune Restrictions to Improve Lentiviral Transduction and Gene Editing In Human Hematopoietic Stem Cells. *Cell Stem Cell* **23**, 820-832.e829 (2018). https://doi.org:10.1016/j.stem.2018.10.008

106 Porteus, M. H. A New Class of Medicines through DNA Editing. *N Engl J Med* **380**, 947-959 (2019). https://doi.org:10.1056/NEJMra1800729

107 Barker, J. N. *et al.* CD34(+) cell content of 126 341 cord blood units in the US inventory: implications for transplantation and banking. *Blood Adv* **3**, 1267-1271 (2019). https://doi.org:10.1182/bloodadvances.2018029157

108 Wilkinson, A. C., Igarashi, K. J. & Nakauchi, H. Haematopoietic stem cell self-renewal in vivo and ex vivo. *Nat Rev Genet* **21**, 541-554 (2020). https://doi.org:10.1038/s41576-020-0241-0

109 Wall, D. A. & Chan, K. W. Selection of cord blood unit(s) for transplantation. *Bone Marrow Transplant* **42**, 1-7 (2008). https://doi.org:10.1038/bmt.2008.100

110 Chen, Y. B. *et al.* GvHD after umbilical cord blood transplantation for acute leukemia: an analysis of risk factors and effect on outcomes. *Bone Marrow Transplant* **52**, 400-408 (2017). https://doi.org:10.1038/bmt.2016.265

111 Zheng, C. *et al.* Comparison of conditioning regimens with or without antithymocyte globulin for unrelated cord blood transplantation in children with high-risk or advanced hematological malignancies. *Biol Blood Marrow Transplant* **21**, 707-712 (2015). https://doi.org:10.1016/j.bbmt.2014.12.023

112 Pascal, L. *et al.* Impact of ATG-containing reduced-intensity conditioning after single- or double-unit allogeneic cord blood transplantation. *Blood* **126**, 1027-1032 (2015). https://doi.org:10.1182/blood-2014-09-599241

113 Pascal, L. *et al.* Impact of rabbit ATG-containing myeloablative conditioning regimens on the outcome of patients undergoing unrelated single-unit cord blood transplantation for hematological malignancies. *Bone Marrow Transplant* **50**, 45-50 (2015). https://doi.org:10.1038/bmt.2014.216

114 Giannotti, F. *et al.* Haploidentical transplantation is associated with better overall survival when compared to single cord blood transplantation: an EBMT-Eurocord study of acute leukemia patients conditioned with thiotepa, busulfan, and fludarabine. *J Hematol Oncol* **11**, 110 (2018). https://doi.org:10.1186/s13045-018-0655-8

115 Fuchs, E. J. *et al.* Double unrelated umbilical cord blood vs HLA-haploidentical bone marrow transplantation: the BMT CTN 1101 trial. *Blood* **137**, 420-428 (2021). https://doi.org:10.1182/blood.2020007535

116 Kindwall-Keller, T. L. & Ballen, K. K. Umbilical cord blood: The promise and the uncertainty. *Stem Cells Transl Med* **9**, 1153-1162 (2020). https://doi.org:10.1002/sctm.19-0288

117 Lund, T. C., Boitano, A. E., Delaney, C. S., Shpall, E. J. & Wagner, J. E. Advances in umbilical cord blood manipulation-from niche to bedside. *Nat Rev Clin Oncol* **12**, 163-174 (2015). https://doi.org:10.1038/nrclinonc.2014.215

118 Cohen, S. *et al.* Hematopoietic stem cell transplantation using single UM171-expanded cord blood: a single-arm, phase 1-2 safety and feasibility study. *Lancet Haematol* **7**, e134-e145 (2020). https://doi.org:10.1016/s2352-3026(19)30202-9

119 Mehta, R. S., Rezvani, K. & Shpall, E. J. Cord Blood Expansion: A Clinical Advance. *J Clin Oncol* **37**, 363-366 (2019). https://doi.org:10.1200/jco.18.01789

120 Lund, T. C. Umbilical Cord Blood Expansion: Are We There Yet? *Biol Blood Marrow Transplant* **24**, 1311-1312 (2018). https://doi.org:10.1016/j.bbmt.2018.05.002

121 Aktipis, C. A. *et al.* Cancer across the tree of life: cooperation and cheating in multicellularity. *Philos Trans R Soc Lond B Biol Sci* **370** (2015). https://doi.org:10.1098/rstb.2014.0219

122 Siegel, R. L., Miller, K. D. & Jemal, A. Cancer statistics, 2020. *CA Cancer J Clin* **70**, 7-30 (2020). https://doi.org:10.3322/caac.21590

123 Kochanek, K. D., Murphy, S. L., Xu, J. & Arias, E. Deaths: Final Data for 2017. *Natl Vital Stat Rep* **68**, 1-77 (2019).

124 Cancer Statistics Center. *2021 Estimates*, <https://cancerstatisticscenter.cancer.org/?_ga=2.233834586.55444370.1627910555-671706030.1627031188#!/> (2021, August 3).

125 Yilmaz, M., Kantarjian, H. & Ravandi, F. Acute promyelocytic leukemia current treatment algorithms. *Blood Cancer J* **11**, 123 (2021). https://doi.org:10.1038/s41408-021-00514-3

126 Welch, J. S. *et al.* The origin and evolution of mutations in acute myeloid leukemia. *Cell* **150**, 264-278 (2012). https://doi.org:10.1016/j.cell.2012.06.023

127 Ganzel, C. *et al.* Extramedullary Disease in Adult Acute Myeloid Leukemia Is Common but Lacks Independent Significance: Analysis of Patients in ECOG-ACRIN Cancer Research Group Trials, 1980-2008. *J Clin Oncol* **34**, 3544-3553 (2016). https://doi.org:10.1200/jco.2016.67.5892

128 Vardiman, J. W. *et al.* The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood* **114**, 937-951 (2009). https://doi.org:10.1182/blood-2009-03-209262

129 Arber, D. A. *et al.* The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood* **127**, 2391-2405 (2016). https://doi.org:10.1182/blood-2016-03-643544

130 Swerdlow, S. H. *et al.* The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood* **127**, 2375-2390 (2016). https://doi.org:10.1182/blood-2016-01-643569

131 Zafar, S. *et al.* Current and future best practice in imaging, staging, and response assessment for Non-Hodgkin's lymphomas: the Specialist Integrated Haematological Malignancy Imaging Reporting (SIHMIR) paradigm shift. *Clin Radiol* **76**, 391.e391-391.e318 (2021). https://doi.org:10.1016/j.crad.2020.12.022

132 Hübschmann, D. *et al.* Mutational mechanisms shaping the coding and noncoding genome of germinal center derived B-cell lymphomas. *Leukemia* **35**, 2002-2016 (2021). https://doi.org:10.1038/s41375-021-01251-z

133 Malcolm, T. I., Hodson, D. J., Macintyre, E. A. & Turner, S. D. Challenging perspectives on the cellular origins of lymphoma. *Open Biol* **6** (2016). https://doi.org:10.1098/rsob.160232

134 Stone, M. J. Thomas Hodgkin: medical immortal and uncompromising idealist. *Proc (Bayl Univ Med Cent)* **18**, 368-375 (2005). https://doi.org:10.1080/08998280.2005.11928096

135 Barwick, B. G., Gupta, V. A., Vertino, P. M. & Boise, L. H. Cell of Origin and Genetic Alterations in the Pathogenesis of Multiple Myeloma. *Front Immunol* **10**, 1121 (2019). https://doi.org:10.3389/fimmu.2019.01121

136 Rajkumar, S. V. *et al.* International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma. *Lancet Oncol* **15**, e538-548 (2014). https://doi.org:10.1016/s1470-2045(14)70442-5

137 Valent, P. *et al.* Proposed Terminology and Classification of Pre-Malignant Neoplastic Conditions: A Consensus Proposal. *EBioMedicine* **26**, 17-24 (2017). https://doi.org:10.1016/j.ebiom.2017.11.024

138 Owen, C. J. *et al.* Five new pedigrees with inherited RUNX1 mutations causing familial platelet disorder with propensity to myeloid malignancy. *Blood* **112**, 4639-4645 (2008). https://doi.org:10.1182/blood-2008-05-156745

139 Hinds, D. A. *et al.* Germ line variants predispose to both JAK2 V617F clonal hematopoiesis and myeloproliferative neoplasms. *Blood* **128**, 1121-1128 (2016). https://doi.org:10.1182/blood-2015-06-652941

140 Valent, P. *et al.* Proposed minimal diagnostic criteria for myelodysplastic syndromes (MDS) and potential pre-MDS conditions. *Oncotarget* **8**, 73483-73500 (2017). https://doi.org:10.18632/oncotarget.19008

141 Steensma, D. P. *et al.* Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. *Blood* **126**, 9-16 (2015). https://doi.org:10.1182/blood-2015-03-631747

142 Valent, P. *et al.* Clonal Hematopoiesis with Oncogenic Potential (CHOP): Separation from CHIP and Roads to AML. *Int J Mol Sci* **20** (2019). https://doi.org:10.3390/ijms20030789

143 Young, A. L., Challen, G. A., Birmann, B. M. & Druley, T. E. Clonal haematopoiesis harbouring AML-associated mutations is ubiquitous in healthy adults. *Nat Commun* **7**, 12484 (2016). https://doi.org:10.1038/ncomms12484

144 Jaiswal, S. *et al.* Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med* **371**, 2488-2498 (2014). https://doi.org:10.1056/NEJMoa1408617

145 Xie, M. *et al.* Age-related mutations associated with clonal hematopoietic expansion and malignancies. *Nat Med* **20**, 1472-1478 (2014). https://doi.org:10.1038/nm.3733

146 Genovese, G. *et al.* Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *N Engl J Med* **371**, 2477-2487 (2014). https://doi.org:10.1056/NEJMoa1409405

147 Cappelli, L. V. *et al.* Indeterminate and oncogenic potential: CHIP vs CHOP mutations in AML with NPM1 alteration. *Leukemia* (2021). https://doi.org:10.1038/s41375-021-01368-1

148 Hoermann, G., Greiner, G., Griesmacher, A. & Valent, P. Clonal Hematopoiesis of Indeterminate Potential: A Multidisciplinary Challenge in Personalized Hematology. *J Pers Med* **10** (2020). https://doi.org:10.3390/jpm10030094

149 Gondek, L. P. & DeZern, A. E. Assessing clonal haematopoiesis: clinical burdens and benefits of diagnosing myelodysplastic syndrome precursor states. *Lancet Haematol* **7**, e73-e81 (2020). https://doi.org:10.1016/s2352-3026(19)30211-x

150 Valent, P. ICUS, IDUS, CHIP and CCUS: Diagnostic Criteria, Separation from MDS and Clinical Implications. *Pathobiology* **86**, 30-38 (2019). https://doi.org:10.1159/000489042

151 Gallì, A. *et al.* Relationship between clone metrics and clinical outcome in clonal cytopenia. *Blood* **138**, 965-976 (2021). https://doi.org:10.1182/blood.2021011323

152 Malcovati, L. *et al.* Clinical significance of somatic mutation in unexplained blood cytopenia. *Blood* **129**, 3371-3378 (2017). https://doi.org:10.1182/blood-2017-01-763425

153 Rumi, E. & Cazzola, M. Diagnosis, risk stratification, and response evaluation in classical myeloproliferative neoplasms. *Blood* **129**, 680-692 (2017). https://doi.org:10.1182/blood-2016-10-695957

154 Patnaik, M. M. & Lasho, T. L. Genomics of myelodysplastic syndrome/myeloproliferative neoplasm overlap syndromes. *Hematology Am Soc Hematol Educ Program* **2020**, 450-459 (2020). https://doi.org:10.1182/hematology.2020000130

155 Greenberg, P. L. *et al.* Revised international prognostic scoring system for myelodysplastic syndromes. *Blood* **120**, 2454-2465 (2012). https://doi.org:10.1182/blood-2012-03-420489

156 Hellström-Lindberg, E., Tobiasson, M. & Greenberg, P. Myelodysplastic syndromes: moving towards personalized management. *Haematologica* **105**, 1765-1779 (2020). https://doi.org:10.3324/haematol.2020.248955

157 Helbig, G., Soja, A., Bartkowska-Chrobok, A. & Kyrcz-Krzemień, S. Chronic eosinophilic leukemia-not otherwise specified has a poor prognosis with unresponsiveness to conventional treatment and high risk of acute transformation. *Am J Hematol* **87**, 643-645 (2012). https://doi.org:10.1002/ajh.23193

158 Morsia, E., Reichard, K., Pardanani, A., Tefferi, A. & Gangat, N. WHO defined chronic eosinophilic leukemia, not otherwise specified (CEL, NOS): A contemporary series from the Mayo Clinic. *Am J Hematol* **95**, E172-e174 (2020). https://doi.org:10.1002/ajh.25811

159 Ruan, G. J. *et al.* A population-based study of chronic neutrophilic leukemia in the United States. *Blood Cancer J* **10**, 68 (2020). https://doi.org:10.1038/s41408-020-0334-1

160 Oran, B. & Weisdorf, D. J. Survival for older patients with acute myeloid leukemia: a population-based study. *Haematologica* **97**, 1916-1924 (2012). https://doi.org:10.3324/haematol.2012.066100

161 Barbui, T. *et al.* The 2016 WHO classification and diagnostic criteria for myeloproliferative neoplasms: document summary and in-depth discussion. *Blood Cancer J* **8**, 15 (2018). https://doi.org:10.1038/s41408-018-0054-y

162 Vardiman, J. W., Harris, N. L. & Brunning, R. D. The World Health Organization (WHO) classification of the myeloid neoplasms. *Blood* **100**, 2292-2302 (2002). https://doi.org:10.1182/blood-2002-04-1199

163 Brehl, N. C. in *Rodak's Hematology (Sixth Edition)* (eds Elaine M. Keohane, Catherine N. Otto, & Jeanine M. Walenga) 589-602 (Elsevier, 2020).

164 Steensma, D. P. *et al.* Common troublesome symptoms and their impact on quality of life in patients with myelodysplastic syndromes (MDS): results of a large internet-based survey. *Leuk Res* **32**, 691-698 (2008). https://doi.org:10.1016/j.leukres.2007.10.015

165 Cogle, C. R. Incidence and Burden of the Myelodysplastic Syndromes. *Curr Hematol Malig Rep* **10**, 272-281 (2015). https://doi.org:10.1007/s11899-015-0269-y

166 Randolph, T. R. in *Rodak's Hematology (Sixth Edition)* (eds Elaine M. Keohane, Catherine N. Otto, & Jeanine M. Walenga) 555-588 (Elsevier, 2020).

167 Szuber, N., Elliott, M. & Tefferi, A. Chronic neutrophilic leukemia: 2020 update on diagnosis, molecular genetics, prognosis, and management. *Am J Hematol* **95**, 212-224 (2020). https://doi.org:10.1002/ajh.25688

168 Quintás-Cardama, A. & Cortes, J. E. Chronic myeloid leukemia: diagnosis and treatment. *Mayo Clin Proc* **81**, 973-988 (2006). https://doi.org:10.4065/81.7.973

169 Vainchenker, W., Constantinescu, S. N. & Plo, I. Recent advances in understanding myelofibrosis and essential thrombocythemia. *F1000Res* **5** (2016). https://doi.org:10.12688/f1000research.8081.1

170 Marcellino, B. K. & Hoffman, R. Recent advances in prognostication and treatment of polycythemia vera. *Fac Rev* **10**, 29 (2021). https://doi.org:10.12703/r/10-29

171 Cerquozzi, S. & Tefferi, A. Blast transformation and fibrotic progression in polycythemia vera and essential thrombocythemia: a literature review of incidence and risk factors. *Blood Cancer J* **5**, e366 (2015). https://doi.org:10.1038/bcj.2015.95

172 Akuthota, P. & Weller, P. F. Spectrum of Eosinophilic End-Organ Manifestations. *Immunol Allergy Clin North Am* **35**, 403-411 (2015). https://doi.org:10.1016/j.iac.2015.04.002

173 Hoffmann, V. S. *et al.* The EUTOS population-based registry: incidence and clinical characteristics of 2904 CML patients in 20 European Countries. *Leukemia* **29**, 1336-1343 (2015). https://doi.org:10.1038/leu.2015.73

174 Srour, S. A. *et al.* Incidence and patient survival of myeloproliferative neoplasms and myelodysplastic/myeloproliferative neoplasms in the United States, 2001-12. *Br J Haematol* **174**, 382-396 (2016). https://doi.org:10.1111/bjh.14061

175 Höglund, M., Sandin, F. & Simonsson, B. Epidemiology of chronic myeloid leukaemia: an update. *Ann Hematol* **94 Suppl 2**, S241-247 (2015). https://doi.org:10.1007/s00277-015-2314-2

176 Hultcrantz, M. *et al.* Incidence of myeloproliferative neoplasms - trends by subgroup and age in a population-based study in Sweden. *J Intern Med* **287**, 448-454 (2020). https://doi.org:10.1111/joim.13019

177 Ruan, G. J. *et al.* A population-based study of chronic eosinophilic leukemia-not otherwise specified in the United States. *Am J Hematol* (2020). https://doi.org:10.1002/ajh.25906

178 Hochhaus, A. *et al.* European LeukemiaNet 2020 recommendations for treating chronic myeloid leukemia. *Leukemia* **34**, 966-984 (2020). https://doi.org:10.1038/s41375-020-0776-2

179 Kantarjian, H. *et al.* Improved survival in chronic myeloid leukemia since the introduction of imatinib therapy: a single-institution historical experience. *Blood* **119**, 1981-1987 (2012). https://doi.org:10.1182/blood-2011-08-358135

180 Pfirrmann, M. *et al.* Prognosis of long-term survival considering disease-specific death in patients with chronic myeloid leukemia. *Leukemia* **30**, 48-56 (2016). https://doi.org:10.1038/leu.2015.261

181 Gotlib, J. World Health Organization-defined eosinophilic disorders: 2014 update on diagnosis, risk stratification, and management. *Am J Hematol* **89**, 325-337 (2014). https://doi.org:10.1002/ajh.23664

182 Finazzi, G., Vannucchi, A. M. & Barbui, T. Prefibrotic myelofibrosis: treatment algorithm 2018. *Blood Cancer J* **8**, 104 (2018). https://doi.org:10.1038/s41408-018-0142-z

183 Barbui, T., Thiele, J., Ferrari, A., Vannucchi, A. M. & Tefferi, A. The new WHO classification for essential thrombocythemia calls for revision of available evidences. *Blood Cancer J* **10**, 22 (2020). https://doi.org:10.1038/s41408-020-0290-9

184 Board, P. D. Q. A. T. E. in *PDQ Cancer Information Summaries* (National Cancer Institute (US), 2002).

185 Kuendgen, A., Kasprzak, A. & Germing, U. Hybrid or Mixed Myelodysplastic/Myeloproliferative Disorders - Epidemiological Features and Overview. *Front Oncol* **11**, 778741 (2021). https://doi.org:10.3389/fonc.2021.778741

186 Patnaik, M. M. & Lasho, T. Myelodysplastic syndrome/myeloproliferative neoplasm overlap syndromes: a focused review. *Hematology Am Soc Hematol Educ Program* **2020**, 460-464 (2020). https://doi.org:10.1182/hematology.2020000163

187 Ricci, C. *et al.* RAS mutations contribute to evolution of chronic myelomonocytic leukemia to the proliferative variant. *Clin Cancer Res* **16**, 2246-2256 (2010). https://doi.org:10.1158/1078-0432.Ccr-09-2112

188 Stieglitz, E. *et al.* The genomic landscape of juvenile myelomonocytic leukemia. *Nat Genet* **47**, 1326-1333 (2015). https://doi.org:10.1038/ng.3400

189 Onida, F. *et al.* Allogeneic stem cell transplantation in patients with atypical chronic myeloid leukaemia: a retrospective study from the Chronic Malignancies Working Party of the European Society for Blood and Marrow Transplantation. *Br J Haematol* **177**, 759-765 (2017). https://doi.org:10.1111/bjh.14619

190 Schwartz, L. C. & Mascarenhas, J. Current and evolving understanding of atypical chronic myeloid leukemia. *Blood Rev* **33**, 74-81 (2019). https://doi.org:10.1016/j.blre.2018.07.004

191 Clara, J. A., Sallman, D. A. & Padron, E. Clinical management of myelodysplastic syndrome/myeloproliferative neoplasm overlap syndromes. *Cancer Biol Med* **13**, 360-372 (2016). https://doi.org:10.20892/j.issn.2095-3941.2016.0043

192 Patnaik, M. M. *et al.* ASXL1 and SETBP1 mutations and their prognostic contribution in chronic myelomonocytic leukemia: a two-center study of 466 patients. *Leukemia* **28**, 2206-2212 (2014). https://doi.org:10.1038/leu.2014.125

193 Itzykson, R. *et al.* Diagnosis and Treatment of Chronic Myelomonocytic Leukemias in Adults: Recommendations From the European Hematology Association and the European LeukemiaNet. *Hemasphere* **2**, e150 (2018). https://doi.org:10.1097/hs9.0000000000000150

194 Patnaik, M. M. *et al.* Predictors of survival in refractory anemia with ring sideroblasts and thrombocytosis (RARS-T) and the role of next-generation sequencing. *Am J Hematol* **91**, 492-498 (2016). https://doi.org:10.1002/ajh.24332

195 Patnaik, M. M. & Tefferi, A. Myelodysplastic syndromes with ring sideroblasts (MDS-RS) and MDS/myeloproliferative neoplasm with RS and thrombocytosis (MDS/MPN-RS-T) - "2021 update on diagnosis, risk-stratification, and management". *Am J Hematol* **96**, 379-394 (2021). https://doi.org:10.1002/ajh.26090

196 Broseus, J. *et al.* Clinical features and course of refractory anemia with ring sideroblasts associated with marked thrombocytosis. *Haematologica* **97**, 1036-1041 (2012). https://doi.org:10.3324/haematol.2011.053918

197 Aldoss, I. *et al.* Therapy-related acute lymphoblastic leukemia has distinct clinical and cytogenetic features compared to de novo acute lymphoblastic leukemia, but outcomes are comparable in transplanted patients. *Haematologica* **103**, 1662-1668 (2018). https://doi.org:10.3324/haematol.2018.193599

198 Noon, M. A. & Hess, C. E. Acute leukemia in adults: comparison of survival between a treated and an untreated group. *South Med J* **69**, 1157-1160 (1976).

199 Dunbar, A. J., Rampal, R. K. & Levine, R. Leukemia secondary to myeloproliferative neoplasms. *Blood* **136**, 61-70 (2020). https://doi.org:10.1182/blood.2019000943

200 Nilsson, C. *et al.* Secondary Acute Myeloid Leukemia and the Role of Allogeneic Stem Cell Transplantation in a Population-Based Setting. *Biol Blood Marrow Transplant* **25**, 1770-1778 (2019). https://doi.org:10.1016/j.bbmt.2019.05.038

201 Brown, A. L., Hahn, C. N. & Scott, H. S. Secondary leukemia in patients with germline transcription factor mutations (RUNX1, GATA2, CEBPA). *Blood* **136**, 24-35 (2020). https://doi.org:10.1182/blood.2019000937

202 Shallis, R. M., Weiss, J. J., Deziel, N. C. & Gore, S. D. Challenging the concept of de novo acute myeloid leukemia: Environmental and occupational leukemogens hiding in our midst. *Blood Rev* **47**, 100760 (2021). https://doi.org:10.1016/j.blre.2020.100760

203 Pendleton, M., Lindsey, R. H., Jr., Felix, C. A., Grimwade, D. & Osheroff, N. Topoisomerase II and leukemia. *Ann N Y Acad Sci* **1310**, 98-110 (2014). https://doi.org:10.1111/nyas.12358

204 Shlush, L. I. *et al.* Tracing the origins of relapse in acute myeloid leukaemia to stem cells. *Nature* **547**, 104-108 (2017). https://doi.org:10.1038/nature22993

205 Ng, S. W. *et al.* A 17-gene stemness score for rapid determination of risk in acute leukaemia. *Nature* **540**, 433-437 (2016). https://doi.org:10.1038/nature20598

206 Eppert, K. *et al.* Stem cell gene expression programs influence clinical outcome in human leukemia. *Nat Med* **17**, 1086-1093 (2011). https://doi.org:10.1038/nm.2415

207 Joudinaud, R. & Boyer, T. Stem Cells in Myelodysplastic Syndromes and Acute Myeloid Leukemia: First Cousins or Unrelated Entities? *Front Oncol* **11**, 730899 (2021). https://doi.org:10.3389/fonc.2021.730899

208 Charaf, L. *et al.* Effect of tyrosine kinase inhibitors on stemness in normal and chronic myeloid leukemia cells. *Leukemia* **31**, 65-74 (2017). https://doi.org:10.1038/leu.2016.154

209 Hadzijusufovic, E. *et al.* STAT5 is Expressed in CD34(+)/CD38(-) Stem Cells and Serves as a Potential Molecular Target in Ph-Negative Myeloproliferative Neoplasms. *Cancers (Basel)* **12** (2020). https://doi.org:10.3390/cancers12041021

210 Jiang, Q. *et al.* Inflammation-driven deaminase deregulation fuels human pre-leukemia stem cell evolution. *Cell Rep* **34**, 108670 (2021). https://doi.org:10.1016/j.celrep.2020.108670

211 Gao, J. X. Cancer stem cells: the lessons from pre-cancerous stem cells. *J Cell Mol Med* **12**, 67-96 (2008). https://doi.org:10.1111/j.1582-4934.2007.00170.x

212 Bennett, J. M. *et al.* Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. *Br J Haematol* **33**, 451-458 (1976). https://doi.org:10.1111/j.1365-2141.1976.tb03563.x

213 Bennett, J. M. *et al.* Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. *Ann Intern Med* **103**, 620-625 (1985). https://doi.org:10.7326/0003-4819-103-4-620

214 Angelescu, S., Berbec, N. M., Colita, A., Barbu, D. & Lupu, A. R. Value of multifaced approach diagnosis and classification of acute leukemias. *Maedica (Bucur)* **7**, 254-260 (2012).

215 Zhang, X., Sun, J., Yu, W. & Jin, J. Current views on the genetic landscape and management of variant acute promyelocytic leukemia. *Biomark Res* **9**, 33 (2021). https://doi.org:10.1186/s40364-021-00284-x

216 Daver, N., Schlenk, R. F., Russell, N. H. & Levis, M. J. Targeting FLT3 mutations in AML: review of current knowledge and evidence. *Leukemia* **33**, 299-312 (2019). https://doi.org:10.1038/s41375-018-0357-9

217 Döhner, H. *et al.* Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood* **129**, 424-447 (2017). https://doi.org:10.1182/blood-2016-08-733196

218 Churpek, J. E. *et al.* Inherited mutations in cancer susceptibility genes are common among survivors of breast cancer who develop therapy-related leukemia. *Cancer* **122**, 304-311 (2016). https://doi.org:10.1002/cncr.29615

219 Schulz, E. *et al.* Germline mutations in the DNA damage response genes BRCA1, BRCA2, BARD1 and TP53 in patients with therapy related myeloid neoplasms. *J Med Genet* **49**, 422-428 (2012). https://doi.org:10.1136/jmedgenet-2011-100674

220 Voso, M. T. *et al.* Fanconi anemia gene variants in therapy-related myeloid neoplasms. *Blood Cancer J* **5**, e323 (2015). https://doi.org:10.1038/bcj.2015.44

221 McNerney, M. E., Godley, L. A. & Le Beau, M. M. Therapy-related myeloid neoplasms: when genetics and environment collide. *Nat Rev Cancer* **17**, 513-527 (2017). https://doi.org:10.1038/nrc.2017.60

222 Walter, R. B. *et al.* Significance of FAB subclassification of "acute myeloid leukemia, NOS" in the 2008 WHO classification: analysis of 5848 newly diagnosed patients. *Blood* **121**, 2424-2431 (2013). https://doi.org:10.1182/blood-2012-10-462440

223 Avni, B. & Koren-Michowitz, M. Myeloid sarcoma: current approach and therapeutic options. *Ther Adv Hematol* **2**, 309-316 (2011). https://doi.org:10.1177/2040620711410774

224 Caldwell, J. T., Ge, Y. & Taub, J. W. Prognosis and management of acute myeloid leukemia in patients with Down syndrome. *Expert Rev Hematol* **7**, 831-840 (2014). https://doi.org:10.1586/17474086.2014.959923

225 Bhatnagar, N., Nizery, L., Tunstall, O., Vyas, P. & Roberts, I. Transient Abnormal Myelopoiesis and AML in Down Syndrome: an Update. *Curr Hematol Malig Rep* **11**, 333-341 (2016). https://doi.org:10.1007/s11899-016-0338-x

226 Béné, M. C. & Porwit, A. Acute leukemias of ambiguous lineage. *Semin Diagn Pathol* **29**, 12-18 (2012). https://doi.org:10.1053/j.semdp.2011.08.004

227 Yan, L. *et al.* Clinical, immunophenotypic, cytogenetic, and molecular genetic features in 117 adult patients with mixed-phenotype acute leukemia defined by WHO-2008 classification. *Haematologica* **97**, 1708-1712 (2012). https://doi.org:10.3324/haematol.2012.064485

228 Matutes, E. *et al.* Mixed-phenotype acute leukemia: clinical and laboratory features and outcome in 100 patients defined according to the WHO 2008 classification. *Blood* **117**, 3163-3171 (2011). https://doi.org:10.1182/blood-2010-10-314682

229 Yates, J. W., Wallace, H. J., Jr., Ellison, R. R. & Holland, J. F. Cytosine arabinoside (NSC-63878) and daunorubicin (NSC-83142) therapy in acute nonlymphocytic leukemia. *Cancer Chemother Rep* **57**, 485-488 (1973).

230 Michaelis, L. C. Cytotoxic therapy in acute myeloid leukemia: not quite dead yet. *Hematology Am Soc Hematol Educ Program* **2018**, 51-62 (2018). https://doi.org:10.1182/asheducation-2018.1.51

231 Wiernik, P. H. *et al.* Cytarabine plus idarubicin or daunorubicin as induction and consolidation therapy for previously untreated adult patients with acute myeloid leukemia. *Blood* **79**, 313-319 (1992).

232 Murphy, T. *et al.* Impact of preleukemic mutations and their persistence on hematologic recovery after induction chemotherapy for AML. *Blood Adv* **3**, 2307-2311 (2019). https://doi.org:10.1182/bloodadvances.2019000306

233 Gunnar Juliusson. *Akut myeloisk leukemi (aml)*, <https://www.internetmedicin.se/behandlingsoversikter/hematologi/akut-myeloisk-leukemi-aml/> (2022, July 15).

234 Vonk, C. M., Al Hinai, A. S. A., Hanekamp, D. & Valk, P. J. M. Molecular Minimal Residual Disease Detection in Acute Myeloid Leukemia. *Cancers (Basel)* **13** (2021). https://doi.org:10.3390/cancers13215431

235 Øvlisen, A. K. *et al.* Stringent or nonstringent complete remission and prognosis in acute myeloid leukemia: a Danish population-based study. *Blood Adv* **2**, 559-564 (2018). https://doi.org:10.1182/bloodadvances.2017007393

236 Burnett, A. K. *et al.* Curability of patients with acute myeloid leukemia who did not undergo transplantation in first remission. *J Clin Oncol* **31**, 1293-1301 (2013). https://doi.org:10.1200/jco.2011.40.5977

237 Sarkozy, C. *et al.* Outcome of older patients with acute myeloid leukemia in first relapse. *Am J Hematol* **88**, 758-764 (2013). https://doi.org:10.1002/ajh.23498

238 Neuendorff, N. R. *et al.* Anthracycline-related cardiotoxicity in older patients with acute myeloid leukemia: a Young SIOG review paper. *Blood Adv* **4**, 762-775 (2020). https://doi.org:10.1182/bloodadvances.2019000955

239 Reville, P. K. & Kadia, T. M. Maintenance Therapy in AML. *Front Oncol* **10**, 619085 (2020). https://doi.org:10.3389/fonc.2020.619085

240 Lachowiez, C. A., Atluri, H. & DiNardo, C. D. Advancing the standard: venetoclax combined with intensive induction and consolidation therapy for acute myeloid leukemia. *Ther Adv Hematol* **13**, 20406207221093964 (2022). https://doi.org:10.1177/20406207221093964

241 Lachowiez, C. A. *et al.* Venetoclax combined with induction chemotherapy in patients with newly diagnosed acute myeloid leukaemia: a post-hoc, propensity score-matched, cohort study. *Lancet Haematol* **9**, e350-e360 (2022). https://doi.org:10.1016/s2352-3026(22)00076-x

242 DiNardo, C. D. *et al.* Azacitidine and Venetoclax in Previously Untreated Acute Myeloid Leukemia. *N Engl J Med* **383**, 617-629 (2020). https://doi.org:10.1056/NEJMoa2012971

243 Hafezi, S. & Rahmani, M. Targeting BCL-2 in Cancer: Advances, Challenges, and Perspectives. *Cancers (Basel)* **13** (2021). https://doi.org:10.3390/cancers13061292

244 Perl, A. E. & Vyas, P. Shall We Dance: Evolving Partnerships of Targeted Therapies for AML. *Clin Cancer Res* **28**, 2719-2721 (2022). https://doi.org:10.1158/1078-0432.Ccr-22-0279

245 Wang, H. *et al.* Venetoclax plus 3 + 7 daunorubicin and cytarabine chemotherapy as first-line treatment for adults with acute myeloid leukaemia: a multicentre, single-arm, phase 2 trial. *Lancet Haematol* **9**, e415-e424 (2022). https://doi.org:10.1016/s2352-3026(22)00106-5

246 Montesinos, P. *et al.* Ivosidenib and Azacitidine in IDH1-Mutated Acute Myeloid Leukemia. *N Engl J Med* **386**, 1519-1531 (2022). https://doi.org:10.1056/NEJMoa2117344

247 Maiti, A. *et al.* Triplet therapy with venetoclax, FLT3 inhibitor and decitabine for FLT3-mutated acute myeloid leukemia. *Blood Cancer J* **11**, 25 (2021). https://doi.org:10.1038/s41408-021-00410-w

248 Dombret, H. & Itzykson, R. How and when to decide between epigenetic therapy and chemotherapy in patients with AML. *Hematology Am Soc Hematol Educ Program* **2017**, 45-53 (2017). https://doi.org:10.1182/asheducation-2017.1.45

249 Cherry, E. M. *et al.* Venetoclax and azacitidine compared with induction chemotherapy for newly diagnosed patients with acute myeloid leukemia. *Blood Adv* **5**, 5565-5573 (2021). https://doi.org:10.1182/bloodadvances.2021005538

250 Estey, E. H. Epigenetics in clinical practice: the examples of azacitidine and decitabine in myelodysplasia and acute myeloid leukemia. *Leukemia* **27**, 1803-1812 (2013). https://doi.org:10.1038/leu.2013.173

251 Boumber, Y. *et al.* A randomized study of decitabine versus conventional care for maintenance therapy in patients with acute myeloid leukemia in complete remission. *Leukemia* **26**, 2428-2431 (2012). https://doi.org:10.1038/leu.2012.153

252 de Lima, M. *et al.* Maintenance therapy with low-dose azacitidine after allogeneic hematopoietic stem cell transplantation for recurrent acute myelogenous leukemia or myelodysplastic syndrome: a dose and schedule finding study. *Cancer* **116**, 5420-5431 (2010). https://doi.org:10.1002/cncr.25500

253 DiNardo, C. D. *et al.* Venetoclax Combined With FLAG-IDA Induction and Consolidation in Newly Diagnosed and Relapsed or Refractory Acute Myeloid Leukemia. *J Clin Oncol* **39**, 2768-2778 (2021). https://doi.org:10.1200/jco.20.03736

254 Brancati, S. *et al.* Venetoclax in Relapsed/Refractory Acute Myeloid Leukemia: Are Supporting Evidences Enough? *Cancers (Basel)* **14** (2021). https://doi.org:10.3390/cancers14010022

255 Arora, S. *et al.* Tumor lysis syndrome and infectious complications during treatment with venetoclax combined with azacitidine or decitabine in patients with acute myeloid leukemia. *Leuk Res* **117**, 106844 (2022). https://doi.org:10.1016/j.leukres.2022.106844

256 Wei, A. H. *et al.* 6-month follow-up of VIALE-C demonstrates improved and durable efficacy in patients with untreated AML ineligible for intensive chemotherapy (141/150). *Blood Cancer J* **11**, 163 (2021). https://doi.org:10.1038/s41408-021-00555-8

257 The United States Food and Drug Administration. *FDA grants regular approval to venetoclax in combination for untreated acute myeloid leukemia*, <https://www.fda.gov/drugs/resources-information-approved-drugs/fda-grants-regular-approval-venetoclax-combination-untreated-acute-myeloid-leukemia> (2020, October 16).

258 European Medicines Agency. *Venclyxto*, <https://www.ema.europa.eu/en/documents/smop/chmp-post-authorisation-summary-positive-opinion-venclyxto-ii-30_en.pdf> (2021, April 22).259 läkemedelsförmånsverket, T. o. *Hälsoekonomisk be-dömning av venclyxto(venetoklax)*, <https://www.tlv.se/download/18.5489dc5a17dbd1f28941a6af/1639746567692/bes211216_venclyxto_underlag_2092-2021.pdf> (2021, December 29).

260 Eyre, T. A. *et al.* Efficacy of venetoclax monotherapy in patients with relapsed chronic lymphocytic leukaemia in the post-BCR inhibitor setting: a UK wide analysis. *Br J Haematol* **185**, 656-669 (2019). https://doi.org:10.1111/bjh.15802

261 Roufayel, R., Younes, K., Al-Sabi, A. & Murshid, N. BH3-Only Proteins Noxa and Puma Are Key Regulators of Induced Apoptosis. *Life (Basel)* **12** (2022). https://doi.org:10.3390/life12020256

262 Jin, S. *et al.* 5-Azacitidine Induces NOXA to Prime AML Cells for Venetoclax-Mediated Apoptosis. *Clin Cancer Res* **26**, 3371-3383 (2020). https://doi.org:10.1158/1078-0432.Ccr-19-1900

263 DiNardo, C. D. *et al.* Molecular patterns of response and treatment failure after frontline venetoclax combinations in older patients with AML. *Blood* **135**, 791-803 (2020). https://doi.org:10.1182/blood.2019003988

264 DiNardo, C. D. *et al.* Clinical experience with the BCL2-inhibitor venetoclax in combination therapy for relapsed and refractory acute myeloid leukemia and related myeloid malignancies. *Am J Hematol* **93**, 401-407 (2018). https://doi.org:10.1002/ajh.25000

265 Chyla, B. *et al.* Genetic Biomarkers Of Sensitivity and Resistance to Venetoclax Monotherapy in Patients With Relapsed Acute Myeloid Leukemia. *Am J Hematol* **93**, E202-205 (2018). https://doi.org:10.1002/ajh.25146

266 Konopleva, M. *et al.* Impact of FLT3 Mutation on Outcomes after Venetoclax and Azacitidine for Patients with Treatment-Naïve Acute Myeloid Leukemia. *Clin Cancer Res* **28**, 2744-2752 (2022). https://doi.org:10.1158/1078-0432.Ccr-21-3405

267 Stevens, B. M. *et al.* Fatty acid metabolism underlies venetoclax resistance in acute myeloid leukemia stem cells. *Nat Cancer* **1**, 1176-1187 (2020). https://doi.org:10.1038/s43018-020-00126-z

268 Pollyea, D. A. *et al.* Impact of Venetoclax and Azacitidine in Treatment-Naïve Patients with Acute Myeloid Leukemia and IDH1/2 Mutations. *Clin Cancer Res* **28**, 2753-2761 (2022). https://doi.org:10.1158/1078-0432.Ccr-21-3467

269 Gilliland, D. G. & Griffin, J. D. The roles of FLT3 in hematopoiesis and leukemia. *Blood* **100**, 1532-1542 (2002). https://doi.org:10.1182/blood-2002-02-0492

270 Larrosa-Garcia, M. & Baer, M. R. FLT3 Inhibitors in Acute Myeloid Leukemia: Current Status and Future Directions. *Mol Cancer Ther* **16**, 991-1001 (2017). https://doi.org:10.1158/1535-7163.Mct-16-0876

271 Bacher, U., Haferlach, C., Kern, W., Haferlach, T. & Schnittger, S. Prognostic relevance of FLT3-TKD mutations in AML: the combination matters--an analysis of 3082 patients. *Blood* **111**, 2527-2537 (2008). https://doi.org:10.1182/blood-2007-05-091215

272 Larson, R. A. *et al.* Midostaurin reduces relapse in FLT3-mutant acute myeloid leukemia: the Alliance CALGB 10603/RATIFY trial. *Leukemia* **35**, 2539-2551 (2021). https://doi.org:10.1038/s41375-021-01179-4

273 Tomlinson, B. K. *et al.* A Phase II Study of Midostaurin and 5-Azacitidine for Untreated Elderly and Unfit Patients With FLT3 Wild-type Acute Myelogenous Leukemia. *Clin Lymphoma Myeloma Leuk* **20**, 226-233.e221 (2020). https://doi.org:10.1016/j.clml.2019.10.018

274 Tzogani, K. *et al.* European Medicines Agency review of midostaurin (Rydapt) for the treatment of adult patients with acute myeloid leukaemia and systemic mastocytosis. *ESMO Open* **4** (2019). https://doi.org:10.1136/esmoopen-2019-000606

275 Abbas, H. A., Alfayez, M., Kadia, T., Ravandi-Kashani, F. & Daver, N. Midostaurin In Acute Myeloid Leukemia: An Evidence-Based Review And Patient Selection. *Cancer Manag Res* **11**, 8817-8828 (2019). https://doi.org:10.2147/cmar.S177894

276 Tandvårds- och läkemedelsförmånsverket. *Rydapt ingår i högkostnadsskyddet*, <https://www.tlv.se/beslut/beslut-lakemedel/generell-subvention/arkiv/2018-01-31-rydapt-ingar-i-hogkostnadsskyddet-.html> (2018, January 31).

277 Smith, C. C. The growing landscape of FLT3 inhibition in AML. *Hematology Am Soc Hematol Educ Program* **2019**, 539-547 (2019). https://doi.org:10.1182/hematology.2019000058

278 Perl, A. E. *et al.* Gilteritinib or Chemotherapy for Relapsed or Refractory FLT3-Mutated AML. *N Engl J Med* **381**, 1728-1740 (2019). https://doi.org:10.1056/NEJMoa1902688

279 Perl, A. E. *et al.* Follow-up of patients with R/R FLT3-mutation-positive AML treated with gilteritinib in the phase 3 ADMIRAL trial. *Blood* **139**, 3366-3375 (2022). https://doi.org:10.1182/blood.2021011583

280 Tzogani, K. *et al.* The European Medicines Agency Review of Gilteritinib (Xospata) for the Treatment of Adult Patients with Relapsed or Refractory Acute Myeloid Leukemia with an FLT3 Mutation. *Oncologist* **25**, e1070-e1076 (2020). https://doi.org:10.1634/theoncologist.2019-0976

281 Pulte, E. D. *et al.* FDA Approval Summary: Gilteritinib for Relapsed or Refractory Acute Myeloid Leukemia with a FLT3 Mutation. *Clin Cancer Res* **27**, 3515-3521 (2021). https://doi.org:10.1158/1078-0432.Ccr-20-4271

282 Tandvårds- och läkemedelsförmånsverket. *Xospata (gilteritinib)*, <https://www.tlv.se/download/18.62b765d3177d2c0ba7b1e2eb/1614683179819/bes210225_underlag_xospata.pdf> (2021, March 28).

283 Wang, E. S. *et al.* Phase 3 Trial of Gilteritinib Plus Azacitidine Vs Azacitidine for Newly Diagnosed FLT3mut+ AML Ineligible for Intensive Chemotherapy. *Blood* (2022). https://doi.org:10.1182/blood.2021014586

284 Burchert, A. *et al.* Sorafenib Maintenance After Allogeneic Hematopoietic Stem Cell Transplantation for Acute Myeloid Leukemia With FLT3-Internal Tandem Duplication Mutation (SORMAIN). *J Clin Oncol* **38**, 2993-3002 (2020). https://doi.org:10.1200/jco.19.03345

285 Xuan, L. *et al.* Sorafenib maintenance in patients with FLT3-ITD acute myeloid leukaemia undergoing allogeneic haematopoietic stem-cell transplantation: an open-label, multicentre, randomised phase 3 trial. *Lancet Oncol* **21**, 1201-1212 (2020). https://doi.org:10.1016/s1470-2045(20)30455-1

286 Bazarbachi, A. *et al.* Clinical practice recommendation on hematopoietic stem cell transplantation for acute myeloid leukemia patients with FLT3-internal tandem duplication: a position statement from the Acute Leukemia Working Party of the European Society for Blood and Marrow Transplantation. *Haematologica* **105**, 1507-1516 (2020). https://doi.org:10.3324/haematol.2019.243410

287 Regionala cancercentrum i samverkan. *Akut myeloisk leukemi (AML) Nationellt vårdprogram*, <https://kunskapsbanken.cancercentrum.se/globalassets/cancerdiagnoser/blod-lymfom-myelom/aml/vardprogram/nationellt-vardprogram-akut-myeloisk-leukemi-aml.pdf> (2022, June 21).

288 Ohanian, M. *et al.* Sorafenib Combined with 5-azacytidine in Older Patients with Untreated FLT3-ITD Mutated Acute Myeloid Leukemia. *Am J Hematol* **93**, 1136-1141 (2018). https://doi.org:10.1002/ajh.25198

289 Cortes, J. E. *et al.* Quizartinib versus salvage chemotherapy in relapsed or refractory FLT3-ITD acute myeloid leukaemia (QuANTUM-R): a multicentre, randomised, controlled, open-label, phase 3 trial. *Lancet Oncol* **20**, 984-997 (2019). https://doi.org:10.1016/s1470-2045(19)30150-0

290 European medicine agency. Refusal of the marketing authorisation for vanflyta (quizartinib). (2019, October 18).

291 Kidoguch, K., Shibusawa, M. & Tanimoto, T. A critical appraisal of Japan's new drug approval process: a case study of FLT3-ITD inhibitor quizartinib. *Invest New Drugs* **39**, 1457-1459 (2021). https://doi.org:10.1007/s10637-021-01151-0

292 Daver, N. *et al.* Venetoclax Plus Gilteritinib for FLT3-Mutated Relapsed/Refractory Acute Myeloid Leukemia. *J Clin Oncol*, Jco2200602 (2022). https://doi.org:10.1200/jco.22.00602

293 Yilmaz, M. *et al.* Hypomethylating agent and venetoclax with FLT3 inhibitor "triplet" therapy in older/unfit patients with FLT3 mutated AML. *Blood Cancer J* **12**, 77 (2022). https://doi.org:10.1038/s41408-022-00670-0

294 Stone, R. M. *et al.* Midostaurin plus Chemotherapy for Acute Myeloid Leukemia with a FLT3 Mutation. *N Engl J Med* **377**, 454-464 (2017). https://doi.org:10.1056/NEJMoa1614359

295 Cerchione, C. *et al.* IDH1/IDH2 Inhibition in Acute Myeloid Leukemia. *Front Oncol* **11**, 639387 (2021). https://doi.org:10.3389/fonc.2021.639387

296 Lu, C. *et al.* IDH mutation impairs histone demethylation and results in a block to cell differentiation. *Nature* **483**, 474-478 (2012). https://doi.org:10.1038/nature10860

297 Choe, S. *et al.* Molecular mechanisms mediating relapse following ivosidenib monotherapy in IDH1-mutant relapsed or refractory AML. *Blood Adv* **4**, 1894-1905 (2020). https://doi.org:10.1182/bloodadvances.2020001503

298 The United States Food and Drug Administration. *FDA granted regular approval to enasidenib for the treatment of relapsed or refractory AML*, <https://www.fda.gov/drugs/resources-information-approved-drugs/fda-granted-regular-approval-enasidenib-treatment-relapsed-or-refractory-aml> (2017, August 1).

299 The United States Food and Drug Administration. *FDA approves ivosidenib for relapsed or refractory acute myeloid leukemia*, <https://www.fda.gov/drugs/resources-information-approved-drugs/fda-approves-ivosidenib-relapsed-or-refractory-acute-myeloid-leukemia> (2018, July 20).

300 The United States Food and Drug Administration. *FDA approves ivosidenib as first-line treatment for AML with IDH1 mutation*, <https://www.fda.gov/drugs/resources-information-approved-drugs/fda-approves-ivosidenib-first-line-treatment-aml-idh1-mutation> (2019, May 2).

301 Roboz, G. J. *et al.* Ivosidenib induces deep durable remissions in patients with newly diagnosed IDH1-mutant acute myeloid leukemia. *Blood* **135**, 463-471 (2020). https://doi.org:10.1182/blood.2019002140

302 The United States Food and Drug Administration. *FDA approves ivosidenib in combination with azacitidine for newly diagnosed acute myeloid leukemia*, <https://www.fda.gov/drugs/resources-information-approved-drugs/fda-approves-ivosidenib-combination-azacitidine-newly-diagnosed-acute-myeloid-leukemia> (2022, May 25).

303 European Medicine Agency. *Tibsovo: Withdrawal of the marketing authorisation application*, <https://www.ema.europa.eu/en/medicines/human/withdrawn-applications/tibsovo> (2021, April 16).

304 European Medicine Agency. Idhifa: Withdrawal of the marketing authorisation application. (2020, January 31).

305 Servier. *Servier submits a Marketing Authorization Application to the European Medicines Agency (EMA) for TIBSOVO® (ivosidenib tablets) for patients with IDH1-mutated Acute Myeloid Leukemia (AML) and Cholangiocarcinoma*, <https://www.prnewswire.com/news-releases/servier-submits-a-marketing-authorization-application-to-the-european-medicines-agency-ema-for-tibsovo-ivosidenib-tablets-for-patients-with-idh1-mutated-acute-myeloid-leukemia-aml-and-cholangiocarcinoma-301500159.html> (2022, March 10).

306 de Botton, S. *et al.* Enasidenib vs conventional care in mutant-IDH2 relapsed/refractory acute myeloidleukemia: a randomized, phase 3 trial. *Blood* (2022). https://doi.org:10.1182/blood.2021014901

307 Botton, S. D. *et al.* Overall survival by IDH2 mutant allele (R140 or R172) in patients with late-stage mutant-IDH2 relapsed or refractory acute myeloid leukemia treated with enasidenib or conventional care regimens in the phase 3 IDHENTIFY trial. *Journal of Clinical Oncology* **40**, 7005-7005 (2022). https://doi.org:10.1200/JCO.2022.40.16_suppl.7005

308 ClinicalTrials.gov. *A Study of Ivosidenib or Enasidenib in Combination With Induction Therapy and Consolidation Therapy, Followed by Maintenance Therapy in Patients With Newly Diagnosed Acute Myeloid Leukemia or Myedysplastic Syndrome EB2, With an IDH1 or IDH2 Mutation, Respectively, Eligible for Intensive Chemotherapy (HOVON150AML)*, <https://clinicaltrials.gov/ct2/show/NCT03839771> (2021, September 1).

309 Stein, E. M. *et al.* Ivosidenib or enasidenib combined with intensive chemotherapy in patients with newly diagnosed AML: a phase 1 study. *Blood* **137**, 1792-1803 (2021). https://doi.org:10.1182/blood.2020007233

310 DiNardo, C. D. *et al.* A phase 1 study of IDH305 in patients with IDH1(R132)-mutant acute myeloid leukemia or myelodysplastic syndrome. *J Cancer Res Clin Oncol* (2022). https://doi.org:10.1007/s00432-022-03983-6

311 Heuser, M. *et al.* Safety and efficacy of BAY1436032 in IDH1-mutant AML: phase I study results. *Leukemia* **34**, 2903-2913 (2020). https://doi.org:10.1038/s41375-020-0996-5

312 Cortes, J. E. *et al.* Olutasidenib (FT-2102) in Combination with Azacitidine Induces Durable Complete Remissions in Patients with mIDH1 Acute Myeloid Leukemia. *Blood* **138**, 698-698 (2021). https://doi.org:10.1182/blood-2021-144905

313 Botton, S. D. *et al.* Effect of olutasidenib (FT-2102) on complete remissions in patients with relapsed/refractory (R/R) mIDH1 acute myeloid leukemia (AML): Results from a planned interim analysis of a phase 2 clinical trial. *Journal of Clinical Oncology* **39**, 7006-7006 (2021). https://doi.org:10.1200/JCO.2021.39.15_suppl.7006

314 Caravella, J. A. *et al.* Structure-Based Design and Identification of FT-2102 (Olutasidenib), a Potent Mutant-Selective IDH1 Inhibitor. *J Med Chem* **63**, 1612-1623 (2020). https://doi.org:10.1021/acs.jmedchem.9b01423

315 De Botton, S. *et al.* Molecular Characteristics of Response to Olutasidenib (FT-2102) in Patients with Relapsed/Refractory mIDH1 Acute Myeloid Leukemia. *Blood* **138**, 2351-2351 (2021). https://doi.org:10.1182/blood-2021-144912

316 Konteatis, Z. *et al.* Vorasidenib (AG-881): A First-in-Class, Brain-Penetrant Dual Inhibitor of Mutant IDH1 and 2 for Treatment of Glioma. *ACS Med Chem Lett* **11**, 101-107 (2020). https://doi.org:10.1021/acsmedchemlett.9b00509

317 Mellinghoff, I. K. *et al.* Vorasidenib, a Dual Inhibitor of Mutant IDH1/2, in Recurrent or Progressive Glioma; Results of a First-in-Human Phase I Trial. *Clin Cancer Res* **27**, 4491-4499 (2021). https://doi.org:10.1158/1078-0432.Ccr-21-0611

318 Adeva, J. Current development and future perspective of IDH1 inhibitors in cholangiocarcinoma. *Liver Cancer International* **3**, 17-31 (2022). https://doi.org:https://doi.org/10.1002/lci2.43

319 Borthakur, G. & Kantarjian, H. Core binding factor acute myelogenous leukemia-2021 treatment algorithm. *Blood Cancer J* **11**, 114 (2021). https://doi.org:10.1038/s41408-021-00503-6

320 Ehninger, A. *et al.* Distribution and levels of cell surface expression of CD33 and CD123 in acute myeloid leukemia. *Blood Cancer J* **4**, e218 (2014). https://doi.org:10.1038/bcj.2014.39

321 Walter, R. B., Appelbaum, F. R., Estey, E. H. & Bernstein, I. D. Acute myeloid leukemia stem cells and CD33-targeted immunotherapy. *Blood* **119**, 6198-6208 (2012). https://doi.org:10.1182/blood-2011-11-325050

322 Burnett, A. K. *et al.* Identification of patients with acute myeloblastic leukemia who benefit from the addition of gemtuzumab ozogamicin: results of the MRC AML15 trial. *J Clin Oncol* **29**, 369-377 (2011). https://doi.org:10.1200/jco.2010.31.4310

323 Cortes, J. E. *et al.* Prevention, recognition, and management of adverse events associated with gemtuzumab ozogamicin use in acute myeloid leukemia. *J Hematol Oncol* **13**, 137 (2020). https://doi.org:10.1186/s13045-020-00975-2

324 Lambert, J. *et al.* Gemtuzumab ozogamicin for de novo acute myeloid leukemia: final efficacy and safety updates from the open-label, phase III ALFA-0701 trial. *Haematologica* **104**, 113-119 (2019). https://doi.org:10.3324/haematol.2018.188888

325 Vogel, W. H. Infusion reactions: diagnosis, assessment, and management. *Clin J Oncol Nurs* **14**, E10-21 (2010). https://doi.org:10.1188/10.Cjon.E10-e21

326 The United States Food and Drug Administration. *FDA Approves Gemtuzumab Ozogamicin for CD33-positive AML*, <https://www.fda.gov/drugs/resources-information-approved-drugs/fda-approves-gemtuzumab-ozogamicin-cd33-positive-aml> (2017, September 1).

327 The United States Food and Drug Administration. *FDA approves gemtuzumab ozogamicin for CD33-positive AML in pediatric patients*, <https://www.fda.gov/drugs/drug-approvals-and-databases/fda-approves-gemtuzumab-ozogamicin-cd33-positive-aml-pediatric-patients> (2020,June 16).

328 European Medicine Agency. *Mylotarg (gemtuzumab ozogamicin) An overview of Mylotarg and why it is authorised in the EU*, <https://www.ema.europa.eu/en/documents/overview/mylotarg-epar-summary-public_en.pdf> (2018, May 5).

329 Hills, R. K. *et al.* Addition of gemtuzumab ozogamicin to induction chemotherapy in adult patients with acute myeloid leukaemia: a meta-analysis of individual patient data from randomised controlled trials. *Lancet Oncol* **15**, 986-996 (2014). https://doi.org:10.1016/s1470-2045(14)70281-5

330 Arain, S. *et al.* Safety and efficacy of gemtuzumab ozogamicin and venetoclax in patients with relapsed or refractory CD33+ acute myeloid leukemia: A phase Ib study. *Journal of Clinical Oncology* **38**, TPS7566-TPS7566 (2020). https://doi.org:10.1200/JCO.2020.38.15_suppl.TPS7566

331 Röllig, C. *et al.* Gemtuzumab Ozogamicin Plus Midostaurin in Combination with Standard Intensive Induction Therapy in Newly Diagnosed AML: Results from a Phase-I Study. *Blood* **138**, 2324-2324 (2021). https://doi.org:10.1182/blood-2021-150069

332 Daver, N. *et al.* A phase II study of decitabine and gemtuzumab ozogamicin in newly diagnosed and relapsed acute myeloid leukemia and high-risk myelodysplastic syndrome. *Leukemia* **30**, 268-273 (2016). https://doi.org:10.1038/leu.2015.244

333 Yamamoto, J. F. & Goodman, M. T. Patterns of leukemia incidence in the United States by subtype and demographic characteristics, 1997-2002. *Cancer Causes Control* **19**, 379-390 (2008). https://doi.org:10.1007/s10552-007-9097-2

334 Watts, J. M. & Tallman, M. S. Acute promyelocytic leukemia: what is the new standard of care? *Blood Rev* **28**, 205-212 (2014). https://doi.org:10.1016/j.blre.2014.07.001

335 de Thé, H. Differentiation therapy revisited. *Nat Rev Cancer* **18**, 117-127 (2018). https://doi.org:10.1038/nrc.2017.103

336 Cicconi, L. *et al.* PML-RARα kinetics and impact of FLT3-ITD mutations in newly diagnosed acute promyelocytic leukaemia treated with ATRA and ATO or ATRA and chemotherapy. *Leukemia* **30**, 1987-1992 (2016). https://doi.org:10.1038/leu.2016.122

337 Burnett, A. K. *et al.* Arsenic trioxide and all-trans retinoic acid treatment for acute promyelocytic leukaemia in all risk groups (AML17): results of a randomised, controlled, phase 3 trial. *Lancet Oncol* **16**, 1295-1305 (2015). https://doi.org:10.1016/s1470-2045(15)00193-x

338 Lo-Coco, F. *et al.* Retinoic acid and arsenic trioxide for acute promyelocytic leukemia. *N Engl J Med* **369**, 111-121 (2013). https://doi.org:10.1056/NEJMoa1300874

339 Head, D. *et al.* Effect of aggressive daunomycin therapy on survival in acute promyelocytic leukemia. *Blood* **86**, 1717-1728 (1995).

340 Stahl, M. & Tallman, M. S. Acute promyelocytic leukemia (APL): remaining challenges towards a cure for all. *Leuk Lymphoma* **60**, 3107-3115 (2019). https://doi.org:10.1080/10428194.2019.1613540

341 Österroos, A. *et al.* A risk score based on real-world data to predict early death in acute promyelocytic leukemia. *Haematologica* **107**, 1528-1537 (2022). https://doi.org:10.3324/haematol.2021.280093

342 Grimwade, D. & Lo Coco, F. Acute promyelocytic leukemia: a model for the role of molecular diagnosis and residual disease monitoring in directing treatment approach in acute myeloid leukemia. *Leukemia* **16**, 1959-1973 (2002). https://doi.org:10.1038/sj.leu.2402721

343 Hsu, K. S. & Kao, H. Y. PML: Regulation and multifaceted function beyond tumor suppression. *Cell Biosci* **8**, 5 (2018). https://doi.org:10.1186/s13578-018-0204-8

344 Rosenbauer, F. & Tenen, D. G. Transcription factors in myeloid development: balancing differentiation with transformation. *Nat Rev Immunol* **7**, 105-117 (2007). https://doi.org:10.1038/nri2024

345 Orfali, N. *et al.* All-trans retinoic acid (ATRA)-induced TFEB expression is required for myeloid differentiation in acute promyelocytic leukemia (APL). *Eur J Haematol* **104**, 236-250 (2020). https://doi.org:10.1111/ejh.13367

346 Lallemand-Breitenbach, V. *et al.* Arsenic degrades PML or PML-RARalpha through a SUMO-triggered RNF4/ubiquitin-mediated pathway. *Nat Cell Biol* **10**, 547-555 (2008). https://doi.org:10.1038/ncb1717

347 Mathews, V. *et al.* Single-agent arsenic trioxide in the treatment of newly diagnosed acute promyelocytic leukemia: long-term follow-up data. *J Clin Oncol* **28**, 3866-3871 (2010). https://doi.org:10.1200/jco.2010.28.5031

348 Ghavamzadeh, A. *et al.* Phase II study of single-agent arsenic trioxide for the front-line therapy of acute promyelocytic leukemia. *J Clin Oncol* **29**, 2753-2757 (2011). https://doi.org:10.1200/jco.2010.32.2107

349 Soignet, S., Fleischauer, A., Polyak, T., Heller, G. & Warrell, R. P., Jr. All-trans retinoic acid significantly increases 5-year survival in patients with acute promyelocytic leukemia: long-term follow-up of the New York study. *Cancer Chemother Pharmacol* **40 Suppl**, S25-29 (1997). https://doi.org:10.1007/s002800051057

350 Nasr, R. *et al.* Eradication of acute promyelocytic leukemia-initiating cells through PML-RARA degradation. *Nat Med* **14**, 1333-1342 (2008). https://doi.org:10.1038/nm.1891

351 Rego, E. M. & De Santis, G. C. Differentiation syndrome in promyelocytic leukemia: clinical presentation, pathogenesis and treatment. *Mediterr J Hematol Infect Dis* **3**, e2011048 (2011). https://doi.org:10.4084/mjhid.2011.048

352 de la Serna, J. *et al.* Causes and prognostic factors of remission induction failure in patients with acute promyelocytic leukemia treated with all-trans retinoic acid and idarubicin. *Blood* **111**, 3395-3402 (2008). https://doi.org:10.1182/blood-2007-07-100669

353 Regionala cancercentrum i samverkan. *Akut promyelocytleukemi (APL)*, <https://kunskapsbanken.cancercentrum.se/diagnoser/aml/vardprogram/akut-promyelocytleukemi-apl/#chapter-13-8-Behandling-av-APL-recidiv> (2022, August 26).

354 Ramos Perez, J. M. *et al.* Value of measurable residual disease monitoring in patients with acute promyelocytic leukemia in the era of frontline 'chemotherapy-free' therapy. *Leuk Lymphoma* **63**, 672-675 (2022). https://doi.org:10.1080/10428194.2021.1992757

355 European medicine agency. *Trisenox arsenic trioxide*, <https://www.ema.europa.eu/en/documents/overview/trisenox-epar-summary-public_en.pdf> (2016, November 1).

356 The asco post staff. *FDA Approves Arsenic Trioxide With Tretinoin for First-Line Treatment of Acute Promyelocytic Leukemia*, <https://ascopost.com/issues/january-25-2018/fda-approves-arsenic-trioxide-with-tretinoin-for-first-line-treatment-of-acute-promyelocytic-leukemia/> (2018, January 25).

357 Regionala cancercentrum i samverkan. *AML Akut myeloisk leukemi–Årsrapport nationellt kvalitetsregister*, <https://cancercentrum.se/globalassets/cancerdiagnoser/blod-lymfom-myelom/aml/dokument/aml-arsrapport-1997-2020.pdf> (2021, August 23).

358 Lengfelder, E. *et al.* Arsenic trioxide-based therapy of relapsed acute promyelocytic leukemia: registry results from the European LeukemiaNet. *Leukemia* **29**, 1084-1091 (2015). https://doi.org:10.1038/leu.2015.12

359 Magnusson, M. *et al.* Expansion on stromal cells preserves the undifferentiated state of human hematopoietic stem cells despite compromised reconstitution ability. *PLoS One* **8**, e53912 (2013). https://doi.org:10.1371/journal.pone.0053912

360 Baudet, A. *et al.* Small molecule screen identifies differentiation-promoting compounds targeting genetically diverse acute myeloid leukaemia. *Br J Haematol* **175**, 342-346 (2016). https://doi.org:10.1111/bjh.13851

361 Döhner, H., Wei, A. H. & Löwenberg, B. Towards precision medicine for AML. *Nat Rev Clin Oncol* **18**, 577-590 (2021). https://doi.org:10.1038/s41571-021-00509-w

362 Kurtz, S. E. *et al.* Molecularly targeted drug combinations demonstrate selective effectiveness for myeloid- and lymphoid-derived hematologic malignancies. *Proc Natl Acad Sci U S A* **114**, E7554-e7563 (2017). https://doi.org:10.1073/pnas.1703094114

363 Swords, R. T. *et al.* Ex-vivo sensitivity profiling to guide clinical decision making in acute myeloid leukemia: A pilot study. *Leuk Res* **64**, 34-41 (2018). https://doi.org:10.1016/j.leukres.2017.11.008

364 Spinner, M. A. *et al.* A Feasibility Study of Biologically Focused Therapy for Myelodysplastic Syndrome Patients Refractory to Hypomethylating Agents. *Blood* **134**, 4239-4239 (2019). https://doi.org:10.1182/blood-2019-129473

365 Baudet, A., Hultmark, S., Ek, F. & Magnusson, M. Small Molecule Screening of Primary Human Acute Myeloid Leukemia Using Co-culture and Multiplexed FACS Analysis. *Bio Protoc* **12**, e4353 (2022). https://doi.org:10.21769/BioProtoc.4353

366 Redig, A. J. & Platanias, L. C. The protein kinase C (PKC) family of proteins in cytokine signaling in hematopoiesis. *J Interferon Cytokine Res* **27**, 623-636 (2007). https://doi.org:10.1089/jir.2007.0007

367 Bedoya, L. M. *et al.* SJ23B, a jatrophane diterpene activates classical PKCs and displays strong activity against HIV in vitro. *Biochem Pharmacol* **77**, 965-978 (2009). https://doi.org:10.1016/j.bcp.2008.11.025

368 Radomska, H. S. *et al.* Block of C/EBP alpha function by phosphorylation in acute myeloid leukemia with FLT3 activating mutations. *J Exp Med* **203**, 371-381 (2006). https://doi.org:10.1084/jem.20052242

369 Shorstova, T., Foulkes, W. D. & Witcher, M. Achieving clinical success with BET inhibitors as anti-cancer agents. *Br J Cancer* **124**, 1478-1490 (2021). https://doi.org:10.1038/s41416-021-01321-0

370 Mochly-Rosen, D., Das, K. & Grimes, K. V. Protein kinase C, an elusive therapeutic target? *Nat Rev Drug Discov* **11**, 937-957 (2012). https://doi.org:10.1038/nrd3871

371 Basu, A. & Pal, D. Two faces of protein kinase Cδ: the contrasting roles of PKCδ in cell survival and cell death. *ScientificWorldJournal* **10**, 2272-2284 (2010). https://doi.org:10.1100/tsw.2010.214

372 Black, J. D. in *Protein Kinase C in Cancer Signaling and Therapy* (ed Marcelo G. Kazanietz) 155-188 (Humana Press, 2010).

373 Black, A. R. & Black, J. D. Protein kinase C signaling and cell cycle regulation. *Front Immunol* **3**, 423 (2012). https://doi.org:10.3389/fimmu.2012.00423

374 Tamargo, J., Le Heuzey, J. Y. & Mabo, P. Narrow therapeutic index drugs: a clinical pharmacological consideration to flecainide. *Eur J Clin Pharmacol* **71**, 549-567 (2015). https://doi.org:10.1007/s00228-015-1832-0

375 Stephen J. Brown, M. H. *Methods of administering prostratin and structural analogs thereof*, <https://patents.google.com/patent/EP2276478A1/en> (2011, January 26).

376 Lebwohl, M. *et al.* Ingenol mebutate gel for actinic keratosis. *N Engl J Med* **366**, 1010-1019 (2012). https://doi.org:10.1056/NEJMoa1111170

377 Ratushny, V., Gober, M. D., Hick, R., Ridky, T. W. & Seykora, J. T. From keratinocyte to cancer: the pathogenesis and modeling of cutaneous squamous cell carcinoma. *J Clin Invest* **122**, 464-472 (2012). https://doi.org:10.1172/jci57415

378 Freiberger, S. N. *et al.* Ingenol Mebutate Signals via PKC/MEK/ERK in Keratinocytes and Induces Interleukin Decoy Receptors IL1R2 and IL13RA2. *Mol Cancer Ther* **14**, 2132-2142 (2015). https://doi.org:10.1158/1535-7163.Mct-15-0023-t

379 European Medicine Agency. *Picato*, <https://www.ema.europa.eu/en/medicines/human/referrals/picato#all-documents-section> (2020, November 16).

380 Schroder, K., Hertzog, P. J., Ravasi, T. & Hume, D. A. Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* **75**, 163-189 (2004). https://doi.org:10.1189/jlb.0603252

381 Healy, F. M., Dahal, L. N., Jones, J. R. E., Floisand, Y. & Woolley, J. F. Recent Progress in Interferon Therapy for Myeloid Malignancies. *Front Oncol* **11**, 769628 (2021). https://doi.org:10.3389/fonc.2021.769628

382 Kiladjian, J. J., Mesa, R. A. & Hoffman, R. The renaissance of interferon therapy for the treatment of myeloid malignancies. *Blood* **117**, 4706-4715 (2011). https://doi.org:10.1182/blood-2010-08-258772

383 Kiladjian, J. J. *et al.* Long-term outcomes of polycythemia vera patients treated with ropeginterferon Alfa-2b. *Leukemia* **36**, 1408-1411 (2022). https://doi.org:10.1038/s41375-022-01528-x

384 Gisslinger, H. *et al.* Ropeginterferon alfa-2b versus standard therapy for polycythaemia vera (PROUD-PV and CONTINUATION-PV): a randomised, non-inferiority, phase 3 trial and its extension study. *Lancet Haematol* 7, e196-e208 (2020). https://doi.org:10.1016/s2352-3026(19)30236-4

385 Berneman, Z. N., Anguille, S., Van Marck, V., Schroyens, W. A. & Van Tendeloo, V. F. Induction of complete remission of acute myeloid leukaemia by pegylated interferon-alpha-2a in a patient with transformed primary myelofibrosis. *Br J Haematol* **149**, 152-155 (2010). https://doi.org:10.1111/j.1365-2141.2009.08029.x

386 Smits, E. L., Anguille, S. & Berneman, Z. N. Interferon α may be back on track to treat acute myeloid leukemia. *Oncoimmunology* **2**, e23619 (2013). https://doi.org:10.4161/onci.23619

387 Luque-Martin, R. *et al.* IFN-γ Drives Human Monocyte Differentiation into Highly Proinflammatory Macrophages That Resemble a Phenotype Relevant to Psoriasis. *J Immunol* **207**, 555-568 (2021). https://doi.org:10.4049/jimmunol.2001310

388 Rosa, F. F. *et al.* Single-cell transcriptional profiling informs efficient reprogramming of human somatic cells to cross-presenting dendritic cells. *Sci Immunol* 7, eabg5539 (2022). https://doi.org:10.1126/sciimmunol.abg5539

389 Fam, C. M. *et al.* PEGylation improves the pharmacokinetic properties and ability of interferon gamma to inhibit growth of a human tumor xenograft in athymic mice. *J Interferon Cytokine Res* **34**, 759-768 (2014). https://doi.org:10.1089/jir.2013.0067

390 ClinicalTrials.gov. *IFN-γ to treat acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) that has relapsed after allogeneic hematopoietic stem cell transplantation*, <https://clinicaltrials.gov/ct2/show/NCT04628338> (2021, December 2).

391 Schwende, H., Fitzke, E., Ambs, P. & Dieter, P. Differences in the state of differentiation of THP-1 cells induced by phorbol ester and 1,25-dihydroxyvitamin D3. *J Leukoc Biol* **59**, 555-561 (1996).

392 Lee, B. H. *et al.* FLT3 mutations confer enhanced proliferation and survival properties to multipotent progenitors in a murine model of chronic myelomonocytic leukemia. *Cancer Cell* **12**, 367-380 (2007). https://doi.org:10.1016/j.ccr.2007.08.031

393 Patnaik, M. M., Mughal, T. I., Brooks, C., Lindsay, R. & Pemmaraju, N. Targeting CD123 in hematologic malignancies: identifying suitable patients for targeted therapy. *Leuk Lymphoma* **62**, 2568-2586 (2021). https://doi.org:10.1080/10428194.2021.1927021

394 Vendramin, R., Litchfield, K. & Swanton, C. Cancer evolution: Darwin and beyond. *Embo j* **40**, e108389 (2021). https://doi.org:10.15252/embj.2021108389

395 Greaves, M. & Maley, C. C. Clonal evolution in cancer. *Nature* **481**, 306-313 (2012). https://doi.org:10.1038/nature10762

396 Cucchi, D. G. J. *et al.* Two decades of targeted therapies in acute myeloid leukemia. *Leukemia* **35**, 651-660 (2021). https://doi.org:10.1038/s41375-021-01164-x

397 Cucchi, D. G. J., Groen, R. W. J., Janssen, J. & Cloos, J. Ex vivo cultures and drug testing of primary acute myeloid leukemia samples: Current techniques and implications for experimental design and outcome. *Drug Resist Updat* **53**, 100730 (2020). https://doi.org:10.1016/j.drup.2020.100730

398 Pabst, C. *et al.* Identification of small molecules that support human leukemia stem cell activity ex vivo. *Nat Methods* **11**, 436-442 (2014). https://doi.org:10.1038/nmeth.2847

399 Hultmark, S. *et al.* Combinatorial molecule screening identified a novel diterpene and the BET inhibitor CPI-203 as differentiation inducers of primary acute myeloid leukemia cells. *Haematologica* **106**, 2566-2577 (2021). https://doi.org:10.3324/haematol.2020.249177

400 Ding, L., Saunders, T. L., Enikolopov, G. & Morrison, S. J. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature* **481**, 457-462 (2012). https://doi.org:10.1038/nature10783

401 Deng, M. *et al.* A motif in LILRB2 critical for Angptl2 binding and activation. *Blood* **124**, 924-935 (2014). https://doi.org:10.1182/blood-2014-01-549162

402 Zhou, B. O., Ding, L. & Morrison, S. J. Hematopoietic stem and progenitor cells regulate the regeneration of their niche by secreting Angiopoietin-1. *Elife* **4**, e05521 (2015). https://doi.org:10.7554/eLife.05521

403 Ding, L. & Morrison, S. J. Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature* **495**, 231-235 (2013). https://doi.org:10.1038/nature11885

404 Zhang, Y. *et al.* CXCR4/CXCL12 axis counteracts hematopoietic stem cell exhaustion through selective protection against oxidative stress. *Sci Rep* **6**, 37827 (2016). https://doi.org:10.1038/srep37827

405 Magnusson, M. *et al.* Expansion on stromal cells preserves the undifferentiated state of human hematopoietic stem cells despite compromised reconstitution ability. *PLoS One* **8**, e53912-e53912 (2013). https://doi.org:10.1371/journal.pone.0053912

406 Moore, K. A., Pytowski, B., Witte, L., Hicklin, D. & Lemischka, I. R. Hematopoietic activity of a stromal cell transmembrane protein containing epidermal growth factor-like repeat motifs. *Proc Natl Acad Sci U S A* **94**, 4011-4016 (1997). https://doi.org:10.1073/pnas.94.8.4011

407 Tsimberidou, A. M., Fountzilas, E., Nikanjam, M. & Kurzrock, R. Review of precision cancer medicine: Evolution of the treatment paradigm. *Cancer Treat Rev* **86**, 102019 (2020). https://doi.org:10.1016/j.ctrv.2020.102019

408 Benedetti, L., Cereda, M., Monteverde, L., Desai, N. & Ciccarelli, F. D. Synthetic lethal interaction between the tumour suppressor STAG2 and its paralog STAG1. *Oncotarget* **8**, 37619-37632 (2017). https://doi.org:10.18632/oncotarget.16838

409 Galeev, R. & Larsson, J. Cohesin in haematopoiesis and leukaemia. *Curr Opin Hematol* **25**, 259-265 (2018). https://doi.org:10.1097/moh.0000000000000431

410 Thol, F. *et al.* Mutations in the cohesin complex in acute myeloid leukemia: clinical and prognostic implications. *Blood* **123**, 914-920 (2014). https://doi.org:10.1182/blood-2013-07-518746

411 van der Lelij, P. *et al.* STAG1 vulnerabilities for exploiting cohesin synthetic lethality in STAG2-deficient cancers. *Life Sci Alliance* **3** (2020). https://doi.org:10.26508/lsa.202000725

412 Romero-Pérez, L., Surdez, D., Brunet, E., Delattre, O. & Grünewald, T. G. P. STAG Mutations in Cancer. *Trends Cancer* **5**, 506-520 (2019). https://doi.org:10.1016/j.trecan.2019.07.001

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Division of Molecular Medicine and Gene Therapy
Department of Laboratory Medicine
Lund University, Faculty of Medicine
Doctoral Dissertation Series 2022:120
ISBN 978-91-8021-281-6
ISSN 1652-8220