The role of stem cell aging in acute myeloid leukemia
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>HSC</td>
<td>Hematopoietic Stem Cell</td>
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<tr>
<td>AML</td>
<td>Acute Myeloid Leukemia</td>
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<tr>
<td>HPC</td>
<td>Hematopoietic Progenitor Cell</td>
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<tr>
<td>LT</td>
<td>Long-Term</td>
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<tr>
<td>BM</td>
<td>Bone Marrow</td>
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<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>HIF</td>
<td>Hypoxia Inducible Factor</td>
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<tr>
<td>FAB</td>
<td>French American British</td>
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<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>LSC</td>
<td>Leukemic Stem Cell</td>
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<td>CSC</td>
<td>Cancer Stem Cell</td>
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<tr>
<td>NOD/SCID</td>
<td>Non-Obese Diabetic and Severe Combined Immunodeficiency</td>
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<tr>
<td>ATM</td>
<td>Ataxia Telangiectasia Mutated</td>
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<tr>
<td>mtDNA</td>
<td>Mitochondrial Deoxyribonucleic Acid</td>
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<tr>
<td>IGF</td>
<td>Insulin Growth Factor</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
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<tr>
<td>FOXO</td>
<td>Forkhead Box Protein O</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>RFS</td>
<td>Relapse Free Survival</td>
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<tr>
<td>ChIP seq</td>
<td>Chromatin Immunoprecipitation Sequencing</td>
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<tr>
<td>RNA seq</td>
<td>Ribonucleic Acid Sequencing</td>
</tr>
<tr>
<td>LIC</td>
<td>Leukemia Initiating Cell</td>
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<tr>
<td>NPM</td>
<td>Nucleophosmin</td>
</tr>
<tr>
<td>BMI1</td>
<td>B cell-specific Moloney MLV insertion site-1</td>
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<tr>
<td>NOG</td>
<td>NOD/SCID/yCnull</td>
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<tr>
<td>NSG</td>
<td>NOD/SCID/Gamma</td>
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<tr>
<td>DDR</td>
<td>DNA Damage Response</td>
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<tr>
<td>GSC</td>
<td>Germinal Stem Cell</td>
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Abstract

Throughout life, the hematopoietic system produces and replenishes blood cells. Hematopoiesis is a tightly regulated process, but alterations and mutations can give rise to tumors. Leukemia is a disease which occurs at all ages, but the incidence of Acute Myeloid Leukemia (AML) strongly increases with age. AML can be defined as a heterogeneous oligoclonal disorder with several different mutations at its origin. It remains under debate whether Hematopoietic Stem Cells (HSC) or Hematopoietic Progenitor Cells (HPC) are the cell of origin in AML. A strong increase in AML incidence is observed with an increase in age, but much remains unclear regarding the role of aging in the development of AML. In contrast to younger patients, AML in the elderly is increasingly resistant to chemotherapy resulting in a poor prognosis. Research has revealed that aging alters stem cell functions. Both extrinsic and intrinsic factors are believed to contribute to aging of the hematopoietic system. Here, we discuss recent developments regarding the role of intrinsic and extrinsic factors in stem cell aging. Moreover, we speculate how these findings can contribute to the increased incidence of AML in the elderly.
1. Introduction

1.1 Hematopoiesis

Hematopoiesis occurs throughout the entire lifespan of an organism, from the embryonic state to adulthood, to produce and replenish the blood system. Hematopoiesis is a tightly regulated process which is accomplished through self-renewal of hematopoietic stem cells (HSC), the proliferation of lineage committed hematopoietic progenitor cells (HPC) and the maturation of differentiated cells. Mature blood cells are produced at high speed, with estimates ranging from $2 \times 10^5$ cells per second to $1 \times 10^6$ per second. However, the HSC they derive from rarely divide. HSC primary reside in G0 and cycle infrequently under normal homeostatic conditions. Thus, an important balance in an adequate pool of HSC in an organism and the continuous demand for replenishment of blood cells should be maintained. In the hematopoietic hierarchy HSC reside at the top, from which they give rise to multipotent progenitors (MPP). These multipotent progenitors (MPP) give rise to oligopotent progenitors (OPP). Common lymphoid progenitors (CLP) give rise to mature B-lymphocytes, T-lymphocytes and natural killer cells (NK). Common myeloid progenitors (CMP) give rise to granulocyte and macrophage progenitors (GMP), which differentiate into monocytes, macrophages and granulocytes. Furthermore, CMP give rise to megakaryocyte and erythrocyte progenitors (MEP), which differentiate into megakaryocytes, platelets and erythrocytes. The development of HSC into MPP, OPP and lineage restricted progenitors (LRP) is generally associated with an increased proliferative index in most stages of development. Subsequently, it should be noted that for some subsets indicated in this figure, the lineage potential and developmental relationships have not been fully resolved. For example, research indicates that the MEP originate from a MPP rather than a CMP. Extensive research is required to determine the origin of the MEP.
1.2 Maintenance of HSC quiescence

The niche of long-term (LT) HSC in the bone marrow (BM) consists of the endosteal surface, where osteoblasts reside and the sinusoidal endothelium, often described as the vascular niche (Fig. 1.2). The vascular niche is thought to house dividing stem and progenitor cells, while the osteoblasts niche reside the more quiescent stem cells. However, the distinct function of these niches is still heavily debated. Recent deep imaging of the BM revealed that more than 85% of HSC reside within 10µM of a sinusoidal blood vessel. These data indicate the presence of quiescent HSC in the vascular niche. Subsequently, the levels of oxygen in the vascular niche were reported to be higher compared to the endosteal niche. If indeed quiescent HSC reside in the vascular niche, oxygen levels should be lower than previously believed. Low levels of oxygen are highly important for the maintenance of HSC self-renewal, low levels of Reactive Oxygen Species (ROS) and survival. Hence, much remains unknown regarding the BM niche and extensive research is required to improve our understanding. Nonetheless, several studies have given insight into the function of the BM niche. From both niches, the osteoblasts niche is the most well studies component. Increased numbers of osteoblasts resulted in a direct increase of HSC numbers without increasing the progenitor numbers. HSC remain in their quiescent state due to close interaction with osteoblasts. This interaction is crucial to attach HSC to the osteoblasts but also maintains HSC function and dormancy. Furthermore, it was reported that most slow cycling HSC are found near the bone, where the environment was believed to be most hypoxic. Recent research challenged this hypothesis due to the presence of LT-HSC in the vascular niche. Nonetheless, LT-HSC reside in the hypoxic BM to diminish proliferation and to regulate further stem cell functions. Due to the hypoxic conditions in the BM, Hypoxia inducible factors (HIF) are stabilized. In vitro research revealed that culturing under hypoxic conditions maintained low HSC numbers and reduced their proliferation rate. Taken together, these data show that the bone marrow niche is of high importance to maintain HSC functionality and dormancy.

Figure 1.2 Schematic overview of the HSC niche. The HSC niche provides physical support, soluble factors and interactions which are required for the maintenance and regulation of HSC functions. This scheme depicts the endosteal niche and vascular niche. Oxygen tension increases closer to the vascular niche in which the dividing HSC are believed to reside.
In steady state HSC a decrease in mitochondria is observed compared to progenitors. The loss of mitochondria can function as a method to decrease reactive oxygen species (ROS). Furthermore, HIF1-α increases glycolysis and thereby is able to minimize ROS generation. Hence, the BM niche is of high importance to maintain stem cell numbers and functions. Low oxygen levels are required to maintain HSC self-renewal, lower the levels of ROS and promote survival. Due to the hypoxic environment in the BM HIF proteins are stabilized which are required to regulate gene expression regarding metabolism and proliferation. Recent research has given insight into the presence of slow cycling HSC in the vascular niche. Further research is required to follow up on this interesting finding.

1.3 Asymmetric and symmetric cell division

1.3.1 Asymmetric division due to extrinsic factors

Whether a stem cell divides symmetrically or asymmetrically depends on intrinsic and extrinsic factors. Research has confirmed that the interplay with the bone marrow niche and the HSC is highly important for the maintenance of the hematopoietic system. Currently, the bone marrow niche remains poorly defined in mammals. Nonetheless, it has been confirmed that osteoblasts residing in the endosteum of the bone marrow and sinusoidal endothelial cells of the bone marrow or spleen regulate the stem cell pool. Osteoblasts express Jagged-1, a ligand which can activate the Notch pathway. The Notch pathway regulates cell fate throughout the body, including HSC self-renewal. Notch is known to maintain a balance in HSC between self-renewal and differentiation. Furthermore, it was demonstrated that Notch signaling increases stem cell numbers without the expansion of mature cells. Taken together, due to the position of a daughter cell closer to the Delta or Jagged-1 expressing osteoblasts or further away, cell fate is determined by extrinsic cues. An example of an asymmetric division regulated by extrinsic factors is the Drosophila germ line stem cells. The germ line stem cell divides with a reproducible orientation to generate two daughter cells, one maintaining its position in the stem cell niche and one that is placed away from the niche.

![Figure 1.3](image)

**Figure 1.3 Controls of asymmetric cell division.**

a) Asymmetric division is initiated due to asymmetric localization of cell polarity regulators. Stem cells are depicted in orange, differentiated cells in green. b) Cell fate determinants are transferred to the cytoplasm of one daughter cell. Other locations are also possible such as the membrane, centrosomes and other structures. c) Orientation causes only one stem cell to be present in the niche. Therefore, only this daughter cell is provided with the required extrinsic signals.

1.3.2 Asymmetric division due to intrinsic factors

Also intrinsic factors, such as cell polarity can determine cell fate (Fig. 1.3). An asymmetric cell division will result in a more differentiated cell and the maintenance of one stem cell. However, symmetric division can also occur, resulting in two differentiated or two stem cells. An example of an asymmetric division regulated by intrinsic factors is the C. elegans zygote. Asymmetric division of the zygote creates one large blastomere for the development of the ectoderm and one smaller blastomere responsible for the production of the mesoderm and endoderm and germine. Furthermore, B cell-specific Moloney MLV insertion site-1 (BMI1), a polycomb protein is highly expressed in HSC. BMI-1 is required during development for the maintenance of gene expression patterns. BMI1 expression gradually decreases upon maturation towards CMP, GMP and
MEP.\textsuperscript{25} In the contrary, BMI-1 expression is maintained in B-cell and T-cell compartments.\textsuperscript{26} Another example of asymmetric divisions due to intrinsic factors is CDC42. CDC42 functions as a regulator of structural elements such as actin and microtubules.\textsuperscript{27} Hereby CDC42 mediates cell polarization, adhesion and migration. Hence, several intrinsic mechanisms have been indicated asymmetric divisions. Nevertheless, BMI1 and CDC42 have also been indicated in aging. Their role in aging will be elaborated further in 2.2.

1.3.3 Symmetric divisions

Nonetheless, also symmetric divisions can occur. Symmetric divisions are common in developing tissues, but also occur in later life. As described above, Drosophila Germline Stem Cells (GSC) normally divide asymmetrically. However, symmetric division can be induced in female germ line stem cells after loss of a stem cell.\textsuperscript{20} Taken together, these data prove that in Drosophila both asymmetric and symmetric division can occur to maintain homeostasis.

In conclusion, both intrinsic and extrinsic factors play an important role in cell fate decisions. The HSC niche is important to maintain stem cell quiescence, to preserve stem cell functions and prevent DNA damage accumulation. Subsequently, intrinsic factors play an important role in maintaining cell polarity. Nevertheless, under certain circumstances such as development or the loss of a stem cell, also symmetric divisions can occur.

1.4 Acute myeloid leukemia

1.4.1 AML: a disease of the hematopoietic progenitor cells

Leukemia is a malignant disease of the bone marrow and the blood which is characterized by the uncontrolled accumulation of immature blood cells. Germline mutations are rare in leukemia and therefore the disease is mostly characterized by somatic mutations. Leukemia is divided into two groups; myeloid and lymphoid, both of which can be chronic or acute. This thesis will mainly focus on Acute Myeloid Leukemia (AML). AML can be defined as a heterogeneous oligo-clonal disorder with several different mutations as its origin (Table 1.3). AML can be described as a disease of the HSC or HPC progenitor cells and is characterized by the accumulation of blasts. These blasts have lost the ability to differentiate fully and fail to respond to normal regulators of proliferation and differentiation.\textsuperscript{28} Bone marrow (BM) failure, which is described as the reduction of erythrocyte, neutrophil and platelet production is often observed at the diagnosis of AML. The loss of mature myeloid cells strongly contributes to the morbidity and mortality due to increased chances of infections and bleedings.\textsuperscript{29} If AML remains untreated, fatal infections, bleedings or organ infiltration will occur within a period of 1-5 years after diagnosis.\textsuperscript{28}

1.4.2 The incidence of AML

AML is the most common myeloid leukemia with an average incidence of 4.1 patients per 100,000 in 2009-2013 (Table 1.1). With an increase in age, the incidence of AML also increases (Table 1.1 and S.Table 1.1). In adults aged 65 and higher, the incidence has increased to an average of 19.1 patients per 100,000. However, the incidence is highest in the age group 80-84 years (Supplementary table 1.1). Moreover, it should be noted that the incidence in men is higher compared to women.\textsuperscript{30}

Table 1.1. SEER Incidence Rates, Age-Adjusted by Sex, 2009-2013.\textsuperscript{30}

<table>
<thead>
<tr>
<th>Age at Diagnosis</th>
<th>All races</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Both Sexes</td>
</tr>
<tr>
<td>All ages</td>
<td>4.1</td>
</tr>
<tr>
<td>Under 65</td>
<td>1.9</td>
</tr>
<tr>
<td>65 and over</td>
<td>19.1</td>
</tr>
<tr>
<td>All ages (IARC world std)</td>
<td>2.8</td>
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</table>
1.4.3 Subtypes of AML

In 1976, the French-American-British Co-operative group developed a uniform system for the classification and nomenclature of acute leukemia, since such a system was lacking. It was believed that this system would provide more insight into the cases entering clinical trials and could provide as a reference standard for new markers. Classification was based on morphology and cytochemical reactions of the bone marrow or peripheral blood. The origin of subtypes M0-M5 lies in the progenitors of white blood cells, while the cell of origin of M6 lies in the progenitor of erythrocytes. Moreover, M7 AML is specifically caused by the progenitor of platelets (Table 1.2). 

<table>
<thead>
<tr>
<th>Nr.</th>
<th>Classification</th>
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<tbody>
<tr>
<td>M0</td>
<td>Myeloid Leukemia with minimal differentiation</td>
</tr>
<tr>
<td>M1</td>
<td>Myeloid Leukemia without maturation</td>
</tr>
<tr>
<td>M2</td>
<td>Myeloid Leukemia with maturation</td>
</tr>
<tr>
<td>M3</td>
<td>Hypergranular Promyelocytic Leukemia</td>
</tr>
<tr>
<td>M4</td>
<td>Myelomonocytic Leukemia</td>
</tr>
<tr>
<td>M4 EO</td>
<td>Myelomonocytic Leukemia with abnormal eosinophils</td>
</tr>
<tr>
<td>M5</td>
<td>Monocytic Leukemia</td>
</tr>
<tr>
<td>M6</td>
<td>Erythroleukemia</td>
</tr>
<tr>
<td>M7</td>
<td>Megakaryocytic Leukemia</td>
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</table>

The FAB method of classification has been used to classify the heterogeneous disease AML for several decades. However, the World Health Organization published a new method for classification in 2001 and further adjustments in 2008, which better suit the recurrent genetic alterations (Table 1.3). These genetic abnormalities are often associated with clinical and pathophysiological features. Subsequently, these abnormalities can be applied as a distinction for diagnosis of different subtypes of disease with widely different prognosis. AML patients with a cytogenetic profile associated with a favorable risk mostly show mutations in PML-RARA, RUNX1-RUNX1T1 or MYH11-CBFβ and respond well to chemotherapy. Patients with an unfavorable risk often show monosomy in their karyotype or complex alterations. The majority of AML patients have an intermediate cytogenetic risk and most commonly have a normal karyotype. Intriguingly, response to treatment greatly differs in these patients. Therefore, recent studies focused on better classification of patients in the intermediate risk group. Targeted sequencing has revealed several recurrent mutations in FLT3, NPM1, KIT, CEPBA and TET2. Other studies reveal recurrent mutations in DNMT3A and IDH1. All together, these mutations might provide more insight into prognostics regarding patients with an intermediate risk. However, current studies revealed that many patients do not show any of these known mutations. These data indicate that a better understanding of the genetic and epigenetic alterations in AML is required to be able better classify disease and increase treatment efficacy. Moreover, with an increase in age an increase in clonal outgrowth was observed. The somatic lesions which cause this clonal outgrowth were observed in 10% of the population with an age higher than 70. However, this number is greatly exceeds the incidence of AML, indicating that mutations for clonal outgrowth do not directly result in tumorigenesis. Nonetheless, clonal expansion does increase the risk of cancer development and is commonly observed in the elderly. The number of somatic mutations that cause clonal expansion increases with age and thereby increases the risk of cancer development.
Table 1.3. The WHO classification of AML, 2008. For the extended table see Supplementary Table 1.2.

<table>
<thead>
<tr>
<th>AML with recurrent genetic abnormalities</th>
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<tr>
<td>AML with t(8;21)(q22;q22); RUNX1-RUNX1T1*</td>
</tr>
<tr>
<td>AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFβ-MYH11*</td>
</tr>
<tr>
<td>Acute promyelocytic leukemia; AML with t(15;17)(q22;q12); PML-RARA and cytogenetic variants</td>
</tr>
<tr>
<td>AML with t(9;11)(p22;q23); MLLT3-MLL</td>
</tr>
<tr>
<td>AML with t(6;9)(p23;q34); DEK-NUP214</td>
</tr>
<tr>
<td>AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1</td>
</tr>
<tr>
<td>AML (megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1</td>
</tr>
<tr>
<td>Provisional entity: AML with mutated NPM1</td>
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<tr>
<td>Provisional entity: AML with mutated CEBPA</td>
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<table>
<thead>
<tr>
<th>AML with myelodysplasia related changes</th>
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<tr>
<td>Therapy-related myeloid neoplasms</td>
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<table>
<thead>
<tr>
<th>Acute myeloid leukemia, Not otherwise specified (NOS)</th>
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<tbody>
<tr>
<td>AML with minimal differentiation</td>
</tr>
<tr>
<td>AML without maturation</td>
</tr>
<tr>
<td>AML with maturation</td>
</tr>
<tr>
<td>Acute myelomonocytic leukemia</td>
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<tr>
<td>Acute monoblastic/monocytic leukemia</td>
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<table>
<thead>
<tr>
<th>Acute erythroid leukemia</th>
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<tbody>
<tr>
<td>- Pure erythroid leukemia</td>
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<tr>
<td>- Erythroleukemia, erythroid/myeloid</td>
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<table>
<thead>
<tr>
<th>Acute megakaryoblastic leukemia</th>
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<tr>
<td>Acute basophilic leukemia</td>
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<tr>
<td>Acute panmyelosis with myelofibrosis</td>
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| Myeloid Sarcoma |

1.5 The Cancer Stem Cell model

1.5.1 The leukemic stem cell in AML and the development of immunodeficient mouse models

The disease AML can be characterized by the accumulation of cells that are unable to differentiate into mature granulocytes or monocytes. These immature blasts possess limited proliferation capacity which indicates towards the presence of a leukemic stem cell. This LSC is believed to possess extensive proliferative capacity and is able to sustain itself via self-renewal. One of the first studies in which myelomonocytic AML cells were engrafted into non-obese diabetic mice with severe combined immuno-deficiency disease (NOD/SCID) revealed that CD34+ CD38−, can be defined as the leukemia initiating cell (LIC). In healthy individuals, the CD34+ CD38− population is able to fully repopulate the blood system in contrast to their progeny. Moreover, transplantation of these SCID leukemia initiating cells were able to propagate AML in a xenograft transplant system. On the other hand, these studies report that SCID leukemia initiating cells are very rare, only one single cell amongst a million of blasts. Subsequently, these leukemic grafts greatly represented the blast morphology and dissemination profiles. Another study performed at the a comparable time confirmed that indeed only a subset of AML cells, CD34+ cells possess the ability to form colonies in culture. Specifically, they discovered that the LSC both in vitro and in vivo capacity
lacked Thy-1 (CD90) expression.\textsuperscript{46} However, the NOD/SCID mice lack a human bone marrow environment, which impedes faithful caputulation of human disease.\textsuperscript{49} Nonetheless, it should be noted that mouse models are rapidly changing and improving.\textsuperscript{50,51}

Figure 1.3. The development of immunodeficient mouse models for xenograft studies.\textsuperscript{52}

Early xenograft studies were performed in SCID mice. Due to residual NK-cell activity the model proved to only be suitable for solid transplantations. The NOD/SCID mouse proved to be better suitable for engraftment of human cells. However, transplanted HSC did not generate cells. NOG and NSG models show no residual NK-cell activity and therefore are often applied to study HSC self-renewal. Furthermore, human cytokines support myelopoiesis and therefore this model is suitable for studying hematological malignancies. Finally, the humanized BLT mouse possess a humanized bone, liver and thymic environment and is often applied for studying infectious diseases. References mentioned do not correspond with this reference list.

Early phase xenograft studies were performed in Severe Combined Immunodeficient (SCID) mice with defects in B-cell and T-cell development. A drawback of the SCID model is that despite their lack of B-cells and T-cell activity, high NK-cell activity remains. Therefore, solid tissues can be transplanted into this model, but single cell suspensions are quickly recognized and lysed by the NK-cells.\textsuperscript{52} Many adjustments were made in the SCID model, ultimately resulting in the Non Obese Diabetic (NOD)/SCID model. In the contrary to SCID mice, NOD/SCID mice proved to be suitable for the engraftment of human cells due to additional defects in NK-cell activity, macrophage activity and circulating complement. The NOD/SCID model is very suitable for the purpose of studying B-cells but to a lesser extend for myeloid output. Subsequently, transplanted HSC do not generate T-cells. It is hypothesized that the lack of T-cells production is due to the residual NK-cell activity.\textsuperscript{53} Current popular strains are the NOD/SCID/γCnul (NOG) and NOD/SCID Gamma (NSG). The NOG and NSG models show no residual NK-cell activity and are often applied for studies regarding HSC self-renewal. Human CD34\textsuperscript{+} cells show a high engraftment rate in NOG and NSG mice, but secondary transplantations often shows low levels of engraftment.\textsuperscript{54,55} A recent study demonstrated that activity of stem and progenitor cells remains for up to eight weeks after engraftment. These data indicate a lack of quiescence as the limiting factor of self-renewal.\textsuperscript{56} Subsequently, the NSG model expressing myelopoiesis supporting cytokines is often applied for studies regarding hematopoietic malignancies.\textsuperscript{55} Finally, a model which is humanized for bone, liver and thymus has been generated and is often used for studying infectious diseases. This model develops mature T-cells and possesses a more complete human immune system.\textsuperscript{57} Thus, dependent on the aim of the study an immunodeficient xenograft mouse model can be selected.
The immunodeficient mouse models have greatly expanded our knowledge regarding leukemic transformation. Research strongly indicates that normal primitive cells are targeted and that also the LSC is organized as a hierarchy. However, the exact phenotype of LSC remains under debate. Recent research revealed that normal primitive cells possess repopulating capacity. In AML, nucleophosmin (NPM) is often mutated and this is associated with low CD34 expression. Therefore, they studied whether CD34+ cells solely initiated leukemia. However, it was discovered that also half of the CD34− cells were able to initiate leukemia. Furthermore, it was confirmed that also CD38− were able to perform as LIC. These new findings can be explained by the presence of an anti-CD38 antibody in previous studies using the NOD/SCID model. Hence, these data indicate that the LIC phenotype is increasingly heterogeneous.

In conclusion, AML is driven by LSC which reside at the top of the hierarchy and are accountable for the production immature cells. Recent research has provided more insight into the heterogeneous phenotype of the LIC. Subsequently, it is likely that LSC are the cause of treatment resistance due to their heterogeneity and cause relapse due to their insensitivity to current treatments.

1.5.2 From hematopoietic stem cell to leukemic stem cells

Currently, it remains unclear whether LSC originate from the HSC themselves or a more downstream early progenitor cell. It proved to be challenging to determine the cell of origin, since leukemogenic events alter surface marker expression. Nonetheless, LSC can be defined by the functional properties of HSC. Leukemic transformation can be described as a multi-step process with chromosomal translocations and mutations. These aberrations result in an incorrect balance of transcription factors which regulate cell-fate decisions. These abnormalities in cell fate decisions are important drivers of myeloid malignancies, especially in the elderly. For example, a single nucleotide polymorphism in the distal enhancer strongly decreases expression of the transcription factor PU.1 by blocking the binding of SATB1 in complex karyotype AML. Subsequently, analysis of an AML patient with t8;21 in the AML-1-ETO oncogene, revealed that the expression of AML-1-ETO resulted in the decrease of the granulocyte differentiation protein CEBP/α. However, in mice the ectopic expression of AML-1-ETO was insufficient to give rise to AML. These data indicate that HSC harbor the original lesions, but additional mutations are required to drive AML transformation in downstream progenitors (Fig. 1.5). Moreover, CSC are believed to be able to generate most or all cell types present in the tumor. Leukemic stem cells are believed to highly express multi drug resistance transporter proteins, like normal stem cells. These proteins mediate drug efflux, a trait that may enable leukemic stem cells to evade the effects of chemotherapy, ultimately causing relapse.

In conclusion, the current evidence points towards the HSC as the origin of mutations. However, this original mutation is insufficient to give rise to AML. Therefore, additional mutations in the downstream progenitors are required. Subsequently, LSC are believed to survive chemotherapy and thereby are able to cause relapse. One of the mechanisms behind this resistance is the high expression of drug efflux transporter proteins. Moreover, like HSC, LSC are believed to be able to circumvent cytotoxic events by elevated apoptosis resistance, enhanced DNA-repair efficiency, detoxification enzyme expression and quiescence. Furthermore, due to their heterogeneity, targeting LSC is increasingly difficult. Hence, to eradicate AML, the leukemic stem cell should be targeted.
Leukemic progression model: HSC accumulate genetic and epigenetic events that lead to blast crisis and leukemia. Heritable mutations are transferred due to self-renewal and differentiation. When stem cells with heritable mutations acquire further lesions, specifically in growth and survival genes, these lesions can promote selection of pre-leukemic clones. This model describes 7 required events to progress from pre-leukemic clones to leukemia. However, the actual number of events depends on the cancer type and the nature of the lesions. The actual mutagenic events occur in HSC, the eventual development of LSC occurs on the level of progenitors which acquired the capacity for unlimited self-renewal. In AML the leukemic clone can arise at the level of multipotent progenitors or further downstream depending on the lesions.

1.6 Scope of this thesis

AML is a disease which occurs in all ages. However, AML is mainly a disease of the elderly, with a median age of 69 in the United States Caucasian population of the Surveillance, Epidemiology and End results database from 1975 to 2004. These data show that age has a strong impact on the incidence of AML. Moreover, age has a major impact on the management and outcome of patients diagnosed with AML. With an increase in age, comorbidity rises while tolerance for intensive treatment decreases. Furthermore, elderly patients have a higher incidence of poor prognostic factors such as secondary leukemia or high risk cytogenetics. Altogether, this leads to a smaller group of patients eligible for remission induction, a lower remission rate and shorter remission duration. Hence, especially in the elderly, treatment for AML proved to be challenging. In the last decade more evidence was shown which describe the presence of a LSC in AML. The incidence of AML strongly increases with age, which can be due to stem cell aging. This thesis will create an overview of the link between stem cell aging and the increased incidence of AML in the elderly. Thereby, the following sub-questions were formulated:

- What are the intrinsic causes of stem cell aging?
- What are the micro-environmental causes of stem cell aging?
- How can stem cell aging contribute to the incidence of AML?
2. The effects of stem cell aging on the incidence of AML

2.1 What are the intrinsic causes of stem cell aging?

2.1.1 Stem cell aging

The HSC which resides in the bone marrow replenishes all cell types in the blood. Thereby, the bone marrow is one of the most highly self-renewing tissues in the human body.\(^{17}\) However, the hematopoietic system also suffers from the detrimental effects of aging. The effects of aging contribute to a strong increase of myeloproliferative diseases, a decline in the adaptive immune system and an increase in the incidence of anemia.\(^{14,75,76}\) In general, aging is associated with a decline in tissue homeostasis and repair after injury, indicating an imbalance in the loss of cells and renewal.\(^{17}\) Moreover, aging causes a proportional lineage shift towards myeloid-biased production in HSC (Fig. 2.1). The relative production of lymphoid cells decreases while the myeloid input is maintained equal or even increases.\(^{77}\) Furthermore, stem cells increase in number with age, but the functionality and quality per stem cell declines.\(^{78}\) It was observed that after serial transplantation of old bone marrow, the HSC were still able to repopulate the blood system.\(^{79}\) These data prove that HSC are able to overcome the detrimental effects of aging. However, it should be noted that several studies reported a decrease in the repopulating capacity of old murine HSC compared to their younger counterpart.\(^{77,80}\) Hence, the hematopoietic system relies on HSC for functional hematopoiesis. Therefore, changes in the hematopoietic system are due to age related alterations in the functionality of HSC. Asymmetric divisions are tightly regulated. However, in drosophila, Germline Stem Cells (GSC) of aged micea strong increase in centrosome misorientation was observed. In addition, loss of orientation was linked to reduced divisions. Thus, misorientation of centrosomes causes a decline in spermatogenesis. Further, this paper demonstrates that GSC partially originate from dedifferentiation and are able to divide with correctly oriented spindles. Since GSC number do not decrease as strongly as expected, it is hypothesized that dedifferentiation functions and the main mechanism to replenish stem cells in Drosophila.\(^{114}\) Subsequently, mouse model studies found that elevated Rho GTPase Cdc42 expression is indicated in aging in HSC. Increased Rho GTPase Cdc42 expression causes a decrease in cell polarity, alters epigenetic modifications and causes functional decline in HSC.\(^{115,116}\) These data show that intrinsic alterations due to aging can cause functional HSC decline. Besides intrinsic factor the HSC exert their function supported by the bone marrow. Therefore, it is likely that aging of HSC consists out of both intrinsic and micro-environmental factors. This thesis aims to point out recent developments and discoveries regarding intrinsic and micro-environmental aging of HSC. Moreover, this thesis highlights recent discoveries regarding the role of HSC aging in tumorigenesis, with a focus on AML.

Figure 2.1 Aging causes alterations in the clonal composition of the HSC compartment.\(^{17}\)
Upon aging a proportional lineage shift toward myeloid-biased balance is observed. Moreover, stem cell numbers increase, but the quality of HSC decreases.
2.1.2 DNA damage causes HSC aging

The vast majority of research has been conducted on the effects of intrinsic aging. In these studies HSC from old donors were transplanted into a young recipient, hereby eliminating the factors of micro-environmental aging. It was hypothesized that accumulated DNA damage could be a mechanism underlying stem cell decline (Figure 2.2). The evidence for indirect involvement of DNA damage in HSC aging was obtained from murine studies. Deficiencies for several genomic maintenance pathways such as nucleotide excision repair, telomere maintenance and non-homologous end-joining were induced. These deficiencies did not deplete stem cell reserves but did result in alterations in the number of HSC and strongly induced functional decline. Furthermore, immunofluorescence for γH2Ax, a DNA damage marker, shows accumulation of endogenous DNA in wild-type HSC. Thus, deficiencies in genomic maintenance pathways are detrimental to the DNA Damage Response (DDR) and induce premature aging in HSC. Recent research in humans revealed that indeed non-telomeric DNA damage accompanies physiological aging stem cells and progenitors. DNA lesions which do not cause apoptosis or senescence can be transferred from HSC to further progeny. In this manner the impact of damage from a lesion in one single HSC can be potentiated to every level of the hierarchy. If the number of functional stem cells decrease below the level of self-renewal and production of mature cells, homeostatic control will diminish accompanied by reduced regenerative potential. Both of these traits can be defined as hallmarks of aging. Furthermore, if DNA damage is unrepaired or incorrectly repaired, this can be sufficiently mutagenic. If indeed the damage is mutagenic, this will result in transformation of the stem cell and its progeny. From this point onwards, the stem cell with unrepaired damage of mutagenic lesions can serve as a substrate for additional hits, driving clonal selection. Nonetheless, it should be noted that potent tumor suppressor pathways are active HSC to ensure that HSC with potential dangerous lesions are eradicated. Nevertheless, with an increase in age DNA damage accumulates, which indicates a decreased ability to resolve DNA damage. Lastly, in many of the described studies great inter-individual differences were observed, indicating that DNA damage itself is not the only cause of intrinsic aging in HSC, but that additional intrinsic or extrinsic mechanisms contribute to stem cell aging.

![Figure 2.2. DNA damage in stem cells.](image)

Depending on the nature of the lesion, different responses are elicited. Mutagenic lesions can result in transformation of HSC or progenitors if no further hits are required. Cytotoxicity and cytostatic can cause apoptosis or senescence of HSC. Furthermore, apoptosis and senescence cause shrinking of the HSC pool. Point mutations can cause altered functional capacity of HSC, thereby altering stem cell biology. Cytotoxic, cytostatic and deregulatory lesions together can disturb homeostasis and reduced regenerative capacity which both are hallmarks of aging.

2.1.3 Tumor suppressor genes p16INK4A and p53 contribute to aging
Somatic cells activate the Retinoblastoma and p53 pathway upon detection of dangerous lesions and thereby provoke apoptosis or a permanent cell cycle arrest. These pathways are highly important in preventing cancer. However, it is becoming increasingly evident that activation of these tumor suppressor pathways contributes to aging. Elevated expression of p16\(^{\text{INK4A}}\) a downstream effector of Rb, was discovered combined with \(\beta\)-galactosidase, a marker for senescence. Moreover, these markers together were implicated as the principal bio-markers of aging.\(^{83,84}\) In addition, a study in mice confirmed that p16\(^{\text{INK4A}}\) expression was elevated in old HSC, but discovered that a p16\(^{\text{INK4A}}\) deficiency in old mice improved age-associated deficits.\(^{85}\) Hence, these studies provide evidence for a causal role of p16\(^{\text{INK4A}}\) in age-associated deficits of HSC. Nonetheless, it remains undefined whether p16\(^{\text{INK4A}}\) induction results in cellular senescence. Several studies confirm that old HSC have the same capacity of producing progeny as young HSC. Furthermore, loss of BMI-1, a polycomb group gene which regulates p16\(^{\text{INK4A}}\) was not sufficient to induce senescence in HSC. HSC without BMI-1 were just as capable of creating progeny as the wild-type. Taken together, these data indicate that cellular senescence and permanent growth arrests are not due to HSC aging. In line with these findings, it is reported that loss of p16\(^{\text{INK4A}}\) in HSC results in a decrease in stress-associated apoptosis rather than senescence.\(^{85}\) Thus, whether stem cell aging is accompanied by increased cellular senescence remains unresolved.

P53 is known as an important tumor suppressor gene and is often lost in tumors, but the role of p53 in aging remains highly complex. For example, homozygous loss of p53 causes predisposition to spontaneous development of several neoplasms.\(^{86}\) Nonetheless, a short isoform or truncated version of p53 suppresses tumorigenesis but causes the early development of degenerative phenotypes implicative for aging. Moreover, increased expression of normal p53 and Arf in mice reduced aging phenotypes, increased age and prevented tumorigenesis.\(^{87,88}\) Furthermore, increased p53 expression due to a hypomorphic allele of MDM2 also prevented tumorigenesis but aging remained normal.\(^{89}\) Thus, p53 can both exert pro- and anti-aging effects. Specifically for the hematopoietic system it was discovered that loss of p53 resulted in an increase of the number of HSC. In addition, p53 deficient HSC were increasingly able to generate copies of themselves after transplantation compared to the controls.\(^{90}\) These data indicate that the capacity for self-renewal is increased. In contrast to these findings, homozygous truncated p53 resulted in reduced proliferative and repopulating capacity in both stem cells and progenitors. On the contrary, a heterozygous truncation in the p53 allele resulted in increased activity in mice.\(^{91}\) Taken together, these studies demonstrate a fragile balance between tumor suppression and stem cell aging. Both tumor suppressor genes p16\(^{\text{INK4A}}\) and p53 play an important role in the aging of HSC. Nevertheless, it remains unclear whether stem cell aging is accompanied by senescence. In addition, p53 can both exert pro- and anti-aging effects, depending on the levels of expression.

### 2.1.4 Telomeres as a possible cause of stem cell aging

The maintenance of telomeres is of high importance for genomic maintenance and prevention of cancer. Many cancers gained the property to maintain telomeres indefinitely.\(^{92}\) In untransformed cells, DNA damage pathways, respectively p53 or p16\(^{\text{INK4A}}\) are activated upon telomere attrition resulting in permanent growth arrests. However, the activation of tumor suppressor genes can be a potential pathway by which stem cell activity is prematurely contaminated with aging. Throughout the process of aging telomere shortening occurs in HSC.\(^{93}\) Therefore, it was proposed that telomerase activity is restricted to primitive stem cells and progenitors.\(^{84}\) Indeed telomerase activity maintains telomeres in ageing HSC, but telomerase is only able to slow down telomere attrition. Moreover, serial transplantation of HSC overexpressing telomerase revealed that telomerase was able to prevent telomere shortening but the HSC were unable to extend their activity beyond their serial transplant capacity. These data reveal that telomerase overexpression does not aid HSC in increasing the number of serial transplants.\(^{94}\) Hence, it was proposed that in serial transplantations, transplantation capacity is telomere length independent. Direct experimental evidence is absent regarding that telomere shortening contributes to aging or influences stem cell
functioning in individual with functional telomerase. Nonetheless, the absence of telomerase has been studies in mice and proved to reduce lifespan and caused progressive diseases in the gut, blood and skin. Stem cells and progenitors were increasingly affected, resulting in reduced replicative capacity and diminished repopulating capacity. The absence of p21 has been indicated in the rescue of stem cell activity and extended longevity. However, loss of p21 does not rescue apoptosis. On the contrary, p53 deficiency has a positive effect in young telomerase deficient mice, while it causes tumorigenesis later in life. P53 rescues both the proliferative and apoptotic effect due to telomere loss. Taken together, complex interaction between tumor suppressor pathways and dysfunctional telomeres regulate stem cells, aging and tumorigenesis.

2.1.5 Reactive oxygen species as a cause of aging

In 1956 it was hypothesized that prolonged exposure to reactive oxygen species (ROS) would contribute to aging. In agreement with the Harman’s hypothesis, a study reports the accumulation of oxidative lesions with age. In addition, caloric restriction resulted in a decrease in oxidative damage which increased longevity. Mutations in pathways involved in the oxidative stress response, such as IGF-1R and p66SHC increased life span. It was hypothesized that this increase in life span was due to a decrease in damage accumulation in the mitochondrial DNA and mitochondrial dysfunction. This hypothesis was confirmed by genetic studies in mice with proof-reading deficient mitochondrial DNA polymerase. These mice showed features of accelerated aging. However, these findings were challenged by a more recent paper in which they describe that accumulation of point mutations in mtDNA does not limit lifespan. Furthermore, in HSC, AtaxiaTelangiectasia Mutated (ATM) proved to be essential for regulating ROS levels. ATM deficient mice showed elevated ROS levels which caused a functional decrease in HSC. Nonetheless, functionality could be restored upon the administration of the anti-oxidant N-acetyl-L-cysteine. It should be noted that contradictory results were published regarding the functions of anti-oxidants in lowering ROS levels and other beneficial effects. In response to ROS in ATM-deficient mice, MAPK p38 is activated and the tumor suppressor gene p16INK4A is induced, which causes loss of activity in HSC. Lastly, FOXO transcription factors were discovered to be required for lowering ROS levels. FOXO showed increased intracellular ROS levels. Nonetheless, these levels could again be decreased by the antioxidant N-acetyl-L-cysteine. In addition, loss of FOXO decreased long-term repopulating activity which can be defined as increased cycling, apoptosis and reduced reserves. In conclusion, ATM, MAPK p38, p16INK4A and the FOXO transcription factors contribute to the maintenance ROS levels, which contributes to the preservation of stem cell functions and reserves.

2.2 What are the micro-environmental causes of stem cell aging?

Even though a large part of the functional decline of stem cells occurs due to intrinsic factors, it is likely that due to the importance of the stem cell niche, the bone marrow extrinsically modulates HSC aging (Fig. 2.3). Especially in HSC it is likely that the micro-environment plays an important role in aging, since HSC increasingly depend on the bone marrow stromal environment. A study in which bone was transplanted subcutaneously from young or old mice into young recipients, showed a decrease in repopulation capacity of young HSC grafted into old bone. Moreover, in vitro studies confirmed that indeed old stroma has reduced capacity to support HSC. Furthermore complex imaging technologies have provided insight into the changes in localization during aging. Intriguingly, it was observed that aged HSC and progenitors reside further away from the endosteum and display a higher cell protrusion. Subsequently, old HSC show reduced adherence to stromal cells combined with reduced cell polarity upon adhesion of aged HSC. Together these data show that changes in the microenvironment are causative for the development of age associated deficits.

Furthermore, with aging a shift in lineage potential is observed which is poorly understood. It was hypothesized that changes in cytokines underlie this differential response. This hypothesis was confirmed by the fact that lineage biased HSC respond differently to TGF-β1. Myeloid based and lymphoid based HSC were purified by their capacity of Hoechst efflux and canonical markers. By both in vivo and in vitro experiments it was demonstrated that TGF-β1 promotes proliferation in myeloid-biased HSC. In addition, TGF-β1 exerts inhibitory effects on lymphoid-biased HSC. Taken together, these data demonstrate the presence of distinct lineage-biased HSC instead of a functionally uniform stem cell pool. Another reason for increase in myeloid-biased HSC with aging, might be the changes in inflammation due to aging. In the elderly, reduced cellularity is observed in the bone marrow. This decrease in cellularity is suggested to be partially due to apoptosis which might be related to a decrease in lymphocytes and macrophages which constitute as a part of the bone marrow microenvironment. Moreover, in murine models it has been observed that adipocytes accumulate in the bone marrow with age. Adipocytes in the bone marrow proved to be negative regulators of the bone marrow micro-environment. Thus, these data indicate that in aging individuals, the amount adipocytes increases which causes alterations in the original bone marrow environment, ultimately affecting HSC functioning. Lastly, old satellite stem cells were exposed to factors in young serum, a method called heterochronic parabiosis. In satellite stem cells Notch signaling is impaired upon aging, resulting in impaired regeneration of muscle. Exposure to young serum restored Notch signaling in old satellite cells. Hence, these results indicate that decline of progenitor activity due to aging can be modulated by systemic factors that change due to aging. Further studies are required to assess whether comparable responses are provoked in HSC.

2.3 How can stem cell aging contribute to the incidence of AML?

2.3.1 AML: A disease of the elderly

Leukemia occurs in all ages, however, AML incidence increases exponentially after 50 years resulting in a median age for AML onset of 69. In addition, with an increase in age, treatment proved to be increasingly challenging. Older patients show a decreased tolerance for intensive treatment and increased comorbidity and cytogenetic risks. Furthermore, in contrast to younger patients, AML in the elderly is increasingly highly resistant to chemotherapy resulting in a poor prognosis. AML patients aged younger than 50 which were treated with curative intent show a 70% median of complete remission. Relapse free survival (RFS) in these patients is 2 years which increases towards 5 years in 25-40% of the patients. In the elderly complete remission ranges from 30 to 50% and RFS in these is strongly decreased toward 9 to 12 months. Subsequently, very few patients
survive beyond 2 years.\textsuperscript{121-124} Hence, these data indicate that severe changes occur due to aging which cause the increase in AML incidence and is eligible for poor response to treatment.

2.3.2 Decreased lymphoid output contributes to the increased AML incidence

Little is known regarding the role of stem cell aging in the incidence of AML. However, recent research describes several factors which can contribute to this phenotype. It was discovered that with an increase in age the hematopoietic system favors myeloid output (Fig 2.1). In mice, a relative decrease in the lymphoid output was observed, while the myeloid output remained equal or even increased.\textsuperscript{77,125,126} Thus, a decrease in lymphoid output causes a decrease in adaptive immunity. This can be increasingly detrimental since the adaptive immune system has been implicated in the inhibition of tumor growth.\textsuperscript{77,127} Hence, loss of lymphoid production can be highly detrimental for the adaptive immune system since these cells are required for tumor suppression. In conclusion, these data indicate that loss of lymphoid production due to age decreases the effectivity of the adaptive immune system. Thereby, the adaptive immune system is less efficient in eradicating mutated cells which may contribute to the increased incidence of AML.

2.3.3 Intrinsic HSC aging contributes to the increased AML incidence

Another factor reported as a cause for stem cell aging is intrinsic aging of HSC. One of these intrinsic factors is DNA damage. It has been proven that with an increase in age DNA damage accumulates in HSC.\textsuperscript{81} The nature of these lesions can be mutagenic, cytostatic, cytotoxic or deregulatory and strongly contribute to the aging of HSC and progenitors (Fig. 2.2).\textsuperscript{82} If these lesions are mutagenic this can lead to transformation of stem cells and their further progeny. From this point onwards, the cell with the lesion is increasingly sensitive for further hits which can ultimately lead to tumorigenic mutations. Due to the fact that HSC mainly reside in G0 it is likely that DNA damage repair is executed via Non-homologous End Joining (NHEJ). However, this system is error prone and can thereby contribute to transformation. Hence, DNA damage accumulates with age and therefore, HSC and progenitors are more prone to oncogenic mutations which can result in AML. Moreover, the accumulation of deregulatory mutations possibly explain the decline in stem cell function with age.

Furthermore, tumor suppressor genes p53 and p16\textsuperscript{INK4A} have been indicated in aging. However, the direct role of p16\textsuperscript{INK4A} remains undefined due to contrary results. In old HSC, p16\textsuperscript{INK4A} was discovered to be elevated. In addition, loss of p16\textsuperscript{INK4A} resulted in a decrease in age associated deficits.\textsuperscript{85} On the contrary, an inverse pattern regarding p16\textsuperscript{INK4A} induction was observed in AML. These data suggest that the age associated p16\textsuperscript{INK4A} might play an important role in tumorigenesis.\textsuperscript{128,129} In addition, p53 can both exert pro- and anti-aging effect, depending on the level of expression. Hence, these data reveal a delicate balance between tumor suppression and aging. Both tumor suppressor genes p53 and p16\textsuperscript{INK4A} play an important role in aging. Nonetheless, extensive research is required to determine the role of senescence in aging. Likewise, the role of telomeres in aging also remains unclear. Nevertheless, it has been show that a complex interaction between tumor suppressor pathways and dysfunctional telomeres regulate stem cells, aging and tumorigenesis.

Furthermore, it has been indicated that ROS increases with age.\textsuperscript{102} Subsequently, damage by ROS proved to decline HSC functionality and reserves. Thus, these data indicate that with an increase in age the amount of mutagenic lesions increases, resulting in HSC and HPC which are more prone to oncogenic mutations. Furthermore, HSC are less able to repair DNA damage due to the error prone NHEJ. Moreover, tumor suppressor pathways are highly active to maintain genomic integrity. However, a tight regulation of tumor suppressor genes is required to maintain a balance between tumor suppression and aging. Subsequently, dysfunctional telomeres activate tumor suppressor pathways and thereby regulate stem cells, aging and tumorigenesis. Lastly, increased activity of HSC in aged individuals results in increased levels of ROS which are detrimental for HSC functionality and reserves.
2.3.4 Bone marrow aging contributes to the increased AML incidence

Moreover, due to aging, the supportive niche of HSC changes. It is known that due to aging bone marrow tissue is replaced by adipocytes, thereby strongly altering the niche.\textsuperscript{119} Furthermore, recent research revealed that bone marrow cellularity decreases upon aging, which might be explained by increased apoptosis.\textsuperscript{120} In addition, aberrations in the niche itself, such as a deletion of Dicer 1 in osteoblast progenitors, disrupts the integrity of hematopoiesis.\textsuperscript{130} Thus, with aging an increase in mutations is observed which can ultimately disrupt hematopoiesis. Moreover, it has been demonstrated that with aging HSC migrate away from the endosteum.\textsuperscript{113} Due to close interaction with osteoblasts HSC maintain their quiescent state. Therefore, we hypothesize that age mediated migration disturbs this direct interaction and thereby decreases HSC quiescence. In addition, residing in the most hypoxic region of the BM decreases the number of HSC and decreases their proliferation rate maintaining their functionality.\textsuperscript{13} Intriguingly, in an aging individual an increase in the number of HSC has been observed. This increase in HSC number is associated with increased self-renewal potential in aged HSC.\textsuperscript{77} Taken together, aging is associated with a migration of HSC away from osteoblasts. This migration possibly decreases stem cell quiescence and increases the chance of mutations.

Hence, this essay summarizes the important role of stem cell aging in the increased incidence of AML. Both extrinsic and intrinsic factors prove to play an important role in the process of aging and increased age associated diseases such as AML. Extensive research is required regarding the role of telomerase and tumor suppressor pathways. In addition, our knowledge regarding the BM niche is limited. To be able to better understand and battle aging and the diseases that come with it, it is required to expand our knowledge.
3. Future directions

The classical model of hematopoiesis, is the model that was believed to be true for decades (Fig. 1.1). Recent research has challenged this classical model in which HSC are mainly responsible for hematopoiesis. In this paper they demonstrated that the classical LT-HSC have little contribution to hematopoiesis in adulthood. In contrast, they showed that long-lived progenitors play a central role in native myelo- and lymphopoiesis.\(^\text{131}\) Hence, the current strong dogma with LT-HSC as the main driver of steady state hematopoiesis is replaced by the discovery that long-lived progenitors play a central role in steady state hematopoiesis. These findings indicate that transplantation and steady state hematopoiesis require the involvement of different cell types. HSC were believed to be ideal candidates for oncogenic transformation due to their life long persistence.\(^\text{64}\) In the contrary, recent data indicate that the large number long lived progenitors are a more accessible pool for oncogenic hits and therefore may be the origin of myeloid malignancies. To be able to successfully combat myeloid malignancies its of high importance to further study the cell of origin. Therefore, functional analysis on genomic, genetic, epigenetic and proteomic level can provide further insight into their origin, how to prevent their formation and how to prevent their progression. To successfully eradicate AML, therapy should target the slow dividing LSC. Current therapies target dividing cells, but to eradicate AML the cell(s) of origin need(s) to be eliminated.

Present-day, more knowledge is gained regarding the characteristics of AML (Table 1.3). However, much remains unclear regarding the biological and clinical features of AML. Therefore, it is of high importance to further characterize the disease to improve treatment and prognosis. A method to achieve this aim is to gain information on patients, including those excluded from studies to detect relevant clinical differences between patients. Trials should be performed in randomized controlled trials with a common endpoint, the quality of life. These kind of trials can provide insight into differences between patients and can contribute to a better understanding of biological and clinical features of AML. In addition, studying the in vivo mechanism of currently used drugs and new drugs can provide more insight into the in vivo mechanisms of these drugs. Due to the oligoclonality of AML, studies regarding combination therapy should be considered. Together, these data can provide new insights in AML in the elderly and increase their prognosis.

Extensive whole genome sequencing (50 patients) or whole exome sequencing (150 patients) combined with RNA sequencing of 200 de novo AML patients provided insight into the mutational landscape of AML. From this point onwards it would be interesting to study the discovered mutations. For example, the use of viral constructs can provide further insights into the effects of these mutations on different cellular processes such as proliferation, survival and metabolism. Subsequently, in vitro studies can be performed to measure the effect of currently applied and new drugs in cells with certain mutations. Nonetheless, it should be noted that AML is an oligo-clonal disease with possibly multiple LSC with different mutations. Therefore, treatment with a certain drug can cause strong remission of one LSC, while the other LSC remains unharmed causing relapse or drug resistance. The oligo-clonal origin of AML can also explain why several drugs were successful in mice but unsuccessful in humans. Combination therapy might resolves this issue.

Subsequently, a better understanding regarding stem cell aging is required. Most studies on the subject of stem cell aging were dependent on murine models. Nevertheless, this knowledge remains partially unlinked to human studies. Furthermore, stem cell aging is indicated to play an important role in the increased incidence of hematopoietic malignancies. However, much remains unclear regarding the extrinsic and intrinsic factors contributing to stem cell aging. First of all, a question that remains unanswered is how HSC become defective with time. In this matter the length of telomeres has been suggested to play a role, but research revealed that aging in HSC is not solely dependent on telomere shortening.\(^\text{125}\) This can be further elaborated since lymphoid output is decreased upon aging, but the myeloid output remains unharmed or even increases.\(^\text{77,125,126}\) In addition, aged HSC show increased self-renewal potential.\(^\text{77}\) Therefore, we hypothesize that with an increase in age, HSC increase the likelihood of symmetric divisions and thereby show increased self-
renewal and a larger stem cell pool. Nonetheless, in homeostatic conditions HSC mostly divide asymmetrically. However, due to loss of HSC or other physiological conditions HSC are able to divide symmetrically. In the case of aged HSC, loss of lymphoid progenitors and output might contribute to this phenomenon. Further studies regarding self-renewal could provide further insight into this hypothesis.

Furthermore, the bone marrow niche remains poorly defined. Creating a better understanding of this supportive niche will provide more insight into the role of the microenvironment in aging. The cellularity of the bone marrow decreases with age and subsequently, adipocytes appear in the bone marrow. This has both strong effects on the niche and on HSC. Further studies are required to elaborate the effects of the changing bone marrow on hematopoiesis.

Taken together, our knowledge regarding AML and stem cell aging only covers the tip of the iceberg. Since, age is proven to be such a strong factor in the increased incidence in AML, extensive research is required to understand the detrimental effects of aging on the hematopoietic system.
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5. Supplementary Figures

**Supplementary Table 1.1. Age-Specific SEER Incidence Rates, 2009-2013**

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<td>70-74</td>
<td>17.1</td>
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<td>22.1</td>
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<td>85+</td>
<td>25.3</td>
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Supplementary Table 1.2. The WHO classification of AML, 2008 extended\textsuperscript{41,42}

<table>
<thead>
<tr>
<th>Category</th>
<th>Subcategory and short description</th>
</tr>
</thead>
</table>
| AML with recurrent genetic abnormalities      | AML with t(8;21)(q22;q22); RUNX1-RUNX1T1*  
AML with inv(16)(p13.1q22) or t(16;16)(p13.1q22); CBFβ-MYH11*  
Acute promyelocytic leukemia; AML with t(15;17)(q22;q12); PML-RARA and cytogenetic variants  
AML with t(9;11)(p22;q23); MLLT3-MLL  
AML with t(6;9)(p23;q34); DEK-NUP214  
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1  
AML (megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1  
Provisional entity: AML with mutated NPM1  
Provisional entity: AML with mutated CEBPA |
| AML with myelodysplasia related changes       | Previous history of myelodysplastic syndrome  
or Myelodysplastic syndrome-related cytogenetic abnormality  
or Dysplasia present in > 50% of 2 or more cell lineages  
**Absence of both**  
Prior cytotoxic therapy for an unrelated disease  
Recurring cytogenetic abnormality as described in AML with recurrent genetic abnormalities |
| Acute myeloid leukemia, Not otherwise specified (NOS) |                                                                                                           |
| AML with minimal differentiation              | <3% of blasts positive for Sudan Black B or MPO.  
Blasts usually express CD13 and/or CD117, with or without CD33 in absence of lymphoid markers cCD3, cCD22 and cCD79a |
| AML without maturation                        | Blasts ≥90% of bone marrow non-erythroid cells (i.e. excluding also lymphocytes, plasmacells, macrophages and mast cells)  
≥3% of blasts positive for Sudan Black B or MPO  
Blasts express MPO and one or more of myeloid-associated antigens such as CD13, CD33 or CD117 |
| AML with maturation                           | ≥10% maturing cells of neutrophil lineage  
<20% bone marrow monocytes |
| Acute myelomonocytic leukemia                 | >20% neutrophils and precursors of marrow cells  
>20% monocytes and precursors of marrow cells |
| Acute monoblastic and monocytic leukemia      | ≥80% of the leukemic cells are monoblasts, promonocytes and monocytes |
| Acute erytroid leukemia                       | **Erythroleukemia (erythroid/myeloid)**  
Presence of medium to large size erythroblasts: ≥ 50% of bone marrow cells  
Blasts: ≥ 20% of the bone marrow nonerythroid cells  
**Pure erythroid leukemia**  
Presence of medium to large size erythroblasts |
| Acute megakaryoblastic leukemia                | >50% of the blasts are of megakaryocytic lineage  
Blasts express CD41 and/or CD61 |
| Acute basophilic leukemia                     | Primary differentiation to basophils; mature basophils are usually sparse |
| Acute panmyelosis with myelofibrosis          | Acute panmyeloid proliferation with accompanying fibrosis |
| Myeloid sarcoma                               | Tumor mass of myeloblasts or immature myeloid cells occurring in an anatomical site other than the bone marrow |