01.

General introduction and scope of this thesis
1.1 The Hematopoietic system

During the lifetime of a human being, steady-state hematopoiesis generates and replenishes all types of mature blood cells. Most mature blood cells are relatively short-lived under steady-state conditions; approximately one trillion \((10^{12})\) cells are formed every day in adult bone marrow [1]. The hematopoietic system is generally envisaged as a cellular hierarchy, starting with the multipotent hematopoietic stem cell (HSC). HSCs can give rise to differentiated progeny belonging to all cell lineages. An unique feature of HSCs is the generation of new multipotent blood stem cells, a process called self-renewal [2]. To sustain adequate levels of mature blood cells, HSCs can either self-renew or differentiate. This ‘decision’ is critical for maintaining life-long hematopoiesis [2]. During differentiation, cells undergo striking changes in shape and homeostasis. In addition, due to stress from severe blood loss or infections, the demand for mature blood cells can change dramatically [3, 4]. Consequently, cell fate decisions of HSCs are tightly regulated by transcription factors, epigenetic modifiers and the surrounding microenvironment [5, 6]. Understanding the mechanisms controlling HSC fate is a central issue in modern stem cell research.

The classical hierarchical model for hematopoiesis is based mainly on in vivo transplantation models of cell populations defined by fluorescence-activated cell sorting (FACS) (Figure 1A, [7]). In this model, multipotent HSCs undergo stepwise differentiation into distinct oligopotent progenitor populations coinciding with progressive loss of multi-lineage potential. In support of the classical model, introduction of barcodes into HSCs and subsequent tracking in mice revealed that most HSC clones give rise to multilineage or oligolineage fates [8]. However this model, in which branching of myeloid and lymphoid cells is the first step in lineage commitment, is currently under debate [9-11].

Single cell transplantation has revealed heterogeneity within the reconstituted HSC population [12, 13]. For example, a study that tracked EGFP labelled progenitors from different cell lineages after single cell transplant found a distinct subgroup of HSCs that differentiated exclusively towards the megakaryocyte/platelet-lineage. However, no HSCs were identified that contributed exclusively to the erythroid, myeloid, or lymphoid cell-lineages [10]. Other studies that combined functional assays with single cell sequencing [9, 14] revealed an early separation of the erythroid and megakaryocytes lineage and cast doubt on the existence of an common myeloid progenitor (CMP, Figure 1B).
In a recent study, index flow sorting was used to separate single cell types based on marker cell expression, followed by single cell sequencing. Using this technique, the molecular profile of each cell could be assigned retrospectively to a classically defined cell type [14]. This study showed that the more immature cell population, unlike more mature progenitors, could not be separated into different sub-populations. This argues against the existence of lineage-biased progenitor cell populations and suggests a more continuous hematopoietic compartment [11]. In summary, the techniques in these studies have provided new understanding of the hematopoietic hierarchy; as a result, new models are still evolving for adult hematopoiesis.

**1.2 The Hematopoietic stem cell niche**

In addition to hematopoietic cells, the bone marrow consists of various non-hematopoietic cells such as endothelial cells, neuronal cells, mesenchymal stem and CXCL12-abundant reticular (CAR) cells [15, 16]. These cells are organized in specific microenvironments called niches, which maintain HSCs homeostasis. These niches provide cytokines, nutrients and cell-cell interactions, which are essential for HSC maintenance and regulation of hematopoiesis (Fig. 2) [16]. Interleukins, granulocyte colony stimulating factor (G-CSF) and erythropoietin (EPO) are cytokines that induce differentiation of HSCs [17]. In contrast, thrombopoietin (TPO), chemokine C-X-C motif ligand 4 (CXCL4), and transforming growth factor beta (TGF-1) and C-X-C motif chemokine 12 (CXCL12) are essential for maintaining stemness of HSC [18-21]. HSCs have been reported to reside in different niches within the bone marrow, although the specific functions of these niches is still unclear and under debate [15, 22]. These various niches may provide different extrinsic signals that regulate stem cell fate [22]. For example, CXCL12 is secreted by endothelial cells, osteoblasts and CAR cells, while TPO is...
produced by osteoclasts and megakaryocytes [23, 24]. HSCs are located near the
trabecular bone (endosteal) or in close proximity to arterioles and/or sinusoids. Most
dormant HSCs are thought to reside in the endosteal niche, which is in close
proximity to bone-lining osteoblasts that produce TPO and osteopontin required
for HSC quiescence [25]. In addition, HSCs are found near small arterioles, lined
with rare NG2⁺ pericytes, which secrete CXL12 and SCF [22]. In contrast, less
quiescent or activated HCSs are thought to reside in the sinusoidal niche, which
is characterized by LEPR⁺ perisinusoidal cells. Although the bone marrow is
believed to be well-vascularized, the blood flow in arterioles and sinusoids is low,
which results in a hypoxic environment due to limited gas exchange [26]. This
vascular network does provide nutrients, enables access to systemic signals and
allows mature blood cells to enter the blood circulation. Moreover, compared
to arterioles, sinusoids are more fenestrated, which allows cells to cross the
sinusoidal barrier [27]. This enables mature blood cells that have been generated
from HSCs to enter the bloodstream.

**Fig 2. The Bone marrow niche.** The more dormant HSCs reside near the endosteal, while less
quiescent HSCs reside near sinusoids. Various cells, such as CAR cells, Megakaryocytes, NG2⁺ or
LepR⁺ perivascular cells, provide different types or quantities of cytokines within the different niches
[22].

### 1.3 Myelodysplasia

Myelodysplastic syndromes (MDS) comprise a heterogeneous group of clonal
stem cell disorders characterized by ineffective and dysplastic hematopoiesis
and peripheral cytopenias. In the Netherlands and other Western countries MDS has a yearly incidence of ~3 cases per 100,000 [28, 29]. However, MDS typically affects older people; the incidence increases strongly after the age of 65 [30]. MDS is diagnosed based on peripheral blood cytopenias, the number of bone marrow blasts and cytogenetic abnormalities. (Table 1 [31]). The survival of MDS patients is extremely variable and ranges between several months to several years [32]. Therefore, prognostic scoring systems have been developed to provide accurate risk-stratification for optimized treatment [33, 34]. MDS patients are categorized into low-risk or high-risk categories according to the International Prognostic Scoring System (IPSS, revised in 2016, [35]). Particularly in low-risk MDS, cytopenias are thought to arise due to the increased susceptibility of early progenitors to undergo cell death [36]. These findings are less pronounced in high-risk MDS but this risk category has a higher risk of developing acute myeloid leukemia (AML), which is associated with a worse prognosis [37, 38]. Chromosomal abnormalities such as del(5q) and trisomy 8 are frequently observed in MDS. Next generation sequencing has identified a number of recurrent somatic mutations in genes involved in RNA splicing, epigenetic modifiers and transcription factors [39]. Recently, various mutations, including ASXL1, EZH2 and TP53 mutations, have been proposed for inclusion in the prognostic scoring system of WHO and are associated with worse prognosis (Table 2, [40]). All observed mutations, except SF3B1 and TET2, were more frequent in high-risk MDS. In addition, the number of different mutations observed in patients is an independent prognostic factor after risk stratification according to the IPSS [41].

<table>
<thead>
<tr>
<th>Table 1, WHO classification 2016 of myelodysplastic syndromes (MDS)</th>
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<td><strong>MDS subtype</strong></td>
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<tr>
<td>MDS-SLD, with single lineage dysplasia</td>
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<tr>
<td>MDS-MLD, with multilineage dysplasia</td>
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<td>MDS-RS, with ring sideroblasts</td>
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<td>MDS-EB, with excess blasts</td>
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<td>MDS with isolated del(5q)</td>
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<td>MDS unclassifiable</td>
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The microenvironment has been implicated in playing a critical role in the pathogenesis of MDS. For example, transcriptome analysis of MSCs from low-risk MDS revealed a common molecular signature, defined by cellular
Table 2, Mutations associated with low-risk and high-risk MDS and/or overall survival

<table>
<thead>
<tr>
<th>Genes mutated</th>
<th>Referances</th>
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<tr>
<td>Enriched in Low-risk MDS</td>
<td>SF3B1</td>
</tr>
<tr>
<td>Enriched in High-risk MDS</td>
<td>U2AF1, STAG2, CBL and KRAS</td>
</tr>
<tr>
<td>Associated with better overall survival, within the same risk group</td>
<td>SF3B1</td>
</tr>
<tr>
<td>Associated with worse overall survival, within the same risk group</td>
<td>CBL, IDH2, ASXL1, DNMT3A, TP53, CUX1, BCOR, RUNX1, U2AF1, SETBP1 and SRSF2</td>
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stress and increased inflammation-signals [44]. In a large cohort study of MDS patients, MSCs were functionally altered in vitro and had a reduced osteogenic differentiation potential, resulting in impaired stromal support for HSPCs [45]. Similarly, MDS-derived MSCs had a reduced expression of cytokines and consequently showed a reduced HSPC support function in vitro [46]. In contrast, inflammatory cytokines were shown to be present in excess in MDS [47]. S100A8 and S100A9 are important regulators of the inflammatory cytokine response and their expression is increased in MDS [48, 49]. Importantly, S100A8 and S100A9 expression has been linked to differentiation defects in the erythroid lineage and to increased pyroptosis, a specific type of cell death induced by inflammation [47, 48]. In vivo studies have shown that MDS xenotransplantation models were often unsuccessful due to limited engraftibility. However, xenotransplantation of MDS cells together with mesenchymal cells led to better survivability of MDS cells in vivo [50]. However, these findings could not be confirmed by Rouault-Pierre K. et al. [51].

1.4 Acute myeloid leukemia

Self-renewal properties of HSCs are tightly regulated and gradually lost during differentiation [2]. In AML the critical balance between self-renewal and differentiation of HSCs is perturbed as result of genetic and epigenetic defects [52-55]. These defects cause a block in differentiation, and consequently result in accumulation of immature cells in bone marrow and peripheral blood. The leukemic cells often have an altered apoptosis programming [56, 57], including a high expression of antiapoptotic proteins BCL-2 and MCL-1, which might promote the survival of the leukemic stem cells [56, 58]. AMLs are classified based on morphological, cytogenetic and molecular abnormalities, which have been linked to disease outcome. Initially, AMLs were classified according to the
French-American-British (FAB) classification based on the differentiation stage of leukemic blasts [59]. Currently, two largely overlapping patient risk stratifications systems, ENL and WHO, are used to predict disease outcome based on a combination of molecular and clinical data [60-62]. Recently, a large number of recurrent genetic mutations have been identified by sequencing a large panel of AML patient cohorts [63-65]. Frequent mutations were detected in the FLT3, NPM1, ASXL1, DMNT3A, NRAS, TET2 IDH1/2, TP53 and CEBPA genes [66]. Based on these mutation analyses, three additional genomic categories were identified, including AMLs with mutation in RNA splicing and chromatin genes, TP53 mutant AMLs with chromosomal aneuploidies and AMLs with IDH2 mutations [63]. Some of these recurrent mutations in AML, such as DNMT3A, ASXL1 and TET2, have also been found in healthy individuals, but at low allele frequency [67, 68]. The presence of somatic mutations in hematopoietic cells without signs of dysplasia is called clonal hematopoiesis of indeterminate potential (CHIP) [69]. Although, DNMT3A, TET2, and ASXL1 mutations are strongly associated with leukemia, individuals with CHIP clones remained healthy for years without detectable expansion of CHIP clones [70]. It is currently unclear if the presence of CHIP is an increased risk factor for leukemia development, although a recent study showed that IDH1, IDH2, TP53, DNMT3A, TET2 and genes involved in spicing were with more than one mutation in leukemia-associated genes or more than one variant of DNMT3A or TET2 had a significantly higher chance of developing AML [71].

AML patients are treated with intensive chemotherapy or hypomethylating agents, with or without an allogeneic stem cell transplantation. Although many patients initially respond well to chemotherapy, the rate of relapse is still high [72], presumably because a small number of leukemic stem cells (LSCs) survived the initial treatment due to intrinsic properties of LSCs [73, 74]. Understanding how genetic and molecular abnormalities in different AML subgroups contribute to leukemia initiation and progression will ultimately help to improve treatment strategies.

1.5 The autophagy mechanism

Autophagy (self-eating in Greek) is a catabolic process whereby damaged or redundant organelles and proteins are sequestered and degraded by lysosomes [75-77]. Different types of autophagy have been described: chaperone-mediated autophagy (CMA), microautophagy and macroautophagy, which is described
in more detail in **Chapter 2**. In brief, CMA is a pathway for selective autophagy-mediated degradation of proteins. [78, 79]. During microautophagy, the cytosolic content in close proximity to lysosomes is invaginated and subsequently degraded. During macroautophagy, double membrane vesicles called autophagosomes are formed, which engulf cytosolic content such as mitochondria. Macroautophagy-dependent removal of mitochondria is also called mitophagy. Studies have shown that two proteins, PINK1 and Parkin, play a central role in the process of mitophagy induction. In depolarized mitochondria, PINK1 accumulates at the outer membrane and can therefore recruit Parkin. In turn, Parkin ubiquitinates different proteins of the outer membrane, resulting in recruitment of p62, and ultimately to autophagy-mediated removal of mitochondria [80, 81]. Throughout this thesis, macroautophagy will be referred to as autophagy.

Autophagy-derived metabolites such as amino acids and lipids can be re-used to generate energy or to serve as building blocks for renewal of cellular components. Although every cell is thought to maintain autophagy at a basal level, autophagic flux can be induced under stress conditions, such as starvation, hypoxia, or DNA damage [82]. In this context, AMPK phosphorylate and activate the ULK complex.
In turn, the ULK complex activates class III autophagy-specific PI3K complex that contains vacuolar protein sorting 34 (VPS34), Beclin1, ATG14L and P150 [84]. Alternatively, under hypoxic conditions, BNIP3 and BNIP3L are expressed in a HIF-1 dependent manner. Both BNIP3 and BNIP3L can bind to Beclin1, thereby activating the PI3K complex [85]. This complex translocates to the endoplasmic reticulum (ER) membrane where PI3K locally produces phosphatidylinositol-3-phosphate (PIP3). This triggers the recruitment of PIP3 effector proteins and ultimately leads to the formation of a specific endoplasmic reticulum (ER) microdomain called the omegasome, which also contains vacuole membrane protein 1 (VMP1) [86, 87]. Maturation of omegasomes into autophagosomes requires the ubiquitin-like conjugation systems. First, ATG12 is covalently bound to ATG5 by ATG7 E1-like enzyme activity [88]. Second, LC3 is covalently linked to phosphatidylethanolamine by both ATG7 and ATG3 E2-like enzyme activity [75]. Finally, the outer membrane of mature autophagosome fuses with lysosomes, after which their content is degraded (Figure 3).

1.6 Autophagy is important in many aspects of hematopoiesis

Although autophagy can be considered to be a cellular housekeeping mechanism, it has become clear that autophagy also fulfils cell-type-specific roles. For example, cellular differentiation requires massive subcellular remodeling. In the final stage of erythropoiesis reticulocytes remove their mitochondria, ribosomes and nucleus (enucleation) in order to fully differentiate to erythrocytes [89]. This also enables a bi-concave cell shape for improved diffusion [90]. Therefore, autophagy is constitutively active during the late stage of erythropoiesis. Inhibition of autophagy results in ineffective removal of mitochondria and severe anaemia in vivo [91-93]. Autophagy has also been shown to be essential for monocyte-macrophage differentiation [94]. During monocyte differentiation, autophagic-flux was increased, while inhibition of autophagy triggered apoptosis. Similarly, during lymphopoiesis deletion of essential autophagy genes resulted in reduced number of T-cells in vivo and an impaired B-cell maturation [95]. Clues that autophagy is also essential for the most immature stem cell fraction have originated primarily from mice knockout (KO) studies. For example, HSCs in ATG7 KO mice failed to reconstitute hematopoiesis upon transplantation in lethally irradiated mice [96]. Moreover in the absence of ATG7, the number of HSCs was reduced, together with reduced production of myeloid and lymphoid progenitors [96]. Recently, Passegué et al, showed that autophagy suppresses oxidative metabolism by clearing mitochondria [97, 98]. Knockout of essential
autophagy genes, such as ATG5, ATG7 or ATG12, resulted in accumulation of mitochondria and consequently ROS accumulation [97, 99, 100]. The increased number of mitochondria were associated with an more activated metabolic state of HSCs, resulting in accelerated myeloid differentiation [96, 97]. When HSCs differentiate, their metabolic rate is increased in order to sustain cell growth [97]. Interestingly, autophagy activity was decreased in two-thirds of HSCs in aged mice [97]. Within the HSC pool of aged mice, HSCs with higher autophagic-flux had a better long-term repopulating potential [97]. This suggests that autophagy in stem cells is essential for balancing self-renewal and differentiation by actively controlling mitochondrial mass, a process that is less efficient in aged mice. In addition, apoptotic proteins are modulated by mitochondria and consequently mitochondrial content within a cell determines if cells undergo apoptosis [101]. Therefore, impaired autophagy resulting in accumulation of detective mitochondria could potentially affect apoptosis.

1.7 Autophagy in AML

Increasing evidence indicates that autophagy plays an important role in leukemia initiation and maintenance. In a recent screen the mutational spectrum of autophagy genes was studied by using whole-exome sequencing in large cohort of cases with myeloid neoplasm. Copy number alteration and missense mutations were detected in ~22% of autophagy-associated genes and in 14% of studied cases [102]. In addition, mutations in splicing factor U2AF35 were frequently detected [103, 104] resulting in the defective processing of ATG7 pre-mRNA and reduced expression of ATG7 [105]. Importantly, the phenotype of ATG7 knockout mice resembles many characteristics of myeloid leukemia, such as anemia and accumulation of myeloid blasts in organs [96, 97, 106]. Mutations observed in the autophagy genes are often hypomorphic, i.e. mutations causing a reduction in gene expression [105], which can prevent the clearance of leukemia-associated oncoproteins such as BCR-ABL, PML/RARA and FTL3-ITD [107-109]. Moreover, impaired mitophagy-dependent clearance of damaged or redundant mitochondria, which leads to accumulation of ROS, might result in increased DNA damage and cellular stress. Although, several mutations detected in leukemia are predicted to repress autophagy, leukemic cells are still highly dependent on their remaining autophagy activity as demonstrated for MLL-AF9 and BCR-ABL model systems [110, 111]. Increased cellular stress and defective removal of oncoproteins as a consequence of impaired autophagy could potentially contribute to leukemia development.
In contrast, in established leukemia increased autophagy can be beneficial for leukemic cells. For example, increased autophagy in leukemic cells could ensure a sufficient supply of metabolites to sustain enhanced proliferation and reduce cellular stress. In addition, autophagy can be upregulated in response to chemotherapy exposure, which could potentially contribute to drug resistance [112-114]. In summary, these findings suggest that during leukemia initiation autophagy is probably repressed, but not completely lost. In contrast during leukemia maintenance, enhanced autophagy contributes to the survival of leukemic cells and to drug resistance.

1.8 Scope of this thesis
Autophagy is an important cellular housekeeping mechanism that allows degradation and recycling of cellular components. In addition, autophagy plays a role in intracellular remodeling during differentiation [115]. So far most studies have been performed with mouse model systems [97, 99, 100, 116], but the consequences for human hematopoietic stem and progenitor cells (HSPC) or AML cells have remained largely elusive. Therefore, this thesis is focused on improving our understanding of autophagy in normal and leukemic hematopoietic cells. Chapter 2 reviews the current understanding of autophagy in cancer cells and cancer stem cells and its multi-faceted role in the tumor microenvironment. Furthermore, therapeutic targeting of autophagy in cancer therapy is discussed.

In Chapter 3, by using human CD34+ cells, we determined the levels of the autophagic flux in normal HSPCs and in more differentiated cells. In addition, based on loss-of-function studies we determined the functional relevance of autophagy in these HSPC for their survival and differentiation. Low-risk MDS is characterized by ineffective and dysplastic hematopoiesis. Increased programmed cell death of hematopoietic bone marrow cells apparently plays a critical role in the observed contradictory phenotype of a hypercellular bone marrow and peripheral blood cytopenias [117]. In Chapter 4 we examined whether an altered dependency on the microenvironment plays a role in the pathogenesis of low-risk MDS by using in vitro culture assays and ultrastructure studies. We also examined whether an aberrant autophagy programming might underlie the observed increased vulnerability to cell death of low-risk MDS progenitor cells.

Autophagy is of importance for maintenance of HSPCs, at least in part by limiting
mitochondrial activity [96, 97, 118]. As shown in Chapter 3, loss-of-function studies in HSPCs show that autophagy is essential for survival of HSPCs. However, it remains unclear whether autophagy acts in a similar fashion in AML. In Chapter 5 we established an *in vitro* model for determining the autophagic-flux in a large panel of primary AML patient cells and leukemic cell lines. Moreover, we examined whether autophagy plays a role in maintenance of AML CD34+ cells *in vitro* as well as *in vivo* by means of genetic or pharmaceutical inhibition of autophagy.

To gain more insight into the mechanism controlling autophagic flux, we investigated the expression pattern of known autophagy associated genes in AML. Expression of a putative autophagy protein vacuole membrane protein (VMP1) was increased at mRNA and protein level in the majority of AML’s compared to normal HSPCs. In Chapter 6 we validated the increased expression of VMP1 in AML. Because limited data is available regarding the role of VMP1 in hematopoiesis, functional *in vitro* and *in vivo* studies were performed to elucidate its function in hematopoiesis and autophagy. Possible survival advantages and drug-resistance due to increased VMP1 expression in AML also were assessed.

Finally in Chapter 7, the most important findings described in this thesis are summarized, and future perspectives are discussed.
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