CHAPTER 1

General introduction and scope of this thesis
INTRODUCTION

Hematopoiesis and ageing of the hematopoietic system

Hematopoietic stem and progenitor cells sustain life-long and continuous replenishment of all mature and differentiated blood cell lineages, a process termed hematopoiesis. Next to differentiation, hematopoietic stem cells (HSCs) are capable of self-renewal. Due to the relatively short lifespan of most mature blood cells, nearly one trillion \((10^{12})\) cells are produced each day.\(^1\) Thereby, the hematopoietic system is one of the most highly proliferative tissues of the human body. The functional number of HSCs is estimated between 20,000 and 200,000.\(^2\)\(^-\)\(^4\) The classical model of hematopoiesis describes a stepwise and hierarchical differentiation structure with HSCs residing at the top of the hierarchy and giving rise to all blood cell lineages through series of progenitor stages, with lineage restriction occurring early in the developmental stage. There is a tightly controlled balance between myeloid and lymphoid lineage output.\(^5\)\(^-\)\(^7\) The lymphoid compartment consists of B-cells, T-cells, natural killer cells and dendritic cells. The myeloid compartment consists of platelets and megakaryocytes, erythrocytes, monocytes/macrophages and granulocytes.\(^1\)

Upon ageing HSCs show a decline in stem cell function with impaired self-renewal and reduced engraftment potential, although their absolute number increases. Hematopoiesis becomes skewed towards production of myeloid cells.\(^8\)\(^-\)\(^12\) The hematopoietic system shows hallmarks of a reduced function, including reduced T- and B-cell function and diminished ability to support erythropoiesis, resulting in a higher prevalence of anemia. Finally, ageing is the main risk factor for myeloid malignancy development.\(^13\)\(^,\)\(^14\)

Accumulation of mutations in healthy tissues with increasing age and emergence of clonal hematopoiesis

The development of cancers, including leukemia, is assumed to be a multi-step process that requires subsequent molecular aberrations and epigenetic changes eventually leading to malignant transformation, clonal selection and outgrowth of overt malignancy.\(^15\) As such, the increasing incidence of cancers with age is suggested to be the result of random accumulation of mutations in replicating tissue.\(^16\) Accumulation of genetic aberrations during ageing has been shown for a wide range of healthy human tissues, in particular those with a high cell turnover.\(^17\)\(^-\)\(^27\) In HSCs, the rate of mutation acquisition is estimated at one exonic mutation per decade of age.\(^28\) The majority of these mutations are presumably ‘neutral hits’ and do not favor clonal expansion. Such mutations may result in diminished HSC function and reduce cell fitness, or even lead to programmed cell death of the mutant stem cell. However, a subset of acquired mutations may confer a selective advantage, resulting in clonal expansion of mutated cells, which is now referred to as clonal hematopoiesis (CH) (Figure 1).\(^29\)\(^-\)\(^32\)
Early evidence for clonality: X-inactivation skewing
The first evidence for clonality in the ageing hematopoietic system was skewing in X-inactivation arising in the blood of older women. This could be detected by a deviation from the theoretical 1:1 maternal-paternal ratio in transcriptional activity at X-chromosome loci. Nearly a decade later, a follow-up study identified the first molecular marker for clonal hematopoiesis: somatic mutations in TET2 were found in a substantial proportion of healthy ageing women with skewed X-inactivation patterns. During the last years, the advent of novel genomic approaches has enabled sensitive and large-scale detection of genetically mosaic tissue with ageing.

Markers for clonal hematopoiesis: copy number alterations and somatic mutations
Chromosomal copy number anomalies were the first marker of widespread CH to be documented by bioinformatic analysis of large-scale single nucleotide polymorphism (SNP) array data. Using this method, clonal populations of cells with chromosomal abnormalities ≥2Mb could be detected in almost 1% of healthy individuals. Chromosomal abnormalities may include deletions or losses of entire chromosomes, duplications or copy-neutral loss of heterozygosity (CN-LOH). In 2014, an important milestone in the field occurred when three large population-based cohort studies reported on abundant somatic mutations in the blood of older people. Classes of somatic mutations included single base substitutions and small insertions or deletions (indels). For all markers of CH, age was the strongest predictor of mosaic status. Using whole-exome sequencing (WES) data, mutated clones with substantial outgrowth in peripheral blood could be detected in 10% of individuals over the age of 65. The rate of detection and frequency of CH markedly increased when more sensitive sequencing techniques were used in follow-up studies, allowing for the detection of mutations at lower variant allele frequencies (VAFs) (Figure 2, Table 1). Such techniques, that include single-molecule molecular inversion probe (smMIP) assays, make use of molecular barcodes and enable correction for low-level sequencing artefacts. Clonal events were identified in nearly all middle-aged individuals, using ultra-deep error-corrected sequencing. Given the accumulation of mutations, combined with our long lifespan, CH could inevitably be observed at relatively young ages using increasingly sensitive sequencing techniques.
The emergence of CH shows a strong relationship with age. Using WES and whole-genome (WGS) sequencing data, these studies were able to detect hematopoietic clones from ~5-7% VAF, corresponding to >10% of blood cells. More recent reports have taken advantage of increasingly sensitive sequencing techniques to detect CH. Targeted next-generation sequencing (NGS) approaches allow for detection of hematopoietic clones at much lower VAF (~1%), with error-corrected techniques going as low as 0.03%. *Only control individuals were included. Adapted from van Zeventer and Buisman et al.*

The landscape of CH is dominated by mutations in epigenetic modifier genes DNMT3A, TET2 and ASXL1. Less frequently, somatic mutations occur in TP53, PPM1D, JAK2, CBL and spliceosome genes (Figure 3). Classical driver genes may account only for a small proportion of clonal events, suggesting many drivers of CH have not yet been identified.

For mosaic chromosomal alterations, more recent techniques using phasing-annotated genomic data have been able to increase detection sensitivity for mosaic events from 50kb occurring in only a small fraction of cells. Sex chromosome losses, and especially mosaic loss of the Y chromosome (mLOY) are the most frequently encountered mosaic event. Autosomal events can be detected in up to ~15% of individuals with a median age of 63 years, based on a large biobank from Japan. Frequently detected abnormalities in the western population include deletions of chromosome 13q or 20q, CN-LOH of 9p or 14q and trisomy 8 or 12. These genes and chromosomal regions are well-known for their occurrence and implications as a potential driver of (myeloid) hematological malignancies.
Figure 3. Overview of recurrent clonal gene mutations contributing to clonal hematopoiesis in the general population
Myeloid driver mutations may be classified into distinct groups based on their cellular function or the functional pathways involved, as indicated. *Including histone/chromatin modification and DNA methylation.

Classification of myeloid malignancies
Hematological malignancies affect the blood, bone marrow and lymph nodes, and may involve the myeloid and lymphoid hematopoietic cell lineages. The 2016 revision of the 4th World Health Organization (WHO) criteria categorizes myeloid malignancies in five major subtypes: acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), myeloproliferative neoplasm (MPN), MDS/MPN overlap syndrome and myeloid neoplasms associated with eosinophilia and abnormalities of growth factor receptors. Myeloid malignancies are rare: based on data from cancer registries in Europe, the population-based incidence is 8.6 per 100,000 per year. Myelodysplastic and myeloproliferative conditions were only classified as malignant disorders and became reportable in cancer registries from 2000 onwards.

Myelodysplastic syndromes
MDS are heterogeneous clonal bone marrow disorders with diverse phenotypes, characterized by ineffective hematopoiesis with morphological abnormalities, resulting in peripheral blood cytopenias. MDS typically occur in the elderly: median age at diagnosis is 71-76 years. Up to 30% of MDS cases progress to AML and this incidence varies across
MDS subtypes.\textsuperscript{59,60} The diagnosis requires ≥1 cytopenia and at least one of the following: 1) ≥10% dysplasia in at least one lineage (examples include erythroid hyperplasia, ring sideroblasts, megakaryocytes with multiple separated nuclei or pseudo-Pelger-Huet anomaly\textsuperscript{61}); 2) 5-19% myeloblasts or 3) presence of an acquired chromosomal abnormality specific for MDS.\textsuperscript{62} The Revised International Prognostic Scoring System (IPSS-R)\textsuperscript{63} may be used to define lower risk or higher risk groups, based on bone marrow blast percentage, number and severity of cytopenias and presence and type of cytogenetic abnormalities. Recently, a new version of the IPSS has been proposed which includes somatic gene mutations (IPSS-M).\textsuperscript{64} While disease-directed treatment in lower risk cases is not necessarily indicated, high-risk patients may be considered for allogeneic hematopoietic cell transplantation (HCT), that remains the only curative treatment to alter the more aggressive course of the disease. Other treatment options include the hypomethylating agent azacitidine and lenalidomide - all aiming at improved quality of life and extension of survival - and supportive therapy with transfusions, iron chelation therapy or growth factors (including erythropoietin, G-CSF and thrombopoietin).\textsuperscript{57}

Acute myeloid leukemia
AML is characterized by expansion of abnormal leukemic myeloid blasts that accumulate in bone marrow, blood and other tissues, resulting in diminished production of mature and functional blood cells. Such immature blasts result from a block in differentiation and subsequently accumulate.\textsuperscript{65} AML is accounting for only about 1% of all cancers.\textsuperscript{66} Although there is no clear causative factor identified, AML incidence increases with advancing age. As for MDS, generally the only curative treatment option is allogeneic HCT. A large fraction of patients is not eligible for transplant, especially the subgroup of elderly AML patients. Treatment options in this population include conventional intensive chemotherapy ('3+7'), low-dose cytarabine, hypomethylating agents and supportive care alone. Survival rates in this older patient population remain very poor.\textsuperscript{65,67,68}

Myeloproliferative disorders
MPN are characterized by malignant proliferation of one or multiple lineages of myeloid precursor cells, resulting in overproduction of mature blood cells. The spectrum of disorders includes chronic myeloid leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). PV and ET result from excessive clonal red cell or platelet production and clinical hallmarks include erythrocytosis and thrombocytosis. PMF is characterized by chronic myeloproliferation and megakaryocytic hyperplasia, resulting in bone marrow fibrosis and impaired hematopoiesis frequently resulting in anemia. A major feature of MPN is the predisposition to thrombotic and hemorrhagic events. PV and ET generally have an indolent clinical course, but may progress to secondary myelofibrosis and AML (2.5%).\textsuperscript{69-72}

MDS/MPN overlap syndromes including CMML
Lastly, MDS/MPN overlap disorders share dysplastic and myeloproliferative features. Such conditions may present with cytopenia as a result of bone marrow dysplasia in addition to elevations in peripheral blood cell counts.\textsuperscript{73} Most common is chronic myelomonocytic...
leukemia (CMML), a chronic myeloid neoplasm characterized by abnormal clonal monocyte proliferation. Median age at CMML diagnosis is ~72 years, with a strong male predominance.74 Treatment options for CMML are similar to MDS: hypomethylating agents, cytoreductive drugs or allogeneic HCT for younger patients with high-risk disease.73,75

**Therapy-related and secondary myeloid neoplasms**

MDS and AML may arise de novo, or as a secondary malignancy. Secondary AML (s-AML) defines a subtype of AML following prior myeloid malignancy. Therapy-related myeloid neoplasms (t-MN) occur after treatment with radiation or chemotherapy. The cumulative incidence of t-MN is increased following autologous hematopoietic cell transplantation.55,76,77 Patients with t-MN have inferior outcomes.78

**Driver genetic lesions in myeloid malignancies**

The clonal nature of cancers was first demonstrated in the 1970s, by cytogenetic analyses.79 Numerous clonal molecular lesions have now been identified in myeloid neoplasms, with potential perturbation of self-renewal, proliferation or differentiation of HSCs. The genomic landscape includes mutations in genes involved in RNA splicing, the cohesin complex, apoptosis and DNA repair, transcription, histone modification, DNA methylation and cell signaling.80 Traditionally, somatic mutations have been held responsible for the abnormal clonal expansion of HSCs in myeloid malignancies and as such are regarded key disease markers. Genetic screening in hematological malignancies has become vital in clinical diagnostic, prognostic and therapeutic evaluation. The spectrum of detected mutations and chromosomal aberrations shows substantial overlap across the different disease conditions.

In MDS, numerous molecular aberrations have been identified and at least one abnormality is detected in ≥95% of patients.80 Large scale genomic studies have revealed more than 40 genes to be recurrently mutated.81-84 Frequently mutated genes include those belonging to the spliceosome complex and epigenetic modifiers, including SF3B1, SRSF2, U2AF1, ZRSR2, TET2, ASXL1, DNMT3A, RUNX1, STAG2 and TP53. SF3B1-mutated MDS is increasingly recognized as a separate disease entity.85 Clonal cytogenetic abnormalities are seen in ~50% of MDS cases.80,86 The presence of a selective set of chromosomal alterations is recognized as presumptive evidence for MDS, even in the absence of dysplasia.55 For example, isolated del5q defines a separate clinical entity, with relatively favorable prognosis and improved response to lenalidomide.87,88 Several other common abnormalities include mLOY, del(20q) or +8. mLOY is not considered a diagnostic clonal marker.55

AML is characterized by a heterogeneous cytogenetic and molecular profile that is used for disease classification and risk stratification.89,90 On average, AML blasts carry 10-13 coding mutations.28,91 Recurrent driver mutations include NPM1, FLT3, CEBPA, IDH1/2, RUNX1 and DNMT3A. Cytogenetic abnormalities in AML are predominantly detected as balanced chromosomal translocations, for example t(8;21), t(15;17) and 11q23 anomalies. The WHO acknowledges several abnormalities as separate clinicopathological entities.55,92
Patients with therapy related MDS (t-MDS) or AML (t-AML) frequently carry TP53 or PPM1D mutations and have higher frequencies of complex karyotypic abnormalities or abnormalities of chromosome 5 or 7. Mutations in SRSF2, SF3B1, U2AF1, ZRSR2, ASXL1, EZH2, BCOR or STAG2 are highly associated with s-AML, and may define a distinct genetic subtype with adverse prognosis even when occurring in the setting of t-AML or de novo AML.

For MPN, driver genetic aberrations are characterized by mutational activation of the JAK-STAT pathway. Key lesions include the BCR-ABL1 fusion gene as a defining lesion for Philadelphia-positive CML, JAK2 V617F or exon 12 mutations in ≥95% of patients with PV and ~50% with ET, and CALR or MPL in ET and PMF. A large fraction of MPN patients present with co-mutations in non-MPN driver genes or accompanying chromosomal aberrations (>60%). The presence of multiple molecular aberrations is associated with adverse disease prognosis.

Finally, in CMML, molecular aberrations occur in up to 90% of patients, most commonly in TET2, SRSF2 and ASXL1 genes. Although there are no disease-defining molecular lesions, the co-occurrence of TET2 and SRSF2 may be highly specific for the myelomonocytic phenotype. Clonal cytogenetic abnormalities are detected in ~30% and can be used for prognostication.

Somatic mutations are increasingly linked to increased sensitivity or resistance to existing therapies and may be used as a target for novel targeted therapeutic interventions. For AML, several targeted therapies have become available during recent years, including FLT3 and IDH1/2 inhibitors that have been shown to augment clinical responses in AML patients with these mutations.

Clonal hematopoiesis as a pre-stage for hematological malignancies in the general population
The risk to develop hematological cancer is roughly 10-times higher for individuals with CH, as estimated in the earliest studies. The rate of progression from CH to hematological malignancy was estimated at 0.5-1% per year. This risk is similar to evolution rates of monoclonal gammopathy of uncertain significance (MGUS) to multiple myeloma (MM) or monoclonal B-cell lymphocytosis to chronic lymphocytic leukemia. Accordingly, most individuals with CH have very low risk of progression. Earlier lines of evidence have demonstrated that preleukemic HSCs may already harbor a proportion of mutations found in leukemic blasts at time of AML diagnosis. Recent case-control studies, facilitated by large-scale NGS, have refined the risk of progression to AML according to the spectrum of driver gene mutations detected in CH. A higher prevalence of CH was found when screening community-dwelling individuals median 6.3 and 9.6 years before diagnosis of AML, and the risk to develop AML was ~3-5 fold higher for CH carriers. Mutations in spliceosome factor genes, IDH1/2 and TP53 conferred highest risks of progression, in addition to multiple driver mutations and higher clone size (VAF>10%). In addition, a recent study has discriminated between CH patterns with increased risk for myeloid versus lymphoid malignancies. Lymphoid drivers predisposed to lymphoid neoplasia and were distinct from...
common drivers associated with myeloid malignancy including DNMT3A, TET2 and ASXL1. CH is proposed to represent an early stage of clonal selection in malignancy development (Figure 4). In 2015, Steensma et al. proposed the term clonal hematopoiesis of indeterminate potential (“CHIP”) to describe CH as a precursor condition (Box 1).

Figure 4. Stepwise progression from clonal hematopoiesis to myeloid malignancy
Many mutations with advancing age are so-called passengers (blue, purple) and do not confer a growth advantage. Other mutations may be detected in the peripheral blood when substantial clonal expansion occurs (yellow, green). A stepwise gain of mutations (orange, red) may eventually prompt malignant transformation. Most clonally expanded mutations will remain detectable over years but do not evolve to malignancy (green). Modified from van Zeventer and Buisman et al.

Hematopoietic (stem) cell transplantation
Transplantation of HSCs, that have self-renewal and pluripotent properties, is a widely used treatment option in clinical hematology. Autologous hematopoietic cell transplantation (ASCT) is frequently applied in patients with lymphoma or MM. Nowadays, HSCs are harvested from the peripheral blood after cytotoxic therapy and application of G-CSF, that initiate their release from the bone marrow compartment. The cell re-infusion is aimed at reconstitution of the bone marrow compartment after myeloablative therapy. Failure to mobilize sufficient numbers of CD34+ blood cells (generally <2 x 10^6/kg body weight) is a major concern and results in potential loss of transplant as a treatment option. Risk factors for poor mobilization include increasing age, diabetes, prior radiotherapy, melphalan, lenalidomide or fludarabine treatment. Direct HSC toxicity or a diminished HSC reserve have been proposed as underlying mechanisms. Allogeneic HCT is a potential curative approach when a graft-versus-tumor effect is warranted for the treatment of various myeloid
malignancies including AML, generally in addition to myeloid toxicity caused by chemo/radiotherapy. HSCs for allogeneic HCT are harvested from a matched related or unrelated donor after stimulation with G-CSF. High-intensity conditioning and a graft-versus-leukemia effect are major mechanisms contributing to the curative effect. The allogeneic procedure is associated with substantial high rates of transplant related morbidity and mortality, including graft-versus-host disease (GVHD).

Clonal hematopoiesis and its relation to cytotoxic therapy and hematopoietic cell transplantation

Individuals treated with cytotoxic chemotherapy or radiotherapy frequently carry somatic mutations indicative for CH. Mutations in DNA damage response genes (TP53, PPM1D, CHEK2) have the strongest association with previous cancer treatment. Pre-existing CH increases the risk of t-MN development after chemotherapeutic treatment. While the traditional hypothesis has been that cytotoxic therapy itself is mutagenic, TP53 mutated clones are frequently detected prior to administration of chemotherapy and gain a selective advantage that allows for outgrowth upon selective pressures by such therapy. A similar mechanism has been proposed for PPM1D mutations, another gene involved in DNA damage response. PPM1D mutant clones have been shown to preferentially gain competitive advantage after genotoxic stress, especially platinum-based. TP53 and PPM1D clones also show selective outgrowth at the expense of other mutated clones. In patients with t-MDS, TP53 and PPM1D mutations are enriched. Thus, such low-level and pre-existing clones may gain clonal advantage and preferentially undergo expansion under selective pressure from therapy, ultimately predisposing to t-MN development.

In the setting of ASCT or allogeneic HCT, donor CH may be an important factor to consider. When HSCs engraft in the recipient, there is extensive self-renewal to reconstitute the bone marrow. This increased proliferative demand might induce selective expansion of the mutated clone and accelerate clonal evolution. Engraftment of mutated clones in the allogeneic transplantation setting is common. Donor-derived CH can be found in recipients with unexplained cytopenia. There is conflicting evidence regarding a potential higher risk for GVHD associated with clonal allogeneic grafts. Although donor-derived leukemia is reported and may result from leukemic evolution of donor-derived CH, its occurrence in the allogeneic setting is rare. After ASCT, t-MN incidence is markedly more frequent. The cumulative incidence of t-MN following ASCT for MM is estimated between 0.5 and 7%, with more recent studies reporting lower cumulative incidences possible due to reduced intensity conditioning. For lymphoma, incidences range from 1-14% depending on follow-up. Age increases the risk of t-MN development. CH is common among patients with lymphoma and MM, and before ASCT ~30% of NHL and 22% of MM patients carry clonal mutations. The risk of subsequent t-MN development after ASCT was increased for patients carrying CH in the context of lymphoma but not MM. Previous chemotherapeutic treatment might have contributed to the preselection or emergence of CH in these individuals.
Clonal hematopoiesis and blood count abnormalities: pre-stage for myeloid malignancies or lower-risk MDS?
The ageing hematopoietic system, despite becoming mosaic in nature, mostly retains its functionality to produce the wide variety of mature peripheral blood cells. An extreme example of oligoclonal hematopoiesis was demonstrated in a 115-year-old woman whose hematopoiesis was driven by two large mutant clones. No abnormalities in peripheral blood counts were detected. Thus, despite the demonstrated ability of such clones to outcompete normal hematopoietic cells, a mutant clone may still be effective at generating a balanced set of mature and differentiated blood cells.

Unexplained blood count abnormalities
Uni- or multilineage cytopenia may result from a wide range of underlying etiologies. The broad differential diagnosis includes nutrient deficiencies, autoimmune disorders, medication or drug-induced cytopenia (e.g. chemotherapy, alcohol use or some anticonvulsants), inflammation or infection and primary bone marrow disorders. The diagnostic approach thus involves distinguishing reactive or nutrient-deficient causes from clonal hematological disorders. Diagnostic and therapeutic challenges may arise: the cause of the cytopenia remains unknown in a substantial proportion of such cases despite careful clinical and laboratory examination. For anemia of the elderly, this proportion approximates one-third of anemic cases. Especially in ageing individuals presenting with unexplained cytopenia(s), the presence of lower risk MDS and related disorders should be considered. Other blood count aberrations that might raise clinical suspicion for myeloid malignancies include unexplained erythrocytosis, thrombocytosis, persistent leukocytosis (or neutrophilia) and monocytosis. A clinical suspicion for MPN is raised when no secondary causes for erythrocytosis or thrombocytosis are identified. Sustained unexplained monocytosis may be a presenting feature of CMML. Some of these conditions may go undiagnosed, especially in the elderly, for example when invasive bone marrow diagnostics are deliberately refrained from.

Somatic mutations detected in patients with unexplained blood count abnormalities
In recent years, sensitive genomic screening methods have progressively been introduced into clinical practice and now allow for the detection of molecular genetic aberrations across a wide range of conditions. For example, JAK2 V617F mutation screening is used for evaluation of patients presenting with unexplained thrombosis occurring at unusual locations, erythrocytosis, thrombocytosis and monocytosis, although it may lack specificity and morphology remains the cornerstone of diagnosis. In patients with unexplained cytopenia presenting in a (tertiary) hematology clinic for evaluation, a high prevalence (28-91%) of somatic mutations was reported. Although the mutational spectrum resembled that of MDS, DNMT3A, TET2 and ASXL1 represented the most commonly observed mutations, similar to CH in the general population. The combined presence of cytopenia and CH conferred a high risk of progression to overt myeloid neoplasm. It was proposed that such individuals suffer from a pre-stage of myeloid neoplasm that resembles low-risk MDS. In contrary, those presenting with nonclonal cytopenias had very low risk of progression. Based on these studies, NGS is increasingly recognized as a valuable component in the
General introduction and scope of this thesis

standard diagnostic work-up of cytopenias and other unexplained blood count abnormalities.\textsuperscript{32,157}

Box 1. Overview of terminology

**CHIP** (clonal hematopoiesis of indeterminate potential)
Presence of one or more clonal myeloid malignancy-associated gene mutations in peripheral blood or bone marrow without evidence for neoplastic disease and in the absence of blood count abnormalities*.

**ARCH** (age-related clonal hematopoiesis)
Alternative terminology for age-related emergence of CHIP.

**ICUS** (idiopathic cytopenia of undetermined significance)
Persistent cytopenia in one or more peripheral blood cell lineages, not explained by any other underlying etiology, with absence of evidence for clonal myeloid mutations (i.e. not tested or not detected).

**CCUS** (clonal cytopenia of undetermined significance)
Persistent cytopenia in one or more peripheral blood cell lineages, with presence of one or more clonal myeloid malignancy-associated gene mutations in bone marrow or peripheral blood*.

**IMUS** (idiopathic monocytosis of unknown significance)
Persistent monocytosis in peripheral blood, with absence of evidence for clonal myeloid mutations (i.e. not tested or not detected).

**CMUS** (clonal monocytosis of unknown significance)
Persistent monocytosis in peripheral blood, with presence of one or more clonal myeloid malignancy-associated gene mutations in bone marrow or peripheral blood*.

In all conditions, criteria for MDS, CMML or other neoplastic hematological disorders are not fulfilled.

*Peripheral blood cytopenias are defined by hemoglobin count <13g/L (males) or 12 g/L (females) for anemia, absolute neutrophil count <1.8 x 10^9/L for neutropenia or platelet counts <150 x 10^9/L for thrombocytopenia.\textsuperscript{114} Clinically relevant monocytosis for diagnosis of CMML according to 2016 WHO definitions was defined as a peripheral blood monocyte count ≥ 1 x 10^9/L and ≥10% of total white blood cell count\textsuperscript{35}, but this cut-off is a topic of debate. *The VAF cut-off to define clinically relevant CHIP and CCUS has been proposed at 2% VAF, meaning that ≥4% of blood cells should carry the respective genetic variant (heterozygosity assumed).\textsuperscript{113}

Acronyms for precursor stages: ICUS, CCUS, IDUS, IMUS, CMUS
Multiple acronyms have been proposed to designate potential precursor states of myeloid neoplasms (Box 1). The term idiopathic cytopenia of undetermined significance ("ICUS") was proposed already in 2005 to define a persistent blood cytopenia without evidence of dysplasia, in the absence of any other secondary causes, that remains unexplained.\textsuperscript{165} In case of peripheral cytopenia(s) and the presence of CH, in the absence of other defining criteria for MDS (such as extensive dysplasia or excess blasts) or another hematological malignancy, this condition is referred to as clonal cytopenia of undetermined potential ("CCUS").\textsuperscript{32,113,166} Other proposed entities include idiopathic monocytosis of unknown significance ("IMUS")
and clonal monocytosis of unknown significance ("CMUS"). The acronym CMUS has been proposed for those not meeting diagnostic criteria for CMML, but carrying CH in combination with unexplained and persistent monocytosis.\textsuperscript{167} Finally, the term idiopathic dysplasia of undetermined significance ("IDUS") is more rarely used to describe abnormal cell morphology or dysplasia in the absence of cytopenia, not meeting diagnostic criteria for hematological disorders.\textsuperscript{168-170} Healthy older individuals may have detectable IDUS similar to dysplastic changes seen in MDS.

![Venn diagram illustrating the relationship and overlap between clonal hematopoiesis, cytopenia and dysplasia](image)

The common feature of all these conditions is the lack of established WHO criteria for myeloid neoplasms. Although all conditions may persist without clinical manifestation, some individuals may progress to overt myeloid malignancy including MDS and CMML. The clinical implications of these precursor or mimicking states remain to be established.
SCOPE OF THIS THESIS

High-throughput and massively parallel sequencing with gene panels focused on genes implicated in MDS, AML and MPN is now often ordered by physicians. The interpretation of somatic mutations in the diagnostic work-up of myeloid malignancy becomes more challenging with the discovery of CH, as such genes are frequently mutated in otherwise healthy ageing individuals. The work presented in this thesis focuses on a better understanding of CH and especially its relation to phenotypic changes in the hematopoietic system, by applying large-scale sensitive sequencing in older individuals, focusing on those with blood count abnormalities, and by the subsequent study of dynamics of mutated clones over time.

In the first part of this thesis, we focused on the relation between blood count abnormalities and CH and especially whether the combination of these age-related phenomena might indicate early stages of myeloid malignancy. We initiated a large-scale NGS project within the prospective population-based Lifelines cohort, that is still ongoing. A sensitive smMIP-based method facilitated reliable VAF estimation for small-sized mutated clones (cut-off used was ≥1% VAF), while controlling for sequencing artefacts (Table 2). By establishing linkage to the national pathology database PALGA, the Netherlands Cancer Registry (NCR) and Statistics Netherlands (CBS), we included reliable coverage of all diagnosed hematological malignancies and causes of death. Chapter 2 focuses on the emergence of peripheral cytopenias and their relationship with changes in peripheral blood counts with advancing age. We explore the impact of cytopenias on overall and cause-specific survival as well as development of hematological malignancies in the general population. Anemia is perhaps the most pertinent clinical hallmark of the ageing hematopoietic system and is still poorly understood. It poses a substantial disease burden in older individuals, with significant morbidity and mortality, even for a mild degree of anemia. In Chapter 3, we investigate the impact and spectrum of age-related CH in older individuals (≥60 years) with anemia. MDS or other myeloid neoplasms may be present in up to 15% of these individuals, as estimated by previous studies. We aimed to answer the question whether CH accounts for “(unexplained) anemia of the elderly”, and whether some individuals might suffer from undiagnosed lower-risk MDS. Chapter 4 subsequently focuses on the occurrence of erythrocytosis, its clinical characteristics and association with CH in community-dwelling individuals of all ages. There is lack of a uniform definition for erythrocytosis; this chapter includes a critical assessment of current clinical cut-off criteria. Chapter 5 covers platelet count abnormalities and their relationship with CH: thrombocytosis and thrombocytopenia. Finally, we aimed to test the hypothesis whether early developmental stages of CMML exist in the general population. Therefore, in Chapter 6 we evaluated proposed cut-offs for a clinically relevant monocytosis and investigated whether the combined presence of peripheral monocytosis and CH may identify such early stages of myelomonocytic malignancy in the general population.

The next projects of this thesis focused on the differential implications of CH when detected in specific subgroups of individuals. Age is the predominant risk factor for developing CH. In
Chapter 7 we investigate the spectrum and consequences of CH in the oldest old population (≥80 years). Failure to mobilize sufficient HSCs for ASCT associates with a higher incidence of t-MN.\textsuperscript{177,178} We questioned whether HSCs from these patients could be already affected by clonal mutations at the time of mobilization. In Chapter 8 we study a consecutive and retrospective cohort of 776 patients undergoing mobilization procedures for ASCT. We describe the biased mutational spectrum and the potential clinical relevance of CH in a nested case-control cohort of poor mobilizers and matched controls.

Next to error-corrected sequencing to detect somatic gene mutations, we applied a sensitive method to detect mosaic chromosomal alterations including mLOY, making use of SNP array data that were generated for a subset of the Lifelines cohort.\textsuperscript{179} In Chapter 9 we investigate the co-occurrence of somatic gene mutations and mLOY in males ≥60 years from the general population.

Most importantly, the final part of this thesis includes a comprehensive assessment of clonal dynamics. Although the trajectory of clonal progression to malignancy likely involves clonal expansion and the acquisition of additional genetic aberrations, little is known about the longitudinal trajectory of CH and clone-specific features that associate with growth and ultimately might drive malignant transformation.\textsuperscript{4} In Chapter 10, we explore the evolutionary landscape of CH in 3359 population-based individuals with and without blood count abnormalities, representing the largest longitudinal cohort so far. We undertook first effort to identify extrinsic drivers of clonal outgrowth.

Finally, a summary of the research in this thesis and its implications are given in Chapter 11. This is followed by a discussion of current evidence and suggestions for future directions in this rapidly expanding area of research.
Table 1. Methods for detection of clonal somatic mutations

<table>
<thead>
<tr>
<th>Method</th>
<th>Droplet Digital PCR (ddPCR)</th>
<th>Whole-exome or whole-genome sequencing</th>
<th>Targeted sequencing</th>
<th>Error-corrected targeted sequencing</th>
<th>Single-cell sequencing</th>
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<tbody>
<tr>
<td>Preprocessing</td>
<td>Massive partitioning of PCR into microdroplets, each followed by separate quantification</td>
<td>Preprocessing without sequence enrichment (WGS) or with exome capture (WES)</td>
<td>Target enrichment (amplicon or hybrid capture based) for specific genes of interest</td>
<td>Target enrichment, together with molecular barcode or tag sequences attached to individual DNA fragments</td>
<td>Sequencing thousands of single cells in parallel</td>
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<tr>
<td>Target region</td>
<td>Selected and known “hotspot” mutations</td>
<td>Exome-wide (all coding regions) or genome wide (also including introns)</td>
<td>Selected panel of genes or regions of interest</td>
<td>Selected panel of genes or regions of interest</td>
<td>Most often targeted gene panel, although whole genome amplification possible</td>
</tr>
<tr>
<td>Sequencing depth*</td>
<td>~10-50x for whole-genome and ~50-100x for whole-exome</td>
<td>500-1000x</td>
<td>Up to 100,000x</td>
<td>Depending on number of cells</td>
<td></td>
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<tr>
<td>VAF limit of detection*</td>
<td>&lt;0.01%</td>
<td>&gt;5%</td>
<td>1-2%</td>
<td>Down to 0.001%</td>
<td>Depending on number of cells</td>
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<td>Advantages</td>
<td>Absolute quantification of mutation frequency in sample</td>
<td>Sequencing of the complete (coding) DNA sequence, suitable for exploratory studies with limited analytical sensitivity</td>
<td>Greater sequencing depth at target loci</td>
<td>Improved sensitivity for low-level mutations by barcode-based distinction between sequencing artefacts and mutations, near-absolute quantification of mutation frequency</td>
<td>Reconstruct subclonal hierarchy, combination with other single cell approaches for ‘multi-omic’ characterization</td>
</tr>
</tbody>
</table>

*Depending on specific sequencing technique used. VAF, variant allele frequency; PCR, polymerase chain reaction; WES, whole-exome sequencing; WGS, whole-genome sequencing.
Table 2. Panel used for targeted smMIP-based next-generation sequencing in the Lifelines cohort

<table>
<thead>
<tr>
<th>Gene</th>
<th>Reference transcript</th>
<th>ENSEMBL reference transcript</th>
<th>Exon</th>
<th>Targeted codons/region</th>
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</thead>
<tbody>
<tr>
<td>ASXL1</td>
<td>NM_015338</td>
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<td>ENST00000316448</td>
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<td>exon 9</td>
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<td>CBL</td>
<td>NM_005188</td>
<td>ENST00000264033</td>
<td>8-9</td>
<td>exon 8 and 9</td>
</tr>
<tr>
<td>CSF3R</td>
<td>NM_156039</td>
<td>ENST00000373103</td>
<td>14, 17</td>
<td>codon 618, 615 and exon 17</td>
</tr>
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<td>DNMT3A</td>
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<td>2-23 (all coding exons)</td>
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<tr>
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<td>ENST00000381652</td>
<td>12, 14 (partially)</td>
<td>codon 617 and exon 12</td>
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<td>NM_000222</td>
<td>ENST00000288135</td>
<td>8 (partially), 17 (partially)</td>
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<td>2-3 (partially)</td>
<td>a.o. codon 12, 13, 61</td>
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<td>ENST00000437180</td>
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<td>ENST00000332351</td>
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<td>NM_003620</td>
<td>ENST00000305921</td>
<td>6</td>
<td>exon 6</td>
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</tbody>
</table>

* Included in panel for Chapter 8. smMIP, single-molecule molecular inversion probe.
REFERENCES

General introduction and scope of this thesis


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