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Clinical and molecular characteristics of Chronic Myelomonocytic Leukemia

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Clinical and molecular characteristics of Chronic Myelomonocytic Leukemia
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By

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“There is nothing like looking, if you want to find something. You certainly usually find something, if you look, but it is not always quite the something you were after.”

— J.R.R. Tolkien, The Hobbit

Popular science summary of the thesis

Blood consists of many different types of cells which are produced by stem cells in the bone marrow. Chronic myelomonocytic leukemia (CMML) is a form of blood cancer originating in the bone marrow stem cells, where driver mutations are acquired. This leads to proliferation of clonal cells originating from the affected stem cells and elevated levels of a type of blood cell called monocytes, in the blood. The most frequently mutated gene in CMML is Tet methylcytosine dioxygenase 2 (*TET2)*. *TET2* mutation (*TET2*^{MT}) is present in about 60% of CMML patients. The median overall survival of CMML patients is 24 months, however survival varies greatly; some patients die within 1-2 months, and some may live over 10 years without treatment.

The aim of this thesis is to better understand how genetic factors (mutations) and epigenetic factors (how DNA is stored, modified and used in the cell) affect the disease.

In paper I, we demonstrated that if mutation information is not available, the prognostic scoring system CPSS best predicted the prognosis of CMML patients. However, mutation status is currently available for most patients, and in paper II we showed that presence of *TET2*^{MT} is associated with longer overall survival. Patients with *TET2*^{MT} also exhibit less signs of inflammation. Paper III assessed platelet size and found that platelets are enlarged in CMML, and that large platelets may indicate a longer overall survival. Finally, in paper IV, we demonstrated that *TET2*^{MT} is important for how DNA is packaged and available in CMML bone marrow cells and that genes coding for inflammatory proteins are switched off in CMML patients with *TET2*^{MT} compared to CMML without *TET2*^{MT}. This could potentially contribute to explaining why CMML patients with *TET2*^{MT} exhibit less signs of inflammation and perhaps also partially explain why *TET2*^{MT} patients live longer than *TET2* wild type (*TET2*^{WT}) patients. These findings may contribute to better management of CMML patients as well as to reveal new potential avenues for treatment targets in CMML.

Abstract

Patients with CMML have a poor but variable prognosis. A deeper understanding of CMML pathobiology is needed in order to improve prognostic scores and better predict the disease course for each individual patient. In this thesis, clinical characteristics such as comorbidities, laboratory values at diagnosis and survival as well as molecular characteristics such as mutations, DNA methylome and chromatin accessibility were assessed to advance the knowledge of CMML pathobiology.

In **paper I,** we found that the CPSS score performed best of available prognostic scoring systems not including myeloid mutations. In **paper II,** the presence of multiple *TET2*^{MT} was significantly associated with longer overall survival. In addition, a cox model including the number of *TET2*^{MT} outperformed the widely accepted CPSS-mol prognostic score. In **paper III,** we demonstrated that platelets are generally enlarged in CMML, and enlarged platelets were associated with longer overall survival. In **paper IV,** unsupervised clustering of exome sequencing data grouped patients into two clusters largely driven by the presence or absence of *TET2*^{MT}, and clusters based on DNA methylation analysis β-values strongly overlapped with mutation clusters. In the *TET2*^{MT} cluster, profound DNA hypermethylation was seen, specifically enriched in inflammatory gene sets and in the enhancer regions of these genes. Integrated analysis of ATAC seq and ChIP seq revealed that DNA coding for these inflammatory gene sets shifted from active chromatin state in *TET2*^{WT} to a repressed state in the *TET2*^{MT} patient cluster.

In conclusion, *TET2*^{MT} especially multihit *TET2*^{MT} is associated with longer overall survival in CMML. In patients with *TET2*^{MT}, genes coding for inflammatory proteins are repressed by several mechanisms. We hypothesize that this renders the *TET2*^{MT} CMML bone marrow more resilient to chronic inflammation and may contribute to a longer overall survival.

List of scientific papers

I. Daniel Moreno Berggren, **Matilda Kjellander**, Ellen Backlund, Marie Engvall, Hege Garelius, Fryderyk Lorenz, Lars Nilsson, Bengt Rasmussen, Sören Lehmann, Eva Hellström-Lindberg, Martin Jädersten, Johanna Ungerstedt, Elisabeth Ejerblad.
Prognostic scoring systems and comorbidities in chronic myelomonocytic leukaemia: a nationwide population-based study.
Br J Haematol. 2021;192(3):474-83.

II. **Matilda Kjellander Kynning**, Ebba Westerberg, Linda Forsell, Maria Creignou, Daniel Moreno Berggren, Bianca Tesi, Elsa Bernard, Elli Papaemmanuil, Yasuhito Nannya, Lucia Cavalier Franco, Davide Valentini, Eva Hellström Lindberg, Seishi Ogawa, Elisabeth Ejerblad, Johanna Ungerstedt.
Comorbidities and mutations including single- and multihit TET2 mutations in relation to outcome in chronic myelomonocytic leukaemia-A population-based study.
Br J Haematol. 2026;208(2):514-23.

III. Ebba Westerberg, Sophia Maassarani, **Matilda Kjellander Kynning**, Anna Ravn Landtblom, Suria Jahan, Cecilia Karlström, Charlotte Gran, Maaike Jongen, Petter Höglund, Niklas Boknäs, Johanna S Ungerstedt.
Platelet size, heterogeneity and turnover in relation to myeloid mutations and outcome in Chronic Myelomonocytic Leukemia.
Manuscript.

IV. **Matilda Kjellander Kynning**, Yasuhito Nannya, Xiangfu Zhong, Yotaro Ochi, Tetsuichi Yoshizato, Gabriele Todisco, Bianca Tesi, Andreas Lennartsson, Teresa Mortera Blanco, Oscar Brück, Kazuma Ohyashiki, Takayuki Ishikawa, Satu Mustjoki, Torsten Haferlach, Eva Hellström Lindberg, Seishi Ogawa, Johanna Ungerstedt.
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Proteomic and transcriptomic screening demonstrates increased mast cell-derived CCL23 in systemic mastocytosis.
The Journal of allergy and clinical immunology, 2023;152(1), 205–213.

III. Lyberg, K., Ali, H. A., Grootens, J., **Kjellander, M.**, Tirfing, M., Arock, M., Hägglund, H., Nilsson, G., & Ungerstedt, J. (2017). Histone deacetylase inhibitor SAHA mediates mast cell death and epigenetic silencing of constitutively active D816V KIT in systemic mastocytosis. *Oncotarget*, *8*(6), 9647–9659. https://doi.org/10.18632/oncotarget.14181

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List of abbreviations

5hmC	5-Hydroxymethylcytosine
5mC	5-Methylcytosine
AID	Autoimmune disease
AIC	Akaike Information Criterion
AML	Acute Myeloid Leukemia
ATAC seq	Assay for Transposase-Accessible Chromatin using Sequencing
AZA	Azacitidine
C-index	Harrell Concordance index
CCI	Charlson Comorbidity Index
CCUS	Clonal Cytopenia of Undetermined Significance
CHIP	Clonal Hematopoiesis of Indeterminate Potential
ChIP seq	Chromatin Immunoprecipitation Sequencing
CML	Chronic Myeloid Leukemia
CMML	Chronic Myelomonocytic Leukemia
CPSS	CMML-Specific Prognostic Scoring System
CPSS-mol	CMML-Specific Prognostic Scoring System–Molecular
CRP	C-Reactive Protein
DMP	Differentially Methylated Probe
ECOG	Eastern Cooperative Oncology Group (Performance Status)
FAB	French–American–British (classification)
FISH	Fluorescence in Situ Hybridization
HCT-CI	Hematopoietic Cell Transplantation–Comorbidity Index
HMA	Hypomethylating Agent
HSC	Hematopoietic Stem Cell
HSPCs	Hematopoietic Stem and Progenitor Cells
HSCT	Hematopoietic Stem Cell Transplantation
ICC	International Consensus Classification

IPF%	Immature Platelet Fraction (%)
IPSS-M	Molecular International Prognostic Scoring System
IPSS-R	Revised International Prognostic Scoring System
ITP	Immune Thrombocytopenia
LDH	Lactate Dehydrogenase
LPS	Lipopolysaccharide
MD-CMML	Myelodysplastic Chronic Myelomonocytic Leukemia
MDAPS	MD Anderson Prognostic Score
MDS	Myelodysplastic Syndrome
MDS-CI	Myelodysplastic Syndrome–Specific Comorbidity Index
MP-CMML	Myeloproliferative Chronic Myelomonocytic Leukemia
MPN	Myeloproliferative Neoplasms
MPV	Mean Platelet Volume
OS	Overall Survival
PCR	Polymerase Chain Reaction
PDW	Platelet Distribution Width
PMF	Primary Myelofibrosis
PMR	Polymyalgia Rheumatica
SMDSR	Swedish MDS Registry
TET2^{MT}	*Tet methylcytosine dioxygenase 2* Mutant
TET2^{WT}	*Tet methylcytosine dioxygenase 2* Wild Type
WHO	World Health Organization

Introduction - chronic myelomonocytic leukemia through history

The disease name "chronic myelomonocytic leukemia" was used for the first time in 1972 in a patient with specific bone marrow morphology and cytology, and peripheral blood monocytosis and neutrophilia (1, 2). However already in 1951 a chronic leukemia associated with monocytosis had been described under the name "chronic monocytic leukemia" (3). In 1982, CMML was included in the disease classification by the French-American-British (FAB) co-operative group as subgroup to myelodysplastic syndrome (MDS), and since the 2001 World Health Organization (WHO) classification, CMML is separated from MDS into its own disease entity (4). The current classification of CMML is based on bone marrow morphology including dysplasia, peripheral blood monocytosis, blast count, clonal driver mutations and absence of signs of other chronic myeloid neoplasm, most recently by the WHO 2022 and International Consensus Classification (ICC) 2022 classifications (Table 1) (5, 6).

Genetic analyses have an increasing role in CMML diagnosis, from negation of Philadelphia chromosome, by cytogenetics, FISH or PCR, to sequencing of large gene panels (5, 6). Mutations are now part of the diagnostic criteria in CMML, and several mutations are linked to prognosis. Mutations in epigenetic modifiers are frequent in CMML, and aberrant DNA methylation patterns have been described, including specific differentially methylated regions that could be linked to response to hypomethylating agent (HMA) treatment (7). Attempts at classifying CMML according to chromatin changes and DNA methylation patterns have been made, however, further studies are needed to elucidate the role of epigenetics in CMML pathobiology and potential targets for novel treatment (7, 8).

There is no specific disease modulating treatment for CMML, and clinical trials including only CMML patients are rare. Overall, improved diagnostic and prognostic tools, a better understanding of CMML pathobiology, and novel CMML-specific treatment, are very much needed.

1 Background

1.1 Demographics and diagnosis

The median age at CMML diagnosis is around 75 years and the incidence is 0,4 per 100 000 inhabitants per year (9, 10). Around two thirds of patients are male, and possibly the CMML incidence is somewhat lower in Asian populations compared to white and black populations (11). The median overall survival is around 24 months and the risk of transforming to acute myeloid leukemia (AML) is 15-20% within 3-5 years (10).

Since 2022 there are two separate classification systems for diagnosing myeloid neoplasms, the ICC and the WHO classification (5, 6). For CMML the differences between these systems are subtle. An absolute monocyte count of ≥0.5x 10^9/L is required according to both ICC and WHO, with monocytes making up more than 10% of the white blood cell count, present for more than 3 months and not explained by inflammation, infection or other causes of leukocytosis. Previously, an absolute monocyte count of ≥1.0 x 10^9/L was required. Since 2022 the requirement was lowered, however, more supporting criteria or additional conditions need to be met for cases with monocytes between 0.5-1.0x10^9/L (Table 1) (5, 6). Criteria for other myeloid or lymphoid neoplasms should not be met, and chronic myeloid leukemia (CML) should be excluded by ruling out the presence of *BCR::ABL1*. The CMML diagnosis is confirmed by a bone marrow examination, with a blast count, including promonocytes, ranging from 0-19%. Clonality, presence of an acquired myeloid mutation or cytogenetic aberrancy, is a supporting criterion in the WHO system and needed for diagnosis of CMML when monocyte count range between 0.5-1.0x10^9/L according to the ICC. See table 1 for comparison of WHO and ICC diagnostic criteria (5, 6).

Flow cytometry of peripheral blood can determine monocyte subsets defined as classical monocytes expressing CD14+/CD16-, intermediate monocytes expressing CD14+/CD16+ and nonclassical monocytes expressing CD14low/CD16+. In healthy, around 85% of circulating monocytes are classical monocytes whereas in CMML, classical monocytes are always >94% of the total monocyte population (12). Peripheral blood monocyte partition with classical monocytes >94% is used in the diagnostic workup of CMML to determine whether bone marrow sampling is warranted (2, 10, 12). Since 2022, the abnormal repartition of monocyte subsets with a threshold of >94% classical monocytes, is included in the WHO diagnostic criteria as a supporting criterion (5, 12).

*Table 1. Comparing diagnostic criteria for CMML according to the 2022 WHO and ICC classifications (5, 6). For WHO Some criteria are prerequisite and some supporting, for ICC all criteria are required. Comparable criteria are listed in the same row. Completely unique criteria for each classification system are only monocyte subsets for WHO and cytopenia for ICC = **

WHO 2022	ICC 2022
Prerequisite criteria	
1. Persistent absolute (≥0.5 × 10^9/L) and relative (≥10%) peripheral blood monocytosis.	Monocytosis defined as monocytes ≥ 0.5 × 10^9/L and ≥ 10% of the WBC
2. Blasts constitute <20% of the cells in the peripheral blood and bone marrow.	Blasts (including promonocytes) < 20% of the cells in blood and bone marrow
3. Not meeting diagnostic criteria of chronic myeloid leukaemia or other myeloproliferative neoplasms.	No *BCR::ABL1* or genetic abnormalities of myeloid/lymphoid neoplasms with eosinophilia and tyrosine kinase gene fusions
4. Not meeting diagnostic criteria of myeloid/lymphoid neoplasms with tyrosine kinase fusions.	
	Cytopenia (thresholds same as MDS)*
Supporting criteria	
1. Dysplasia involving ≥1 myeloid lineages.	Bone marrow examination with morphologic findings consistent with CMML (hypercellularity due to a myeloid proliferation often with increased monocytes), and lacking diagnostic features of acute myeloid leukemia, MPN or other conditions associated with monocytosis
2. Acquired clonal cytogenetic or molecular abnormality.	Presence of clonality: abnormal cytogenetics and/or presence of at least one myeloid neoplasm associated mutation of at least 10% allele frequency
3. Abnormal partitioning of peripheral blood monocyte subsets.*	
Requirements for diagnosis	
- Pre-requisite criteria must be present in all cases.	
- If monocytosis is ≥ 1 × 10^9/L: one or more supporting criteria must be met.	
- If monocytosis is ≥0.5 and <1 × 10^9/L: supporting criteria 1 and 2 must be met.	In cases without evidence of clonality, monocytes ≥ 1.0 × 10^9/L and > 10% of the WBC, and increased blasts (including promonocytes), or morphologic dysplasia, or an abnormal immunophenotype consistent with CMML would be required for its diagnosis.

The WHO also subgroups CMML based on myeloblast count, including blast equivalents, which are promonocytes, monoblasts and myeloblasts. And patients with up to 9% bone marrow blasts are defined as CMML-1 and patients with 10% to 19% and/or when any Auer rods are present as CMML-2 (5). In addition, CMML can also be subtyped according to the French-American-British classification, first described in 1994, in dysplastic (MD-CMML) and proliferative (MP-CMML) subtypes, based on white blood cell count $\leq 13\times10^9$/l and $>13\times10^9$/L (13). MP-CMML is known to have an adverse prognosis compared to MD-CMML (2, 10, 14).

1.2 Treatment

There is to date no CMML-specific treatment. The only curative treatment is allogeneic hematopoietic stem cell transplantation (HSCT). Due to high age, comorbidities and low performance score, most CMML patients are not eligible for HSCT, and overall only about 10% of CMML patients undergo HSCT treatment (10, 15). In patients not eligible for HSCT, treatment options consist of supportive care using transfusions or EPO if anemia is present (2, 16). For cases with profound peripheral blood leukocytosis but no increase in bone marrow blasts, cytoreductive treatment with hydroxyurea is recommended (2, 16). For high-risk MD-CMML, hypomethylating agents (HMA) is the standard of care (2, 10, 16). Azacitidine (AZA), a HMA, was approved for MD-CMML with increased blast count, however, AZA can also be used in MP-CMML and have a known effect on inflammatory symptoms even though the disease response is considered lower in MP-CMML compared to MD-CMML (16-18).

In the, to our knowledge, first and thus far only prospective clinical trial in CMML, MP-CMML patients were randomized to either treatment with hydroxyurea or HMA (decitabine), with a primary endpoint of difference in event free survival (19, 20). There was no difference in event free survival between the groups, although decitabine treated patients had better response rate and less AML transformation, and hydroxyurea treated patients had less adverse events (19).

Recently AZA was also used in combination with ruxolitinib with promising effect on response, however, the study only included 23 CMML patients and further studies are needed (21).

Ultimately, HMA can result in temporary hematological response but is not disease modifying (2, 10, 17). Some novel treatments are being investigated, such as monoclonal antibodies Lenzilumab (anti-GM-CSF) and IO-202 (anti-LILRB4), but further studies are needed in search of CMML-specific disease-modifying therapy (22, 23).

1.3 Genetic abnormalities

About 30% of CMML patients have cytogenetic abnormalities, of which the most common are trisomy 8 and loss of chromosome Y, seen in ~5% respectively (24-27). Trisomy 8 is associated with high risk-disease and isolated -Y is associated with a favorable outcome (24, 27). Complex karyotype, e.g. three or more cytogenetic aberrations, is rare in CMML (24).

Somatic myeloid mutations are found in around 95% of CMML patients, the most frequently mutated genes are presented in table 2 (9, 28-30). The most common functions affected involves DNA methylation (*TET2, DNMT3A*), histone marks (*ASXL1, EZH2*), spliceosome components (*SRSF2, U2AF1*) and cell signaling (*NRAS, KRAS, JAK2*) as well as transcription factors (*RUNX1, SETBP1*) (9, 28-31).

The most frequently mutated gene in CMML, *TET2*, encodes a protein catalyzing the conversion of 5-methyl-cytosine (5mC) to 5-hydroxymethylcytosine (5hmC), and eventually the demethylation of DNA (Figure 1) (32). Loss-of-function $TET2^{MT}$ increase stem cell renewal in the hematopoietic compartment and is associated with a favorable prognosis (33-35). Apart from regulating differentiation through epigenetic modulation, both positive and negative regulation of inflammatory immune responses have been attributed to TET2 protein (36).

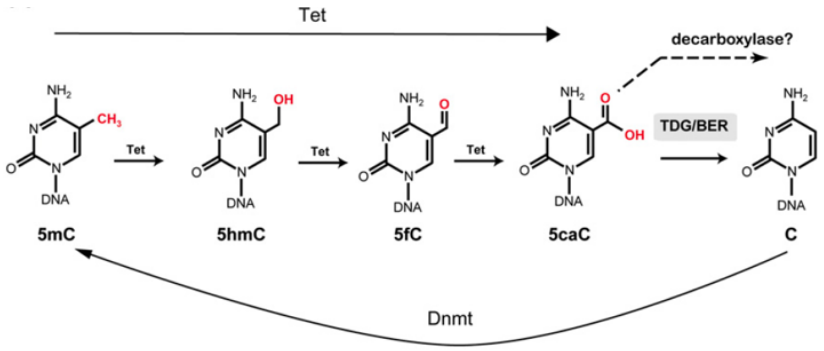


Figure 1. Demethylation by Tet-family and base excision repair. Methylation by Dnmt-family. BER = Base excision repair, TDG = Thymine-DNA glycosylase. Reprinted with permission (32).

Table 2. Prevalence of mutations in the most frequently mutated genes in CMML and the biological functions affected.

Mutated Gene	Prevalence	Affected biological machinery	References
TET2	50-60%	DNA methylation	(26, 29, 37, 38)
SRSF2	30-50%	Splicing	(26, 29, 37, 38)
ASXL1	30-40%	Histone marks	(26, 29, 37, 38)
NRAS	10-20%	Cytokine Signaling	(26, 38)
CBL	10-20%	Cytokine Signaling	(26, 38)
RUNX1	10-20%	Transcription	(9, 26, 29, 38)
KRAS	10-15%	Cytokine Signaling	(26, 38)
U2AF1	5-10%	Splicing	(26, 38)
JAK2	5-10%	Cytokine Signaling	(26, 29, 38)
SETBP1	5-10%	Histone Marks/Transcription	(9, 26)
ZRSR2	5-10%	Splicing	(26, 38)
DNMT3A	5-7%	DNA methylation	(26, 29, 38)
EZH2	4-8%	Histone Marks	(26, 38)
IDH2	5%	DNA methylation	(26, 38)
SF3B1	5%	Splicing	(26, 29, 38)

The *ASXL1* gene codes for a polycomb group protein, playing a critical role in gene silencing, through histone modifications, resulting in chromatin condensation (39). Loss-of-function mutations in *ASXL1* hence lead to the reduction of repressive marks and decondensation of chromatin (39, 40). Mutations in *ASXL1* are known to confer an adverse prognosis in CMML, as well as in other myeloid malignancies, and it is included in several prognostic scores (41-44).

SRSF2 codes for a protein important in pre-RNA splicing and mutations in the *SRSF2* gene lead to abnormal splicing patterns (33).

Mutations affecting cell signaling pathways include RAS-pathway mutations (eg. *NRAS*^{MT}, *KRAS*^{MT}) and *JAK2* mutations. RAS-pathways mutations as well as *JAK2* mutations have been associated with proliferative features in CMML (9, 33).

1.4 Prognostic scoring systems

The reported median overall survival for CMML is 24 months but varies greatly between patients, and some individuals can live for more than a decade without treatment, reflecting the heterogeneity of disease and the need of powerful prognostic tools to correctly predict prognosis in a unique individual (9, 10, 15).

A widely used prognostic scoring system for CMML is the CMML-Specific Prognostic Scoring System-molecular (CPSS-mol), first published in 2016 (41). CPSS-mol includes mutations in *ASXL1*, *NRAS*, *RUNX1* and *SETBP1* in addition to cytogenetic risk, bone marrow blast percentage, white blood cell count and transfusion dependency that made up the previous CPSS (45). The Revised International Prognostic Scoring System for Myelodysplastic Syndromes molecular (IPSS-M) includes clinical parameters and mutations in 31 genes and has since it was developed been used for MD-CMML with leukocyte count ≤13×10^9/l as 272 MD-CMML patients were included in the training cohort (42). However, recent evidence suggests IPSS-M could also be used in MP-CMML (30). Palomo et al showed that IPSS-M had comparable prognostic power and improved discrimination compared to CPSS-mol, with c-indices of 0.678 for IPSS-M and 0.629 for CPSS-mol for their study cohort of 511 mixed MD-CMML and MP-CMML patients (30). Recently the BLAST-mol system was developed using only information about blasts, leukocyte count and anemia as well as mutation status with performance comparable to CPSS-mol, with AUC at 5 years at 0.86 compared to 0.75 for CPSS-mol in their cohort (46). iCPSS is also a novel prognostic model discerning subgroups of differing overall survival as well as leukemia free survival (47).

Previously, when mutation status was not readily available for all patients, scoring systems including primarily clinical parameters were more useful. The most

prevalent were the CPSS, the MD Andersson Prognostic Scoring system (MDAPS) the Mayo prognostic scoring system and the revised International prognostic scoring system (IPSS-R) and they all included in some variation information about bone marrow blast count, white blood cell count, and hemoglobin level with some also including karyotype as well as other peripheral counts (45, 48-50).

With highly variable outcome and prognosis, reliable prognostic scoring systems are important in CMML management. Despite genetic information improving prognostic scoring systems over the last decade, the most accurate scoring systems still only have c-indices of approximately 0.6-0.7 and further studies are needed to improve the prediction of prognosis for an individual patient. (2, 10, 30, 51).

1.5 Autoimmune comorbidities

Autoimmune disease (AID) is seen in 10-25% of CMML patients (52-55). AID has been reported to be associated with lower risk disease; however no study has demonstrated a significant impact of AID on overall survival (52-55). The most frequent concomitant AID are rheumatoid arthritis, vasculitis, and immune mediated thrombocytopenia (ITP), however polymyalgia rheumatica (PMR), gout, psoriasis, inflammatory bowel disease and uveitis have also been reported (52, 55-57). The majority of concomitant AID are diagnosed 0.5 months prior to the CMML diagnosis, with a range of 0 to 44 months (52, 57). Some AID diagnosed prior to the CMML diagnosis are arguably actually early CMML disease manifestations, such as "undefined inflammatory syndromes", the most common AID in a study by Zahid et al (52). However, other AID, defined with positive serological testing are also more frequent in CMML than in the general population and if the AID manifestations are driven by CMML or if CMML is caused by an underlying chronic AID needs further investigations.

1.6 Platelet characteristics

Platelet size, size variation and immature platelet fraction can be measured in clinical routine by established parameters mean platelet volume (MPV), platelet distribution width (PDW) and immature platelet fraction (IPF%). The IPF% represent the fraction of platelets newly released in periferal blood by bone marrow megacaryocytes, and could potentially be used to discriminate causes of thrombocytopenia (58). Increased levels of MPV, indicating large platelets, have been described in myeloid neoplasms MDS and primary myelofibrosis (PMF) and is possibly associated with inferior overall survival in PMF (59). Dysplastic megakaryocytes are frequently found in CMML, however to our knowledge,

platelet size, variation and immature platelet fraction has not been assessed in CMML.

1.7 Alterations in the epigenome

Hematopoiesis is a process where dynamic regulation of stem cell differentiation and self-renewal is required for physiological maintenance of blood cell production during a lifetime of challenges, from bleeding to infection and injury. Epigenetic markers, both DNA methylation and histone modifications are known to regulate gene expression and differentiation (60). The most common form of DNA methylation is the addition of a methyl group in the C5 position of cytosine, particularly common on a cytosine preceding guanine, called a CpG-site, and early studies showed that about ~1% of DNA was 5mC (60-62). Genomic imprinting and X-chromosome inactivation are among the first phenomena found to be regulated by DNA-methylation, but DNA methylation also regulates hematopoiesis and DNA methyltransferases (DNMT-family) play a crucial role in regulating hematopoietic differentiation (63).

Global DNA hypomethylation was one of the first epigenetic aberrancies described in cancer, however, local hypermethylation of eg. promoters have repeatedly been linked to disease (62, 64). CpG sites are enriched in promoter regions and aberrant DNA methylation is thought to particularly affect promoters, attracting methyl binding proteins which repress gene expression, hence silencing tumor suppressor genes in cancer (8, 65).

As DNA methylation is a mechanism through which differentiation is controlled, aberrant DNA methylation could potentially have different effects in separate cell populations depending on the differential status (61). However, DNA-methylation analysis of bulk bone marrow mononuclear cells (MNCs), including all stages of progenitors and mature cells, have been able to correlate global DNA-methylation status to both disease severity and prognosis in CMML (8). *TET2*^{MT} resulted in the largest number of differentially methylated probes (DMPs) compared to any other mutation. *TET2*^{MT} patients displayed significantly higher methylation levels and interestingly the *TET2*^{WT} patient group had the most advanced disease features such as higher CPSS groups, as well as inferior overall survival, while having a methylation signature most similar to controls (8). Further studies are needed to elucidate the implications of aberrant DNA methylation for the pathobiology of CMML by e.g. combining RNA expression analyses as well as epigenetic and DNA sequencing.

1.8 Clonal development

CMML is a stem cell disease and the disease driving mutations arise in the hematopoietic stem cell (HSC) compartment (26, 66). Clonal HSCs harboring these mutations have a competitive advantage in the HSC compartment comparted to normal HSC which allows the disease to propagate (66). Various distinct molecular trajectories have been described to result in the granulomonocytic bias. For instance, RAS-pathway mutations (e.g. *NRAS*^{MT}, *KRAS*^{MT}, *CBL*^{MT}, *PTPN11*^{MT}, *NF1*^{MT}) are known to result in granulomonocytic expansion and is associated with MP-CMML and leukocytosis while *TET2*^{MT}/*SRSF2*^{MT} as founding mutations also results in granulomonocytic differentiation but is rather associated with MD-CMML and dysplastic features (26, 67). However, neither RAS-pathway lesions nor *TET2*^{MT} completely corresponds to either subtype of CMML and the relatively homogenous mutational landscape of CMML can not explain the more heterogenous clinical features and prognosis of CMML (9).

Palomo et al aimed to link morphological features to mutations and combinations of mutations but included different diagnoses of MDS/MPN, including CMML, demonstrating that that multiple *TET2*^{MT}, *SRSF2*^{MT} and *RUNX1*^{MT} in combination correlated to a CMML phenotype and was less frequently seen in other MDS/MPN subtypes (26). Other phenotype-genotype correlations made within the CMML population are *JAK2*^{MT} and proliferative features as well as RUNX1 and thrombocytopenia (9, 26).

However, the presence of mutations alone cannot explain the CMML clinical presentations, but patterns of clonal expansion and clonal branching must be considered, as well as other non-genetic mechanisms such as transcriptional or epigenetic aberrations. For instance, in a study of 19 CMML patients and 12 age-matched controls CMML HSCPs were shown to have a downregulation of proinflammatory genes and increase in the repressive H3K9me2 mark (68). Further, cytokine mediated clonal expansion should also be considered, in mice *TET2*^{MT} has been shown to result in increased macrophage secretion of proinflammatory cytokines interleukin-6 as well as interleukin-1β, the latter being known to contribute to leukemic clonal expansion (9, 69, 70).

In order to explain the discrepancy between relative mutational homogeneity but clinical heterogeneity in CMML, not only are more extensive mutational studies needed but also their combination with other techniques such as analysis of transcription (RNA sequencing) and DNA methylation analysis.

1.9 *TET2*^{MT} in Clonal Hematopoesis of Indeterminate Potential

Clonal hematopoesis of indeterminate potential (CHIP) is the presence of at least one somatic mutation in absence of cytopenia and MDS and all other hematopoietic neoplasms and disease. Mutations in *DNMT3A*, *TET2* and *ASXL1* are most frequent (71–73). CHIP is present in around 10% of persons over 70 years old, and around 20% of persons over 90 years old and is associated with an increased risk of developing hematologic cancer (71–73). Tulstrup et al demonstrated that in CHIP as well as in clonal cytopenia of undetermined significance (CCUS), *TET2*^{MT} associated DNA hypermethylation is enriched in enhancer regions and genes involved in leukocyte function and immune responses, and the majority of *TET2*^{MT} associated methylation changes are shared between CHIP and AML (74). Persons with *TET2*^{MT} CHIP have been described to have a higher incidence of cardiovascular events as well as diabetes type 2 and accelerated artherosclerotic development have been described in *TET2*^{KO} mice (69, 71, 75, 76).

To further understand the role of *TET2* in CHIP, Huerga Encabo et al studied human *TET2*^{WT} and *TET2*^{MT} human stem and progenitor cells (HSPCs) in vitro and found that upon long term stimulation with lipopolysaccharide (LPS), mimicking chronic inflammation, the progenitor cells attenuated inflammatory stimuli while mature monocytes showed hyperactivation of inflammatory response pathways (Figure 2) (77). If it is the case that *TET2*^{MT} CMML evolves from CHIP and CCUS, these features may be shared between CHIP and CMML. If the same patterns of attenuation to inflammatory stimuli in progenitors are true also for CMML, studies are needed to elucidate what implications that would have for prognosis, subgrouping and management of *TET2*^{MT} CMML in contrast to *TET2*^{WT} CMML.

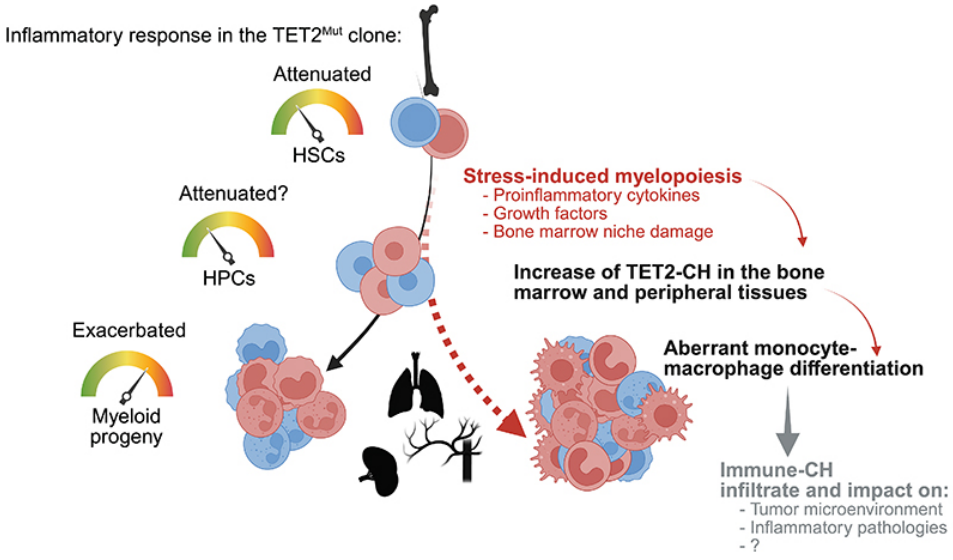


Figure 2. Schematic overview of potential mechanisms of how TET2^{MT} in CHIP may affect differentiation as well as cause differential phenotype in progenitors and mature cells respectively. Reprinted with permission (77).

2 Research aims

Paper I

To perform, in a nationwide population-based cohort, 'real-world' comparison of scoring systems and comorbidity indices in CMML and to also present detailed data on incidence of CMML, clinical characteristics including cytogenetics, treatments, comorbidities, AML-transformations and survival.

Paper II

To explore the relation between disease characteristics, comorbidities, mutations and overall survival (OS) in CMML in a population-based cohort.

Paper III

To investigate platelet size, size variation and immature platelet fraction (MPV, PDW, and IPF%) in relation to disease characteristics, mutation status, and clinical outcome, in treatment naive CMML patients.

Paper IV

To understand the association between DNA methylation patterns, myeloid mutations and chromatin state as well as subsequent effect on transcriptional output and disease outcome, in CMML.

3 Materials and methods

Paper I. In paper I, we performed a detailed retrospective chart review of 359 patients with CMML, collected at 53 hospitals in Sweden. These patients represent all CMML cases diagnosed between 2009 and 2015 in Sweden and reported to the Swedish MDS registry (SMDSR), which have an estimated coverage of 96% of all diagnosed MDS patients (78). Information collected from the charts included laboratory parameters at diagnosis, prior history of chemotherapy and radiation, comorbidities, transfusions, diagnostic procedures including cytogenetics and bone marrow morphology. The cytogenetic report was reviewed centrally by a clinical geneticist. To confirm information about transformation to AML, patients were cross referenced to the Swedish Adult Acute Leukemia Registry.

The fraction of patients with somatic mutations analysed as part of the routine diagnostic workup was low at this time, and mutation status was therefore not included in this study.

Statistical analyses in paper I included a cox regression model to explore the independent effect of comorbidity indices on survival. Significant variables from the univariate analyses were considered in the multivariable analyses. The final variables were chosen using backward elimination and included age, monocyte count, CPSS-group and the comorbidity index Charlson Comorbidity Index (CCI-score). To evaluate the cox-model, the Akaike information criterion (AIC) was calculated, and including the CCI score improved the AIC. The risk transformation to AML was calculated using the cumulative incidence function to account for the competing risk of death. To evaluate the prognostic scoring systems in paper I the comorbidity indices, the Harrell concordance (C) index was used (79). The C-index ranges between 0.5 and 1, where 1 stand for perfect discrimination and 0.5 for no discrimination at all. All analyses were performed using R version 3.2.2 and 3.5.1 (R foundation for statistical Computing, Vienna, Austria) and SPSS statistics for Windows, Version 24 (IBM, Armonk, NY, USA).

Paper II. For this study, we performed a thorough, retrospective chart review of all patients diagnosed with CMML at Karolinska University hospital between 2002 and 2019 (n=149). Extensive information was collected from the charts, including information on laboratory parameters at diagnosis, prior history of chemotherapy, irradiation, thrombosis, bleeding, atrial fibrillation as an indication of cardiovascular risk and gout. We also collected data on transfusions, diagnostic procedures including cytogenetics and bone marrow morphology and outcome. Cytogenetic

analyses were performed at regional clinical genetic laboratories according to clinical routine and reviewed centrally by one of the investigators. We also were able to collect information about somatic mutations for this cohort. Cases that had not undergone sequencing analysis as part of the clinical diagnostic routine for Karolinska University Hospital for this time period were submitted for a myeloid panel as part of this research study.

To assess the distribution of baseline patient characteristics, we performed standard descriptive analyses including the Mann–Whitney U-test and Fisher's exact test. Kaplan–Meier curves were compared using the log-rank test. To investigate the independent effect of clinical and molecular parameters on OS, a Cox regression model was developed. Variables of interest and/or of clinical relevance that were also significant ($p < 0.05$) from the univariable analyses were used in the Cox regression model. We also included the 10 most common mutations, to ensure that potentially relevant factors were not omitted, despite not all being significant with $p < 0.05$ in univariable analysis. The final Cox regression model was defined using stepwise selection, and AIC was used to assess it. All analyses were performed using R version 4.4.2 (R Foundation for Statistical Computing, Vienna, Austria).

Paper III. In paper III, we performed a single-center, retrospective, explorative study including all patients diagnosed with CMML at the Karolinska University Hospital within the time period of January 2019 to October 2023. Patients were only included in the cohort if they were not under treatment with any cytoreductive therapy for CMML, at the time of MPV, PDW and IPF% sampling. Follow-up samples were collected for the 12 months following diagnosis in patients who remained untreated during this time. Information collected from the charts included routine peripheral blood counts. Pathology reports were reviewed for bone marrow blast count, megakaryocyte dysplasia, cytogenetics and somatic myeloid mutations. Mutation annotations were limited to the five most mutated genes in larger CMML cohorts, due to the small cohort size.

Statistical analyses in paper III were performed using R version 4.4.1 (R Foundation for Statistical Computing, Vienna, Austria). Discrete variables were compared using Fisher’s exact test or Pearson's Chi-square test. Continuous variables were assessed for normality with the Shapiro-Wilk test when appropriate, and for homogeneity of variances using Levene’s test. Non-normally distributed data were compared using Wilcoxon rank sum test while normally distributed data (all

with equal variances) were compared using Student's t-test. Heatmaps were produced with ComplexHeatmap, using Spearman's rank correlation as distance metric and average linkage as hierarchical clustering of rows and columns. Kaplan-Meier survival analysis was performed using log-rank test. All-cause mortality was used as measure of clinical outcome and patients were censored in case of allogeneic stem cell transplant or at the end of study period (June 17, 2025), whichever came first.

Paper IV. In this paper we investigated diagnostic bone marrow samples from an international cohort of CMML patients, from biobanks in Karolinska Institutet, Sweden; University of Helsinki, Finland; Münchner Leukämielabor (MLL), Germany and Kyoto University, Japan. For each CMML patient, either buccal swab or CD3+ sorted cells were used as germline control. Samples were subjected to whole exome sequencing performed in Kyoto University and samples were analysed using the Genomon pipeline (genomon pipeline 2.6.3, https://github.com/Genomon-Project) and paired germline samples to filter somatic variants. DNA methylation analysis was performed using the Illumina Infinium MethylationEPIC BeadChip array (Illumina, Inc., San Diego, CA, USA). For a subset of 5 *TET2*mt and 5 *TET2*wt samples assay for transposase-accessible chromatin using sequencing (ATAC seq), chromatin immunoprecipitation sequencing (ChIP seq), with H3K4me3, H3K27ac, and H3K27me3 histone modification antibodies, and RNA sequencing was also performed in Kyoto university. For the whole cohort clinical information was collected, including information on laboratory parameters at diagnosis, karyotype and information of transformation to AML.

To define chromatin states across the genome, specific to the CMML samples in the cohort, the ChromHMM model (80) was used with the ATAC seq and ChIP seq data as input to define chromatin states for the study specific cohort. ChromHMM were used to learn models based on 5 to 16 chromatin states. We selected the model of 7 chromatin states generated by ChromHMM, based on correlations between models (5 states model to 16 states model).

Statistical analyses were performed using R version 4.4.2. Unsupervised clustering of mutation data was made using Hclust stats and the Ward D method. Unsupervised clustering of normalized b-values was made using consensus clustering (81). Survival analyses were made using survfit() from survival (82) and OR was calculated using oddsratio() from epitools (83). Enrichment analyses for hypermethylated genes, genes associated to hypermethylated enhancers and

genes that shift in chromatin state were made using enricher clusterProfiler (84). GSEA of RNAseq and ATACseq data was made using hypeR (v 2.0.0) (85).

Ethical Considerations

The conceivable ethical risk of note in all four studies was that of potential breach of integrity of the patient by handling sensitive personal data. The data was of course anonymised after collection but as long as the key exists this data must still be considered as sensitive and personal. In paper I, because of the nationwide scale, the ethical board accepted the study without participants providing written consent. All patients were included in the national MDS registry. Patients are informed that they are included in this registry upon MDS diagnosis, but written consent is not required, so there is a risk of patients being included despite them not explicitly agreeing to it. In papers II and III written consent was collected for alive patients, but deceased patients were included without written consent, which implies a risk some of these patients were included against their will. In paper IV all patients had provided written consent. Overall, even though real, the risk of actual breach of integrity was low in these studies as no single patient was analysed in specific and only larger groups and compiled data was used. The benefit of gained knowledge should in these cases largely outweigh the low risk of beached integrity in these studies.

In paper IV, an additional potential risk of note is that of handling extensive genetic data, including germline data. The germline data, however, was only used as background to filter out somatic variants. We only used the germline data for filtering in an automated pipeline, it was never considered on its own, never individually reviewed by a human, and therefore the risk of discovering any potential incidental finding of e.g. a hereditary disease was never there. However, as long as the whole exome data files from germline samples exists, they are sensitive personal data that must be handled properly.

4 Results and Discussion

4.1 Clinical characteristics and comorbidities – papers I-IV

A clear strength of the cohorts in paper I, II and III is their population-based nature. Paper I is nationwide and paper II and III represent unselected cohorts of all CMML patients diagnosed within the catchment area of Karolinska Hospital in Stockholm. As the median age at diagnosis in papers I, II and III was 75-76 years, slightly older than previously reported and in paper I a slightly higher national incidence of 0.51 compared to 0.4 per 100'000 reported in literature as well as longer overall survival and lower frequency of AML transformation, we believe these cohorts reflect a 'real-world' dataset, including to a large extent also low risk and potentially older patients that studies based on cohorts from large referral centres might miss (Table 3) (9, 10).

A majority of CMML cases in all papers were CMML-1 (70-94%) and MP-CMML (56-63%) (Table 3). Previous studies have reported different distributions of WHO-groups and FAB-groups. Palomo et al presented 20% CMML-2 in their recent study and 33% MP-CMML (30). Coltro et al also described 20% CMML-2 in their large cohort of over 1000 patients, and 50% MP-CMML (35). In papers I and II the study cohorts are unselected, and few diagnosed cases are unlikely missed. We see a slightly higher incidence of MP-CMML in papers I and II than in previous studies, but we can not know if our cohorts represent the true distribution of CMML or e.g. if some cases with lower WBC are misdiagnosed as MDS instead of MD-CMML. MP-CMML is known to be associated with shorter overall survival but despite this we see slightly longer overall survival in all our papers (Table 3). All papers are based on patients diagnosed before 2022 when the cutoff for monocyte levels at diagnosis was lowered from 1 to 0.5 x 10^9/L.

The majority of patients in our studies had a normal karyotype, as is expected for CMML. In papers I, II and IV about one third of patients had an abnormal karyotype, which is line with previous studies (10). The prevalence of cytogenetic abnormalities is considerably lower in CMML compared to MDS where around 50% of patients have abnormal karyotype (86).

Papers I and II studied comorbidities. In paper I cardiac conditions was the most prevalent comorbidity (34%), followed by autoimmune disease (25%). In paper II AID was the most common comorbidity, present in 34% of patients. The most frequent AID found in both independent cohorts were Polymyalgia rheumatica,

Hashimoto's thyroiditis and psoriasis. It is clear that AID is overrepresented in CMML, compared to the general population where the incidence is reported to be 3% (87). AID is also overrepresented in CMML compared to MDS, where AID can be seen in 10% of patients, including studies in which CMML and MDS are grouped together, arguably making the real difference in AID presence between CMML and MDS even larger (53, 54). In paper II, patients with concomitant AID (n=50) had a median overall survival of 20 months compared to 32 months for non-AID patients (n=99). This difference was not statistically significant as the Kaplan Meier curves crossed after 6 years of follow-up, at a time when only 14 patients were still alive. Previously, concomitant AID has been associated to a lower Mayo clinic prognostic score, but no significant difference in overall survival depending on AID (52). Our paper II was based on in depth patient chart review and reported a higher incidence of concomitant AID, and the available studies on AID and CMML have not used exactly the same definition of AID, which makes results difficult to interpret. Larger studies are needed to elucidate if this overrepresentation of AID is a trigger and driver of CMML or rather a secondary symptom and paramalignant phenomenon of the CMML disease itself. In paper II thrombosis, also known to be a paramalignant symptom, was present in 15% of patients and was borderline significantly associated with shorter overall survival.

In paper I CCI, HCT-CI and MDS-CI, comorbidity scoring tools often used in HSCT work up, was applied to all patients at diagnosis and had C-index of 0.62, 0.61 and 0.59 respectively. Their performance improved for the subgroup of the cohort <70 years of age and in low risk patients, indicating that comorbidities have a larger prognostic impact in low risk disease and the added disadvantage of the comorbidity is of less importance in advanced disease. The most frequent comorbidities are inflammatory, and it could be discussed if these are comorbidities acquired prior to CMML diagnosis at all or actually early manifestations of the CMML disease.

Table 3. Compilation of cohorts studied.

	Paper I	Paper II	Paper III	paper IV
Cohort	Sweden (2009-2015)	Stockholm-Karolinska (2002-2019)	Stockholm-Karolinska (2019-2023)	Samples from Biobanks in Stockholm, Helsinki, Kyoto, MLL
Inclusion criteria	All CMML patients included in SMDSR	All CMML patients with consent	All CMML patients with consent and untreated at lab-sampling	Samples available from CMML patients with informed consent
Total number of patients	337	149	55	152
Median follow up time, years	5.6	6.5	na	3.4
Median OS, months (95% CI)	21.3 (17.8–24.8)	29.6 (20.4 - 36)	45.7	35.6 (29.8 – 44.8)
Sex				
Male	61%	65%	60%	66%
Female	39%	35%	40%	34%
Age at diagnosis, years Median (range)	76 (28-97)	75 (24 - 97)	76 (28-90)	72 (24 - 91)
Hemoglobin, g/dl Median (range)	108 (30-168)	113 (64 - 153)	118 (63-168)	107 (8 - 153)
WBC, x 10^9/L Median (range)	19.2 (1.2 - 213.2)	15.9 (3.2 - 152)	11 (5-78)	18.2 (3.10 - 373)
Platelet count, x 10^9/L Median (range)	113 (8 - 928)	123 (15 - 1080)	120 (35 - 596)	105 (7.00 - 1230)
Monocyte count, x 10^9/L Median (range)	3.9 (0.1 - 91.0)	3.6 (0.3 - 62)	3 (1-16)	4.6 (0.28-15.8)
WHO subtype				
CMML 1	80%	70%	94%	73%
CMML 2	20%	30%	6%	27%
FAB				
CMML-MD	37%	44%	na	38%
CMML-MP	63%	56%	na	62%
Karyotype				
Normal	69%	69%	92%	73%
Abnormal	31%	31%	8%	27%
RBC transfusion dependency at diagnosis				
No	69%	93%	na	na
Yes	31%	7%	na	na
AML transformation				
Yes	18%	29%	na	29%

4.2 Paper I

In paper I, scoring systems available that did not include mutation data for CMML were evaluated. The overall survival according to the risk groups in each prognostic scoring system is presented in figure 3. The C-index of the CPSS had a slightly better C-index of 0.69, compared to MDAPS and Mayo score (C-index of 0.65 and 0.66 respectively) but these differences were non-significant. The C-index of CPSS was however significantly better than that of the IPSS-R which had a C-index of 0.60 (P = 0.004).

Next, in subgroup analysis of patients aged <70 years the C-indices were 0.78, 0.67, 0.66 and 0.65 for the CPSS, IPSS-R, MDAPS and Mayo score, respectively and the CPSS C-index was significantly better than the c-indices of the other scoring systems. This is of note, as it is most commonly in the younger age group that HSCT is considered and reliable prognostic tools are of especially important. For patients aged ≥70 years all scoring systems were borderline significantly better than the IPSS-R. The IPSS-R was also tested in the subgroup of patients with MD-CMML, but the prognostic power did not increase and thus in paper I it was concluded that IPSS-R should not be used in CMML.

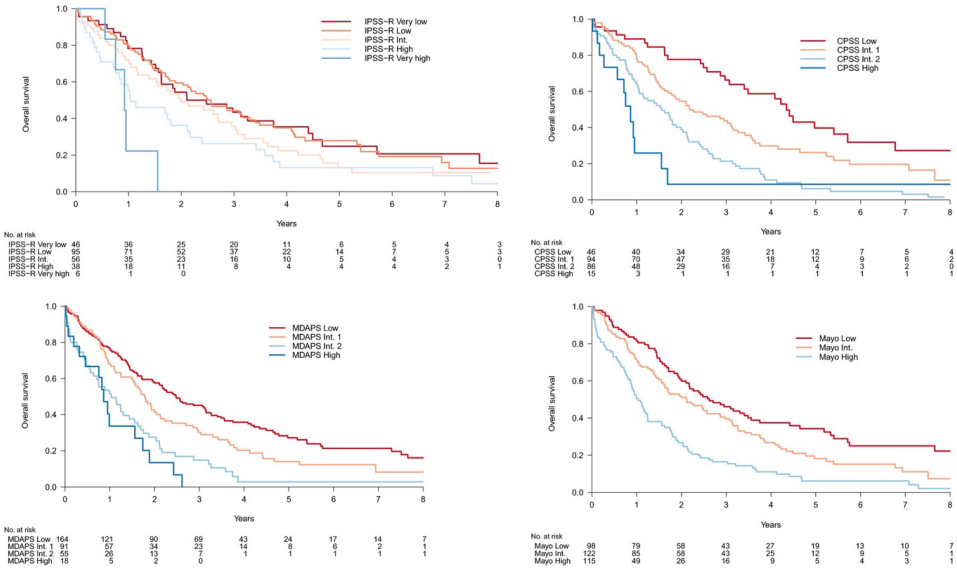


Figure 3. Overall survival categorised according to risk group in the prognostic scoring systems; IPSS-R, CPSS, MDAPS and Mayo score. Reprinted with permission from British Journal of Haematology.

In literature, there are conflicting results for which scoring system is performing best in CMML. Calvo et al found similarly that CPSS performed better than MDAPS and Mayo-score (88). Padron et al however, found that both CPSS and IPSS-R had the highest C-index in their study (89).

The scoring systems' performance for predicting transformation to AML was also evaluated. 18% of patients transformed to AML after a median of 16 months from diagnosis and the C-index of the CPSS, IPSS-R MDAPS and Mayo score was 0.68, 0.64, 0.66 and 0.62, respectively; these differences were non-significant. The median age of transformation was 72 and the prognosis after transformation was very adverse, with a median OS of 83 days after transformation.

To conclude findings in paper I, CPSS was found to perform best, especially in younger patients. Based on the findings, the use of IPSS-R should not be recommended in CMML. However, the prognosis of CMML is still very varying and better prognostic tools are needed. Scoring systems including mutations are developed but could not be evaluated in this study as mutation status was not included in the routine clinical diagnostic workup for a majority of the patients at the time during which these patients were diagnosed and no mutation data was available.

4.3 Paper II

In paper II information about mutations was available. It was found that *TET2*^{MT} was the most common mutation, found in 77/119 patients (64.7%), followed by *SRSF2*^{MT} 54/119 (45.4%) and *ASXL1*^{MT} 52/119 (43.7%). Patients with *TET2*^{MT} were significantly older, with a median age at diagnosis of 77 years compared to 69 years for *TET2*^{WT} patients. The *TET2*^{MT} patients had higher haemoglobin levels, more frequently CMML-MD and lower leucocyte and monocyte counts compared to *TET2*^{WT} patients (Figure 4). There was no difference in AID prevalence between *TET2*^{MT} and *TET2*^{WT} patients; however of note, CRP levels were significantly lower in *TET2*^{MT} than in *TET2*^{WT} patients. *TET2*^{MT} was also associated presence of *SRSF2*^{MT} and absence of *ASXL1*^{MT} and *SETBP1*^{MT}. Of the patients carrying *TET2*^{MT} a majority (66/77 patients) had multihit *TET2*^{MT}. The associations between clinical manifestations and *TET2*^{MT} were even stronger for multihit *TET2*^{MT}. In addition, multihit *TET2*^{MT} was associated with lower LDH and less monocytosis. So overall the *TET2*^{MT} patients displayed clinical features associated with favourable prognosis as well as less elevated inflammatory parameters and these patterns were even more clear in multihit *TET2*^{MT} than in singlehit *TET2*^{MT} patients. Presence *TET2*^{MT} and *ASXL1*^{MT} were

significantly inversely correlated, but not mutually exclusive, meaning some patients had both mutations. It could be discussed if further mutational subgroups would correlate even more with some clinical features, e.g multihit *TET2*^{MT}/*ASXL1*^{WT}, but our study was limited by too small numbers for these analyses.

Despite being 8 years older at diagnosis, *TET2*^{MT} patients lived longer with a median OS of 37 months compared to 20 months in the *TET2*^{WT} group (Table 4). Interestingly, when separating *TET2*^{MT} patients into groups of single-hit (n = 11) and multihit *TET2*^{MT} (n = 66), in fact *TET2*^{WT} (n = 42) and single-hit *TET2*^{MT} patients had a similar OS whereas multihit *TET2*^{MT} was associated with a significantly longer OS (Figure 5). These results imply that multihit *TET2*^{MT} acts as a separate disease entity in CMML, similar to a finding in a recent large analysis of mixed chronic myeloid neoplasms, where a subgroup of cases with multihit *TET2*^{MT} was associated to *SRSF2*^{MT}, older age, milder anaemia and diagnosis of CMML (90).

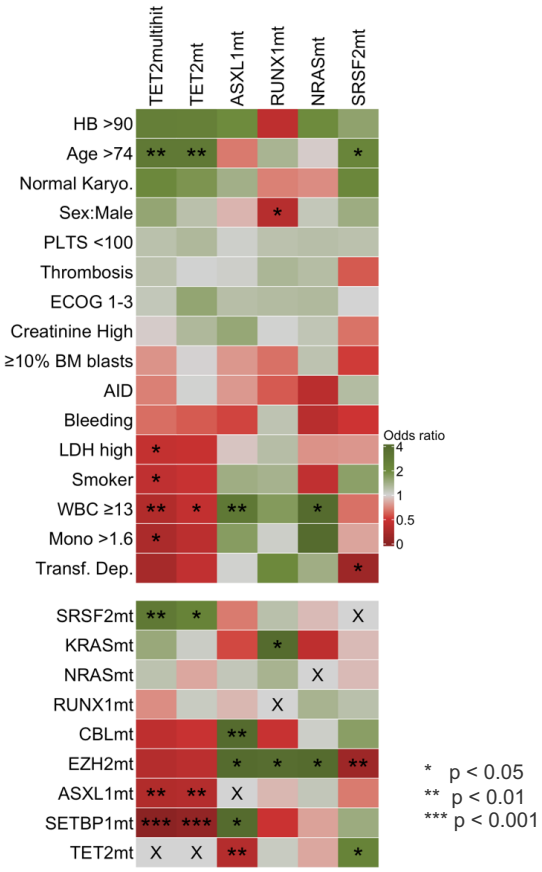


Figure 4. Associations between myeloid mutations and clinical parameters. Reprinted with permission from British Journal of Haematology.

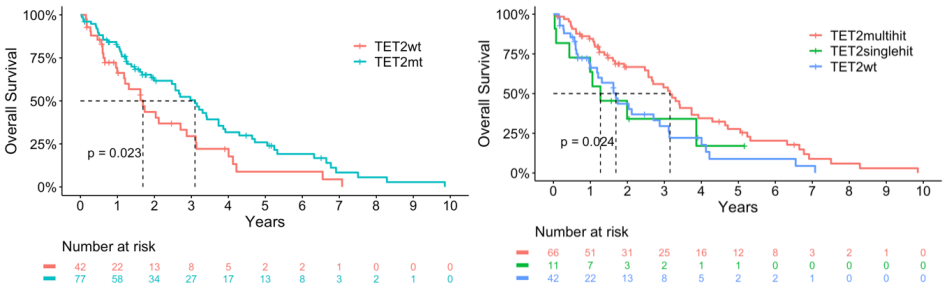
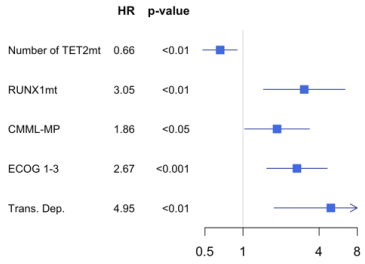


Figure 5. Overall survival depending on TET2 mutation status. Reprinted with permission from British Journal of Haematology

Next, a cox model was developed using the 10 most prevalent mutations as well as variables that had a significant impact on OS in univariable analysis, namely CMML-MP/ MD (as a categorical variable, proxy for leucocyte count), thrombosis, haemoglobin ≤90 g/L, age >74 years, ECOG >0 and transfusion dependence. Variables with too many missing values were excluded. After stepwise selection, number of *TET2*^{MT} remained an independent significantly beneficial parameter, and presence of *RUNX1*^{MT}, CMML-MP, ECOG >0 and transfusion dependence were independent factors for adverse prognosis (Figure 6). *RUNX1*^{MT} and MP-CMML are known to be associated with an adverse prognosis and both *RUNX1*^{MT} and WBC are included in CPSS-mol (41, 44). The ECOG performance status reflect the overall fitness of the patient and transfusion dependence is a proxy for severe anaemia. *RUNX1*^{MT}, WBC and severity of anaemia have been presented in prognostic scoring systems previously, although to our knowledge not in this combination and never with the combination of *TET2*^{MT} or the number of *TET2*^{MT}. The *TET2*multihit cox model was then evaluated against the currently recommended prognostic model for CMML, CPSS-mol (41). Overall, the *TET2*multihit model had superior discriminative power with C-index 0.72 compared to 0.64 for CPSS-mol, and significantly better calibration, as well as significantly superior overall performance compared to CPSS-Mol (Figure 6). Results in this paper suggest that *TET2*^{MT} and in particular multihit *TET2*^{MT} CMML acts as separate disease entity in CMML and the prognostic power of multihit *TET2*^{MT} should be considered for inclusion in novel prognostic scoring systems.



Analysis	Model	Score	
C-index	TET2multihit-model	72.4	
	CPSS-mol	63.8	
AUC at 5 years (95% CI)	TET2multihit-model	84.2 (71.9-96.5)	p < 0.01
	CPSS-mol	52.1 (30.7-73.5)	
Brier score at 5 years (95% CI)	TET2multihit-model	10.3 (5.7;14.8)	p < 0.01
	CPSS-mol	16.1 (9.0;23.1)	

Figure 6. Significant variables included in the TET2multihit cox model (left) and parameters of the TET2multihit model in comparison to CPSS-mol (right). Reprinted with permission from British Journal of Haematology

4.4 Paper III

Laboratory parameters at diagnosis were collected for 55 CMML patients. 94% of this cohort consisted of CMML-1 patients because CMML-2 patients were to a larger degree treated at time of first sampling and thus not included in the cohort. Overall, 62% (33/53), 68% (36/53), and 79% (26/33) of patients had MPV, PDW, and IPF% values above the reference range. Thus, the majority of the CMML patients in our cohort had elevated MPV, PDW and IPF% values, indicating that platelets are large, heterogeneous in size, and have a high turnover compared to patients with values within normal range.

Unsupervised clustering based on MPV, PDW, and IPF% values generated two distinct clusters (Figure 7). Cluster one consisted of patients with high MPV, PDW, and IPF% and low platelet count. While this combination has not been reported in healthy subjects (91), it is well established in patients with ITP, an immune-mediated disease with high platelet turnover (58, 92). These findings suggest that a combination of high IPF% and low platelet count may identify a subset of CMML patients with a paraneoplastic autoimmune, ITP-like disease phenotype that could conceivably be responsive to immunosuppressive therapy. This cluster was also enriched for *SRSF2*^{MT} and *TET2*^{MT}. Separately MPV and PDW levels positively correlated with presence of either *SRSF2*^{MT} or *TET2*^{MT}, but not with presence of any other mutation. Only the positive correlation with presence of *SRSF2*^{MT} remained significant after adjusting for multiple testing though. The tendency of correlating with *TET2*^{MT} could likely be explained by the well-established co-occurrence of *TET2*^{MT} and *SRSF2*^{MT} in CMML (93). The association between MPV and PDW and presence of *SRSF2*^{MT} has also been reported in subjects with clonal haematopoiesis, where mutations in splicing factors (*SRSF2*, *SF3B1*, and *U2AF1*) is

also associated with thrombocytopenia (94). An association between *SRSF2*^{MT} and megakaryocyte dysplasia has recently been described in MDS patients (95), and in primary human CD34+ cells, *SRSF2*^{MT} promotes megakaryocytic differentiation (96). These findings provide a mechanistic rationale for the association between megakaryocyte dysplasia, thrombocytopenia and changes in platelet phenotype that were observed *SRSF2*^{MT} CMML patients in this study.

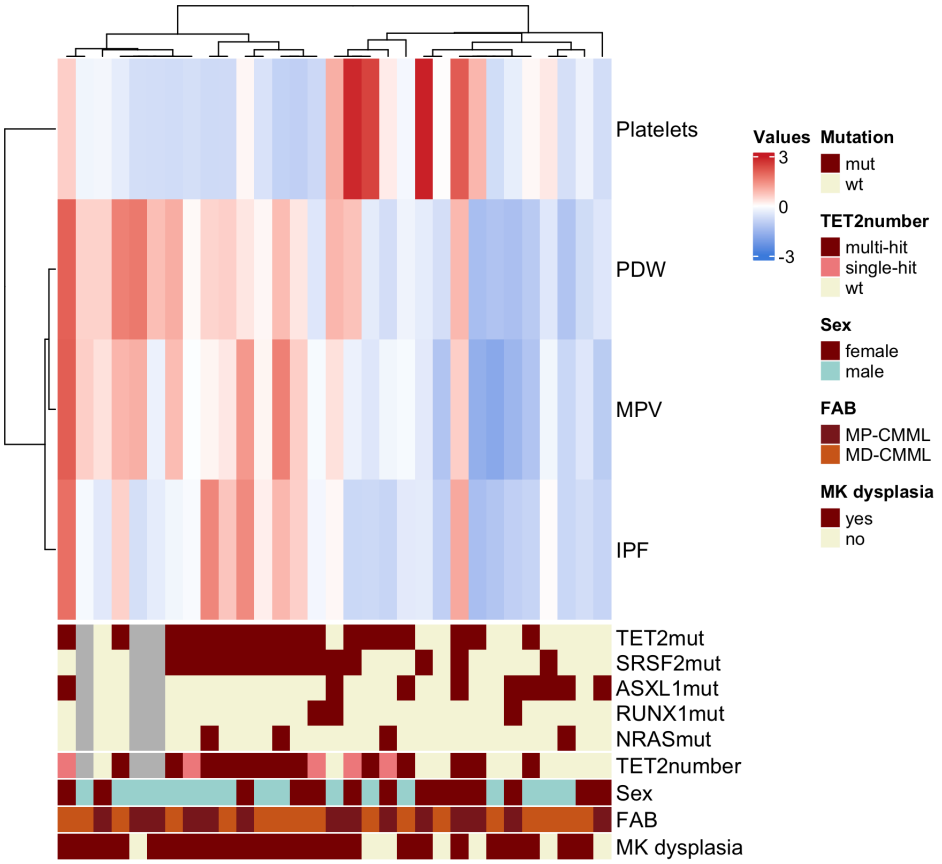


Figure 7. Unsupervised clustering of PDW, MPV, IPF%, and platelet count, showing two distinct clusters.

Median overall survival in the cohort was 45,7 months, considerably longer than in previous studies, likely due to the absolute majority of the cohort, 94%, being CMML-1. Patients with an elevated MPV had a significantly longer overall survival compared to patients with normal MPV levels (56 months compared to 22

months, p<0.05) (Figure 8). In a longitudinal assessment of MPV, PDW, IPF%, and platelet count we found no significant changes over time when comparing values at 3, 6 and 12 months to values at first sampling (0 months), indicating that MPV, PDW and IPF are markers of disease biology and that platelet dysregulation is a characteristic feature of CMML. Whether elevated MPV could be a clinically useful prognostic marker in CMML remains to be evaluated in larger independent studies, specifically including also more CMML-2 patients.

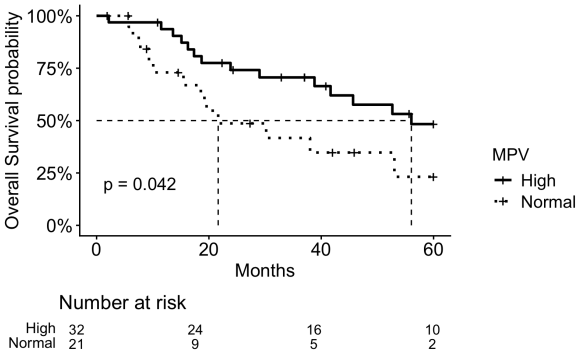


Figure 8. Overall survival depending on elevated (black line) vs normal (dotted line) MPV

4.5 Paper IV

In paper IV whole exome sequencing analysis of 141 paired tumor and germline CMML patients could confirm presence of mutations in *TET2* (59%), *SRSF2* (44%) and *ASXL1* (29%) and an especially high fraction of patients harboured multihit *TET2*^{MT}, that is 66% (41 of 62) of all *TET2*^{MT} patients. Clustering the patients based on the presence of recurrent mutations resulted in two distinct clusters heavily defined by *TET2*^{MT} status and *SRSF2*^{MT} and *TET2*^{MT} were commonly co-mutated (Figure 9). Next, patients were clustered using unsupervised clustering based on the beta values from DNA methylation analysis (Infinum EPIC array, Illumina) only, which also resulted in two distinct clusters (Figure 9). When fusing the methylation and mutation cluster data of these patients, we found a large overlap, indicating that the DNA methylation patterns are largely driven by *TET2*^{MT} status (Figure 10). Considering the function of the TET2 protein and that *TET2*^{MT} is known to cause a large number of DMPs in CHIP, it is not surprising that *TET2*^{MT} have an effect on the DNA methylome in CMML. Separation of CMML patients into two clusters based

on beta values have also been described by Palomo et al however our results show even clearer separation according to *TET2*^{MT} status (8). It is previously known, and as shown in paper II, that *TET2*^{MT} confers a favourable prognosis, in line with this it was seen that the *TET2*^{MT} cluster had a longer OS than the TET2wt cluster (Figure 10).

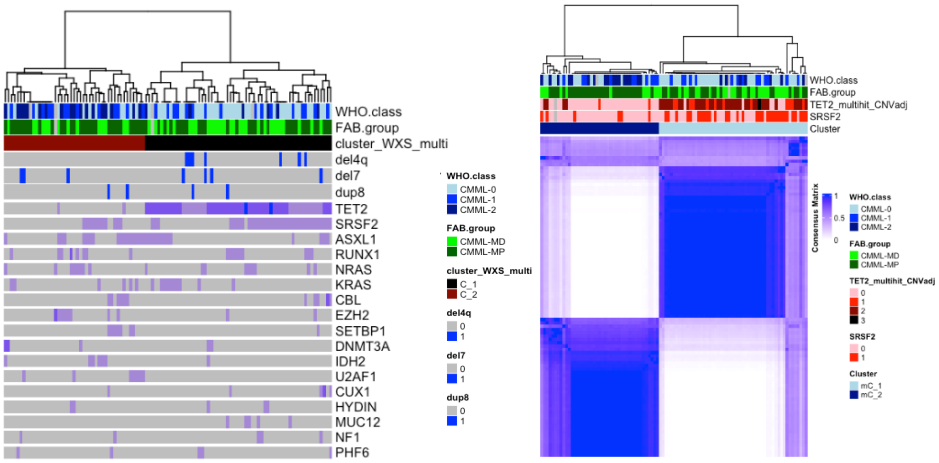


Figure 9. Unsupervised clustering based on mutations (left) or based on B-values only (right).

Analysis of differential methylation between the clusters showed that the *TET2*^{MT} cluster exhibited extensive hypermethylation (Figure 11). This has previously been shown in *TET2*^{MT} CHIP cases (74). A total of 9694 genes had at least one hypermethylated probe and in enrichment analysis of hypermethylated genes 12 significantly differentially methylated Hallmark pathways were found, among which several were inflammatory, e.g. the Hallmark Inflammatory Response

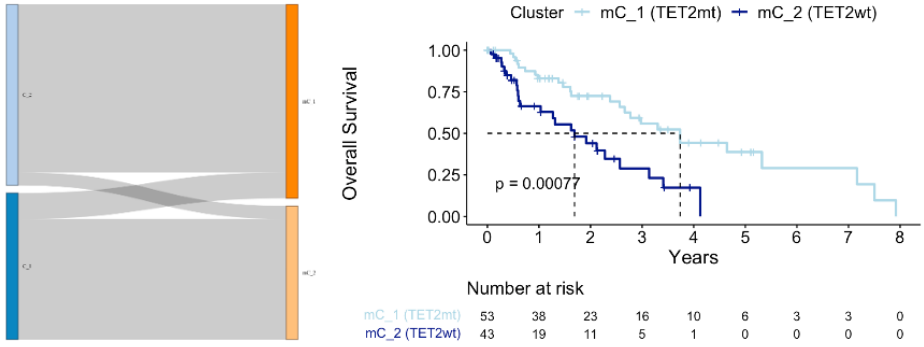


Figure 10. Riverplot of overlap between mutation and methylation cluster (left) and overall survival of methylation clusters (right).

pathway, IL2 Stat5 Signalling, and TGF beta Signalling pathways (Figure 11). These findings indicate an epigenetic silencing of inflammatory pathways in *TET2*^{MT} CMML compared to *TET2*^{WT} CMML. In the previous study by Palomo et al hypermethylation in the TET2mt cluster was also seen, and genes coding for inflammatory cytokines were hypermethylated in CMML compared to healthy and hypermethylation of immune response pathways in *TET2*^{MT} CMML patients compared to *TET2*^{WT} CMML patients (8). It was also seen that the hypermethylation found in the *TET2*^{MT} cluster was enriched in enhancer regions, similarly to what has been previously shown in *TET2*^{MT} CHIP cases (Figure 12) (74). Next, to discern the effect of hypermethylation of enhancers, genes associated to the hypermethylated enhancers were examined and found to belong to the Hallmark Inflammatory response and Hallmark TNFA signalling via NFKB pathways. Thus, indicating that these inflammatory gene pathways are silenced through hypermethylation of their enhancers. This is of note, as we observed cases where the enhancer methylation seemingly impacted the gene expression more than the methylation status of the gene itself. That the aberrant hypermethylation seen in the *TET2*^{MT} cluster is specifically enriched in inflammatory gene sets as well as enhancers indicate it is not random. It could be discussed if this specific aberrant methylation pattern seen in CMML is related to the function of the TET2 protein and further studies are needed to investigate the role of both *TET2*^{MT} and *TET2*^{WT} in CMML and in healthy and if it would be a potential treatment target.

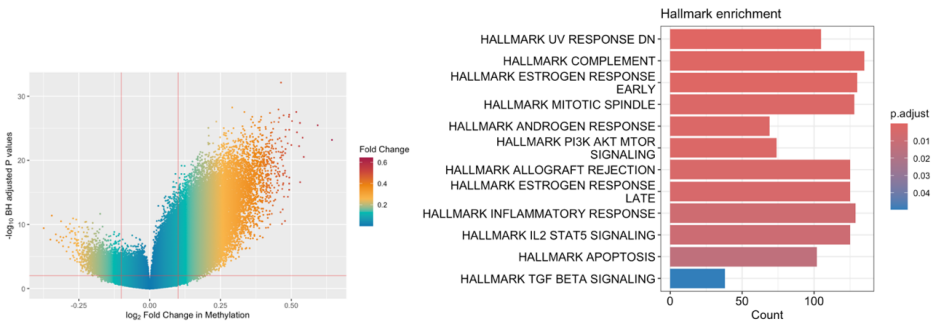


Figure 11. Volcano plot of DMPs found in TET2^{MT} *CMML compared to TET2*^{MT} *CMML (left) and Hallmark pathways significantly enriched in hypermethylated DNA (right).*

Figure 12. Showing enrichment of DNA methylation array probes and DMPs across chromatin states.

Tulstrup et al used the ChromHMM model based on previously published Roadmap Epigenomics data to find chromatin states in their CHIP cohort (74). Integrating the ATACseq and ChIP seq data from 5 *TET2*^{WT} and 5 *TET2*^{MT} CMML cases, we could define chromatin states were across the genome, in our cohort specifically. Among the chromatin states, some states were active and accessible, e.g. active promoters or accessible unmarked, while some states were repressed, eg the quiescent state. We subsequently used the learned model in analysis of *TET2*^{WT} CMML and *TET2*^{MT} CMML separately, finding genes exhibiting a chromatin state shift between *TET2*^{WT} and *TET2*^{MT} cases. Of particular interest were genes that shifted from an active state in *TET2*^{WT} patients to a repressed state in *TET2*^{MT} patients and among these genes, Hallmark Inflammatory Response pathway was specifically enriched, indicating a specific repression of inflammatory genes in *TET2*^{MT} CMML (Figure 13). This change in chromatin states suggests a global reprogramming to a chromatin state less responsive to inflammatory stimuli. In CMML, with an inflammatory microenvironment, increased resilience to inflammation would potentially be advantageous and give a clonal advantage and selection over time.

The findings in this study indicate that *TET2*^{MT} CMML share several features with *TET2*^{MT} CHIP, strengthening the notion that *TET2*^{MT} CMML evolves from CHIP, as opposed to *TET2*^{WT} CMML, in which plausibly, disease evolves through other mechanisms. It should be investigated further if this implies that CMML development could be hindered by potential treatments, targeting either *TET2* itself or e.g inflammatory pathways seen to be altered in *TET2*^{MT} CMML.

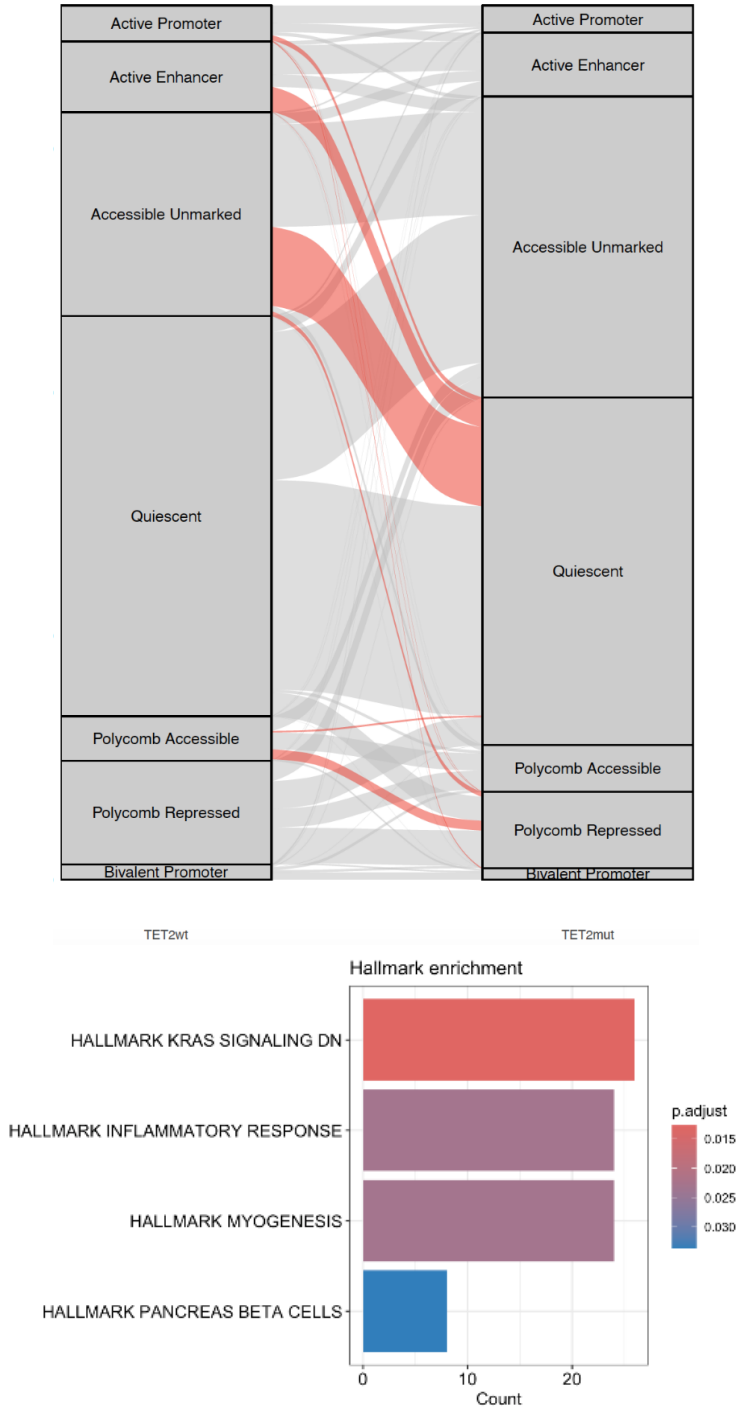


Figure 13. Showing genes having an active state in TET2^{WT} CMML and a repressed state in TET2^{WT} CMML (top) and Hallmark pathways significantly enriched among these, highlighted, genes (bottom).

5 Conclusions

We conclude that in population based CMML cohorts, AID is even more frequent than previously described, present in up to 33.6% of patients. We confirm that *TET2*^{MT} confers a favourable prognosis in CMML, and that the number of *TET2*^{MT} was an independent significantly beneficial parameter in multivariable analysis. A cox model including number of *TET2*^{MT} outperformed CPSS-mol, concluding that that the number of *TET2*^{MT} should be considered for inclusion in future prognostic scores. In addition, platelet size is enlarged in CMML, and large platelets measured as MPV may be a potential prognostic marker as it was associated with a longer overall survival. *TET2*^{MT} is associated with profound DNA hypermethylation localized to genes encoding inflammatory proteins and enhancer regions of these genes. Integrated analysis of ATAC seq and ChIP seq revealed that the chromatin of inflammatory gene DNA switched from an open state in *TET2*^{MT} to repressed in *TET2*^{WT}, indicting that *TET2*^{MT} represses inflammatory responses and makes the bone marrow microenvironment more resilient to chronic inflammation, prolonging overall survival.

Overall, this thesis suggests the importance of *TET2*^{MT} in CMML pathobiology and that *TET2*^{MT} CMML should be considered as a separate disease entity from *TET2*^{WT} CMML.

6 Points of perspective

Results in this thesis suggests *TET2*^{MT} CMML is a separate disease entity from *TET2*^{WT} CMML. If it is the case that *TET2*^{MT} CMML evolves from CHIP, it could be discussed if it would be possible to intervene in the CHIP-stage, already before CMML is developed, with e.g treatment hampering clone expansion by either targeting *TET2* or the inflammatory microenvironment.

However, not all CMML is *TET2*^{MT} CMML and I think further characterization of the clonal architecture might reveal additional insights into CMML pathobiology. Palomo et al reported *TET2*^{MT}, *SRSF2*^{MT} and *ASXL1*^{MT} as the most frequent ancestral mutations in CMML and that other common mutations in CMML, such as *RUNX1*^{MT}, RAS-pathway mutations or *JAK2*^{MT} most often were secondary (26). However, a significant fraction of CMML patients, 12% in paper II and 23% in paper IV are wild type for all *TET2*, *SRSF2* and *ASXL1*, meaning some other lesion must be founder. *TET2*^{MT} is also not unique to CMML but is found in other myeloid neoplasms and diseases. Also, what are the interactions between mature and immature cells of the clone and how do they impact each other? Studies at single cell level and preferably including consecutive samples would be of importance and might reveal novel potential avenues for treatment.

I also think further studies of the inflammatory microenvironment of CMML are needed. Understanding of the interplay between the remodelled bone marrow microenvironment in which the *TET2*^{MT} clone has a competitive advantage and the clonal HSPCs as well as more mature clonal cells might be key to how to intervene in disease development.

It is clear in this thesis that DNA methylation and chromatin patterns can discern subgroups within CMML and reveal insights beyond the molecular landscape. With regards to the impact of *TET2*^{MT} on CMML epigenome and the substantial differences observed in DNA methylation patterns between *TET2*^{MT} and *TET2*^{WT} CMML, is a CMML classification based on DNA methylome the future?

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8 Declaration about the use of generative AI

No AI assisted tools were used in the writing of the kappa or in any part of the generation of the contents of this thesis. I take full responsibility for the content of the thesis kappa.

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