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From Macrophages to Mutations

Tumour Microenvironment and Genetic Evolution in Mantle Cell Lymphoma

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Abstract

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Mantle Cell Lymphoma (MCL) is, despite recent advances, still not considered curable. The aim of this thesis is to increase the understanding of MCL tumour biology by describing the impact of the immune microenvironment and tumour genetics on the outcome for the patient.

In a first study (paper I) we described the composition of the tumour immune microenvironment in MCL tumour tissue. We found that CD3+ T-cells were the most abundant cell type in the tumour tissue, and that high numbers of FOXP3+ regulatory T-cells and CD163+ macrophages were associated with shorter survival.

The negative prognostic impact of macrophages in MCL was confirmed in a second study (paper II) where we investigated the soluble macrophage marker sCD163 and found that high serum levels correlated with worse prognosis in both newly diagnosed and relapsed MCL patients.

The third study (paper III) further underscores the importance of macrophages in MCL. In this study, we analysed differences in the plasma proteome between patients that did or did not relapse before 24 months (POD24). Among 1463 proteins, the most differentially expressed were two macrophage markers, sCD169 and sVSIG4.

In the fourth study (paper IV) we focused on tumour genetics, using sequential tumour biopsies. We showed that MCL tumours accumulate high-risk genetic alterations over time, and that the type of treatment the patient received affected the risk of developing such alterations.

Treatment is also the focus of the last study (paper V), where we described a higher risk of acute haematologic side effects in patients with clonal haematopoiesis.

In summary, this thesis has provided new insights about the tumour microenvironment in MCL and advanced the understanding of how anti-tumour treatments interact with tumour biology as well as with patient intrinsic factors.

Keywords: Mantle cell lymphoma, Microenvironment, Macrophage, Regulatory T cell, Mutation, Copy number alteration, Prognosis, Toxicity, Treatment, Clonal haematopoiesis

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Till Morfar.
”Det är roligt att leva, för då får man se hur det går.”

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. Joana de Matos Rodrigues*, Anna Nikkarinen*, Peter Hollander, Caroline E Weibull, Riita Räty, Arne Kolstad, Rose-Marie Amini, Anna Porwit, Mats Jerkeman, Sara Ek, Ingrid Glimelius. *contributed equally
Infiltration of CD163-, PD-L1- and FoxP3-positive cells adversely affects outcome in patients with mantle cell lymphoma independent of established risk factors. *Br J Haematol.* 2021 May;193(3):520-531.

II. Anna Nikkarinen, Lavanya Lokhande, Rose-Marie Amini, Daniel Molin, Gunilla Enblad, Anna Porwit, Mats Jerkeman, Caroline E. Weibull, Peter Hollander, Sara Ek and Ingrid Glimelius
Soluble CD163 predicts outcome in patients with mantle cell lymphoma both in chemoimmunotherapy and targeted therapy treated patients. *Blood Adv. 2023 Sep 26;7(18):5304-5313.*

III. Patrick Nylund, Tove Selvin, Anna Nikkarinen, Ann-Marie Ly, Mattias Berglund, Kossi D. Abalo, Daniel Molin, Gunilla Enblad, Peter Hollander, Mats Hellström and Ingrid Glimelius
Comprehensive blood plasma proteomics identifies VSIG4 and CD169 as prospective biomarkers for high-risk of early relapse in mantle cell lymphoma. *Manuscript.*

IV. Anna Nikkarinen, Jonas Almlöf, Albin Österroos, Claes Ladenvall, Rose-Marie Amini, Peter Hollander, Kristin Ayoola Gustafsson, Panagiotis Baliakas, Ingrid Glimelius.
Accumulation of Aggressive Genetic Features after Chemoimmunotherapy: A Longitudinal Study in Mantle Cell Lymphoma. *Accepted for publication in Leukemia.*

V. Anna Nikkarinen, Tove Selvin, Jonas Almlöf, Albin Österroos, Claes Ladenvall, Peter Hollander, Rose-Marie Amini, Panagiotis Baliakas, Ingrid Glimelius.
Impact of Clonal Haematopoiesis on Haematological Treatment toxicity in Mantle Cell Lymphoma Patients. *Manuscript.*

Manuscripts not included in the thesis

The following manuscripts have been authored during the PhD period but are not included in the thesis.

I. Nikkarinen A, Glimelius I.
Aplastic anemia triggered by the Bruton tyrosine kinase inhibitor acalabrutinib in two patients with mantle cell lymphoma - A case report. *EJHaem. 2024 Jun 16;5(4):820-824.*

II. de Matos Rodrigues J, Lokhande L, Olsson LM, Hassan M, Johansson A, Janská A, Kumar D, Schmidt L, Nikkarinen A, Hollander P, Glimelius I, Porwit A, Gerdtsson AS, Jerkeman M, Ek S.
CD163+ macrophages in mantle cell lymphoma induce activation of pro-survival pathways and immune suppression. *Blood Adv. 2024 Aug 27;8(16):4370-4385*

III. Nylund P, Nikkarinen A, Ek S, Glimelius I.
Empowering macrophages: the cancer fighters within the tumour microenvironment in mantle cell lymphoma. *Front Immunol. 2024 Mar 19;15:1373269*

IV. Juliana Imgenberg-Kreuz, Cecilia Fugmann, Anna-Maja Molin, Carin Backlin, Alina Johansson, Milica Vranic, Anna Nikkarinen, The Autolymphoma study group, Per Eriksson, Christopher Sjöwall, Eva Baecklund, Gunnel Nordmark
Targeted proteomics identifies differentially expressed proteins in Sjögren's disease with incident lymphoma.
RMD Open. 2025 Oct 23;11(4):e005897

Contents

Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
ASCT	Autologous stem cell transplantation
BTKi	Bruton's tyrosine kinase inhibitor
CAR T	Chimeric Antigen Receptor T-cell
CCUS	Clonal cytopenia of undetermined significance
CH	Clonal haematopoiesis
CI	Confidence interval
CNAs	Copy number alterations
CRS	Cytokine release syndrome
CSF-1	Colony stimulating factor 1 (macrophage growth factor)
ELISA	Enzyme-linked immunosorbent assay
FFPE	Formalin-fixed paraffin-embedded
G-CSF	Granulocyte-colony stimulating factor
HR	Hazard ratio
ICAHT	Immune effector cell–associated haematologic toxicity
ICANS	Immune effector cell–associated neurotoxicity syndrome
IHC	Immunohistochemistry
Ki-67	Proliferation marker
MCL	Mantle cell lymphoma
MIPI	Mantle Cell Lymphoma International Prognostic Index
MDS	Myelodysplastic syndrome
MRD	Minimal residual disease
M2	Alternatively activated macrophage phenotype
NK-cells	Natural killer cells
OS	Overall survival
PFS	Progression-free survival
POD24	Progression of disease within 24 months
TAM	Tumour-associated macrophage
TME	Tumour microenvironment
TTP	Time to progression
Treg	Regulatory T-cell
VAF	Variant allele frequency
WES	Whole exome sequencing

Sammanfattning på svenska

Mantelcellslymfom (MCL) är en ovanlig och ofta aggressiv form av B-cellslymfom. De som insjuknar drabbas inte sällan av upprepade återfall och behandlingskomplikationer. Trots betydande framsteg med intensiv kemoterapi, tyrosinkinas-hämmare och T-cellsterapier är sjukdomen fortfarande obotlig för de flesta patienter. Utfallet i MCL beror inte bara på tumörens genetiska egenskaper, utan påverkas även av tumörens mikromiljö samt patientens förutsättningar.

Syftet med denna avhandling var att fördjupa förståelsen för tre centrala och delvis sammanlänkade biologiska områden i MCL:

1. den immunologiska tumörmikromiljön
2. tumörens genetiska utveckling över tid
3. betydelse av klonal hematopoes (CH)

I avhandlingens första del definierar vi tumörens immunologiska sammansättning med fokus på den typ av immuncell som kallas makrofager. Genom analys av tumörvävnad, serum och proteomikdata visar vi att hög förekomst av M2-associerade makrofagmarkörer – CD163, VSIG4 och CD169 – är starkt kopplade till sämre överlevnad. Resultaten visar att makrofager är viktiga i sjukdomsutvecklingen vid MCL och pekar ut nya möjligheter för immunbaserad riskstratifiering.

I den andra delen undersöker vi MCL:s genetiska utveckling över tid genom helexomsekvensering av tumörprov från diagnos och flera återfall. Studien visar att högriskförändringar, särskilt *TP53*-mutationer och deletioner i *CDKN2A*, ackumuleras över tid och att nytillkomna förändringar är fler hos de patienter som behandlats med kemoterapi än hos de som fått målinriktade behandlingar eller de som endast övervakats (watch and wait). Resultaten tyder på att behandling med kemoterapi kan bidra till utveckling av mer aggressiva tumörkloner, och stödjer en övergång mot målinriktade terapier istället för intensiva cytostatikabehandlingar.

I avhandlingens tredje del analyserar vi klonal hematopoes hos patienter med MCL. Vi visar att förekomsten av klonal hematopoes är förknippad med ökad risk för hematologiska biverkningar, inklusive behov av blodtransfusioner och risk att behöva avbryta behandlingen i förtid.

Sammanfattningsvis visar denna avhandling att förloppet i MCL påverkas av samspelet mellan tumörens immunmikromiljö, dess genetiska utveckling över tid och patientens biologiska förutsättningar. Genom att integrera dessa perspektiv kan framtida behandlingar optimeras för att bättre identifiera högriskpatienter, minska biverkningar och i förlängningen förbättra överlevnaden för patienter med MCL.

Introduction

Mantle cell lymphoma (MCL) is an aggressive B-cell lymphoma characterized by repeated relapses and biological heterogeneity. Over the past 15 years, survival has improved markedly through intensified immunochemotherapy, Bruton tyrosine kinase inhibitors (BTKi), and more recently Chimeric Antigen Receptor T-cell (CAR-T cells) and bispecific antibodies, yet the disease remains incurable for the majority of patients.

While recent research has primarily focused on tumour genetics and drug development, it is increasingly clear that MCL biology is not only shaped by the malignant clone, but also by the tumour immune microenvironment (TME) and the interactions between tumour cells and the TME. Especially macrophages are emerging as important players in tumour evolution and prognosis in several malignancies. In MCL, however, these aspects remain less well understood.

At the same time, accumulating evidence suggests that treatment itself may drive genomic evolution, potentially contributing to shorter remissions at each relapse. Despite this, the patterns of tumour evolution over time and how genetic alterations relate to treatment pressure, have not been systematically studied in MCL.

Finally, even highly effective treatments can be limited by toxicities. Systemic therapies are associated with acute haematologic toxicities, but we lack tools to predict which patients are most vulnerable. Clonal haematopoiesis (CH) has emerged as a factor that increases the risk of haematologic toxicity, yet its clinical relevance in MCL has not been fully explored.

MCL represents a biologically and clinically complex malignancy in which outcome depends on tumour biology, immune composition, and host-related factors. A deeper understanding of these interactions is necessary to develop more individualized and less toxic treatment strategies.

This thesis investigates these three layers of MCL biology: the tumour microenvironment, the genetic evolution of the tumour under therapeutic pressure, and the role of CH in treatment-related toxicity.

Background

Epidemiology and treatment

MCL is a B-cell lymphoma that constitute between 5-7% of all malignant lymphomas in Western Europe. The incidence is 1-2 per 100 000 person years, median age is 72 years and the male to female ratio is 3:1(1).

MCL is a heterogeneous disease with survival ranging from weeks to decades and the treatment landscape is evolving fast. Median survival for MCL has radically improved, from historically 3-5 years to 12.5 years among younger patients treated with the Nordic protocol (2), and is likely to improve further after implementation of BTKi-based regimens (3).

The Nordic protocol includes a rituximab (R)-chemotherapy combination with cyclophosphamide, doxorubicin, vincristine and prednisone (R-CHOP) alternating with cytarabine, followed by autologous stem cell transplantation (ASCT) and maintenance treatment with rituximab for three years. The recently finished TRIANGLE study has shown that addition of the BTKi ibrutinib to this concept, with or without the ASCT, is improving the outcome and these results are now changing the standard of care for younger MCL patients (3).

Older or fragile patients have historically been treated with other chemotherapy regimens, including R-bendamustine, R-CHOP or combinations with bortezomib (VR-CAP). Studies of BTKi are changing the treatment landscape also for this group of patients and combinations with CD20-ab and BTKi are emerging as first-line treatment (4).

In the relapsed setting, BTKi is standard for patients that did not receive this in the first line. CAR T-cells and bispecific antibodies are also increasingly used. These treatments are often effective but complicated by both early and late toxicities and they come with a non-MCL mortality rate of 10% within the first year (5, 6) and patients with relapse after CAR T have particularly poor outcomes (7).

In summary, despite the recent progress, there is a great need for better risk stratification and toxicity management as well as more efficient treatments.

Tumour microenvironment

The tumour microenvironment (TME) is composed of a mix of stromal cells, blood vessels, extracellular matrix, immune cells and tumour cells and plays a critical role in lymphoma biology, influencing tumour survival, immune escape, and therapy resistance (8-10).

Early MCL studies suggested that T-cell infiltration may mark more indolent disease and that MCL cells recruit T-regulatory cells (Tregs) through CCL4/CCL5 expression (11, 12). Immune checkpoint markers such as PD-1 and PD-L1 have been investigated with inconsistent results, likely due to methodological differences (13-17).

The tumour-associated macrophages (TAMs) are important in regulating immune response (9, 10). The surface markers CD163, VSIG4 and CD169 are membrane-bound proteins that can be shed into the circulation as soluble forms (sCD163, sVSIG4 and sCD169) upon macrophage activation (18-20). High infiltration of these M2-like, anti-inflammatory TAMs, has been linked to poor prognosis in some lymphoma subtypes (21, 22) but they had not been systematically studied in MCL when the work with this thesis began.

Table 1. The different cell types and immune markers discussed in this thesis

Marker	Cell type
T-cell markers	
CD3	T-cell marker (pan-T)
CD4	T-helper cells
CD8	Cytotoxic T-cells
FOXP3	Forkhead box P3, Regulatory T cells (Tregs)
Immune checkpoint markers	
PD-1	Activated T-helper cells, NK-cells
PD-L1	Activated T-cells, macrophages, tumour cells
PD-L2	Activated T-cells, macrophages, tumour cells
CD70	Co-stimulatory ligand binding CD27 (tumour cells)
CD27	Co-stimulatory T-cell receptor (Tregs)
CD47	“Don’t-eat-me” signal on tumour cells
Macrophage markers	
CD163	M2-like macrophages
CD169	Macrophages of undetermined polarization
VSIG4	M2-like macrophages

Key genetic alterations in MCL

The characteristic translocation in the malignant B cells in MCL, t(11;14)(q13;q32), leads to constitutively overexpressed Cyclin D1, resulting in unregulated cell cycle and cell division. Expression of the epigenetically controlled transcription factor SOX11 determines if the MCL cell will develop into non-nodular, indolent MCL or nodular and more aggressive MCL (23) (Figure 1). SOX11 overexpression also leads to CD70 upregulation, a surface protein that interacts with CD27 on FOXP3^{+} regulatory T-cells (24). Secondary genetic aberrations are common and probably needed for malignant development. The most common secondary mutations are *ATM* (38%), *TP53* (22%) and *CCND1* (16%). Other frequently mutated genes are *KMT2D* (15%) and *NSD2* (13%). Frequent copy number alterations (CNAs) are 3q^{gain} (40%), *CDKN2A*^{del} (31%), *ATM*^{del} (27%), *RB1*^{del} (25%) and *TP53*^{del} (23%) (25).

While these risk factors are well established at diagnosis, much less is known about how they evolve during the course of disease or in response to therapy.

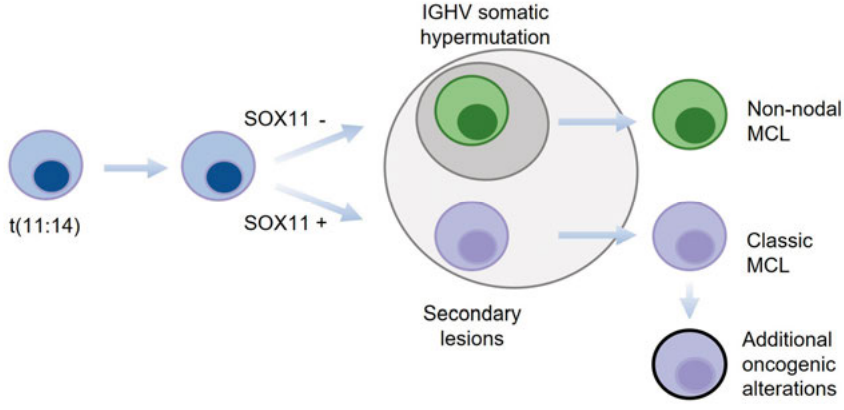

Figure 1. Early evolution of MCL. A pre-B-cell gains the 11:14 translocation, and depending on SOX11 expression, either enters the germinal centre for immunoglobulin heavy chain variable region gene (IGHV) somatic hypermutations, or remain IGHV unmutated, but acquires secondary oncogenic lesions such as *ATM* or *TP53* mutations.

Clonal haematopoiesis

Clonal haematopoiesis (CH) is a common age-related phenomenon in which blood stem-cell populations acquire somatic mutations and expand clonally. CH can occur in both lymphoid and myeloid lineages and is associated with increased risk of cardiovascular disease, haematologic malignancies, and

overall mortality (26-28) (Figure 2). When CH is accompanied by persistent cytopenia, a condition termed clonal cytopenia of undetermined significance (CCUS), the risk of progression to myeloid neoplasms is further elevated (29). CCUS also pinpoints the close relations between myeloid CH and an insufficient production or survival of mature blood cells.

Beyond its pre-malignant potential, CH can affect a patient's ability to tolerate treatment. Evidence from other malignancies shows that CH impairs hematopoietic recovery after radiotherapeutic treatments, chemotherapy, stem cell mobilization, and CAR-T cell therapy (30-33). In MCL, this connection had not been thoroughly investigated prior to this thesis and we lack biological markers predicting the risk of toxicity.

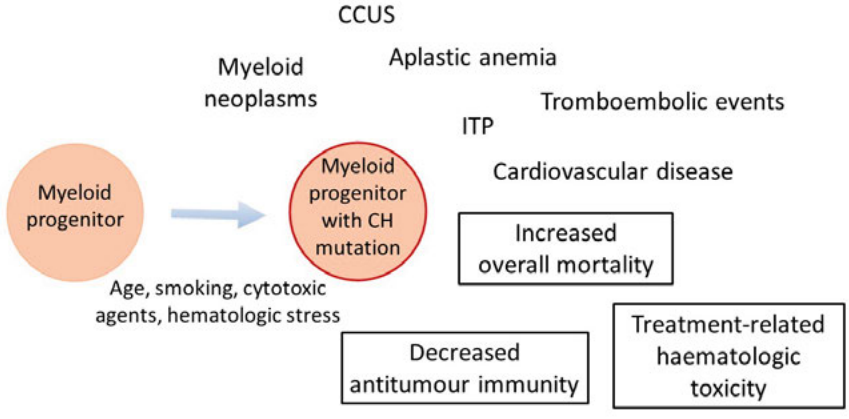

Figure 2. Overview of clonal haematopoiesis and some of the associated conditions. Conditions marked with boxes are discussed in detail in paper V.

Aspects of MCL treatment in relation to TME and CH

All current MCL therapies either rely on the TME for their efficacy or modulate it in clinically meaningful ways. This includes both newer immune-based strategies and established treatments.

T-cell engaging therapies

CAR T cells and bispecific antibodies exert their anti-lymphoma effects through direct immune engagement within the TME. In CAR-T cell treatment, T-cells are harvested from the patient, modified and returned to take part in the TME in targeting the lymphoma cells. The bispecific antibodies (anti-CD20/CD3) bind T-cells to lymphoma cells and facilitate T-cell engagement

and activation of antibody-dependent cellular cytotoxicity (ADCC) against the lymphoma cells (34).

Classical immunochemotherapy

Even standard regimens depend on immune effector mechanisms. Rituximab relies on ADCC, engaging antigen-presenting cells and macrophage-mediated cytotoxicity (35). Macrophages can modulate chemotherapy responses, and experiments in DLBCL have shown enhanced sensitivity to CHOP when macrophages are stimulated with granulocyte-macrophage colony-stimulating factor (GM-CSF) (36). Bendamustine has both direct cytotoxic and immunomodulatory properties, including effects on T-cell function, which has led to recommendations against its use prior to apheresis for CAR T-cell production (37, 38).

Targeted agents and the TME

Modern targeted therapies influence not only MCL cells but also the surrounding immune compartment. The BTKi **ibrutinib** downregulates PD-1 on T cells and disrupts macrophage–tumour signalling. The bcl-2 inhibitor **venetoclax** reduces Tregs, decreases PD-1 expression, and enhances natural killer (NK) cell function and the immunomodulatory drug **lenalidomide** promotes Th1 polarization and increases macrophage phagocytosis (39). These effects suggest that part of their clinical activity is mediated through TME reprogramming.

Clonal haematopoiesis and treatment resistance

Evidence is emerging that, beyond the above-mentioned toxicity, CH may contribute to treatment resistance. CH-associated macrophages show impaired antigen presentation, and CH-mutated Tregs promote T-cell exhaustion (40). Cytotoxic therapy further accelerates CH clone expansion, creating a feedback loop that may deepen immune dysfunction (41, 42).

Prognostic and predictive factors

Risk stratification in MCL is traditionally based on clinical variables such as the mantle cell international prognostic index (MIPI), which includes age, performance status, white blood cell count, and lactate dehydrogenase (43). Biological variables, including Ki67 proliferation index, histological subtype (classic, blastoid or pleomorphic morphology) and p53 overexpression, are also widely used as prognostic markers (44, 45).

At the molecular level, *TP53* alterations are the strongest predictors of poor outcome, with both mutations and deletions leading to aggressive disease even though their respective contribution remains disputed (45-50). Secondary genetic alterations, including *CDKN2A*, *NOTCH1* and *RB1* deletions further

modulate prognosis, particularly when co-occurring with *TP53* alterations. These high-risk features define an "ultra-high-risk" subgroup with particularly poor overall survival (45). While *ATM* mutations are frequent, their prognostic impact is less clear and may depend on mutual exclusivity with *TP53* mutations (51).

Very few biomarkers reliably predict treatment response in MCL beyond indicating a generally poor prognosis. Mutations in *CARD11* and *BIRC3*, which affect pathways targeted by ibrutinib and lenalidomide, are of theoretical interest as predictive markers (52). Similarly, *SMARCA4* mutations may influence response to venetoclax (1). Other proposed markers for resistance to BTKi or BCL2 inhibitors remain difficult to interpret, as it is often unclear whether they reflect true resistance or simply aggressive tumour biology unlikely to respond to any therapy (53, 54).

Currently, no biomarkers from the tumour microenvironment are used for estimating prognosis, and patient-specific factors beyond age and frailty are not routinely applied to predict treatment toxicity.

Summary and knowledge gaps

In summary, the biology of MCL is dependent on complex interactions between tumour intrinsic factors such as genetic alterations, the tumour microenvironment and host-related factors. The genetic risk factors are increasingly well-characterized at diagnosis, but several key knowledge gaps remain:

- The composition and clinical relevance of the tumour microenvironment, particularly macrophages, were largely unknown when this work began.
- While several high-risk mutations are established, little was known about how these genetic features evolve during the course of disease, and their possible relation to treatment.
- Although clonal haematopoiesis is common in aging individuals and linked to a vast range of haematologic conditions, its role in MCL had not been systematically explored.

Aims

The overall aim of this thesis is to describe the impact of tumour biology, including the microenvironment and genetic landscape, on prognosis and treatment outcomes in mantle cell lymphoma (MCL).

Specific aims:

1. To describe the tumour microenvironment in MCL and to evaluate the impact of macrophages and other immune cells in the tumour microenvironment on prognosis
2. To assess the evolution of high-risk features in MCL tumours over time
3. To explore whether clonal haematopoiesis contributes to treatment-related toxicity

Patients and methods

Paper I-III

Study populations

For paper I, we included 176 patients from the Swedish lymphoma register and 106 patients from the clinical trials Nordic Mantle Cell Lymphoma study 2 and 3.

Paper II included a total of 131 patients. 81 newly diagnosed patients from the population-based biobanks UCAN and VIOLA and 50 relapsed patients from UCAN and the clinical phase II study Philemon where patients were treated with lenalidomide, venetoclax and rituximab. Tissue samples were available from the patients in the UCAN cohort.

For paper III we collected plasma samples from 83 untreated MCL patients included in UCAN.

Samples and techniques

Paper I was based on immunohistochemistry. Formalin-fixed, paraffin-embedded (FFPE) tumour tissue samples were stained for seven different immune markers. The expression of the macrophage marker CD163 was evaluated manually whereas the other markers were evaluated using digital pathology scoring (Figure 3). For paper II, serum samples were evaluated for soluble CD163 (sCD163) by enzyme-linked immunosorbent assay (ELISA) and in paper III we used the Olink proteomics platform and analysed 1463 different proteins with an antibody-based technique called proximity extension assay.

Analyses

In paper I, optimal cut-offs for the different markers were established with maximally selected rank statistics.

In paper II, the median level of sCD163 was decided a priori as a cut-off. sCD163 levels were evaluated in correlation to other risk factors as well as progression and survival. After showing the prognostic value of sCD163 by median, and in order to suggest a clinically useful cut-off, the largest curve separation was evaluated with maximally selected rank statistics in R.

In paper III, the proteins were evaluated for differential expression between patients that did or did not progress within 24 months (POD24). The most

differentially expressed proteins (VSIG4 and CD169) were evaluated regarding their correlation to clinical parameters as well as IHC staining for tissue macrophages and prognosis.

The Kaplan-Meier method and Cox proportional hazards models (univariable and multivariable) were used for survival analysis in all three papers.

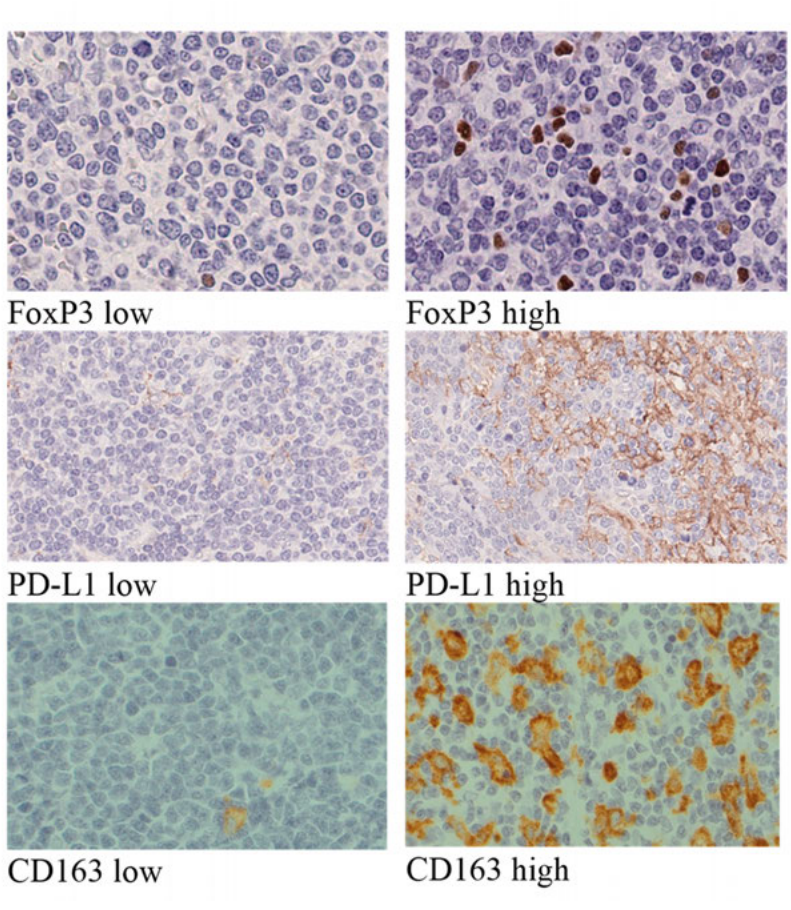

Figure 3. Representative stainings of FoxP3, PD-L1 and CD163.

Paper IV

Study population, samples and techniques

We included 70 patients with MCL and available tumour tissue from diagnosis and at least one relapse. FFPE samples were stained and analysed for morphology, p53 and Ki-67%. 31 of the patients had available fresh frozen tumour cells from two or more time points (Figure 4). We analysed the cells with whole exome sequencing (WES).

Analysis

Any changes in mutations (single nucleotide variants and small indels) and CNAs were investigated and related to clinical characteristics, including what type of treatment the patient had received, and to prognosis.

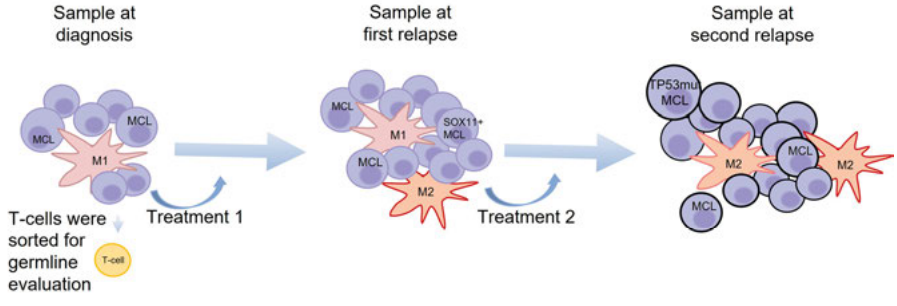

Figure 4. We analysed sequential tumour tissue samples with whole exome sequencing, clinical and pathological risk factors and related the results to given treatment

Paper V

Study population

We included 53 patients that were treated for MCL between 2002-2020. Detailed information was available over a total of 103 systemic treatment occasions in 46 of the patients. Medical records were evaluated for interventions related to haematological toxicity.

Samples and techniques

WES was performed on live frozen cells from bone marrow or peripheral blood. CH was defined as presence of pathogenic or likely pathogenic mutations listed as myeloid chip driver mutations by the world health organization (WHO) (29) and/or the UK biobank (55).

Analysis

The CH mutations were analysed for correlation to acute haematological toxicity and prognosis. Since one patient could contribute with multiple treatments, odds ratios were calculated with sandwich estimator, using ID as the cluster variable.

Results

Paper I

Expression pattern

The pan T-cell marker CD3 was the most commonly expressed marker in MCL tumour tissue, with a median of 10% positive cells out of all cells in the stained cores. The most abundant T-cell subtype was T cells expressing CD8 with a median of 6% positive cells. The Treg marker FoxP3 was expressed in 2% of the cells. PD-1 and PD-L1 were expressed at 10% and 1%, respectively. The macrophage marker CD163 was expressed in very low numbers with a median of 0.06% positive cells of all cells within the tumour tissue (Figure 5).

Survival analysis

For outcome analysis, overall survival (OS) was analysed for the whole cohort whereas PFS data was available in the study cohort. FoxP3, PD-L1 and CD163 above cut-off were associated with poor prognosis.

Combined signatures

A synergistic effect was seen when combining the cell types into immune signatures, and patients with presence of $CD163^{+}PD\text{-}L1^{+}$ cells (above cut-off) in the TME had shorter OS than patients with none or only one of the cell types above cut-off (Figure 6a). A similar synergism was seen for PFS in TMEs with the $CD163^{+}FoxP3^{+}$ cells above cut-off in the study cohort (Figure 6b).

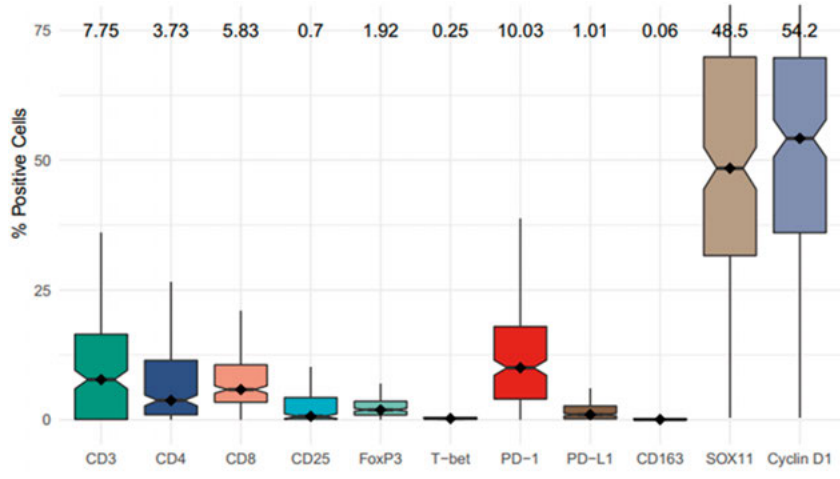

Figure 5. Number of positive cells/total number of cells for each marker. Boxes represent interquartile range and median value for each marker is shown.

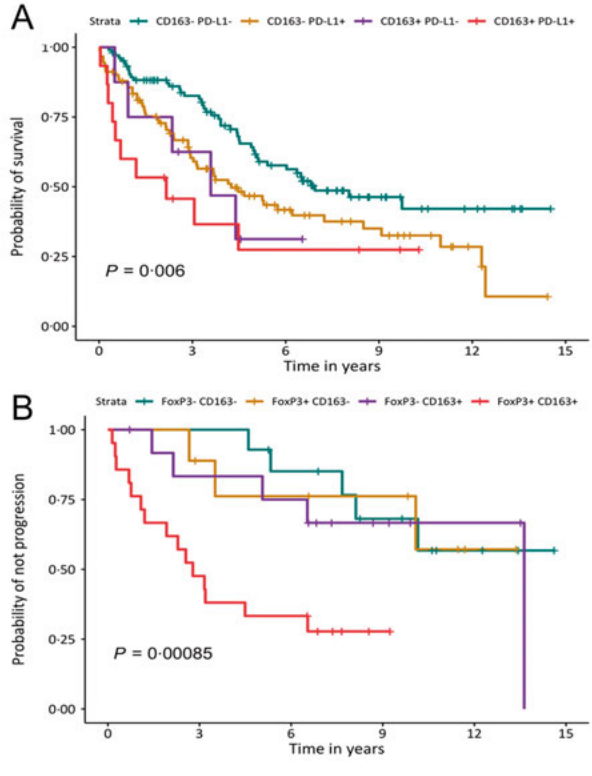

Figure 6. Kaplan-Meier curves showing the markers combined into immune signatures. a. $CD163^{+}PD\text{-}L1^{+}$ cells and b. $CD163^{+}FoxP3^{+}$ cells.

Paper II

Survival analysis

Soluble CD163 above median was associated with shorter PFS and shorter OS in both newly diagnosed and relapsed MCL. (Multivariable Cox proportional hazard adjusted for age and sex: HR_{PFS} 3.13, 95% CI: 1.83-5.36, and HR_{OS} 4.10, 95% CI: 2.06-8.16). 5-year survival among patients with sCD163 levels below median at diagnosis was 97% (Figure 7).

Correlations

sCD163 was higher in patients with low haemoglobin (Hb), high lactate dehydrogenase (LDH) and p53 overexpression and/or TP53 mutations. There was a moderate correlation (r=0.64, p=0.014) between the levels of sCD163 and the number of $CD163^+$ cells in the tissue. There was no systematic change in sCD163 levels during or after treatment.

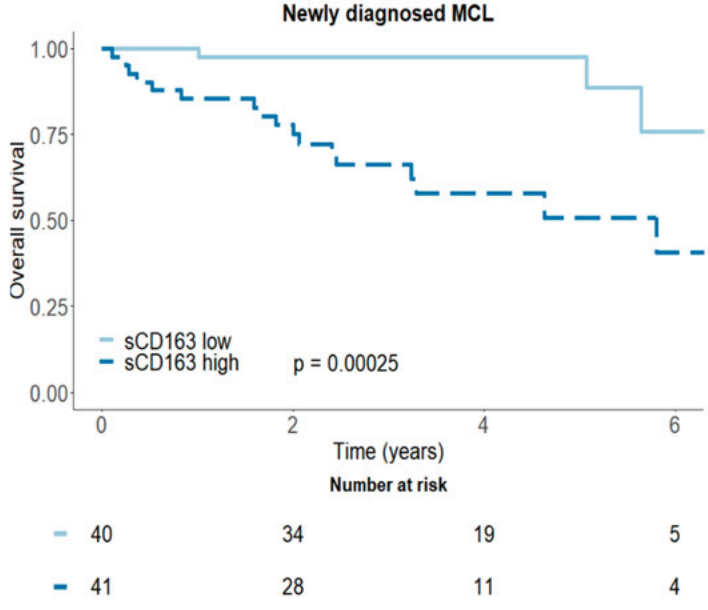

Figure 7. Kaplan-Meier curve showing the difference in overall survival for patients with sCD163 levels above or below median.

Paper III

Differentially expressed proteins

Among the 1463 evaluated proteins, 261 were differentially expressed between patients that did ($POD24^+$) and did not ($POD24^-$) progress within 24 months.

Enrichment analysis of the differentially expressed proteins, using human cell atlas Tabula Sapiens in Enrichr, showed an enrichment of macrophage-associated proteins in $POD24^+$ patients (Figure 8a). In line with this, several macrophage-associated proteins were differentially expressed, including sCD14, CSF-1, sCD163, IL-10, CCL18, Galectin 3, TNF-α, IL-4R, IL-6, IL-18, CCL3 and CXCL8. The two proteins that differed the most between the two groups where soluble sialic acid binding Ig like lectin 1 (sCD169) and soluble V-set and immunoglobulin domain containing 4 (sVSIG4) (Figure 8b-d).

Correlation to clinical characteristics

Patients with high levels of sCD169 and sVSIG4 generally had high MIPI, reflecting both higher white blood cell count, higher LDH and older age in patients with one or both of the macrophage markers overexpressed. There was also a strong correlation to low haemoglobin levels and low albumin levels but no statistically significant association to the tumour characteristics, *TP53* mutational status, Ki-67% or morphology (Figure 9). Using the same method as previously used for CD163 (manually counting the percentage of positive cells within tumour areas), there was a correlation between the soluble and tissue markers for CD169 ($r=0.63$, $p<0.001$). The VSIG4 tissue staining failed and positive cells were found only in a minority of the samples, hence this was not evaluable.

Correlation to prognosis and sensitivity analysis

Since these markers were chosen based on the differential expression by POD24, any prognostic relevance needs to be validated in another cohort, and data gathering is ongoing. With that in mind, the correlation to prognosis was strong also beyond POD24, and adjusted for potential confounders. Cox proportionale hazard ratios (HR) for OS for the combination of high sVSIG4 and/or high sCD169 were univariable HR (95% CI) 4.6 (1.9-11.1), multivariable HR 3.0 (1.2-7.5) adjusted for MIPI and sex and in a fully adjusted model HR 3.5 (1.03-11.1) adjusted for age, sex, p53, ki-67% and morphology. In this last model, 30 patients were excluded due to missingness.

A negative value of either marker is strongly associated with good prognosis, defined as no POD24. Based on the whole cohort n=83: negative predictive value of no POD24 was 93% for either marker individually and 97% for sVSIG4 and sCD169 combined. A positive value is less specific, with a positive predictive value of POD24 of 51% for sVSIG4, 49% for sCD169 and 46% for the combination.

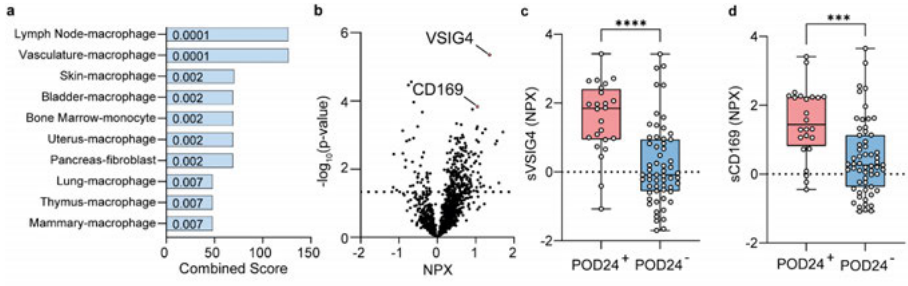

Figure 8. a. enrichment of different types of macrophage-associated proteins in POD24+ patients. b. The two most differentially expressed proteins were sCD169 and sVSIG4. c-d. The expression of sCD169 and sVSIG4 in patients with POD24+ vs POD24-.

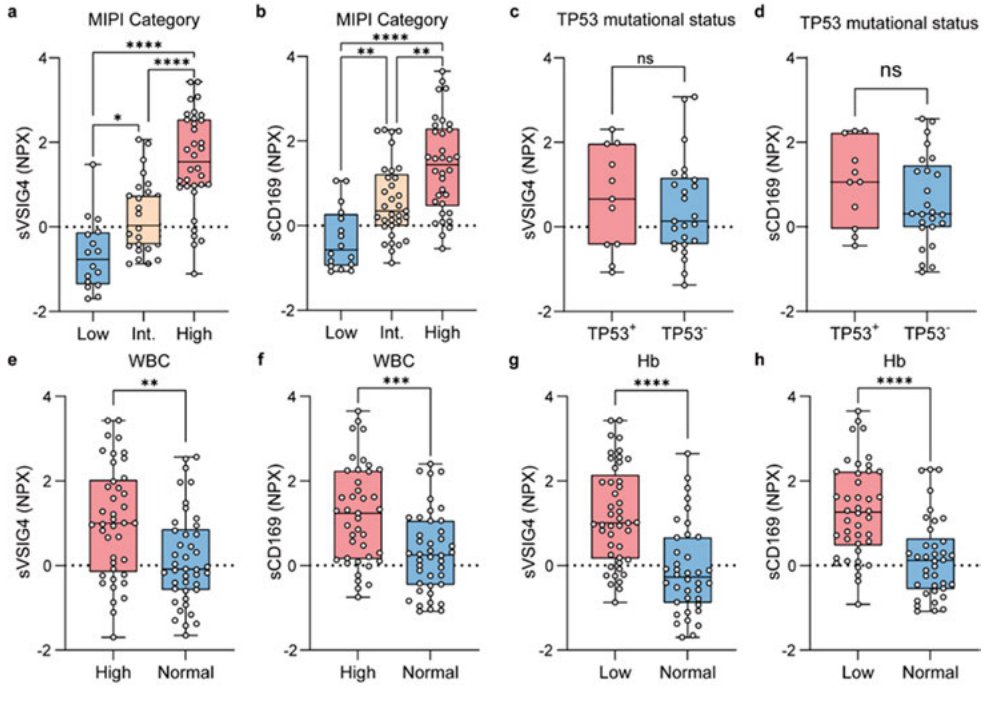

Figure 9. Association between sCD169 and sVSIG4 and selected clinical parameters.

Paper IV

Genomic evolution

Comparing paired tumour samples from diagnosis and relapse, we found that high-risk features accumulated with time. The most frequent new alteration was *CKDN2A* deletions, found in 29% of previously wild type patients. *TP53* alterations also accumulated with time. Together, these high-risk features were found in 67% of the patients at the last evaluated sample.

Looking beyond the established MCL-associated alterations, we detected new somatic alterations at relapse in all patients. New somatic mutations were detected in 100% and new CNAs in 53% of the patients.

Survival analysis

The development of new alterations was associated to worse prognosis and patients that presented with two or more new somatic mutations and/or any number of new CNAs at relapse had both shorter remaining OS and shorter time to progression (TTP) than patients without new alterations (univariable HR for OS (95% CI) 1.8 (1.1-2.9), multivariable HR 1.9 (1.1-3.1) adjusted for sex, age and *TP53* mutation at relapse).

Treatment type and genomic evolution

Importantly, we found that the number of both new mutations and new CNAs correlated with the type of treatment the patient had received. The average number of new alterations was 7.2 vs 3.1 vs 1.5, for patients treated with chemoimmunotherapy, targeted therapy and watch and wait (Figure 10). The average number of new mutations was highest among patients treated with bendamustine, while the number of new CNAs was highest in patients treated with high-dose chemotherapy and ASCT.

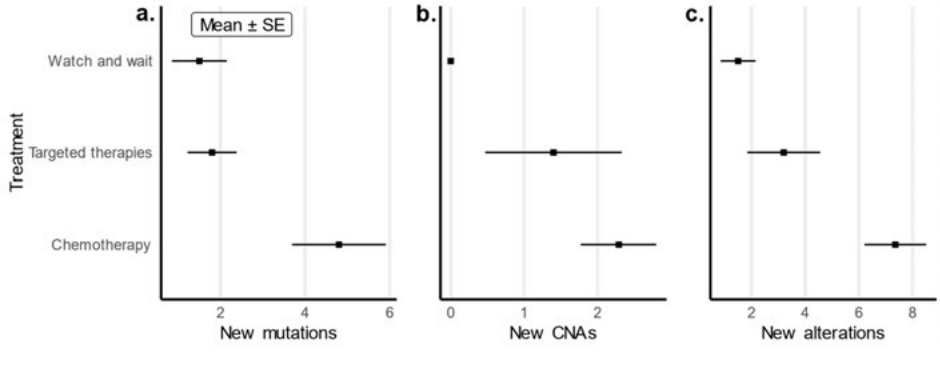

Figure 10. The number of new alterations at relapse was higher in patients that had been treated with first-line chemoimmunotherapy than in patients that had been treated with first-line targeted therapies or only been on watch and wait.

Paper V

Identification of clonal haematopoiesis

We detected one or more CH mutation in 15 of the 53 patients (28%). Among the genes associated with myeloid CH mutations, low variant allele frequency (VAF) *TP53* mutations were the most frequent followed by *DNMT3A, ASXL1, CREBBP, CUX1, ETV6, JAK2, KRAS, NOTCH1, PRPF8, SF3B1 and TET2*.

Patients with CH at diagnosis were more likely to require interventions for haematologic toxicity than patients without CH. The difference in toxicity was most pronounced in the incidence of anaemia and consequential erythrocyte transfusions (67% vs 24%, p=0.013), unplanned administration of granulocyte-stimulating factor (G-CSF) (58% vs 21%, p=0.027), and treatment dose reduction or discontinuation due to toxicity (92% vs 44%, p=0.004).

The increased risks were confirmed in odds ratio (OR) analysis over all 103 treatment lines. The increased incidence of interventions among patients with CH was only seen in those receiving chemotherapy and lenalidomide combinations and not in patients treated with BTKi (Figure 11).

CH at diagnosis was associated to shorter PFS, and so was the need of multiple interventions, dose reduction or termination of treatment.

In order to evaluate if the impact on haematologic toxicity and prognosis was driven by *TP53* CH, we repeated the analyses excluding *TP53* as CH mutations. The results were similar. Patients with non-*TP53* CH needed more interventions and more often required dose-reductions or preterm termination of treatment than patients without CH [OR (95% CI) 3.9 (1.1-14.1)] and [OR (95% CI) 4.4 (1.3-13.1)] respectively, adjusted for age, sex, treatment type and *TP53* alteration (Figure 12). Patients with non-*TP53* CH also had shorter PFS than patients without CH (log rank p=0.019).

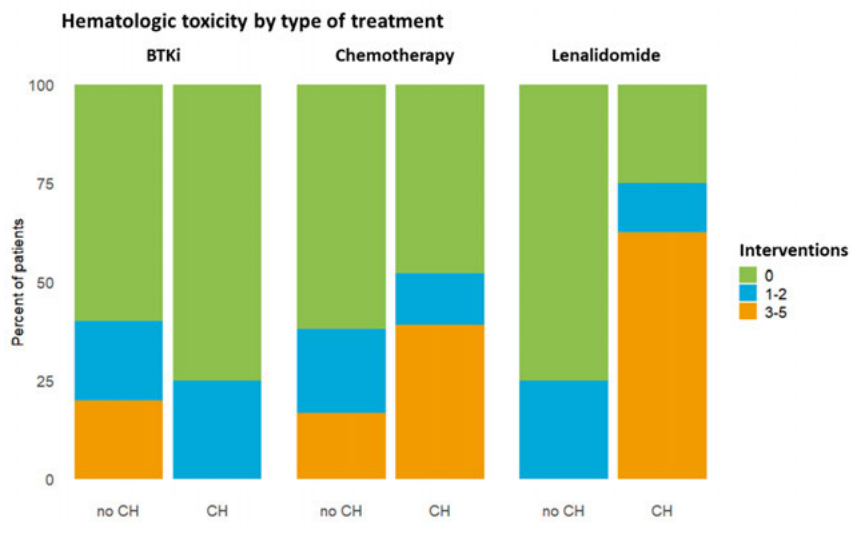

Figure 11. Haematologic toxicity measured as the need for different types of interventions, by type of treatment.

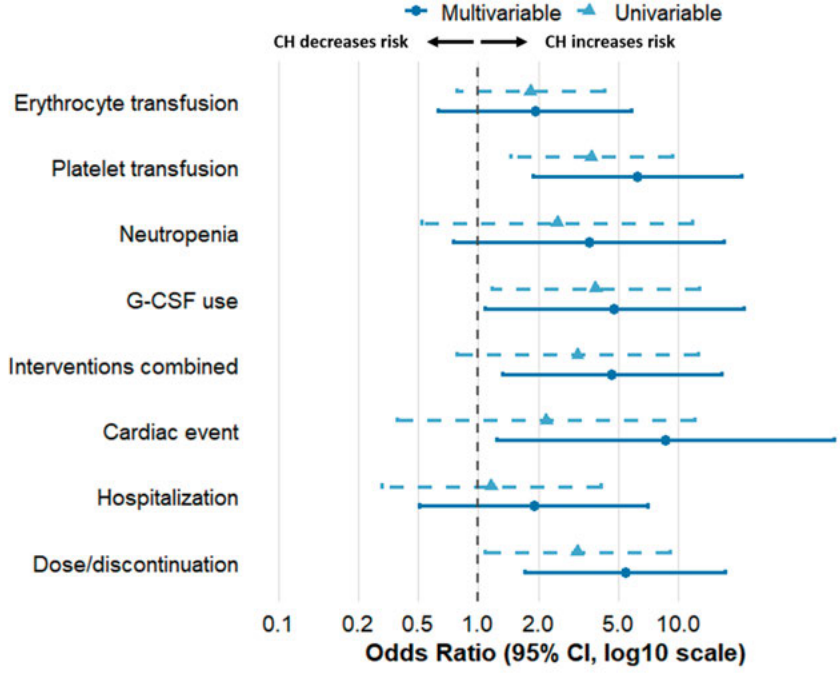

Figure 12. Risk for interventions, hospitalization and dose reduction or discontinuation in patients with and without non-*TP53* clonal haematopoiesis. Odds ratios with sandwich estimator. Multivariable model adjusted for age, sex, treatment type and *TP53* alteration.

Discussion and conclusions

Summary of main findings

In Paper I-V, we show that M2-like macrophages predict adverse prognosis in MCL, that genetic evolution is influenced by therapy, and that clonal haematopoiesis is a significant host factor shaping treatment toxicity.

Immune biology in MCL

Across three papers and five cohorts, we consistently showed that M2-like macrophages—captured by CD163 IHC, sCD163, sVSIG4 and sCD169—are associated with inferior prognosis in MCL. While similar findings exist in other malignancies (21), this is the first time this has been demonstrated in MCL.

Prognostic immune signatures

The macrophage markers CD163, CD69 and VSIG4 as well as the checkpoint ligand PD-L1 and regulatory T-cell marker FoxP3, all indicate cells that have properties that regulate and suppress inflammation. TAMs actively affect the tumour microenvironment and also support the tumour with angiogenesis, modulation of the stroma and immune evasion.

One interesting result in paper II was that the levels of sCD163 measured in complete remission were associated to the risk of relapse, and no patient with low levels relapsed within 24 months. A similar pattern was seen in paper III. This could indicate a more competent immune system in the patients with low levels of the macrophage markers, or continued stimulation of the TME by residing tumour cells among patients with high levels.

Mechanistic links -TP53 and macrophages

One very interesting finding is the correlation between macrophages and *TP53* mutations. *TP53*-mutated MCL samples had higher levels of CD163 and sCD163, indicating a link between *TP53* status and an M2-polarized microenvironment (Figure 13). Mechanistically, mutant p53 can promote immunosuppressive macrophage phenotypes via multiple pathways, including IL-34

and extracellular vesicle signalling (56-61). The correlation was not seen in paper III, something that could reflect that this correlation is specific to CD163, but the relation between *TP53* and sVSIG4 and sCD169 could not be fully evaluated due to many missing values and a correlation also among these markers cannot be excluded.

Both *TP53* and macrophages play important roles in MCL tumour progression, and the correlation could open up for treatment of *TP53*-altered MCL through the TME.

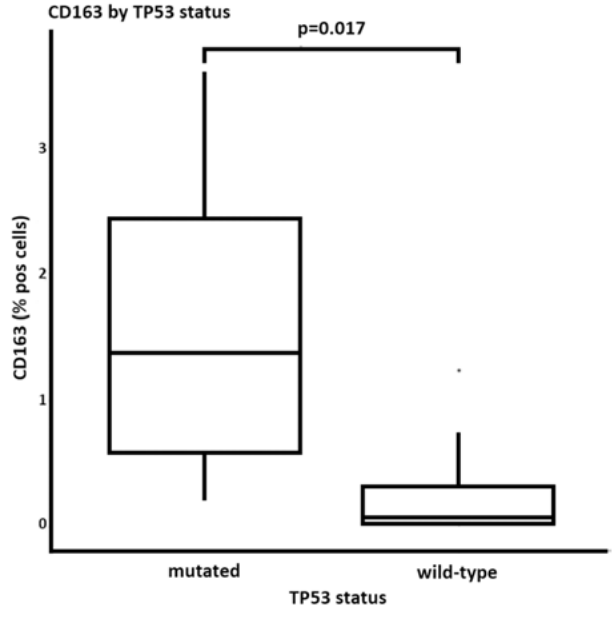

Figure 13. Percent of CD163 positive cells in *TP53* mutated versus *TP53* wild type tumours. CD163 values from lymph node samples, cohort from paper IV.

Clinical implementations of immune markers

The most immediate clinical implication of our findings lies in the use of soluble macrophage markers as prognostic tools. Low levels of sCD163, as well as sVSIG4 and sCD169, identify patients with excellent outcomes and may help define a group that could safely receive less intensive treatment to reduce toxicity. To become clinically useful, these markers must be standardized and validated across laboratories, and potential confounding factors, such as infection, taken into account. Further work is also needed to determine optimal cut-offs and to validate sVSIG4 and sCD169 in independent cohorts.

In addition to their role as biomarkers, macrophages and other immunoregulatory cells represent potential therapeutic targets. In paper I, combining

$CD163^+$ macrophage levels with other immune markers into composite immune signatures strengthened their prognostic value and underscored the importance of the immunosuppressive tumour microenvironment in MCL.

Immune checkpoint blockade against PD-1 or PD-L1, is successful in several other lymphomas, but has thus far yielded disappointing results in MCL (62). This may reflect both lower expression of these checkpoints in MCL (63) and the possibility that they are largely expressed by immune cells rather than tumour cells. Moreover, frequent 9p21 deletions in MCL may include the gene position of PD-L1 and PD-L2, forcing MCL cells to rely on alternative suppressive pathways for immune evasion.

Other immunoregulatory mechanisms may therefore be more relevant in MCL. One example is $FOXP3^+$ Tregs, which we identified in paper I as a negative prognostic marker. Their relevance is further supported by recent findings that SOX11 activate Tregs through CD70-CD27 signalling (64), and by the increasing interest in targeting Treg-associated checkpoints — an area highlighted by the 2025 Nobel Prize (65).

Altogether, these findings suggest that immune profiling may be useful in risk stratification and could help guide future therapeutic strategies in MCL.

Genetic evolution

In the largest longitudinal sequencing study of MCL so far, we show that high-risk alterations — particularly *TP53* and *CDKN2A* — accumulate over time. Importantly, the extent of new alterations correlated with treatment type, being highest after chemoimmunotherapy and lowest in watch-and-wait patients. We also found that pathology-based methods underestimate the prevalence of negative prognostic factors.

Mechanisms

Our findings are consistent with known mutational footprints of cytotoxic therapies as chemotherapy contribute to DNA damage and cytotoxic effects in both malignant and healthy cells as part of their mechanism of action. Chemotherapy induces mutations similar to those induced by aging, but about 100 times more during the period of exposure (41). They contribute to a well-known risk of secondary malignancies (41), as well as an increased incidence of CH after intense chemotherapy (66). These findings would support the theory of sequential clonal evolution rather than the "born to be bad" model(67), but we cannot rule out the presence of pre-existing high-risk alterations that emerges after treatment.

Limitations

Even though this was the largest study investigating therapy-associated genetic alterations in MCL so far, the numbers are still limited, especially among

patients with targeted therapies and waw. The results need to be validated in larger cohorts of paired biopsies, with formal statistical tests, also confirming that treatment, rather than tumour biology, explain the increase in alterations.

Clinical implication

The results of this study support the ongoing transitioning away from chemotherapy in order to not induce new alterations in already aggressive tumours. Many of the new clinical studies in MCL are investigating combinations of chemotherapy-free treatments such as BTKi + bispecific antibodies. This might not be sufficient for patients with rapidly progressing tumours and high-risk factors such as *TP53* alterations and Ki-67 >50%, where an induction with chemotherapy is probably still needed.

In order to identify patients in need of intense treatment or high risk of progression on standard treatments, gene sequencing is crucial. Pathology-based methods identified only a small part of the alterations, and patients should be evaluated with genome-based methods both at diagnosis and relapse.

Clonal haematopoiesis and treatment-related toxicity

We found that CH is a strong predictor of haematologic toxicity in MCL, independent of age and tumour characteristics. Patients with CH had markedly higher rates of anaemia, transfusion need, and treatment discontinuation.

Among CH carriers, 83% required clinical interventions and 92% needed dose reductions or treatment discontinuation, underscoring the challenges in safely delivering therapy to these patients. Our findings are consistent with previous studies, particularly those highlighting excess toxicity in patients with CH during lenalidomide-based regimens (68, 69).

Distinguishing CH from tumour-derived mutations

TP53 represents a particular challenge, as it is both a common driver mutation in MCL and a well-known CH-associated mutation (42, 68, 69). Current methods to differentiate CH from tumour mutations lack consensus. Some groups apply variant allele frequency cut-offs, while others rely on DNA fragmentation patterns or remission samples (42, 68, 70). Taking advantage of our sequential samples, we classified *TP53* mutations based on whether they tracked with the clonal dynamics of the tumour. Although this approach reduces misclassification, small tumour subclones may still be mistaken for CH.

Importantly, *TP53* mutations did not correlate with increased toxicity, and the association between CH and treatment-related toxicity was stronger when *TP53* CH cases were excluded. This suggests that part of the *TP53* mutations initially attributed to CH may represent tumour-derived mutations rather than true CH.

CH within the tumour microenvironment

Emerging evidence suggests that CH also infiltrate and shape the tumour microenvironment. Studies pairing tumour-free material with tumour have demonstrated myeloid cells carrying CH mutations, including *TP53*, within tumour stroma, where they can worsen clinical outcomes (71). Experimental models show that CH-bearing immune cells can promote tumour progression by suppressing antigen presentation, inducing T-cell exhaustion, and skewing macrophages toward an immunosuppressive M2 phenotype (40, 72-74).

Bidirectional interactions may also exist, where chronic inflammation within the tumour niche promotes the expansion of CH clones, establishing a self-reinforcing cycle of immune dysfunction and tumour growth (75) (Figure 14).

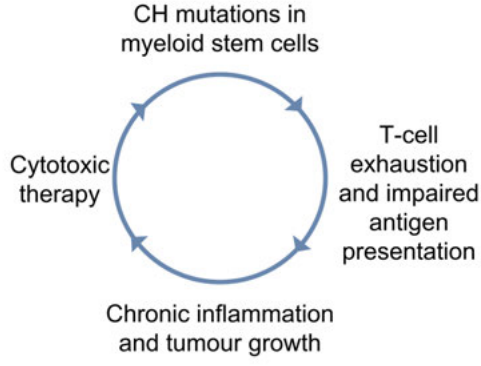

Figure 14. Self-reinforcing cycle of CH and tumour growth.

Clinical implications

CH status may help identify patients at higher risk for treatment-related toxicity, allowing pre-emptive supportive measures or the avoidance of high-risk therapies. Incorporating CH screening into clinical decision-making could therefore support a more individualized treatment strategy and ultimately reduce morbidity.

A related toxicity phenomenon is immune effector cell–associated haematotoxicity (ICAHT), characterized by prolonged anaemia, thrombocytopenia, or neutropenia after CAR T-cell therapy. The CAR-HEMATOTOX score, which incorporates blood counts, ferritin, and CRP, predicts this risk (76). Notably, these same parameters are frequently altered in CH-driven inflammation and CCUS (77, 78), raising the possibility that the score may serve as a surrogate marker for underlying CH. Supporting this, patients with CH and elevated ferritin do exhibit delayed haematologic recovery after CAR T-cell therapy (33). CH has also been associated with higher rates of CRS, ICANS, and post–CAR T-cell MDS (79, 80). If confirmed in larger studies, CH could

be useful for prediction of risk for haematologic toxicity also after T cell-engaging therapies.

Overall conclusion

This thesis illustrates how the outcome for MCL patients is shaped by the dynamic interplay between tumour biology, immune microenvironment, and individual patient factors. High levels of M2-like macrophages identify patients with biologically aggressive disease and a need for intensified therapy. At the same time, treatment itself may drive genomic evolution — not only within the tumour clone but also in non-malignant hematopoietic cells, leading to clonal haematopoiesis that can further alter the tumour microenvironment and impair treatment tolerance.

Together, these findings highlight that treatment outcomes and prognosis in MCL are influenced not only by the tumour itself, but also by the surrounding immune environment and patient-related factors. This means that future treatment strategies should combine genetic markers with immune-based biomarkers in order to better predict risk, choose the right treatment intensity, and reduce toxicity (Figure 15).

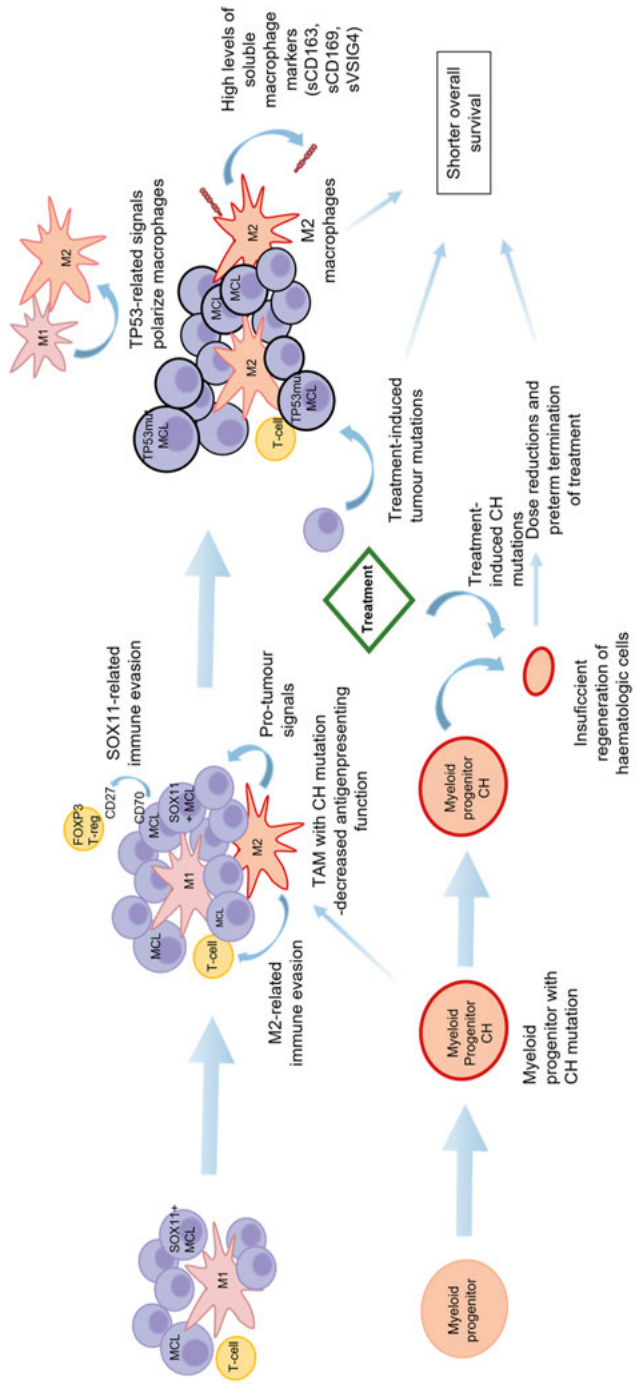

Figure 15. Illustration of the interplay between tumour microenvironment, clonal haematopoiesis, tumour genetics, treatment and outcome.

Future perspective

In the near term, clinical validation and implementation of the macrophage markers, preferably soluble CD163, may provide an important addition to the prognostic markers for risk-adapted treatment. Additionally, as gene panels become increasingly affordable and accessible, tumour genetic evaluation should be offered MCL patients at both diagnosis and relapse. CH mutations could easily be included in the gene-panels and the emerging knowledge about CH and treatment-related toxicity integrated into clinical decision-making.

Our results of targeted therapies being associated both with less genetic alterations at a subsequent relapse, and with less haematological toxicity in patients with CH, calls for a continued effort to treat MCL patients with chemotherapy free concepts in first line.

(Epi)genetic alterations and the TME

Moving forward, the interaction between M2 macrophages, the gene *TP53* and its protein p53 should be explored in more detail. Whether dysfunctional p53 drives macrophages toward a pro-tumoral M2 phenotype, or whether functional p53 actively supports anti-tumoral immunity (or a combination of both) is not established, but both p53 and M2 macrophages are key variables in MCL, and understanding this relationship may lead to new opportunities to therapeutically modulate the TME in high-risk MCL.

Another interesting step would be to map mutations to specific cell types in MCL, preferably through single-cell approaches. Such analyses could clarify whether presumed tumour mutations actually belong to immune cells, and whether certain CH-mutated clones contribute disproportionately to toxicity or immune dysfunction.

Epigenetic dysregulation is another largely unexplored dimension of MCL biology. Both chromosomal instability and SOX11 upregulation are important in MCL pathogenesis and may reflect early epigenetic events. Defining these changes may deepen the understanding of MCL pathogenesis and potentially lead to epigenetic therapies.

Macrophages and treatment-related complications

During the collection of data for Paper V, it became evident that MCL patients experience a vast range of treatment-related side-effects, also beyond the haematologic toxicity.

Infections are frequent. During and after treatment, increased infection rates can at least partly be attributed to B-cell aplasia, an intentional consequence of the treatment, but the increased susceptibility emerges at least four years before diagnosis and persists throughout follow-up, suggesting underlying immune dysregulation rather than treatment effects alone (81). Macrophages are an important part of the innate immune system and highly involved in responses to infection (82). An important next step is investigating the relations between M2 macrophages and risk of infection in MCL, and to what extent any correlations could be overcome by immunoglobulin supplementation.

T cell-engaging therapies and CD20-antibodies are associated with severe toxicity in MCL (83, 84). It has been speculated that the high incidence in CRS and ICANS is due to frequent bone marrow involvement, high tumour burden or specific to the drugs used in MCL. However, macrophages play a central role in the inflammatory cascade that drives CRS and ICANS (85-87). Given the importance of macrophages in MCL, they might also influence the risk of immunologic reactions to therapy. If a correlation between the TME composition and the risk of immunologic reactions was shown, this would open up for preventive measures.

Final summary

MCL remains a challenging disease to treat, but the landscape is rapidly changing. With new therapies and a better understanding of the immune microenvironment and genetic alterations, we are moving closer to individualized treatment. The work presented in this thesis highlights how factors beyond the tumour itself, including the immune system and clonal haematopoiesis, shape both the disease course and the treatment tolerance.

As treatment options expand, so does the challenge to use them wisely. The future of MCL care will depend on integrating a broad range of information: tumour biology, immune composition, patient factors, and the impact of treatment on each of these elements.

Ultimately, the goal is to offer patients longer, better lives — with treatments that are not only effective, but also thoughtfully tailored to those who receive them.

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