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## Molecular Investigations of high-risk Mantle Cell Lymphoma

### Genetic factors and the impact of the microenvironment

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2022

#### Document Version:

Publisher's PDF, also known as Version of record

[Link to publication](#)

#### Citation for published version (APA):

de Matos Rodrigues, J. (2022). *Molecular Investigations of high-risk Mantle Cell Lymphoma: Genetic factors and the impact of the microenvironment*. [Doctoral Thesis (compilation), Department of Immunotechnology]. Department of Immunotechnology, Lund University.

#### Total number of authors:

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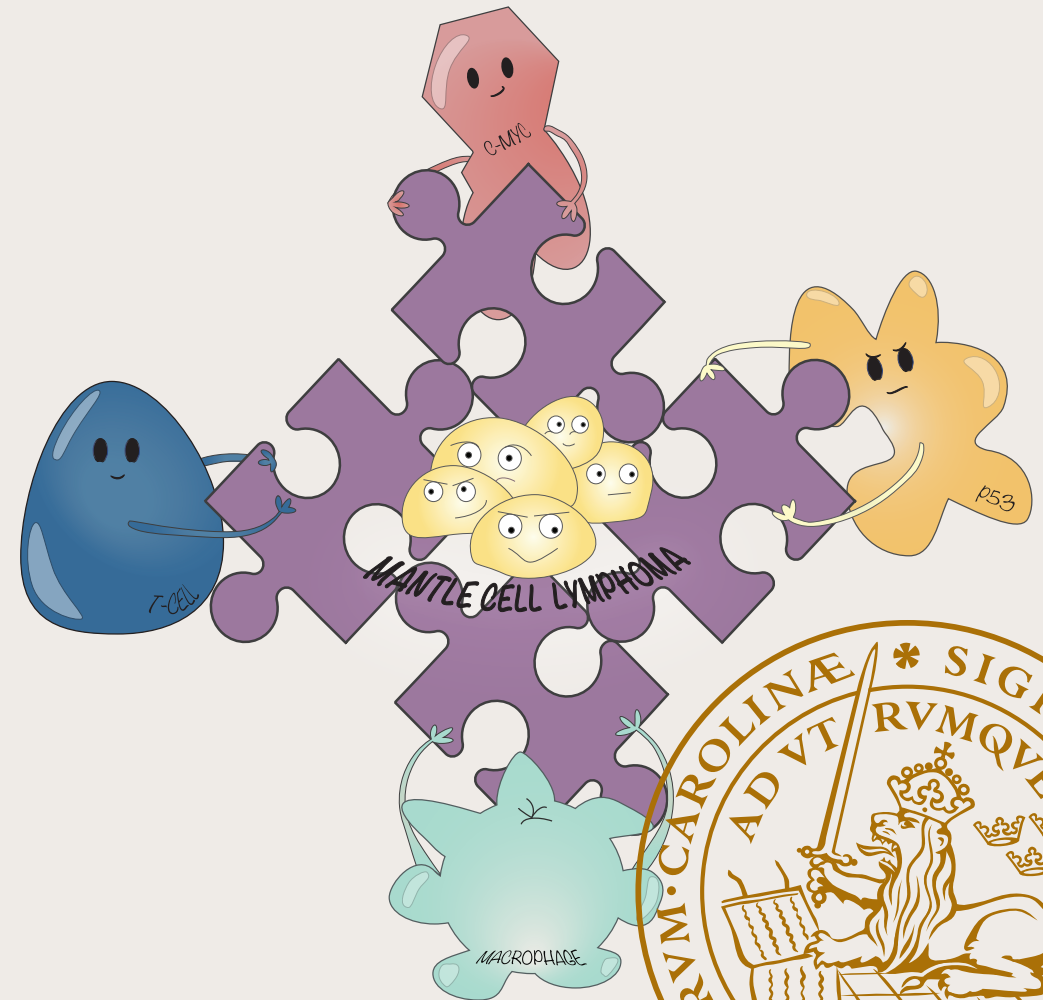
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# Molecular Investigations of high-risk Mantle Cell Lymphoma

Genetic factors and the impact of the microenvironment

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## Molecular investigations of high-risk mantle cell lymphoma



# Molecular Investigations of high-risk Mantle Cell Lymphoma

Genetic factors and the impact of the  
microenvironment

Joana de Matos Rodrigues



**LUND**  
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## DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the  
Faculty of Engineering at Lund University to be publicly defended on 17<sup>th</sup> of  
June at 09.00 in Sharience, Medicon Village,  
Scheeleorget 1, Lund

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| <b>Organization</b><br>LUND UNIVERSITY<br><br>Department of Immunotechnology<br>Medicon Village (building 406)<br>SE-223 81 Lund, Sweden<br><br>Author<br>Joana de Matos Rodrigues                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |                           | <b>Document name</b><br>DOCTORAL THESIS<br><br><b>Date of issue</b><br>17 <sup>th</sup> June 2022<br><br>Sponsoring organization |
| <b>Title and subtitle</b><br>Molecular Investigations of high-risk Mantle Cell Lymphoma<br><i>Genetic factors and the impact of the microenvironment</i>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |                           |                                                                                                                                  |
| <b>Abstract</b><br><p>Mantle cell lymphoma (MCL) is an aggressive disease, with variable clinical course and heterogenous molecular characteristics. In this thesis, which is based upon six original papers, we aimed to understand MCL deregulations, both at intrinsic and extrinsic levels, to identify companion biomarkers that would allow patient stratification. With the aim to bridge the gap between research and clinical application, the current work took advantage of major technological breakthroughs to advance the biological understanding of MCL. Paper I showed the applicability to apply next-generation sequencing to formalin-fixed paraffin-embedded (FFPE) MCL tumor samples, enabling clinical implementation. The workflow allowed for the identification of recurrent and new mutations that can be used to guide treatment and to investigate the efficacy of new compounds in MCL. Despite major clinical benefits, the current standard of care shows varying treatment responses, with most patients eventually relapsing. In paper II we explored the transcriptomic profile of patients submitted to this regimen and identified metabolic changes in patients with worse treatment outcome. We focused on CPT1A, a rate-limiting enzyme in fatty acid oxidation, as a biomarker for MCL, and showed that it has a negative prognostic impact. <i>TP53</i> mutations have been accepted as markers of poor prognosis in MCL, but genetic evaluation remains a challenge in many clinical facilities. Thus, in paper III we explored the possibility of using p53, evaluated by immunohistochemistry, as a surrogate marker for <i>TP53</i> missense mutation. We showed a high concordance between p53 overexpression and <i>TP53</i> missense mutation. In paper IV we intended to understand the role of c-Myc in MCL. We concluded that overexpression of c-Myc at protein and mRNA level was associated with poor clinical outcomes and, contrary to other B-cell lymphomas, <i>MYC</i> was not often translocated nor showed amplifications in MCL. Further, we describe a synergistic negative effect of dual aberrations in <i>MYC</i> and <i>TP53</i>. Paper III and paper IV findings strengthened the need for patient stratification in MCL for effective clinical management of the disease. Paper V and paper VI focused on the tumor-extrinsic factors in MCL. In paper V, we characterized the immune composition of MCL, particularly the presence of T-cell subtypes and the M2-like macrophages (CD163+ cells). We concluded that FoxP3+ cells were associated with shorter time to progression in patients treated with standard of care and that the presence of CD163+ cells was a negative prognostic marker, irrespective of treatment. In paper VI we were interested in understanding the crosstalk between tumor cells, T-cells and CD163+ cells in MCL in a spatial context. We showed that CD163+ cells are affected by their distance to tumor cells and that tumor microenvironments with presence of CD163+ cells show higher levels of MAPK activation. In summary, this work provided a comprehensive analysis of the contribution of molecular and tumor-extrinsic factors to treatment outcome in MCL and advanced the current knowledge of the disease, by highlighting additional therapeutic targets.</p> |                           |                                                                                                                                  |
| <b>Key words</b> CPT1A; c-Myc; Immune microenvironment; Macrophages; Mantle Cell Lymphoma; <i>TP53</i>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |                           |                                                                                                                                  |
| Classification system and/or index terms (if any)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |                           |                                                                                                                                  |
| Supplementary bibliographical information                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |                           | <b>Language: English</b>                                                                                                         |
| ISSN and key title                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |                           | <b>ISBN</b><br>978-91-8039-275-4 (electronic)<br>978-91-8039-276-1 (print)                                                       |
| Recipient's notes                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | <b>Number of pages</b> 93 | Price                                                                                                                            |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | Security classification   |                                                                                                                                  |

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# Molecular Investigations of high-risk Mantle Cell Lymphoma

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microenvironment

Joana de Matos Rodrigues



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Faculty of Engineering

Department of Immunotechnology

ISBN 978-91-8039-275-4 (electronic)

ISBN 978-91-8039-276-1 (print)

Printed in Sweden by Media-Tryck, Lund University  
Lund 2022



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*“Remember to look up at the stars and not down at your feet.*

*Try to make sense of what you see and wonder about what makes the universe exist. Be curious. And however difficult life may seem, there is always something you can do and succeed at.*

*It matters that you don't just give up.”*

***Stephen Hawking***



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Papers I-VI

# Original Papers

This thesis is based on the following papers, which will be referred to in the text according to their roman numerals (I-VI).

- I Rodrigues JM, Porwit A, Hassan M, Ek S, Jerkeman M. Targeted genomic investigations in a population-based cohort of mantle cell lymphoma reveal novel clinically relevant targets. *Leuk Lymphoma*. 2021;62(11):2637-47.
- II Gerdtsen AS, Rodrigues JM, Eskelund CW, Husby S, Grønbæk K, Rätty R, Kolstad A, Geisler C, Porwit A, Jerkeman M, Ek S. Overexpression of the key metabolic protein CPT1A defines mantle cell lymphoma patients with poor response to standard high dose chemotherapy independent of MIPI and complement established high-risk factors. *Manuscript submitted for publication*
- III Rodrigues JM, Hassan M, Freiburghaus C, Eskelund CW, Geisler C, Rätty R, Kolstad A, Sundström C, Glimelius I, Grønbæk K, Kwiecinska A, Porwit A, Jerkeman M, Ek S. p53 is associated with high-risk and pinpoints *TP53* missense mutations in mantle cell lymphoma. *Br J Haematol*. 2020;191(5):796-805.
- IV Rodrigues JM, Hassan M, Hollander P, Gkika E, Andersson S, Ek F, Olsson R, Geisler C, Rätty R, Kolstad A, Sundström C, Glimelius I, Porwit A, Jerkeman M, Ek S. High-risk MCL with *MYC* deregulation: molecular aberrations and treatment alternatives. *Manuscript*
- V Rodrigues JM\*, Nikkarinen A\*, Hollander P, Weibull CE, Rätty R, Kolstad A, Amini RM, Porwit A, Jerkeman M, Ek S\*, Glimelius I\*. 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;193(3):520-31.

- VI     Rodrigues JM, Lokhande L, Gerdtsen AS, Nikkarinen A, Hollander P, Porwit A, Glimelius I, Jerkeman M, Ek S. Impact of spatial localization and presence of macrophages on immune suppression in mantle cell lymphoma. *Manuscript*

\* Indicates equal authorship contribution

# My contribution to the papers

- I I was involved in the planning of the study, performed data and statistical analysis, and wrote the manuscript.
- II I was involved in the design of the gene expression microarray practical work. I was responsible for the immunohistochemistry work, and I performed the respective data analysis and interpretation. I contributed to the writing of the manuscript.
- III I performed data and statistical analysis and took an active part in writing the manuscript.
- IV I was involved in the planning and design of the work. I took a role in the experimental *in vitro* work. I performed data interpretation and statistical analysis and wrote the manuscript.
- V I participated in the planning of the study, performed data and statistical analysis and took an active role in the writing of the manuscript.
- VI I designed the study and was involved in the planning of the work. I was involved in the selection of regions in the study. I was the main responsible for data analysis and interpretation. I wrote the manuscript.

# Additional Publications

Merrien M, Wasik AM, Ljung E, Morsy MHA, de Matos Rodrigues J, Carlsten M, Rassidakis GZ, Christensson B, Kolstad A, Jerkeman M, Ek S, Herold N, Wahlin BE, Sander B. Clinical and biological impact of SAMHD1 expression in mantle cell lymphoma. *Virchows Arch*. 2021; Epub ahead of print.



# Abbreviations

|              |                                           |
|--------------|-------------------------------------------|
| ASCT         | Autologous Stem Cell Transplantation      |
| <i>ATM</i>   | ATM Serine/Threonine kinase               |
| BCL-2        | Apoptosis Regulator Bcl-2                 |
| BCR          | B-cell receptor                           |
| BLISS        | Biobank of Lymphomas in Southern Sweden   |
| BTK          | Bruton's Tyrosine Kinase                  |
| B7-H3        | B7 homolog 3                              |
| CAR          | Chimeric Antigen Receptor                 |
| <i>CCND1</i> | Cyclin D1                                 |
| CD           | Cluster of Differentiation                |
| c-Myc        | Myc Proto-oncogene Protein                |
| CNS          | Central Nervous System                    |
| <i>CPT1A</i> | Carnitine Palmitoyltransferase 1A         |
| CSF1         | Macrophage Colony-stimulating Factor 1    |
| CTLA-4       | Cytotoxic T-lymphocyte protein 4          |
| CXCL12       | Stromal Cell-derived Factor 1             |
| CXCR4        | C-X-C Chemokine Receptor type 4           |
| DLBCL        | Diffuse Large B-cell Lymphoma             |
| ERK          | Extracellular Signal-regulated Kinase     |
| FAK          | Focal Adhesion Kinase                     |
| FAO          | Fatty Acid Oxidation                      |
| <i>FEN1</i>  | Flap Structure-specific Endonuclease 1    |
| FFPE         | Formalin-fixed Paraffin-embedded          |
| FISH         | Fluorescence <i>in situ</i> Hybridization |
| FL           | Follicular Lymphoma                       |
| FoxP3        | Forkhead Box Protein P3                   |
| ICB          | Immune Checkpoint Blockade                |
| IDO          | 2,3-Dioxygenase                           |
| <i>IGH</i>   | Immunoglobulin                            |

|                      |                                                                     |
|----------------------|---------------------------------------------------------------------|
| IHC                  | Immunohistochemistry                                                |
| IL                   | Interleukin                                                         |
| JNK                  | c-Jun N-terminal Kinase                                             |
| Ki-67                | Proliferation Marker Ki-67                                          |
| MAPK                 | Mitogen-activated Protein Kinase                                    |
| MCL                  | Mantle Cell Lymphoma                                                |
| <i>MEF2B</i>         | Myocyte Enhancer Factor 2B                                          |
| MIPI                 | Mantle cell lymphoma International Prognostic Index                 |
| mTOR                 | Mammalian Target of Rapamycin                                       |
| mutp53               | Mutated p53 protein                                                 |
| <i>MYC</i>           | MYC proto-oncogene                                                  |
| NF- $\kappa$ B       | Nuclear Factor Kappa-light-chain-enhancer of Activated B-cells      |
| NGS                  | Next-Generation Sequencing                                          |
| NHL                  | Non-Hodgkin Lymphoma                                                |
| NK cells             | Natural Killer cells                                                |
| NLG                  | Nordic Lymphoma Group                                               |
| N-MCL2/3             | Nordic Lymphoma Group MCL2 and MCL3 clinical trials                 |
| OS                   | Overall Survival                                                    |
| OXPPOS               | Oxidative Phosphorylation                                           |
| p53                  | Cellular Tumor Antigen p53                                          |
| PAX5                 | Paired Box Protein Pax-5                                            |
| PD-1                 | Programmed Cell Death Protein 1                                     |
| PD-L1                | Programmed Cell Death 1 Ligand 1                                    |
| PI                   | Proliferation Index                                                 |
| PI3K                 | Phosphoinositide 3-kinase                                           |
| PLK1                 | Serine/Threonine-protein Kinase PLK1                                |
| R/R                  | Relapsed/Refractory                                                 |
| R-CHOP               | Rituximab, Cyclophosphamide, Doxorubicin, Vincristine, Prednisolone |
| SIRP $\alpha$        | Signal Regulatory Protein Alpha                                     |
| SOX11                | Transcription Factor SOX-11                                         |
| STAT                 | Signal Transducer and Activator of Transcription                    |
| T <sub>c</sub> cells | Cytotoxic T-cells                                                   |
| T <sub>H</sub> cells | Helper T-cells                                                      |
| <i>TP53</i>          | Tumor Protein p53                                                   |

|                   |                                                                            |
|-------------------|----------------------------------------------------------------------------|
| T <sub>regs</sub> | Regulatory T-cells                                                         |
| TTP               | Time to Progression                                                        |
| VISTA             | V-type Immunoglobulin Domain-containing Suppressor of<br>T-cell Activation |
| <i>WEE1</i>       | G2 Checkpoint Kinase                                                       |
| WEE1              | Wee1-like Protein Kinase                                                   |
| WT                | Wild-type                                                                  |
| XPO1              | Exportin 1                                                                 |



# 1. Introduction

The incredible breakthroughs and technological advancements in biomedical research in the last years have prompted healthcare management to shift towards precision medicine approaches (1). Precision medicine aims to facilitate clinicians' decisions by efficiently and accurately predicting the best available clinical workflow for an individual patient. Hence, there is a need for developing/adapting tools that can be easily implemented in a clinical setting with a low economic burden. The identification of companion diagnostic, prognostic and therapeutic biomarkers needs to be achieved for each group of patients. This thesis is focused on the B-cell malignancy mantle cell lymphoma (MCL). The main aim was to contribute to the molecular knowledge of this complex disease while identifying companion biomarkers, for clinical implementation, to allow patient stratification.

Cancer is a major public health issue worldwide and is estimated that 19.3 million cases were identified in 2020. Cancer is the attributed cause of death to 10 million people in 2020 (2) and the burden of cancer incidence and mortality is still growing in society. This is partly caused by an aging population. Treatment approaches to cancer today are still based on clinical factors, such as the age and fitness of the patient, not taking fully into account molecular characteristics of the tumor. Increased biology knowledge is expected to promote a shift towards tailored approaches. The last decades have brought massive advancements in technologies that allow clinicians to improve clinical management for specific diseases. The overall result is a significant reduction in mortality and a lower burden in health care, with less patients relapsing and in need of further treatment, and potentially lower costs for society (3-6). However, many patients still fail to achieve the desired outcome with current therapeutic strategies. The goal is to identify patterns of deregulation that can enable clinicians to stratify patients efficiently and accurately into tailored treatment approaches. An important feature of such research is the need to adapt complex molecular traits in tools that

are compatible with the clinical workflow, as well as maintaining a low economic impact on healthcare.

MCL is an aggressive B-cell lymphoma for which afflicted patients have a short life expectancy of five to seven years after diagnosis (7). In the last decades, an improvement in overall survival (OS) has been observed (8). This is largely due to the implementation of rituximab, an anti-human cluster of differentiation (CD) 20 antibody, in combination with high dose cytarabine and autologous stem cell transplantation (ASCT) when deemed possible (9, 10). However, this approach is primarily used in young and fit patients. Thus, MCL treatment decision is still limited by the patients' age and fitness.

It is a disease characterized by a highly heterogeneous clinical course, with some patients achieving long and durable responses, while others failing to respond to first-line standard treatments. However, long-term, most patients will relapse (11). At relapse, no standard treatment for relapsed/refractory (R/R) disease is defined and little information is available to guide treatment options (12).

In paper I-IV of this thesis, the focus was to explore tumor intrinsic factors in MCL. In Paper I, we aimed to demonstrate the applicability of a Next-Generation Sequencing (NGS) panel of lymphoma targets developed for formalin-fixed paraffin-embedded (FFPE) samples in the clinical workflow of MCL management. The study confirmed that targeted sequencing of FFPE samples could be an option for tailored treatment approaches, but it remains to be shown which treatments will benefit most tumor protein p53 (*TP53*) mutated and other high-risk groups. In Paper II, the transcriptomic landscape was explored. MCL is commonly categorized by low proliferation index (PI) (low proliferation marker protein Ki-67 (Ki-67) expression) or high PI (high Ki-67 expression); and with classical morphology or non-classic morphology (blastoid and pleomorphic variants). Highly proliferative tumors and tumors with non-classic morphology are commonly associated with extremely aggressive disease and poor prognosis, being resistant to current therapies (13). The goal was to move beyond this classification, often susceptible to inter-observer variability, and identify additional molecular traits that could be used as companion biomarkers and alternative targets in these subgroups. Traditionally, genetic deregulation in MCL has been focused primarily on proliferation. However, targeting proliferation alone has proven insufficient to treat MCL. In this study, we revealed

an additional association between treatment failure and deregulation of metabolic pathways. Our analysis pinpointed carnitine palmitoyltransferase 1A (*CPT1A*), an enzyme in fatty acid oxidation (FAO), as a poor prognostic marker, similar to previous reports in other cancers (14).

*TP53* mutations are considered an independent prognostic factor in MCL (15), but their assessment in clinical routine is still hampered due to the lack of routine target sequencing approaches in the clinical workflow, particularly outside the major hospitals. In paper III we focused on filling this need. Our results showed that, when sequencing data is not available, immunohistochemistry (IHC) evaluation of cellular tumor antigen p53 (p53) expression is a reliable surrogate for identifying missense mutations in *TP53*. c-Myc has an important role in B-cell development and identifies high-risk groups in other lymphomas (16). However, the role of myc proto-oncogene protein (c-Myc) in MCL is far from understood and the frequency at which it is affected shows conflicting results in different studies. Thus, in paper IV, we aimed to clarify the impact of c-Myc molecular aberrations in MCL. We showed that c-Myc deregulations commonly appear at the transcriptomic and protein level, rather than involving chromosomal rearrangements, and could be considered a companion diagnostic marker with prognostic value in MCL.

Papers V and VI focused on the impact of the microenvironment in MCL. The role of the microenvironment in the proliferation and drug resistance mechanisms in MCL is well accepted (17). Nonetheless, the composition of the microenvironment in MCL remains poorly studied. The goal of Paper V was to characterize the immune microenvironment in MCL and identify prognostic markers beyond the current tumor intrinsic molecular risk factors. This study showed the strong impact of the MCL composition in disease and treatment response, highlighting an immune suppressive microenvironment as a negative prognostic factor and opening doors to exploiting immune oncologic strategies in the clinical management of MCL. We showed that the presence of forkhead box protein P3 (FoxP3)<sup>+</sup> cells was associated with a shorter time to progression (TTP) in patients treated with the current standard protocol. CD163<sup>+</sup> macrophages showed the strongest prognostic impact in MCL among the phenotypes explored, independent of the treatment approach and other high-risk markers in MCL. Thus, in paper VI we explored the crosstalk between MCL tumor cells and CD163<sup>+</sup> M2-like macrophages. CD163<sup>+</sup> M2-like macrophages have different

phenotypic profiles depending on their spatial localization and the presence of macrophages in the tumor microenvironment translated into an increased expression of the mitogen-activated protein kinase (MAPK) pathway.

In essence, the following chapters will put the findings of the present work into a broader context. Initially, an introductory chapter about the current clinical setting of MCL will be presented, with a brief description of the cohorts used throughout the studies (Chapter two). Chapter three will focus on the tumor intrinsic factors that are associated with poor patient prognosis and the identification of prognostic and companion biomarkers that allow for patient stratification, as an essential step towards precision medicine. In chapter four the tumor microenvironment will be discussed, with a focus on T-cells and macrophages as biomarkers beyond the traditional tumor-intrinsic factors and potential targets of immunomodulatory treatments. Lastly, the main conclusions will be presented in chapter five.



## 2. Mantle Cell Lymphoma

In western countries, the yearly incidence of lymphoma is estimated to be 20/100 000 people (18). In Sweden, 2 000 people are diagnosed with a subtype of lymphoma every year (19). Traditionally, lymphomas have been categorized in Hodgkin and non-Hodgkin lymphomas (NHL). Worldwide, more than 250 000 people died of NHL in 2020, whereas more than 500 000 were newly diagnosed (2). MCL represents 3-10% of all NHL and is the focus of this work. MCL will be briefly presented in this chapter and discussed in the following chapters in the context of the novel findings of the different papers that constitute this thesis.

### 2.1 B-cell lymphomas

Lymphomas represent a highly heterogeneous group of malignancies that arise from lymphocytes (7). This heterogeneity is due to the biological development and differentiation of lymphocytes in the human body, which is a stepwise process of genetic events that lead to an extensive and diverse B and T-cell repertoire (20). Most lymphomas arise in B-cells and bear phenotypic resemblances to a specific stage in B-cell differentiation, commonly termed as “cell of origin” (21, 22). B-cell lymphomas are commonly divided into indolent and aggressive subtypes, depending on clinical and biological characteristics. Indolent variants are for example follicular lymphoma (FL) and marginal zone lymphoma. Diffuse large B-cell lymphoma (DLBCL) and MCL are classified as aggressive lymphoma variants (7, 22).

MCL has an increasing incidence worldwide (23), with an estimation of 3 320 newly diagnosed patients in 2016 (24). The Nordic Lymphoma Group (NLG) reported an increased incidence in both Sweden and Denmark between 2001 and 2010 (25). To address the proposed aim of this thesis, clinical material from two cohorts of diagnostic MCL patients was used. The first cohort named

NLG MCL2 and MCL3 (N-MCL2/3) is part of two clinical trials conducted by the NLG that established the current standard of care (9, 10). The second cohort, Biobank of Lymphomas in Southern Sweden (BLISS) is a population-based cohort of patients diagnosed with MCL in Southern Sweden between 2000-2014.

## 2.2 Molecular events characteristic of MCL

The t(11;14)(q13;q32) is the hallmark of MCL and is considered the primary genetic event (26). This aberration juxtaposes the cyclin D1 (*CCND1*) gene to the immunoglobulin (*IGH*) gene, leading to constitutive expression of cyclin D1 (27-29). Most MCL tumors acquire the translocation in the pro/pre-B-cell stage of B-cell differentiation, during V(D)J recombination. Alternatively, the translocation can be acquired during somatic hypermutation or class switch recombination in the mature B-cell stage (28, 30). *CCND1* is a proto-oncogene that regulates the cell cycle transition G1-S (31). Overexpression of cyclin D1 promotes proliferation independently on external stimuli, conferring these cells a growth advantage. Cyclin D1 is capable of binding to several promoters, leading to an abnormal transcriptome in neoplastic cells, as well as being involved in DNA-damage response and apoptosis regulation (31, 32).

Transcription factor SOX-11 (SOX11) is a disease-specific antigen in MCL, reported to be overexpressed in 90% of MCL patients (33-36). SOX11 expression is epigenetically regulated (37-39), with the promoter region being unmethylated in MCL tumors, as opposite to SOX11 negative B-cell lymphomas. SOX11 reduces BCL6 expression, hindering entrance into the germinal center (40). Additionally, SOX11 is also reported to promote paired box protein Pax-5 (PAX5) expression, which blocks the cell in the mature B-cell stage (41). It is then postulated that epigenetic deregulation of SOX11 may be an early and important player in MCL initiation.

## 2.3 Clinical presentation and pathobiology evaluation

Diagnosis of MCL is performed according to the World Health Organization Classification of Hematological Neoplasms (7). Patients that

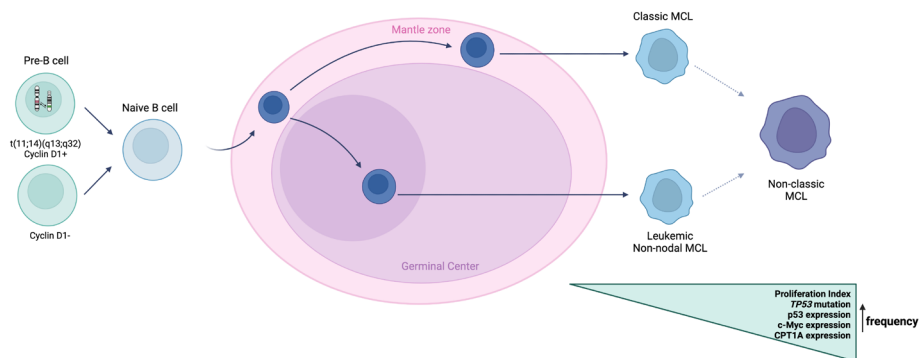
develop MCL are elderly, with a median age at diagnosis of >70 years, and often male (7, 25, 27). Our population-based cohort (BLISS) has a median age at diagnosis of 71 years (range from 45-94) and 76% of male predominance. N-MCL2/3 is representative of clinical trials focused on young patients ( $\leq 65$  years), as so, age at diagnosis was considerably lower (median of 57 years, ranging from 28 to 65) than the median diagnostic age for MCL. Nonetheless, the male predominance is still represented, with 73% of patients being male.

MCL is a highly heterogeneous disease and patients diagnosed with MCL show a variety of clinical presentations. Most of the patients are diagnosed with advanced and disseminated disease (Ann Arbor stage III and IV), showing lymphadenopathy, cytopenia, splenomegaly and extra-nodal manifestations (12). Extra-nodal manifestations commonly appear in the bone marrow, blood, and gastrointestinal tract (7, 42). Involvement of the central nervous system (CNS) is rare at the time of initial diagnosis, with some studies reporting a negative effect on survival, yet scarce information is available (43-45).

The advancements in the last decades have expanded the knowledge on the cell of origin and the current classification of MCL into two main categories: conventional and leukemic non-nodal MCL (Figure 1). Subtyping of MCL aids in understanding the heterogeneous clinical characteristics reported in MCL and the subtypes are described as having different cells of origin (7, 12). Most diagnosed MCL are of conventional presentation. The leukemic non-nodal MCL variant, which is reported with highly variable frequency, is indolent and often asymptomatic at diagnosis (7). Both cohorts used throughout our studies are mostly representative of the classical MCL, with patients presented as symptomatic at the time of diagnosis. Most tissue samples are from an affected lymph node, followed by biopsies from bone marrow and the gastrointestinal tract. Moreover, an additional entity, *in situ* mantle cell lymphoma, is reported (7), associated with accidental findings and believed to have a low rate of progression that may not require therapeutic intervention (46).

From the histological point of view, three subtypes of MCL are distinguished: classical, pleomorphic and blastoid. Classical MCL is traditionally cyclin D1 positive (7, 12), but cyclin D1 negative cases do exist (47). These cases are often cyclin D2 or cyclin D3 positive, highlighting the impact of D-type cyclins in the development of MCL (47-50). SOX11 works as a supportive diagnostic biomarker for cyclin D1 negative cases, since its expression is not connected with

of cyclin D1 immunoreactivity (7, 34, 36). Above 90% of MCL as SOX11 positive. Assessment of t(11;14)(q13;q32) is done by fluorescent *in situ* hybridization (FISH). MCL cells are positive for common B-cell antigens, namely, CD19, CD20, CD22, and CD79. Usually, MCL cells are also positive for FMC-7, CD5 and CD43, but negative for CD3, CD23, CD11c, CD10 and negative for CD200 (7, 12). Aberrations of the common immunophenotype of MCL have also been reported, such as CD5 negativity, CD11c, CD23 and CD200 positivity (51, 52).



**Figure 1 Mantle cell lymphoma pathogenesis.** MCL is characterized by Cyclin D1 reactivity due to the hallmark t(11;14)(q13;q32) that juxtaposes the *CCND1* gene to the *IGH* gene. Although less frequent, Cyclin D1 negative MCL cases are reported in the literature, frequently overexpressing other members of the Cyclin D family. WHO classification currently identifies two clinical subtypes of MCL: Leukemic non-nodal MCL and conventional MCL, which are described to have a different cell of origin. Histologically, MCL is classified into three subtypes: classic, pleomorphic, and blastoid, with the latter two commonly referred to as non-classic MCL. Non-classic MCL is more aggressive and associated with a higher proliferation index, higher frequency of *TP53* mutations, p53 and c-Myc overexpression and increased levels of CPT1A protein expression. These high-risk factors, although more frequent in non-classic MCL are not exclusively of this histological subtype.

## 2.4 Prognosis

The MCL International Prognostic Index (MIPI) is currently the only prognostic score used in the clinical workflow. It is calculated based on age, performance status, lactate dehydrogenase, and leukocyte counts. MIPI stratifies the patients into three risk categories, low, intermediate, and high-risk patients. These three groups were initially reported to have an average OS not reached, 51 months and 29 months, respectively (53). In a more recent update, the five-year OS rate was 83%, 63% and 34% for low, intermediate and high-risk patients,

respectively (54). Variations of MIPI have also been explored with combinations with other markers, such as MIPI-B (including Ki-67 expression) (55) MIPI-B-miR (including microRNA-18b expression) (56), and including p53 and SOX11 overexpression (57), among others, but they remain to be applied in the clinical setting as routine. Patients with non-nodal MCL are hypothesized to have an indolent disease associated with longer survival (58). The blastoid and pleomorphic histological variants of the disease, also termed non-classic morphology, are associated with inferior prognosis (7, 15, 59). Other commonly attributed markers of poor prognosis are high PI, measured by Ki-67 expression (60), *TP53* mutations (15), and c-Myc aberrations (61). In chapter three, these high-risk markers will be further described.

MCL patients have a median of 5-7 years of OS after diagnosis, with age being one of the strongest prognostic markers (7, 12). The BLISS cohort reflects real-world patient data, with a median OS of 4.5 years. The NLG clinical trials MCL2 and MCL3 (9, 10) showed that young and fit patients could achieve a median OS of 12.7 years and a median TTP of 9.9 years. This cohort is a source of valuable biological material to be used to understand possible mechanisms that predict relapse and pinpoint prognostic indicators.

## 2.5 Current treatment strategies in MCL

Nowadays, treatment management is based upon the patients' age at diagnosis, evaluation of physical fitness, and underlying comorbidities. Clinicopathological traits, such as morphology assessment and Ki-67 positivity, can be included. Overall and disease-free survival has increased considerably in the last decades. Nevertheless, MCL is still deemed an incurable disease with a high frequency of relapses and an insufficient response to salvage treatment (62).

### 2.5.1 First-line treatment approaches

As a first-line treatment stratification, young and fit patients receive intensive combinatorial chemotherapy approaches, that include rituximab and cytarabine, and will be evaluated for consolidation with ASCT. Twenty years ago, the addition of rituximab to CHOP (cyclophosphamide, doxorubicin, vincristine,

and prednisolone), R-CHOP, showed increased survival in MCL (63). More recently, the NLG demonstrated in the MCL2 and MCL3 clinical trials, that the administration of high-dose cytarabine with CHOP and rituximab as induction therapy, followed by high-dose cytarabine prior to ASCT, led to a remarkable improvement in survival and TTP in young and fit patients (9, 10, 15, 64). Unfortunately, a high frequency of relapses is still observed (11).

At diagnosis, most patients are above 65 and might not be eligible for high-intensive chemotherapy or ASCT. Old and fragile patients are frequently treated with other chemoimmunotherapies, such as VR-CAP (65) (Bortezomib, rituximab, cyclophosphamide, and doxorubicin) and R-BAC (rituximab, bendamustine, and cytarabine) (66, 67) and Rituximab and Bendamustine (R-Bendamustine) (68). Of note, such old and fragile patients are in majority in the real-world clinical setting. This is also noted in our BLISS cohort, where a large fraction of patients was considered older than N-MCL2/3 patients, and thus were more frequently treated with R-Bendamustine. An approach of “watch and wait” is accepted in the non-nodal MCL subtype which corresponds to less than 20% of patients (25, 69-71).

### **2.5.2 Relapse and refractory disease: the current role of target agents**

There is no standard therapy for relapsed MCL (12). Treatments are selected based on individual factors, including symptomatology, prior therapy (both in number and category), TTP, age, comorbidities, and performance status (72). A tendency is a high number of relapses per patient and a progressive shortening of duration between relapses (62).

R/R patients may receive the same chemoimmunotherapy regimens as above but are nowadays also commonly treated with targeted agents against molecules of deregulated pathways in MCL, often in combination with rituximab. The development of these regimens is due to the increased understanding of MCL pathogenesis. The most frequent therapy options include Bruton’s tyrosine kinase (BTK) inhibitors, such as ibrutinib (73) which showed impressive activity in MCL (74). The apoptosis regulator Bcl-2 (BCL-2) inhibitor venetoclax and the immunomodulatory agent lenalidomide have also been extensively explored in R/R patients (75-77). Other explored target therapies are phosphoinositide 3-kinase (PI3K) inhibitors (e.g., idelalisib) (78), mammalian target of rapamycin

(mTOR) inhibitors (e.g., everolimus) (79), proteasome inhibitors (e.g., bortezomib) (80), and histone deacetylase inhibitors (e.g., vorinostat) (81).

## 2.6 Towards personalized treatment approaches

In the last years, there has been a significant shift in the treatment strategies in MCL. Previously, options were restricted to the use of intensive chemoimmunotherapies, associated with high levels of toxicity. These options are continuously being challenged towards chemotherapy-free approaches, both at the frontline and R/R stage. The high heterogeneity of the disease and the complex crosstalk of malignant cells with its surroundings challenges the treatment outcomes and forges the need to develop tailored-treatment approaches.

### 2.6.1 Risk-stratified treatment strategies

So far, high-risk variants of MCL have an inferior OS and TTP irrespective of the treatment approach chosen (13, 15). This highlights the urgent need for tailored approaches for these subgroups of patients.

The current guidelines still support the treatment of high-risk patients with standard-of-care, despite the poor outcome. However, recommendations are to include these patients in novel clinical trials when available (13, 67). Novel agents are explored for de novo high-risk MCL and R/R MCL, which is also associated with an increased frequency of high-risk variants (82-84). A clinical trial with acalabrutinib demonstrated a similar overall response for patients, irrespective of high-risk features, albeit still considered short (85). A hopeful result came from the AIM clinical trial, which showed that venetoclax in combination with ibrutinib was effective in *TP53* mutated patients (83). However, a promising trial for *TP53* mutated tumors is the ZUMA-2 with chimeric antigen receptor (CAR)-T-cell therapy, where an objective response was achieved in most patients, irrespective of *TP53* mutation status (86).

A lesser established group of high-risk patients is the one that carries c-Myc aberrations, where no proposal on treatment in the clinic has been made. In paper IV, we explored the possibility of administering serine/threonine-protein kinase

PLK1 (PLK1) inhibitors to high c-Myc tumors. PLK1 is a mitotic kinase with central roles in the cell cycle, DNA damage response and DNA repair pathways (87). We showed in paper IV that its expression correlated with c-Myc expression. Others have shown that PLK1 effectively reduced survival in *in vitro* MCL models. PLK1 inhibitors have already been explored in other preclinical studies in MCL in combination with ibrutinib, belinostat and copanlisib (88-90). Further investigations will be required to fully demonstrate the effect of PLK1 inhibitors in MCL as treatment approaches and as a tool to overcome the aggressiveness of c-Myc deregulation.

### **2.6.2 Novel immunotherapy strategies: are chemo-free approaches the future of MCL treatment?**

Worldwide, the interest in immunotherapeutic strategies has increased, mostly due to the success of immune checkpoint blockade (ICB) in specific subtypes of patients (91). Thus, there is a hope that such treatment approaches will benefit high-risk MCL patients.

Programmed cell death protein 1 and its ligand (PD-1/PD-L1) is among the most common targets for ICB and expression of these markers is inconsistently reported in MCL (92-97). In paper V, however, we show that a small subset of patients expresses PD-1 and PD-L1. Others have shown that MCL cells that express PD-L1 were able to inhibit T-cell proliferation and the blocking of PD-L1 in MCL cells leads to increased T-cell responses *in vitro* and *in vivo* (94). Nonetheless, when PD-1 blockade was administered in NHL patients, the therapeutic response was not achieved (98). A small number of patients diagnosed with MCL were included in the studies and the results were discouraging, with experts suggesting that the low expression of PD-1/PD-L1 could explain the poor results. Of note, PD-L1 upregulation is proposed to be dependent on a functional ATM/ATR/CHK1 axis (99), and ATM serine/threonine kinase (*ATM*) is the most frequently mutated gene in MCL (paper I). Interestingly, PD-L1 levels tended to be higher in p53 overexpressing tumors in our study, which often do not have a mutated *ATM* (paper V). PD-1/PD-L1 ICB needs to be further explored in MCL, as it could be a therapeutic strategy for a specific subgroup of patients.



To overcome rituximab relapse, second-generation anti-CD20 monoclonal antibodies have started to be explored, including ofatumumab (100), obinutuzumab (101-103), and ublituximab (104). Another approach being explored is bispecific T-cell engagers (105). Blinatumomab, a CD3/CD19 BiTE was used in 24 R/R MCL and showed an overall response rate of 71% (106). Glofitamab, a CD3/CD20 BiTE has also been shown to induce high response rates in R/R MCL patients, with an overall response rate of 81% (107).

One of the most promising immunotherapy strategies in MCL has been CAR-T-cell therapy. Brexucabtagene autoleucel is an anti-CD19 CAR-T that was explored in the ZUMA-2 trial. A 93% of overall response rate was reported, with 67% of patients having a complete response. At a median follow-up of 17.5 months, 48% of the patients still had ongoing responses. Patients with high-risk variants, e.g., high PI, non-classic morphology, and *TP53* mutations had similar initial responses as non-aggressive variants (86, 108), but blastoid cases were a representative subgroup that had had disease progression within 24 months (109).

Therapies targeting macrophages are underexplored but are a promising approach in MCL. As shown in paper V, the frequency of CD163<sup>+</sup> cells in the tumor region was associated with worse survival in MCL. CD163 is a common marker of M2-like macrophages. One suggested strategy to overcome the crosstalk between macrophages and cancer cells is the inhibition of CD47. CD47 is an immunoglobulin that binds to signal regulatory protein alpha (SIRP $\alpha$ ) and enables CD47<sup>+</sup> cells to escape macrophage-mediated phagocytosis. CD47 has shown overexpression in NHL, with the highest value associated with MCL (110, 111). Chao and colleagues showed the synergetic effect of anti-CD47 antibodies and rituximab, through a mechanism leading to stimulation of phagocytosis and elimination of lymphoma in a mice model (110). ALX148, a fusion protein between a CD47 blocker and an inactive human immunoglobulin fragment crystallizable region, in combination with rituximab, leads to a 41-64% overall response rate in highly aggressive high-grade B-cell lymphoma, including MCL, at a median follow-up of 14 months (112, 113). Moreover, ibrutinib and macrophage colony-stimulating factor 1 (CSF1) inhibitors have shown preclinical potential in MCL (114). CSF1 is involved in the survival and proliferation of macrophages (115). Altogether, our finding of the prognostic role of macrophages with these previous studies supports the novel approach of disrupting MCL and macrophages crosstalk as therapeutic strategies in MCL.



### 3. Tumor intrinsic mechanisms and molecular deregulation

The heterogeneity in MCL also manifests at the molecular level. The advancements of biotechnology and bioinformatics have brought the possibility to study the different deregulations at a deeper level and exploit the interactions and regulatory mechanisms that lead to specific clinical phenotypes. The study of the different omics allows for a deeper understanding of the complexity of MCL and sheds light on targetable aberrations. This translates into new opportunities for clinical management and the development of tailored treatment approaches that prompt better outcomes for patients.

Although overexpression of cyclin D1 remains characteristic of MCL, this phenomenon by itself is incapable of leading to overt lymphoma. This has been supported by the finding of t(11;14)(q12;q32) in the blood of healthy individuals (116, 117), as well as previous mice studies (118-120). Secondary aberrations and deregulations must happen for the malignant transformation to occur. These deregulations occur at distinct molecular levels.

#### 3.1 The genomic landscape in MCL portrays a highly heterogeneous disease

Numerous studies in the past years have consistently shown the genomic heterogeneity in MCL, regarding mutated genes and their incidence (30, 121-128), and deregulation of epigenetic mechanisms (124, 129-132), providing important insights into the molecular profile and pathogenesis of the disease. MCL is characterized by frequent chromosomal imbalances. Common cytogenetic abnormalities, besides the t(11;14)(q13;q32), include: the 3q25-q29,

7p22 and 8q24 (MYC proto-oncogene (*MYC*) gene locus) gains and 1p32, 13q33-q34, 17p13 (*TP53* gene locus) and 6q loss (30, 133-135). Among the most mutated genes in MCL, *ATM* is reported at a higher frequency, with roughly 50% of the tumors harboring at least one mutation. Other frequently mutated genes identified are *TP53*, *CCND1*, *KMT2D*, *KMT2C*, *UBR5*, *NOTCH1*, *NSD2*, *BIRC3*, and *NOTCH2* (30, 121, 123-127).

In Paper I, we intended to demonstrate the applicability and potential benefits of including sequencing approaches in the clinical workflow for MCL. We evaluated a target panel of 200 genes in a total of 77 biopsies of diagnostic MCL from the population-based BLISS cohort and confirmed the most commonly recurring mutated genes in MCL could be found. 70% of the analyzed tumors had at least one mutation in a gene involved in cell cycle regulation. This observation strengthens the importance of cell cycle deregulation in MCL. Commonly affected pathways include apoptosis and *TP53*-transcriptional pathways (Paper I)(136). Pararajalingam *et al.* have proposed in their study that two myocyte enhancer factor 2B (*MEF2B*) recurring mutations were MCL-specific (137). In our study we found four samples matching these mutations in *MEF2B*. *MEF2B* codes a protein that is hypothesized to drive lymphomagenesis and be involved in the germinal center formation. *ATM* and *TP53* have shown a tendency to be mutually exclusive (30, 138), which is also demonstrated in our study (Paper I). Nonetheless, a small subset of patients can have mutations in both genes (15). We showed that a significant number of mutations in these two genes are driver mutations, thus it can be hypothesized that either *ATM* or *TP53* mutations might be required for malignant development. Further, we show that 60/77 patients studied harbored at least one mutation with the potential to be included in the management and treatment decisions in the clinics in the foreseeable future.

In this study, we used FFPE samples, which are the golden standard for IHC-based routine pathology diagnosis, but are characterized by partly degraded nuclei acids. The partial DNA degradation in FFPE samples reduces their quality and has hampered their use in molecular technologies. It is to be noted that a significant number of samples failed quality control and were not sequenced. It is possible to iterate reasons for this phenomenon, such as the lack of guidelines and protocols for the fixation and handling of FFPE samples, particularly in more aged blocks. Nonetheless, we showed in paper I, the applicability and potential to bring

target NGS approaches to clinical management in the context of MCL using archival FFPE samples, although only 50% of our samples passed quality control. We believe that this potential is increased with the standardization of protocols for FFPE handling in the clinical setting.

Epigenetic deregulation is characteristic of cancer cells. MCL cells seem to have epigenetic imprinting similar to the germinal center-experienced B-cells, which suggests that MCL is antigen-driven. Identical to the heterogeneity observed at the genomic level, DNA methylation changes are highly variable, and many alterations are only seen in one or a few cases. (132). Several deregulated epigenetic mechanisms have been described (131) and genes involved in such mechanisms are commonly subjected to mutations in MCL (30, 124). Such examples are *NSD2*, *KMT2D*, and genes involved in the SWI/SNF chromatin remodeling complex, that we showed to be mutated in paper I.

## 3.2 The transcriptome in MCL – from deregulated signaling pathways to treatment response prediction

Understanding the mechanisms behind the deregulation of MCL-associated pathways and increased proliferation has been the focus of several studies in MCL, with the goal of developing alternative treatment strategies.

In addition to deregulation of the cell cycle (139-141), MCL seems to be dependent on constitutive B-cell receptor (BCR) activation (142-145). The impact of BCR deregulation in patient management was shown in Bomben *et al.* study that separated high and low BCR activation tumors by a signature containing six representative genes, which was then reported to have a prognostic impact (146). Apoptotic mechanisms are also impaired and involve *BCL-2* mRNA overexpression as described in 3-17% of MCL tumors (147, 148). Activation of the PI3K/AKT/mTOR pathway (149, 150) and JAK/Signal transducer and activator of transcription (STAT)3 (151) has been observed in MCL. Other reported deregulations include constitutive activation of the classical and alternative nuclear factor kappa-light-chain-enhancer of activated B-cells (NF- $\kappa$ B) pathways (124, 144, 152), deregulation of Notch (126), and WNT

pathways (150). These studies have ultimately led to the development of targeted treatment strategies.

### **3.2.1 Proliferation and treatment outcome signatures**

Cell cycle deregulation is a key feature of MCL and the development of technologies that allowed analysis of the full transcriptome of a sample has led to an avalanche of global gene expression profiling investigations dedicated to understand the underlying biology and variability in MCL.

In 2003, Rosenwald and colleagues pioneered the work on defining a proliferation signature that correlated with the frequency of Ki-67 positive cells and the mitotic index in MCL (48). This proliferation signature was later in 2008 used as a basis for the development of a five-gene list that could be applied to fresh frozen and archival samples for use in pathology departments (153). Several other proliferation signatures have been proposed, with little overlap between them (48, 69, 144, 146, 150, 153-157). This variability reflects the strong effect proliferation has on the transcriptome.

In 2017, the MCL35 assay, an amplification-free Nanostring-based 17-gene signature, was developed. This signature includes both genes that are up and down-regulated in proliferative tumors, and although it has been validated, evaluation of Ki-67 is still the prevailing method for proliferation assessment. The MCL35 assay (155) is the most promising gene expression signature reported to date (158-160). It was able to stratify patients into three risk groups: high, standard, and low, with a median OS of 1.1, 2.6, and 8.6 years, respectively. Nonetheless, MCL35 failed to stratify patients treated with the N-MCL2/3 protocols, the current gold standard treatment, based on TTP (158). In Paper II we aimed at filling this gap. We used the homogeneously treated N-MCL2/3 cohort, which has a significantly higher median follow-up than the cohort used to establish MCL35 (8, 11, 15). With this strategy, we identified several genes able to distinguish short versus long TTP. As expected, the cell cycle pathway was enriched among the ones associated with TTP. Thermogenesis, fatty acid degradation, and oxidative phosphorylation (OXPHOS) were additional pathways associated with inferior survival.

Current clinical management in most centers does not include gene expression analysis, which contributed to the lack of current clinical use of any of

the developed signatures. The identification of surrogate markers of easy applicability in the clinic is thus a great need. One such technique that is part of the clinical routine is IHC. *CPT1A* was upregulated in patients with poor prognoses in our analysis, and it was part of the thermogenesis/OXPHOS cluster (Paper II). We validated, in the population-based BLISS cohort and in a subset of patients of the N-MCL2/3 cohort, the prognostic value of CPT1A at the protein level, through IHC. CPT1A is a key rate-limiting enzyme in FAO that is ubiquitously expressed in the body and highly regulated (14). Recent studies have shown that CPT1A is a marker of worse prognosis in other malignancies, such as breast cancer (161) and acute myeloid leukemia (162, 163), and targeting *CPT1A* alone or in combination with cytarabine treatment could be a therapeutic option in hematological malignancies (164). Of note, we also found that *SLC25A20*, which is involved in the transport of long-chain fatty acids into mitochondria (165), was also upregulated, suggesting that the carnitine cycle may have a role in MCL development that has not been identified before.

### 3.3 High-risk variants of Mantle Cell Lymphoma

The avalanche of advancements in technologies has boosted our understanding of the underlying mechanisms governing MCL lymphomagenesis. This has encouraged the identification of distinct subtypes characterized by different molecular features and/or commonly associated with poorer prognosis. Several studies have reported high-risk features for MCL, namely: advanced age (166, 167), significant comorbidities, poor performance status (168), non-classic morphology (169), high Ki-67 expression (55), and *TP53* aberrations (15).

With increased knowledge of the disease came the development of improved therapies and the strive to move past conventional chemotherapy, with often intensive regimens and high cytotoxic effects. Considering this, other markers of high-risk have been proposed, such as c-Myc aberrations (61, 170), complex karyotype (171), minimal residual disease-positive (172), and several genomic mutations (121, 126, 128, 173, 174). Nonetheless, most of these molecular traits have not been validated in prospective studies and a few remain challenging to implement in the clinical setting due to lack of more recent technologies in the hospital setting and further clinical validation.

Here, highly proliferative tumors, non-classic morphology, *TP53*, and c-Myc aberrations in MCL will be discussed, reflecting upon the translational application of our findings, seeking the establishment of prognostic markers of easy evaluation in the clinical workflow.

### **3.3.1 Highly proliferative tumors – Ki-67 overexpression**

To this date, the proliferation index (PI) in MCL is commonly evaluated using Ki-67 expression as measured by IHC (60). A negative prognostic value associated with high PI at diagnosis has been repeatedly demonstrated in large patient cohorts by both us (Paper III, Paper V) and others (10, 15, 175-179). Also at relapse, high PI is associated with inferior prognosis (180, 181). Thus, the prognostic impact of PI is well defined and seems to be independent of treatment approaches, even in the rituximab era. However, poor reproducibility of this marker due to interobserver variability is a strong concern among the expert community (60, 182).

In our cohorts, around 20% of the patients showed Ki-67 expression  $\geq$  30%, the established cut-off for classification as highly proliferative tumors. Frequent studies showed a strong overlap between high PI and non-classic morphology (175, 180, 183). This could also be seen in our studies (paper I-V). In paper I, only nine patient samples overexpressed Ki-67, but we observed that all tumors either harbored *ATM* or *TP53* mutations. p53 overexpression is also associated with highly proliferative tumors (paper III), which is consistent with previous reports (179, 183, 184). In paper II and paper IV we report a correlation between high *MYC* mRNA and c-Myc protein with Ki-67 overexpression.

Given the prognostic impact of PI in MCL, it is of major interest to understand the molecular features that characterize this subgroup of patients, as well as to identify alternative treatment approaches for patients with highly proliferative tumors. In paper II, we showed that WEE1 G2 checkpoint kinase (*WEE1*) and flap structure-specific endonuclease 1 (*FEN1*) mRNAs were highly upregulated in tumors with Ki-67  $\geq$  30%. The association was validated on the protein level in an independent cohort of MCL patients. Wee1-like protein kinase (*WEE1*) has a critical role in the cell cycle and DNA damage response. *WEE1* is believed to be a pseudo-oncogene in malignant cells that sustains proliferation upon oncogene activation while maintaining a high genetic instability and



enhancing DNA repair upon chemo and radiotherapy treatments (185). A screening approach previously published also identified WEE1 inhibition as a complement to rituximab (186). It has also been shown in preclinical models that the combination of CHK1 and WEE1 inhibition is a promising therapy in MCL (187), but so far WEE1 inhibition has not been assessed in clinical trials.

FEN1 is involved in DNA replication, namely at Okazaki fragment maturation and in the base excision repair pathway (188). Interestingly, FEN1 is necessary for the repair of oxidative DNA damage (189) and OXPHOS represented one cluster that was enriched in high Ki-67 tumors (Paper II). Additionally, FEN1 is not expressed in quiescent cells (190), and it is hypothesized to support the increased proliferation of cancer cells (191). Studies have shown that mice carrying FEN1 mutations had early-onset of B-cell lymphomas (192). These data support the role of FEN1 in highly proliferative cells and as a putative target for MCL tumors with high Ki-67 expression.

### **3.3.2 Non-classic morphology: Blastoid and pleomorphic variants**

Blastoid and pleomorphic are cytological variants of MCL, characterized by blastic morphological features and high proliferation, with abysmal prognosis (15, 55, 59, 193, 194). Blastoid cells resemble lymphoblasts, with fine chromatin and round nuclei, often medium-sized. Pleomorphic cells are large with irregular nuclei and prominent nucleoli (7). Non-classic morphology comprises these two cytological MCL variants, that constitute 10-20% of all MCL cases (15, 55, 195). In our cohorts, the frequency of non-classic morphology tumors was 12%, with the highest frequency observed in the N-MCL2/3 cohort where eligibility criteria included age < 65 years. Some studies have reported that patients with blastoid variants are slightly younger at diagnosis (59, 194).

Blastoid morphology frequently arises *de novo*, but it may also evolve from classical disease during progression (177, 196, 197). A single study has reported that transformation occurred in up to 35% of patients (198), but this remains to be validated. Clinically, these patients are similar to those with classic MCL, however, they are described as more often presenting extranodal (59, 194) and CNS involvement (199). Non-classic morphology is associated with *TP53* aberrations and high proliferation (55, 183, 193, 200) (Paper III). However, none of these characteristics are specific nor all-encompassing for non-classic

morphology variants. Non-classic morphology remains a strong prognostic factor in MCL (13) and pinpoint therapeutic alternatives is a current unmet need. In paper I and paper II we address this issue by exploring genomic and transcriptomic deregulations in non-classic MCL.

In paper I, we saw that similar to highly proliferative tumors, blastoid tumors had either *ATM* or *TP53* mutations and we report two blastoid cases with a very short OS that harbored the exportin 1 (*XPO1*)<sup>E571K</sup> mutation. *XPO1* encodes the exportin-1 protein that mediates the translocation of RNAs and proteins from the nucleus (201). This protein is highly expressed in MCL, with a possible role in pathogenesis and an association with poor prognosis (202). This mutation is hypothesized to be lineage-specific and almost exclusive of B-cells (203). The role of the mutation is not clear, but recent studies believe that it can alter the nuclear translocation dynamics and could promote lymphomagenesis driven by BCL-2 and c-Myc (203, 204). c-Myc aberrations are reported to be enriched in blastoid morphology cases (Paper IV) (170, 205). *XPO1* is a targetable gene, and several selective inhibitors of nuclear export compounds are being explored in different studies (201).

Interestingly, Streich and colleagues (200) recently observed that chromothripsis occurred in 62% and was exclusive of blastoid MCL. Chromothripsis is a complex phenomenon that leads to patterns of alternating gene copy number changes within chromosomes. The presence of this phenomenon highlights the high genomic instability and replication stress in these tumors (206, 207). In paper II, when evaluating the gene expression profiling of blastoid tumors, *MAP2K6*, *MAP3K8*, *PPP2R1B*, and *CUL5* were exclusively deregulated in these tumors. These four genes have roles in the regulation of cell growth and proliferation, with the first being involved in the activation of the MAPK signaling pathway in response to environmental stress (208-210). Several metabolic pathways showed higher deregulation in non-classic vs classic morphology in our approach. These data strongly suggest that non-classic morphology MCL is associated with higher cellular stress and genomic instability, likely contributing to their adverse prognosis.

### 3.3.3 *TP53* mutations and p53 overexpression

*TP53* is the strongest negative prognostic marker in MCL (15, 57, 138, 174, 211-216), with currently no adequate treatment approach. *TP53* is a key tumor suppressor gene and is one of the most important genes involved in regulating apoptosis. *TP53* encodes a transcription factor, p53, which binds to its target genes through the DNA binding domain and regulates their expression. p53 has a pleiotropic effect and, besides DNA repair, is also involved in apoptosis, senescence, antioxidant defense, cell metabolism, and cell cycle arrest. *TP53* deregulation is described in lymphomas at the DNA, RNA, and protein levels (217).

#### 3.3.3.1 *TP53* deregulation in MCL

*TP53* is reported to be deregulated at the genomic level due to point mutations, deletions, and chromosomal alterations, and at the protein level, with overexpression of the p53 protein (15, 57, 174, 212). *TP53* mutations are estimated to be present in 10-20% of patients diagnosed with MCL (15, 212, 214, 218). The majority are missense mutations and affect mostly the DNA binding domain (Paper III), leading to a protein with only one amino acid substitution, called mutp53 (219). Mutations seem to be more frequent in relapsed tumors compared to diagnostic samples (82, 83). Deletions are also frequently reported in diagnosed patients (10-30%) and reports of concomitant aberrations have also been published (15, 212-215, 220).

*TP53* mutations have been consistently shown to be associated with a worse prognosis, irrespectively of current treatment approaches (138, 174, 213, 218, 221). The NLG studied the N-MCL2/3 cohort and showed that the median OS time for mutated patients was 1.8 years, compared with 12.7 years in wild-type (WT) patients (15). In our population-based cohort, the median OS time for patients carrying *TP53* mutated tumors was similar, 1.4 years, however, the WT patients had a shorter OS of 6.2 years. Deletions involving this gene have also been shown to carry a prognostic impact in MCL, but they seem to be less significant than mutations (15, 125, 212, 214). We showed that p53 protein expression is present in 13% of the patients diagnosed with MCL (Paper III). Overexpression of p53 protein, defined as  $\geq 30\%$  of positive cells, is associated

with a poor prognosis and in our population-based cohort, patients with p53 overexpression had a median OS of 0.9 years, with a hazard risk of 3.1 (Paper III).

*TP53* aberrations are associated with non-classic morphology (183, 215, 222) and high Ki-67 expression (15, 223). Additionally, it has been proposed that *TP53* mutations may represent non-nodal MCL that, after an indolent phase, evolved to a highly aggressive variant (224). These cases are frequently reported as SOX11 negative in literature. However, in Paper III, we could not confirm an association between the two markers, *TP53* and SOX11. Despite *TP53* aberrations being enriched in other markers of aggressive disease, mutated *TP53* tumors can be of classic morphology and classified as low proliferative tumors. Thus, there is a need for an assessment of *TP53* aberrations for improved risk stratification in MCL patient management, beyond the current guidelines.

#### *3.3.3.2 From mutations to overexpression of the protein: applicability in the clinical routine*

As targeted sequencing remains to be fully implemented in clinical routine, a strategy to stratify patients with *TP53* mutations, who seem to require alternative treatments, is warranted. Immunostaining for p53 as a surrogate marker for *TP53* mutations is suggested in other tumors (225-228), since mutp53 protein is stabilized and overexpressed in the cancer tissue. Hence, in paper III, we aimed at evaluating the feasibility of applying p53 immunoreactivity as a tool to identify *TP53* mutations. We showed that positive p53 expression is an accurate method to identify *TP53* missense mutations, with an area under de curve of 0.96 for the population-based material. We reached 100% specificity and 82% of sensitivity when evaluating p53 immunostaining of tissue microarrays and complementing with whole tissue sections. Of note, in our study, bone marrow samples did not always reflect the *TP53* mutational status on the tumor, with a higher level of concordance when the same biopsied material was considered for both analyses. Albeit not identifying all mutation types, one advantage of IHC analysis was the visualization of mutated subclones, which can easily be missed by sequencing due to low allele frequency. Thus, immunostaining of p53 was able to identify poor prognosis patients with *TP53* missense mutations and to single out cases with the presence of clones for p53, making it an important tool in the clinical workflow of MCL.

### 3.3.4 c-Myc aberrations in B-cell lymphoma and MCL

Deregulation of c-Myc is reported in most B-cell lymphomas (229-233). The *MYC* gene is located at 8q24 and was first identified as an oncogene in Burkitt lymphoma, due to the characteristic t(8;14)(q24;q32) (234), that juxtaposes *MYC* with the *IGH*, leading to overexpression of c-Myc (235). c-Myc is a transcriptional factor able to regulate 10-15% of the human genome and affect both protein-coding genes and non-coding RNA products (236-238).

c-Myc has a pivotal role in B-cell proliferation, being expressed in the initiation events of the germinal center. c-Myc transcriptional targets are involved in the regulation of cellular metabolism, DNA replication, and telomerase function (21). c-Myc driven malignant B-cells are associated with disease aggressiveness and involved in the transformation of indolent lymphomas (16, 239-241). c-Myc constitutive expression leads to genomic instability (242), cell proliferation (243), and overall deregulation of *MYC* physiological targets (244).

The frequency of *MYC* aberrations in MCL is far from understood and at which molecular level it occurs remains to be elucidated. *MYC* is commonly deregulated in many cancers (245, 246), but rarely by oncogenic mutations. Instead, aberrant activation of c-Myc is due to amplification (247), insertional mutagenesis (238, 248), chromosomal translocations (234, 235) and transcriptional/post-transcriptional events (249, 250). In paper I, we confirm that the *MYC* gene is rarely mutated in MCL. Then, in paper IV we investigated the presence of additional abnormalities of *MYC* in diagnostic MCL. We showed that 15% of the samples had more than 20% of positive c-Myc cells, and were considered to overexpress the protein (MYC<sup>+</sup>). However, chromosomal alterations were scanty observed in our study. Of note, we were not able to evaluate all samples through FISH analysis, so it is possible that some chromosomal rearrangements were missed, as it has been described that translocation of *MYC* might not lead to c-Myc overexpression. Ongoing investigations will include FISH analyses of all cases. Through mRNA *in situ* hybridization, we assessed the expression of *MYC* mRNA and showed a strong correlation between protein and mRNA, which indicates that part of the deregulation of c-Myc happens at the transcriptomic level. To be noted that this evaluation was particularly challenging in samples with a high frequency of autofluorescent cells, such as bone marrow biopsy samples.

Most patients with c-Myc deregulated tumors will have some additional known aggressive characteristics. c-Myc has been associated with disease progression, relapse, and transformation of classic to non-classic variants (61, 232, 240, 251, 252). In paper IV we show that commonly reported aggressive variants of MCL, namely highly proliferative tumors, non-classic morphology, and high-risk MIPI cases, were enriched for c-Myc aberrations. *MYC*<sup>+</sup> was associated with a worse prognosis, with *MYC*<sup>+</sup> tumors having an OS of 1.5 years and a four-fold risk of dying. This negative effect was independent of other high-risk markers of aggressiveness (Paper IV). With respect to the impact of *MYC* rearrangements, although less frequent, they remain associated with a poorer prognosis (61, 231, 253).

### **3.3.5 c-Myc<sup>+</sup> and *TP53*/p53<sup>+</sup>: a new high-risk group?**

Concomitant alterations in *TP53* and/or p53 and c-Myc seem to be associated with a strongly increased risk of dying (Paper IV). Patients with tumors harboring alterations in both molecules showed a median OS of only 0.9 years after diagnosis and were associated with aggressive variants of the disease. Although other studies had noticed the association between c-Myc and p53 in MCL (213, 254-256), no comprehensive study on the prognostic effect of concomitant alterations has been made before our report. Interestingly, in the a study that focused on molecular subtyping MCL, the clusters with shortest survival identified harbored *TP53* mutations and had a strong active *MYC* pathway (257).

The additive negative effect of p53 and c-Myc has also been noticed in DLBCL (258, 259). The mechanism behind the negative effect is unclear, and studies have pointed to the regulation of apoptosis by c-Myc. Further, *MYC*-induced genomic instability and cell cycle progression seem to be enhanced when p53 is absent (242, 260-262). Additionally, different mutp53 have been shown to affect and be affected by c-Myc, e.g., R172H mutp53 was stabilized by c-Myc (263). However, the concrete mechanism behind c-Myc and *TP53*/p53 additive negative effect remains to be fully elucidated in B-cell lymphomas.

## 4. Tumor microenvironment in MCL

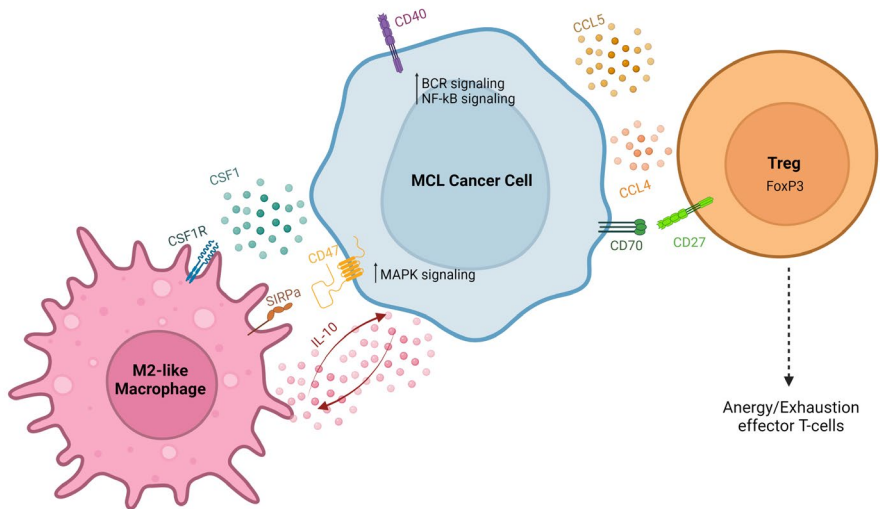
The microenvironments in B-cell lymphomas can be divided into three categories; recruitment, re-education, and effacement as proposed by Scott and Gascoyne (264). The proposal emphasizes that the microenvironment attributes resemble a continuous spectrum, rather than three very distinct microenvironment subtypes. The determinants of the different classification stages included tumor-intrinsic characteristics, the dependence on external stimuli for tumor proliferation, survival and immune invasion, and the host inflammatory response. The MCL microenvironment is placed between re-education and effacement microenvironments, but resembles more a re-educated microenvironment. This pinpoints a complex network between tumor intrinsic factors and the surrounding environment. The MCL microenvironment is referred to as a dynamic niche, with crosstalk between the tumor cells, accessory cells, and soluble factors.

In this chapter, the MCL tumor immune microenvironment will be discussed, mentioning the different components and their postulated role in the disease. In light of the conclusions of papers V and VI, a special focus on regulatory T-cells ( $T_{\text{regs}}$ ) and macrophages will be presented.

### 4.1 The immune microenvironment in MCL

The importance of the immune microenvironment is already established in B-cell lymphoma, but there is a lack of knowledge into its composition and role in treatment response in MCL. In paper V we aimed at filling this gap. There we showed that there was high heterogeneity in the immune microenvironment in MCL and that T-cells were the most common, albeit inter-patient variability.

The main mechanisms postulated to drive immune evasion in lymphoma are: i) defective immune recognition, such as mutations in specific genes that impair recognition, including *MEF2B*; ii) aberrant co-stimulatory/co-inhibition signals, e.g., expression of PD-1 and PD-L1 in the microenvironment; iii) presence of immune suppressive cells, like macrophages and T regulatory cells ( $T_{regs}$ ); iv) secretion of immune regulatory factors that suppress T-cell effector functions, such as interleukin-10 (IL-10) and indoleamine 2,3-dioxygenase (IDO) (265-269). Interestingly, in paper I, we showed that seven patients carried mutations in *MEF2B*, in paper V we reported that PD-1 had an average expression of 10% in our MCL cohorts. Although to a low extent, but with a negative impact on prognosis, macrophages and  $T_{regs}$  were found in a proportion of tumor samples (Figure 2). This data suggests that different mechanisms are involved in MCL evasion from immune cells.



**Figure 2** Mantle cell lymphoma cells interact with T regulatory cells and M2-like macrophages in the tumor microenvironment. MCL cancer cells express CD40 and interact with CD154+ cells, leading to increased BCR and NF- $\kappa$ B signaling. MCL cells secrete cytokines, such as CCL5 and CCL4 that can attract Tregs to the microenvironment. Tregs, which are FoxP3+ cells, interact with MCL cancer cells through CD70/CD27 axis and can promote increased anergy and/or exhaustion in effector T-cells. MCL cancer cells interact with macrophages in a loop involving IL-10 and CSF1 secretion. MCL express CD47, a “do not eat me” signal, that binds to SIRP $\alpha$  on macrophages, as an evasion mechanism. The presence of M2-like macrophages in the tumor microenvironment in MCL is associated with an increase in MAPK signaling.



#### 4.1.1 T-cell subsets and their impact on MCL

T-cells seem to be the most frequent immune population infiltrated in MCL tissue (270). This is also supported by the observation that soluble CD40 ligand (CD154) was present at higher levels in peripheral blood of MCL patients (271). It is thus hypothesized that MCL cells, which express CD40, interact with CD154<sup>+</sup> T-cells, leading to aberrant expression of BCR, NF- $\kappa$ B pathway, and survival factors, promoting lymphomagenesis and drug resistance (17, 272).

In paper V, the majority of CD3<sup>+</sup> cells were cytotoxic T-cells (T<sub>C</sub> cells), rather than helper T-cells (T<sub>H</sub> cells), with an average of 5.8% and 3.7% of positive cells, respectively. An increase in CD3<sup>+</sup> cells within the tumor had a positive impact on prognosis, most likely due to its negative association with age and the presence of a higher number of T<sub>C</sub> cells. Nygren *et al.* reported a lower T-cell level in MCL compared to reactive lymph nodes, with a tendency of a decrease in T-cell numbers when the pattern of malignant growth was diffuse rather than mimicking mantle zone growth. Further, they showed that the decline was due to a loss of CD4<sup>+</sup> cells (273). In our study, an increased frequency of CD4<sup>+</sup> cells was associated with short OS (Paper V). Nonetheless, the prognostic impact of CD4<sup>+</sup> cells show conflicting results, with a low absolute count in MCL peripheral blood samples and a high CD4:CD8 ratio previously being associated with longer OS (273, 274). Higher CD8:CD3 ratios have been frequently associated with high-risk MIPI cases. A higher ratio of CD8:CD4 central memory T-cells has also been connected to shorter TTP and OS in patients treated with ASCT (275).

A study in MCL has pointed out that lymph nodes with high proliferation showed higher levels of CD8, cytotoxic T-lymphocyte protein 4 (CTLA-4), PD-1, and PD-L1 expression, which points towards the exhaustion of T<sub>C</sub> cells in this subgroup of patients (276). Given the high complexity of the subtypes of T-cells and their range of activation status, these conflicting results are, to a certain degree, expected. One of the strongest limitations of the study in paper V is the use of single IHC staining. By only evaluating one cell marker is not possible to confidently clarify which cell type is presented, as the same marker can be expressed by more than one cell type. Thus, the role and impact of both T<sub>C</sub> and T<sub>H</sub> cells remain controversial and elusive in MCL, with a need for further studies and a deeper molecular characterization of specific subsets.

#### 4.1.1.1 *T<sub>regs</sub> are associated with sorter TTP in MCL*

A strong correlation between CD4<sup>+</sup> cells and FoxP3<sup>+</sup> cells was observed in paper V, which suggests that a large number of CD4<sup>+</sup> T-cells are T<sub>regs</sub>. T<sub>regs</sub> are immunosuppressive cells, hampering the function of effector T-cells, antigen-presenting cells, and NK cells. Common mechanisms involved in T<sub>reg</sub> immune suppression include upregulation of immune checkpoints inhibitors, consumption of IL-2 leading to effector T-cell deprivation, and release of suppressive molecules, such as IL-10 (277). IL-10 seems to be important in MCL development (278). T<sub>regs</sub> appear enriched in B-cell lymphoma compared to lymph nodes and inflammatory tonsils (279). In MCL lymph node biopsies, evaluated by flow cytometry, around 11% of CD3<sup>+</sup> cells showed a CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> phenotype, a similar frequency to DLBCL, but significantly lower than in FL (280).

MCL cells secrete CCL4 and CCL5 (281), which can attract T<sub>regs</sub> to the microenvironment (282). Overexpression of CD70 on MCL cells is correlated with T<sub>reg</sub> levels (283). CD70 is the unique ligand for CD27, a co-stimulatory molecule expressed in lymphocytes, whose expression on T<sub>regs</sub> has been correlated to a strong suppressive function (284). The increased expression of CD70 has been proposed as a mechanism of immune escape either by induction of immunosuppressive cells or anergy/exhaustion in effector T-cells (285, 286). T<sub>regs</sub> in MCL have also been shown to express the transmembrane protein CTLA-4, reported to be constitutively expressed by this cell type (283). CTLA-4 binds to the co-stimulatory molecules CD80 and CD86 on antigen-presenting cells leading to degradation of these molecules and inhibiting dendritic cell expression of IL-6 and TNF- $\alpha$  (287, 288).

Furthermore, we showed that higher levels of FoxP3<sup>+</sup> cells predict a worse prognosis in patients treated with R-CHOP, hinting that the impact of these cells may be dependent on the treatment approach (paper V). Similar findings have also been reported in DLBCL (289, 290) and FL in the rituximab era (291). Higher frequency and ratios of FoxP3<sup>+</sup> cells in relation to both CD3<sup>+</sup> and CD4<sup>+</sup> cells have been further associated with worse OS in MCL (270, 283). Nonetheless, T<sub>regs</sub> have not been sufficiently explored in MCL, and due to their role in

modulating the immune response, they may be an interesting therapeutic target in this disease.

#### **4.1.2 Macrophages: key players in carcinogenesis and immune evasion**

Studies have shown that macrophages appear to be crucial in lymphomagenesis, as support for tumor growth (292, 293). Macrophage presence seems to be transversal to all B-cell lymphomas (264, 294-297), including MCL, as we showed in paper V.

In physiologic conditions, macrophages are involved in homeostasis, inflammatory responses, and immune responses, serving as links between intrinsic and adaptive immunity (298). Historically, macrophages have been categorized into classic or alternatively activated macrophages, M1 and M2, respectively. Increased efforts have been made to understand the polarization process of macrophages (299, 300). Transcription and translation are tightly regulated in these cells to fine-tune cellular function and the modulatory effects of macrophages are defined based upon activation of specific transcription factors, histone modifications, changes in DNA methylation patterns, and regulation by different non-coding RNAs (301, 302). The deeper knowledge of this process has led to the realization that M1 and M2 are the extremes of the polarization spectrum (303, 304). In the carcinogenesis process, M1 macrophages are considered anti-tumor with cytotoxic capabilities, whereas M2 macrophages have strong immunosuppressive properties, like the production of IL-10 and IL-13 (305).

Macrophages are hypothesized to be part of carcinogenesis already at early stages, by producing reactive oxygen species, thus leading to genomic instability, and interacting with cancer stem cells, promoting protective conditions for cancer development (306, 307). Additionally, macrophages are associated with resistance to treatments, by blocking cytotoxic effects from T-cells and NK cells due to the expression of PD-L1 and V-type immunoglobulin domain-containing suppressor of T-cell activation (VISTA) (308-310). Importantly, the tumor microenvironment also supports the immunosuppressive properties of macrophages. The acidic pH of the tumor microenvironment modulates macrophages towards the promotion of immune evasion (311), the presence of T<sub>H</sub> type 2 cells, and consequent secretion of type 2 cytokines (312), and T<sub>regs</sub>

attraction (313). Tumor cells are also known to release cytokines that attract and modulate macrophages. CD47, a 'do not eat me' signal that is expressed in many tumor cells including MCL cells, interacts with SIRP $\alpha$  on macrophages contributing to immune evasion (110, 111, 314).

#### *4.1.2.1 Crosstalk between Macrophages and tumor cells in MCL*

MCL interacts closely with macrophages, but the modulatory effects between these two cell types have not been explored. Paper VI was focused on characterizing the crosstalk between tumor cells and macrophages and identifying targets for the modulation of macrophages. In paper VI, we used the new digital space profiling technology from Nanostring to spatially characterize the expression of 69 proteins in CD163<sup>+</sup>, CD3<sup>+</sup> and CD20<sup>+</sup> cells. This technology provided a platform for multiplex protein profiling, allowing for discrimination between spatially localized CD163<sup>+</sup> cells. With this approach, we were able to phenotypically profile M2-like macrophages and changes associated with their localization in the tissue. M2-like macrophages not in contact with tumor cells expressed increased levels of the immune-checkpoint regulators B7 homolog 3 (B7-H3) and VISTA. VISTA is highly expressed in myeloid cells (315) and studies have shown a co-inhibitory role in the tumor microenvironment (316). B7-H3 is often associated with worse prognosis in cancer and its expression has been previously reported in MCL (317, 318). The increased expression of these two molecules suggests that M2-like macrophages in regions adjacent to tumor-rich areas have a role in T-cell proliferation inhibition.

Macrophages within the MCL microenvironment have been reported previously (177, 319). In our study, the frequency was low, with an average of 0.06% positive cells, but a higher number of CD163<sup>+</sup> M2-like macrophages, was associated with more aggressive disease (paper V). M1-like macrophages can also be found within the tumor microenvironment of MCL, reported more frequent than M2-like macrophages (319, 320). Interestingly, both types of macrophages have been shown to express PD-L1 (319). Papin *et al.* showed that macrophages in MCL were more M2-like but were able to express both M1 and M2 soluble factors (114). Koh *et al.* showed that CD163<sup>+</sup> cells were more frequent in patients with bone marrow involvement (321). We showed that bone marrow samples had an average higher frequency of CD163<sup>+</sup> cells than compared to the lympho nodes,

0.42% and 0.05%, respectively (Paper V). *In vitro* studies showed that macrophages/monocytes were necessary for the establishment of long-term culture of MCL cells and hypoxic conditions favored macrophage activation and potentiate cancer cells survival (322, 323). Further, myeloid precursor cells provide favorable conditions for the growth and establishment of MCL cells in the bone marrow (323-325). Unfortunately, a large part of the bone marrow samples had to be excluded from the study presented in paper VI due to increased levels of background and autofluorescence.

Current discoveries suggest that monocytes may be able to differentiate or be reprogrammed into macrophages in the tumor microenvironment. B1 lymphocytes, described as the normal counterpart of MCL cells, are capable of recruiting monocytes due to secretion of relevant cytokines, such as IL-10, and promote their programming towards alternative activated M2 phenotypes (323, 326). Papin *et al.* demonstrated this phenomenon in MCL, with reprogramming being attributed not only to IL-10 but also to CSF1. IL-10 and CSF1 plasma levels are high in MCL patients (114). Le *et al.* showed that IL-10 secreted by macrophages polarized by MCL cells lead to STAT1 activation and MCL growth (320). These studies suggest a strong loop between MCL cells and macrophages, via IL-10 secretion, that allows for immune evasion and malignant growth.

We report an increase in MAPK activation in tumors with macrophages in the tumor microenvironment (Paper VI). MAPK pathway activation is known to be involved in macrophage polarization (327). Further, the MAPKs extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNK), and p38 are reported to be activated upon CSF1 treatment (328-330). We showed that expression of phosphorylated ERK1/2 in tumor cells was increased in the presence of macrophages (Paper VI) and it is suggested by previous studies that inhibition of ERK decreases the expression of IL-10 (330), making it an interesting target for modulation. Additionally, increased basal levels of phosphorylated-ERK and p38 have been previously associated with poor survival in MCL (145).

We showed that the presence of CD163<sup>+</sup> macrophages within the tumor area was associated with a worse prognosis, irrespective of the treatment approach (paper V). There is an association between poor prognosis and high monocyte counts in the peripheral blood (321). It has thus been hypothesized that monocyte counts can be surrogate markers of alternatively activated M2 macrophages in the tumor and predict worse survival (331). Koh *et al.* did not report an association

between CD163<sup>+</sup> macrophages and survival, instead, a positive correlation between absolute monocyte counts and CD163<sup>+</sup> cells was observed, with the first associated with worse outcome (321). The discrepancy in results might be related to the method of staining and evaluation, as in paper V we focused on macrophages within the tumor area and, as we show in paper VI, CD163<sup>+</sup> macrophages are phenotypically different in different spatial localizations. The presence of CD163<sup>+</sup> cells in the MCL tumor might be a surrogate marker for an immunosuppressive environment. Moreover, a significant correlation was observed in our study between the expression of CD163<sup>+</sup> cells and FoxP3<sup>+</sup> cells. Tumors with high numbers of CD163<sup>+</sup> and FoxP3<sup>+</sup> cells had a worse TTP when treated with R-CHOP (Paper V). Streich *et al.* (200) also reported the presence of tumor-infiltrating macrophages positive for PD-L1 in blastoid tumors. In paper V, non-classic morphology tumors showed a higher frequency of PD-L1<sup>+</sup> and CD163<sup>+</sup> cells than classic morphology tumors, which shows that these variants may be surrounded by a highly immunosuppressive environment.

Mainly, our data together with other findings strengthen the role of M2-like macrophages in MCL and underline the crosstalk between malignant cells and M2 macrophages in the development and treatment response in MCL.

## 5. Concluding remarks

A major goal in MCL management is to reduce treatment failure and relapse frequency and potentially develop curative strategies. The implementation of new immunotherapy approaches compared to the current strategies has the potential to induce long-term remissions and to cure patients. To reach this, identification of companion biomarkers that allow for patient stratification and that can be easily implemented in the current clinical workflow, is urgently warranted.

The last years have brought incredible advances in technology that revolutionized biomedical research and allowed us to study diseases at unparalleled resolution, promoting an increment in knowledge into the cellular mechanisms that contribute to cancer development, progression, and treatment failure. The work developed for this thesis has benefitted from such advancements, one of the most obvious being the adaptability of these techniques to challenging samples such as FFPE samples, which were our main source of patient samples. FFPE samples are still the preferred archival sample format in hospitals worldwide, making them a valuable source of information. However, there has been a lack of guidelines for processing FFPE samples, which led to limited use of these samples in molecular studies, as the fixation and preparation fragments and highly degrade biomolecules.

This thesis is based on six original papers that focused on understanding specific deregulations within MCL biology while bridging the gap between research and clinical application. In Paper I we demonstrated the applicability of an NGS panel in the clinical setting, providing relevant information for further tailored treatment strategies in MCL. The massively reduced costs of sequencing and the potential of clinical targetable mutations have boosted the interest in evaluating specific mutations in the context of clinical management. We showed that FFPE samples, despite the fragmented DNA, can be used in MCL for target sequencing and that several genes that appear mutated in the samples could be

used as targets for therapy. Thus, this pipeline can be easily implemented in the current patient management setting. Further investigations on the efficacy of compounds targeting these mutations are warranted, e.g., compounds targeting *XPO1*.

To date, the mechanism behind treatment response and drug resistance is not fully understood, but the identification of patients that do not benefit from the current standard of care is essential to further develop alternatives more beneficial for them. In paper II we reported that global gene expression changes in non-treatment responders pinpointed metabolic alterations in these tumors, namely OXPHOS and FAO, as potential deregulated mechanisms that help tumors overcome high-intense chemotherapeutic approaches. Among the different targets, *CPT1A* was further explored at the proteomic level, underlying the significance of metabolism in treatment response.

Unfortunately, the clinical implementation of the rapid knowledge originated from advanced technologies has not kept pace with the current discovery rate. The ability to translate complex findings obtained with more powerful technologies into guidance for clinicians' decisions has been nothing but challenging. High costs and the requirement of specialized personnel are some of the reasons behind the challenge. In an attempt to minimize this gap, we aimed at validating our most promising findings through IHC. IHC is the standard diagnostic technique in lymphomas, thus it is readily available in the current clinical workflow. This makes IHC the optimal choice to bridge the gap between the information obtained through the cutting-edge technologies applied in the research and the clinical setting. In paper III we successfully showed the use of p53 IHC as a surrogate marker for identifying mutations in the *TP53* gene. Given the strong prognosis of these mutations, their assessment remains a crucial step to be included as standard of protocol.

The goal of paper IV was to understand the role of another possible high-risk factor in MCL, c-Myc. Resorting to a vast number of MCL samples, we unraveled the negative prognostic role of c-Myc overexpression in MCL. Interestingly, this negative effect was reinforced when there was a combination of aberrations in c-Myc and *TP53*, suggesting an interplay between the two proteins in MCL. These two studies (paper III and paper IV) have shown that patient stratification is an unmet need in MCL and an effective clinical management



workflow is dependent on understanding disease drivers and identifying the best approaches to diminish their effects in disease aggressiveness.

In the last two papers of this thesis (paper V and paper VI), the focus shifted towards the immune microenvironment in MCL. As current treatment investigations have a strong focus on cellular therapies and immune modulation, a deeper understanding of the microenvironment plays a pivotal role. Thus, in paper V we aimed at providing a comprehensive characterization of the immune composition of MCL. We showed the strong impact of the MCL composition in disease and drug response, highlighting that an immune suppressive microenvironment has a negative prognosis, opening to exploiting immune oncologic strategies in the clinical management of MCL, such as PD-1/PD-L1 inhibitors for patients that showed expression of these markers. The presence of FoxP3<sup>+</sup> cells was associated with a shorter TTP in patients treated with the N-MCL2/3 protocol in our analysis. Of all phenotypes interrogated, CD163<sup>+</sup> macrophages showed the strongest prognostic impact in MCL, independent of the treatment approach and other high-risk markers in MCL. Thus, in paper VI we aimed to understand the crosstalk between MCL tumor cells and CD163<sup>+</sup> M2-like macrophages and identify strategies to modulate their interaction towards a beneficial outcome. We were able to profile how macrophages modulated their immediate environment in this disease, with the most interesting finding being the upregulation of the MAPK pathway in tumors with CD163<sup>+</sup> cells. Interestingly, this exploratory study also showed that macrophages in contact with tumor cells differ from macrophages that do not directly interact with the tumor cells. The spatial architecture is thus an important component in tumor development and modulation of these immune cells can be a target in MCL clinical management.

Throughout this thesis, I mainly used two MCL cohorts and took advantage of the development of new technologies to further identify biomarkers that can be easily accessible in the clinical setting, with the ultimate goal of contributing towards personalized approaches in MCL. The studies included investigations at different molecular levels, that combined provide a complex view of this disease and highlight the need to consider the different deregulations when evaluating treatment response and/or understanding the lymphomagenesis process. Further studies are warranted to validate the biomarkers here identified and to explore the feasibility of using them as targets for future stratified treatment

strategies The continuous understanding of the microenvironment contributions, namely regarding the crosstalk between macrophages and MCL cells, hopefully, will potentiate new immunotherapeutic strategies that will effectively kill the cancer cells.

The current flow of data that is possible to obtain from these new technologies and the current computational power available, position modern research at the crossroads of understanding how the different omics deregulations can, jointly, determine patient management. It is to be believed that the future will bring molecular signatures that originate from distinct molecular levels and take into account the interactions between tumor cells and their surroundings, and focus on how these collectively contribute to disease development and treatment response. Hopefully, this work contributed to this end. I would like to finish with a sentence that I have heard a few times throughout these four years, from clinicians treating patients with this disease, one of those my co-supervisor Mats Jerkeman: “These are exciting times to be working with B-cell lymphomas”.

# Popular Science Summary

Cancer is a devastating disease worldwide. The goal is to treat each patient individually while minimizing secondary effects. Such an approach is called precision or personalized medicine. In this work, we aimed at identifying biomarkers that could sort patients based on their treatment response to different therapeutic approaches. Biomarkers are biological characteristics, such as alterations in a specific gene or expression of a protein, that can be measured and associated with a particular disease state. Our focus was on a subtype of blood cancer that is difficult to treat and where patients suffer from relapses.

The currently available treatment options for this cancer type give a wide range of responses: some patients will be cancer-free for several years, others will have relapses within shorter times, and some will not respond at all to the treatment. We try to identify which patients belong to which group to optimize treatment choices and thus to improve the life expectancies. We found out that metabolism, which is responsible for transforming food into energy in cells, is altered in groups of patients that do not respond well to the current treatment. Moreover, we identified a specific protein, called c-Myc, which could be used as a biomarker for disease prognosis. We showed that patients with high levels of this protein in tumor cells were more likely to die from the disease.

For these patients, immunotherapy treatments are one of the future strategies that can postpone disease outbreaks. Such strategies take advantage of human immune cells which are programmed to attack cancer cells. Immune cells are responsible for protecting our bodies from diseases and fighting infections, and cancer cells are known to be able to deceive them. Part of our study aimed at understanding how these immune cells are affected in this cancer subtype and how we can use them to fight it. A specific type of immune cell, called macrophages, showed in our studies to have an impactful role. Macrophages are cells that recognize and kill their targets, but their effect is context dependent. We discovered that macrophages express different protein markers when they are in

close contact with tumor cells and seem to provide them support for treatment resistance. Tumor cells were also affected if macrophages were present. This signals a conversation between macrophages and tumor cells which opens the door to exploring immunotherapy strategies targeting macrophages.

In the last years, the scientific world has experienced major technological breakthroughs that allow studying cancer at a deep level. Unfortunately, these technologies are not implemented in the clinical setting. The findings obtained from these studies need to be adapted to currently clinically available technologies. Thus, throughout our work, we put an effort into providing affordable and easily implemented methods which can identify already known biomarkers in the clinical setting. We managed this by confirming our findings through a widely used clinical technique called immunohistochemistry. Consequently, we believe that our findings can be rapidly implemented and benefit nowadays patients.

In short, our work contributes to the knowledge of one of the blood cancer subtypes and bridges the gap between the technological advancements in research and the clinical application of such discoveries. We expect to have contributed to a future where patients get tailored treatments that will benefit them most.

# Resumo em Português

O cancro é uma doença mundialmente devastadora. Atualmente, o objetivo é tratar cada doente e, simultaneamente, minimizar possíveis efeitos secundários. Tal estratégia é denominada medicina personalizada. O principal intuito deste projeto foi a identificação de biomarcadores com potencial para classificar doentes de acordo com a resposta individual a diferentes linhas terapêuticas. Biomarcadores são características biológicas, tais como alterações na expressão de genes ou proteínas, mensuráveis, e podem ser associadas a determinados estádios da doença. O foco deste projeto foi um tipo de cancro do sangue considerado de tratamento difícil e com os doentes a sofrerem frequentemente recidivas.

As terapias atualmente disponíveis para este tipo de cancro traduzem-se num elevado espectro de respostas: alguns doentes mantêm-se livres de doença por vários anos, outros doentes sofrem recidivas num curto espaço de tempo, e outros não apresentam qualquer tipo de resposta à terapia. Tentámos identificar quais os doentes que pertencem aos diferentes grupos de forma a otimizar a escolha de terapias, aumentando a esperança de vida nestes doentes. Descobrimos que o metabolismo, mecanismo responsável nas células por transformar alimento em energia, apresenta alterações em grupos de doentes com fraca resposta à atual linha terapêutica. Identificámos uma proteína, chamada c-Myc, que pode ser utilizada como um biomarcador de prognóstico nesta doença. Num dos estudos conseguimos comprovar que elevados níveis desta proteína expressos nas células cancerígenas estavam associados a uma maior probabilidade de sucumbir à doença.

Para estes doentes, tratamentos de imunoterapia são vistos como estratégias futuras para prolongar o tempo até uma possível recidiva. Estas estratégias utilizam a capacidade das células do sistema imunitário estarem programadas para atacar e eliminar as células cancerígenas. As células do sistema imunitários são as responsáveis por proteger o corpo humano de doenças, assim como combater

infecções. Contudo, as células cancerígenas desenvolvem frequentemente mecanismos para as enganar. Uma parte dos nossos estudos teve como objetivo perceber como é que estas células do sistema imunitário estão alteradas neste tipo de cancro e qual a possibilidade de as usar para combater o desenvolvimento desta doença. Um tipo de células do sistema imunitário, denominado macrófagos, células que reconhecem e destroem os seus alvos, demonstrou ter um papel importante. O seu efeito está, no entanto, dependente do contexto em que se encontram, pois estas células expressam diferentes proteínas dependendo da sua proximidade às células cancerígenas e parecem ter um papel de suporte, levando a que se verifique uma resistência terapêutica. As células cancerígenas mostraram também ser afetadas pela presença de macrófagos. Estes resultados indicam que existe uma interação entre ambas as células, macrófagos e células cancerígenas, o que proporciona uma base para a exploração e desenvolvimento de imunoterapias direcionadas aos macrófagos.

Nos últimos anos, a comunidade científica experienciou grandes avanços tecnológicos que permitiram estudar o cancro numa elevada complexidade. Infelizmente, estas tecnologias não se encontram em prática clínica. Como tal, todas as descobertas deste projeto necessitam de ser adaptadas às tecnologias disponíveis hoje em contexto de prática clínica. Ao longo do desenvolvimento deste trabalho, foram feitos esforços significativos para providenciar métodos de baixo custo e de fácil acesso que permitam a avaliação dos biomarcadores em contexto clínico. Para isso, confirmámos as nossas descobertas mais relevantes através de uma técnica recorrente na prática clínica, chamada imuno-histoquímica. Consequentemente, acreditamos que o nosso trabalho pode ser rapidamente implementado e passível de vir a beneficiar os doentes atuais.

Concluindo, o nosso trabalho contribui para um maior conhecimento acerca deste tipo de cancro do sangue e preenche a lacuna entre os avanços tecnológicos da investigação e a sua aplicação na prática clínica. Esperamos vir a contribuir para um futuro em que os doentes obtenham as terapias que mais os venham a beneficiar.

# Acknowledgments

“All our dreams can come true, if we have the courage to pursue them.”

Walt Disney

My dream was not possible to be achieved without the contribution of so many. As I close another chapter, I feel an enormous gratitude for everyone that at different stages contributed to my success.

First and foremost, I need to thank **Sara**. Without you this journey would not have started. Thank you for picking me, for bringing me to the cold and dark Sweden and to allow me to be part of the group. Thank you for the fruitful conversations, for the constant availability and for all the learnings, for pushing me to be a better scientist and person, and for never stop believing in me. I have learned so much from your example, as a scientist and a true leader. You are an amazing mentor and I just hope one day I will be able to inspire anyone half as much as you inspire me. Thank you for making this journey so fun and investing all the time you did in me. I am extremely grateful.

**Mats Jerkeman**, thank you for the fast answers, the availability, for the inclusion in all steps of the different processes. You guided so many of the questions we tried to answer here and were my first door to the clinic world, to understand the needs and I have learned so much from you. To **Catja** and **Anders** for always having a door open for questions, help and guidance. To **Anna S. Gerdts**, thank you for a constant positivity and reinforcement. You have been a true inspiration in the last thesis sprint!

A big big thank you to the **Cancer Target** group, past and present members. For all the help and ideas exchange. Being part of a group with so much variety in expertise and personalities contributed for my growing, professionally, and personally. It has been so much fun being on board!

I also want to acknowledge the **CREATE Health/CanFaster** group, for the opportunity, for opening doors and providing me with tools and soft skills that will allow me to thrive in different positions. A word of acknowledge to **Jana** that takes in all our grumpiness and complaints and is still standing – I really appreciate the patience you had with me.

To everyone at the **Department of Immunotechnology** (past and present), for welcoming me with opening arms, for the constant English-speaking fikas and for hearing me constantly complaining about the terrible Swedish weather. I would like to thank every single person that, at any stage helped me, either to find a reagent or to figure out all the bureaucracy around different issues! I am truly grateful for all of you, and I wish you all the best. **Magdalena**, thank you for always picking up the phone and being my go-to person every time things went wrong in the lab – and well they went wrong a lot of times! **Tim**, I am very grateful for your kind words, for reading my very long kappa and always keeping the good spirit. To **Kristina Lövgren** and **Björn Nodin** for the help with IHC, to **Lina Olsson** and **May Hasson** for the GeoMx help. To **Fredrik Ek** for the small molecule library and suggestions. To **Peter Hollander** and **Anna Nikkarinen** for help with IHC and FISH. To **Agi** and **Anna** for HALO help and being available to open the door any time!

**Anna Porwit**, for all the discussions and feedback, for answering my endless questions with a great patience, and including me in the pathology rounds, a great way to learn more about lymphoma! To **Ingrid Glimelius**, for always being so supportive and enthusiastic with all research plans. To all other **co-authors** that promoted my growing by exchanging ideas and feedback in the different projects.

To the **Nordic MCL group**, which has been a source of knowledge, fun and discussion. Also, for the opportunity to use the valuable clinical material. To the **European MCL network**, I am grateful for the inclusion, for being so onboard with us, young researchers, coming to the discussions. Within both groups I gained a deeper insight into the challenges you face in the clinical setting.

Thank you to **May, Hanna, Stina, Lina** and **Renos**, my master (and bachelor) students. It was great having you on board and witness you growing. A special thanks to **May**, that thought me how to help, how to become a supervisor and kept by my side all these years. I will never forget all the constant exchange of ideas and gossip/laugh.



A very warm and big thank to **Lavanya**, for challenging me and helping me with the annoying and problematic R, for the cover, for explaining any advance modeling that came into my way. Thank you for taking this journey with me and for always being there to help, even when our personalities clashed. I can't think of a better person to have by my side throughout the years and I will forever carry the memories of our trips within Europe. Thank you for the friendship. A big thank you to **Aastha**, that became my "bestie at work". I had so much fun with the endless talks, the hugs and I am truly grateful for how much you always cared for and helped me. I don't think I would've endured the last couple of months without you, at least I am sure I would've not smiled as much. To **Sergio**, I can't stop thinking that, from all of us, you had the hardest journey but not for one second stopped smiling and caring for us, thank you for being a great friend. Thank you to the three of you. I am so grateful that you crossed and became a part of my life.

To **Sah, Claudxi, Titi, Ana Beatriz, Ana Inês, Kika, Ni, Marta, Carolina** (and **Manoel**) **Xana, Ilda**, and so many others, for making sure that distance was never an issue. I never felt truly apart. To **Cat** and **Alex** for never getting mad at me when I constantly said no to plans or left early. You made my life in Sweden so much better every day. Thank you all for making sure I did not get lost in academia.

To **Mom** and **Dad**. What I am, everything I achieved I owe you both. Thank you for setting the example, for all the sacrifices I know you made for me and for never allowing the distance to be that big. I hope I made you proud. **Afonso**, you will always be my little baby brother, the annoying one that turned into a teenager enclosed in his own room, but who still is the joy of my life. I hope this book and my journey will show you that you can always achieve what you set on yourself to do, and that the three of us will be around to support and help you no matter what.

Last, but not least, to **Rúben**. You deserve a full page of thank you. I am forever grateful to you, for having your constant support, for taking care of me, for enduring my worst side and still staying. You supported and helped me every day (even on my extremely grumpy days) and I could not ask for better. I will never be able to pay you back the kindness – and patience, I know! To the moon and back, always <3



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