Mantle cell lymphoma strategies in primary treatment

Mantle cell lymphoma-strategies in primary treatment

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Abstract

Mantle cell lymphoma (MCL) is associated with poor prognosis due to an aggressive clinical course. Being a rare disease, there are few randomized trials in MCL and there is no defined golden standard in primary treatment.

This works aimed to (I) investigate outcome in relation to primary treatment in MCL based on population-based registry data, (II) to evaluate tolerability and efficacy of lenalidomide-rituximab-bendamustine (LBR) in newly diagnosed MCL patients within a phase I/II trial (MCL4) including (III) outcome in relation to genetic alterations, and (IV) to study how novel agents interfere with response to anti-CD20 antibodies.

Our results showed that survival in MCL patients improved during 2000-2011, which partly could be explained by the introduction of rituximab and intensified treatment with high dose chemotherapy consolidation. We also found that treatment with radiotherapy to limited-stage disease and observation in non-symptomatic MCL were associated with long-term survival. In paper II and III, LBR was found to be an active combination in untreated MCL patients, except for cases harboring *TP53* mutations, but associated with significant toxicity including second primary malignancies. In paper IV, we showed that the BTK-inhibitor ibrutinib, negatively affected the immune mediated cell death induced by a type I or II anti-CD20 antibody in MCL cell lines, not restored by addition of lenalidomide, a potential sensitizer to anti-CD20 ab.

This work has provided data on important factors for outcome in MCL that may be taken into clinical use, such as active observation in non-symptomatic patients and rituximab and intensified approaches in primary treatment. Moreover, the addition of lenalidomide to BR could not be recommended as first-line treatment in MCL due to excessive toxicity and novel combinations with activity in elderly patients as well as in *TP53* mutated MCL are highly warranted. Future studies, including in vitro models on drug interaction will clarify how novel agents should be combined for optimal use in MCL.

Key words: mantle cell lymphoma, prognostic factors, in vitro model, ADCC, rituximab, bendamustine, lenalidomide, ibrutinib, watch and wait,

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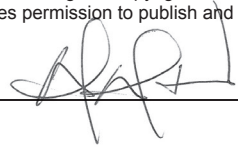
Signature  Date 2018-03-08

Mantle cell lymphoma-strategies in primary treatment

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If a man will begin with certainties, he shall end in doubts; but if he will be content to begin with doubts, he shall end in certainties."

Francis Bacon, the Advancement of Learning

Content

List of Papers....13
My contributions to the papers....14
Paper I....14
Paper II....14
Paper III....14
Paper IV....14
Populärvetenskaplig sammanfattning....15
List of abbreviations....19
Introduction....21
Historical review....21
Clinical characteristics and diagnosis of MCL....21
Epidemiology....21
Etiology....22
Clinical presentation....22
Diagnosis and staging procedure....22
Morphological subtypes and immunohistochemistry profile....23
Pathobiology of MCL....25
Cell of origin....25
Genetic alterations....26
Cell cycle regulation....26
DNA damage response....27
Cell survival and proliferation via membrane receptor pathways....28
Apoptosis....28
Epigenetic modification....29
MicroRNA in MCL....29
Alterations in gene expression....30
G1-S transition via p16/p19-p53 pathway....31
Impact on cell survival and apoptotic signal pathways....31
DNA damage repair/cell cycle arrest....32
Selected altered protein expression in MCL....32
SOX11....32
Cyclin D1....33
The role of microenvironmental factors in MCL....34
Prognostic factors....35
Ki-67....35
Blastoid MCL....35

MIPI-mantle cell lymphoma international prognostic index....36
Molecular prognostic factors....36
Minimal residual disease....37
Treatment of mantle cell lymphoma....39
Indolent MCL....39
Local treatment in limited stage disease....41
Systemic therapy....41
Treatment with anti-CD20 antibodies....43
Mechanism of action....43
NK-cell mediated ADCC....43
Rituximab....44
Resistance to rituximab....45
Second generation anti-CD20 antibodies....45
Obinutuzumab....46
Other anti-CD20 antibodies....46
Primary treatment of young patients....47
What is the optimal induction in young patients?....47
High-dose chemotherapy with autologous stem cell support....50
The support for HD-ASCT in MCL....50
What is the optimal consolidation?....50
Remission status prior to ASCT is prognostic....51
Maintenance with rituximab after ASCT....51
Primary treatment of elderly patients....53
Induction with immunochemotherapy....53
Maintenance with rituximab....55
Salvage treatment....57
Immunochemotherapy and novel agents....57
Maintenance with rituximab in relapsed MCL....58
Is there a role for allogenic stem cell transplant in MCL?....58
Novel agents....59
Targeting B cell receptor signaling....59
Inhibition of Bruton´s tyrosine Kinase by ibrutinib....59
Resistance to ibrutinib....60
Off-target effects....60
Other BTK-inhibitors....61
Lenalidomide-an immune-modulating agent....62
Mechanism of action....62
Sensitizing to antibody treatment....63

Lenalidomide in MCL 63
Other small molecule inhibitors 64
PI3K inhibitors 64
mTOR inhibitors 65
Bcl-2 inhibitor 65
Proteasome inhibitor 65
Aims 67
Material and Methods 69
Nordic Lymphoma Group 69
Paper I-an observational study on MCL 69
Hypothesis 69
Swedish and Danish lymphoma registries 69
Study design and patients 70
Paper II – a phase I/II trial investigating a new combination for elderly patients with untreated MCL 70
Hypothesis 70
Trial design 70
Treatment 71
Phase I 71
Phase II 71
Patients 72
Evaluation of response and safety analysis 72
Paper III-mutational profile as prognostic marker 73
Hypothesis 73
Patients and genes selected for study 73
NGS and ddPCR 73
Evaluation of ADCC on MCL cell lines (paper IV) 74
Hypothesis 74
Assessment of ADCC by flow cytometry 74
Statistics 76
Paper I 76
Paper II 76
Paper III 76
Paper IV 77
Results 79
Paper I 79
Paper II 80
Paper III 83
Paper IV 84

Discussion 87
Understanding the improved outcome in MCL 88
What is the optimal induction for elderly patient with MCL? 89
Can LBR be recommended to elderly untreated patients with MCL? 90
Is LBR a feasible regimen? 90
Is LBR an active regimen for untreated patients with MCL? 93
Does the addition of lenalidomide overcome poor prognosis in TP53-mutated MCL? 94
What is the optimal partner for R-B and R-L? 95
How can *in vitro* models be used in design of regimens? 96
Future perspectives 99
MRD as a prognostic and treatment-stratifying marker in MCL 99
Is there any hope for TP53-mutated MCL? 100
Chemo-free strategies in MCL 101
Possibilities with *in vitro* models 102
Evolving strategies in clinical research 102
Acknowledgements 105
References 107

List of Papers

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

I. Real world data on primary treatment for mantle cell lymphoma: a Nordic Lymphoma Group observational study.

Anna Abrahamsson, **Alexandra Albertsson-Lindblad**, Peter N. Brown, Stefanie Baumgartner-Wennerholm, Lars M. Pedersen, Francesco D'Amore, Herman Nilsson-Ehle, Paw Jensen, Michael Pedersen, Christian H. Geisler, and Mats Jerkeman. Blood. 2014 124:1288-1295

II. Lenalidomide-bendamustine-rituximab in patients older than 65 years with untreated mantle cell lymphoma.

Alexandra Albertsson-Lindblad, Arne Kolstad, Anna Laurell, Riikka Räty, Kirsten Grønbæk, Jan Sundberg, Lone Bredo Pedersen, Elisabeth Ralfkiær, Marja-Liisa Karjalainen-Lindsberg, Christer Sundström, Mats Ehinger, Christian Geisler, and Mats Jerkeman. Blood. 2016 128:1814-1820

III. The addition of lenalidomide to rituximab-bendamustine does not overcome the poor prognostic impact of *TP53* mutations in mantle cell lymphoma.

CW Eskelund*, **A Albertsson-Lindblad***, A Kolstad, A Laurell, R Räty, L B Pedersen, C Geisler, M Jerkeman, K Grønbæk. *Manuscript submitted to British Journal of Hematology,* * contributed equally to this work

IV. Ibrutinib inhibits antibody dependent cellular cytotoxicity induced by rituximab or obinutuzumab in MCL cell lines, not overcome by addition of lenalidomide.

Alexandra Albertsson-Lindblad, Catja Freiburghaus, Mats Jerkeman, Sara Ek. *Manuscript*

My contributions to the papers

Paper I

I participated in the analysis of the data and was responsible for writing of manuscript.

Paper II

I participated in collecting the data and was responsible for analysis of data and writing of manuscript.

Paper III

I participated in design of the study, collecting of the data and was responsible for writing of manuscript in collaboration with the other first co-author.

Paper IV

I participated in design of the study, collecting and analysis of the data and writing of manuscript

Populärvetenskaplig sammanfattning

Mantelcellslymfom (MCL) är en typ av cancersjukdom som utgår från immunsystemets celler och drabbar ca 100 personer per år i Sverige, varav majoriteten är äldre. Sjukdomen karaktäriseras av ett aggressivt förlopp med tidig spridning till organ utanför lymfbanorna som t. ex benmärg och magtarmkanalen. Med nuvarande behandlingsstrategier, som innefattar regelbunden infusion av rituximab, en antikropp mot CD20 -molekyl på lymfomcellens yta, och en eller flera cellgifter i kombination, betraktas ändå sjukdomen som icke botbar och färre än hälften av patienterna överlever längre än fem år efter diagnos. Det råder inte konsensus kring vilken som är den mest lämpliga behandlingsregimen av patienter med MCL med avseende på effekt och biverkningsprofil. Således föreligger ett stort behov att studera nya behandlingskombinationer samt att identifiera nya effektiva substanser med effekt på MCL för att förbättra prognosen hos patienter som drabbats av sjukdomen.

Syftet med det här avhandlingsarbetet har varit att undersöka om man, genom att kombinera populations-baserad registerinformation, utfallet av en klinisk prövning och *in vitro* studier på hur läkemedel interagerar, kan dra slutsatser om viktiga faktorer för överlevnad vid MCL, hur nya läkemedel kan kombineras med avseende på effektivitet, tolerabilitet och interaktioner, för att förbättra prognosen för patienter med MCL.

I avhandlingen ingår fyra arbeten, varav tre är gjorda i samarbete med Nordiska lymfomgruppen (NLG), ett nordiskt samarbetsorgan som arbetar med att upprätta kvalitetsregister, vårdprogram och kliniska prövningar för patienter med lymfom.

I det första arbetet har vi med hjälp av Svenska och Danska lymfomregistret och uppgifter från patientjournaler skapat en databas för nästan 1400 patienter som diagnosticerats med MCL under åren 2000-2011. I studien har vi bland annat kunnat visa att överlevnaden förbättrats för patienter med MCL under tidsperioden vilket, utifrån vårt material, delvis kan korreleras till introduktion av CD20-antikropp och intensiv cellgiftsterapi med stamscellstöd. Dessutom har vi identifierat två grupper med mycket lång tids överlevnad utan behandling med cellgifter; en grupp med begränsad spridning av sjukdomen som erhållit lokal strålbehandling och en grupp med stillsam sjukdom utan symtom som kunnat följas med regelbundna kontroller, så kallad ”aktiv exspektans”.

I det andra arbetet har vi undersökt utfallet av en klinisk studie, initierad av NLG, för nydiagnostiserade patienter >65 år med MCL. Studien, NLG/MCL4"Lena-Berit", utprovade en ny kombination av läkemedel "LBR" som innefattar tre olika typer av verkningsmekanismer, ett immunstimulerande läkemedel, lenalidomid, en typ av cellgift, bendamustin, samt, en CD20-antikropp, rituximab. Målsättning med studien var att ta reda på vilken dos av lenalidomid som är lämplig vid kombination med BR samt att undersöka effektivitet, biverkningsprofil, överlevnad, livskvalité och påverkan på immunförsvaret hos patienter som fått behandling enligt regimen. Totalt inkluderades 50 patienter från Sverige, Norge, Danmark och Finland. Studien har visat att behandlingskombinationen är effektiv vid MCL med hög andel patienter som svarat på behandling inklusive uppnått molekylär remission, dvs. utan mätbar sjukdomsaktivitet i blod eller benmärg. Emellertid utvecklade en stor andel av patienterna i studien biverkningar, främst i form av kvarstående nedsatt immunförsvar med risk för svåra infektioner, hudutslag, och uppkomst av andra tumörformer.

I ett tredje delprojekt har vi analyserat förekomst av särskilda genetiska förändringar i tumörcellerna i relation till prognos hos de patienter som ingick i Lena-Berit-studien. Tidigare arbeten har visat att förekomst av genetiska förändringar i två gener med koppling till cellens egen kontroll av tillväxt vid exempelvis DNA skada, *TP53* och *CDKN2A*, har kopplats till sämre överlevnad efter behandling med CD20-antikropp och cellgifter vid MCL. Proverna, som samlades in före start av behandling analyserades för utläsning av deletioner (bortfall av gen) samt mutationer (enstaka fel i gen) av ett urval gener, däribland *TP53* och *CDKN2A*. Studien, som också inkluderade en långtidsuppföljning av studieresultatet, visade att patienter med förekomst av mutation i *TP53* aldrig uppnådde molekylär remission med LBR och hade betydligt kortare överlevnad än övriga patienter.

I det fjärde arbetet har vi utarbetat en modell för studier av hur nya läkemedel som binder till specifika molekyler utanpå eller inuti cellen påverkar effekten av CD20-antikroppar vid MCL. CD20-antikroppars effekt vid lymfom tillskrivs bl. a. "antikroppsmedierad celldöd" (ADCC) vilket sker via bindning och aktivering av kroppens egna (NK-) immunceller som då attackerar och dödar tumörcellen. Vidareutveckling av CD20-antikroppar har bl. a inneburit modifiering för att förstärka ADCC, s.k. typ II antikroppar.

Tidigare studier på cellinjer från en annan typ av blodcancer (kronisk lymfatisk leukemi) har visat att tillägg av ibrutinib, ett nytt läkemedel som är effektivt vid MCL genom att det bl.a hämmar celltillväxt, till CD20-antikropp kan minska ADCC genom att ibrutinib hämmar immuncellernas aktivering. Tvärtom har lenalidomid visats kunna förstärka CD20-antikroppars effekt.

Vi har visat att obinutuzumab, en typ II antikropp, inducerar ADCC i större utsträckning än en typ I antikropp som rituximab. Vidare har vi påvisat att ibrutinib

minskar effekten av både typ I och II CD20 antikroppar med avseende på ADCC vid MCL och att den negativa effekten av ibrutinib inte hävs genom tillägg av lenalidomid.

Sammantaget visar arbetena i avhandlingen att även om prognosen vid MCL har förbättrats, tack vare introduktion av målinriktade läkemedel som CD20 antikropp och intensiv behandling vid diagnos, finns det grupper av patienter i stort behov av effektivare behandlingsalternativ; äldre med MCL samt patienter med MCL med förekomst av TP53 mutationer. Vidare har vi kunnat dra slutsatsen att cellgifter i kombination med flera läkemedel som stimulerar immunförsvaret bör ges med försiktighet, i synnerhet till patienter som inte tidigare fått någon cellgiftsbehandling och att interaktioner mellan läkemedels effekt på immunförsvaret kan påvisas med prekliniska modeller. Framtida studier som integrerar populations-baserad data med kliniska prövningar, molekylär karaktärisering av MCL vid diagnos samt vidare utveckling av läkemedelskombinationer kommer att ge vidare vägledning av hur prognosen för patienter med MCL kan förbättras.

List of abbreviations

7-AAD	7-Aminoactinomycin D
ADCC	antibody-dependent cell-mediated cytotoxicity
AE	adverse event
Ara-C	cytarabine
ATM	ataxia telangiectasia mutated gene
BCL	B cell lymphoma
BCR	B cell receptor
BEAM	carmustine, etoposide, cytarabine, melphalan
BEAC	carmustine, etoposide, cytarabine, cyclophophamide
BTK	bruton's tyrosine kinase
CD	cluster of differentiation
CDC	complement-dependent cytotoxicity
CDK	cyclin dependent kinase
CDKN2A	cyclin dependent kinase inhibitor 2 A
CFSE	carboxyfluorescein succinimidyl ester
CHOP	cyclophosphamide, doxorubicin, vincristine, prednisone
CLL	chronic lymphatic leukemia
CNS	central nervous system
CR	complete remission
CVP	cyclophosphamide, vincristine, prednisone
dd-PCR	digital droplet PCR
DHAP	dexamethasone, high-dose cytarabine, cisplatin
DLBCL	diffuse large B cell lymphoma
DNA	deoxyribonucleic acid
FC	fludarabine, cytarabine
FcR	Fc receptor
FL	follicular lymphoma
(HD-)ASCT	high-dose consolidation with chemotherapy and autologous stem cell support
hyper-CVAD	doxorubicin, cyclophosphamide, vincristine, dexamethasone (CHOP) alternating with high-dose cytarabine and methotrexate
IFN-α	interferon alpha
IGHV	immunoglobulin variable heavy chain
INK4A	inhibitor of cyklin-dependent kinase 4

LDH	lactate dehydrogenase
LRI	lenalidomide, rituximab, ibrutinib
mAb	monoclonal antibody
MCL	mantle cell lymphoma
MIPI	mantle cell lymphoma international prognostic index
MRD	minimal residual disease
mTOR	mammalian target of rapamycin
NFkB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	next generation sequencing
NHL	non-Hodgkin lymphoma
OS	overall survival
PBMC	peripher blood mononuclear cells
PCR	polymerase chain reaction
PDGF A	platelet-derived frowth factor A
PFS	progression-free survival
PI3K	phosphatidylinositol-3-kinase
PD	progressive disease
PR	partial remission
R-B/BR	rituximab, bendamustine
R-BAC	rituximab, bendamustine, cytarabine
RBi	rituximab, bendamustine, ibrutinib
RiBVD	rituximab, bendamustine, bortezomib
RIT	radioimmunotherapy
R-L/R2	rituximab, lenalidomide
R/R	relapsed/refractory
RCT	randomized clinical trial
RIST	reduced intensity allogenic transplant
RT	radiotherapy
SNP	small nucleotide polymorphism
SOX11	SRY (sex-determing region Y) box 11
TBI	total body irradiation
TGF B	transforming growth factor B
TP53	tumor protein p53
WaW	watch and wait
WBC	white blood count

Introduction

Historical review

Mantle cell lymphoma (MCL) is a malignant B cell lymphoma, defined by the presence of translocation on chromosome 11 and 14, t(11;14)(q13;q32) [1]. The name refers to the observation of malignant lymphoid cells arising from the mantle zone surrounding the follicle zone in lymph nodes.

The translocation t(11;14)(q13;q32) was first described in 1979, then associated with a heterogenic group of non-Hodgkin lymphomas [2]. Several entities were used to describe lymphomas of mantle origin, including diffuse small cleaved cell lymphoma, centrocytic lymphoma (the European Kiel classification) and intermediately differentiated lymphocytic lymphoma (in US). The association between t(11;14)(q13;q32) and a specific type of non-Hodgkin lymphoma was finally made in early 1990s, which led to the proposal of MCL as a specific entity. MCL was adopted into the Revised European American Lymphoma (REAL) classification of lymphoid neoplasms in 1994 [3-5].

Clinical characteristics and diagnosis of MCL

Epidemiology

In Sweden, 80-120 patients are diagnosed with MCL each year, representing approximately 5% of all lymphomas, according to the Swedish lymphoma registry data from 2000-2013[6]. 72% of patients are males and the median age at diagnosis is 70 years. An increased incidence rate of MCL has been reported from population-based data, even after the introduction of immunohistochemistry staining for cyclin D1 and detection of t(11;14)(q13;q32) in routine practice [7-9].

Etiology

In general, there is no strong correlation between MCL and any biological or environmental risk factor and the disease is regarded as arising *de novo*.

Unlike other B cell lymphomas like diffuse large B cell lymphoma (DLBCL), follicular lymphoma (FL) and Burkitt lymphoma (BL), risk factors such as chronic infection, immunosuppression, auto-immune diseases, sun-exposure, smoking or body mass index (BMI) have not been associated with increased risk of MCL [10-12].

The presence of another hematological malignancy in a first degree relative has been associated with up to two-fold risk and hay fever has been associated with decreased risk for MCL, suggesting that the pathogenesis may be a combination of environmental and host-related factors. It has been discussed whether atopy, as hay fever, could be associated with earlier detection and eradication of cancer-antigen by higher release of cytokine response [13].

Clinical presentation

Most patients with MCL present with aggressive disease, typically manifested with enlarged lymph nodes and > 80% of MCL cases are disseminated (stage III-IV) at diagnosis. Extranodal involvement is common, most frequently in bone marrow (80%), peripheral blood (34%) and gastrointestinal tract but may also be detected in other loci, such as lung, pleura, liver, eye, CNS and bone tissue [6, 14]. Gastrointestinal involvement has been detected in >80%, although less than 30% have macroscopic lesions or presence of gastrointestinal symptoms [15].

A more indolent course of MCL has been observed in a small subset of patients, commonly diagnosed with non-nodal, leukemic disease with bone marrow involvement and splenomegaly [16, 17].

Diagnosis and staging procedure

According to the WHO classification, the diagnosis of MCL is based on histological examination and immunohistochemistry staining of a tissue biopsy by detection of a lymphoid malignancy with overexpression of cyclin D1 and/or the presence of t(11;14)(q13;q32), showed by karyotyping or fluorescence in situ hybridization (FISH) [1].

The routine investigation also includes a diagnostic bone marrow biopsy and aspirates, peripheral blood samples and computer tomography (CT-scan)

(neck/chest/abdomen/pelvis) for staging. Further investigations may include upper/lower endoscopy, CT-scan of CNS and CSF (cerebro-spinal fluid) cytology in presence of CNS symptoms and/or high-risk disease. PET/CT is recommended to define lower stage disease (stage I/II), prior to local radiotherapy [18].

Staging is based on Ann Arbor classification, through subsequent revision, latest in Lugano classification 2014 [19]. Stage I and II represent limited disease (one and two nodal sites respectively on ipsilateral side of diaphragm) whereas stage III and IV denote disseminated disease with involved nodes both sides of diaphragm (stage III) or involvement of one or more extranodal sites (stage IV).

Morphological subtypes and immunohistochemistry profile

Four morphological subtypes of MCL are described based on the histological growth pattern; diffuse, nodular, mantle zone or a combination of the three. The cytomorphology in MCL includes a range of variants, including small round lymphocytes, marginal zone like, intermediate sized, pleomorphic and blastoid cells [1].

Cyclin D1 is overexpressed in 95% of MCL cases. Cyclin D1-negative MCL may be positive for cyclin D2 and cyclin D3 [20, 21].

In addition to cyclin D1, the immunohistochemical profile is characterized by positivity for typical B cell markers, such as CD19, CD20, CD5, FCM, CD79a, CD43, Bcl-2 and negativity for CD23, CD10, BCL6 and CD200 [1].

SOX11, a member of the SOX family of transcription factors, is overexpressed in nearly 100% of MCL and is useful to differentiate MCL from related lymphomas such as CLL, MZL and FL as well as to verify the diagnosis in cyclin D1-negative cases [22-24].

Further, the diagnostic examination should include evaluation of Ki-67, a proliferation marker, with prognostic value in MCL [25].

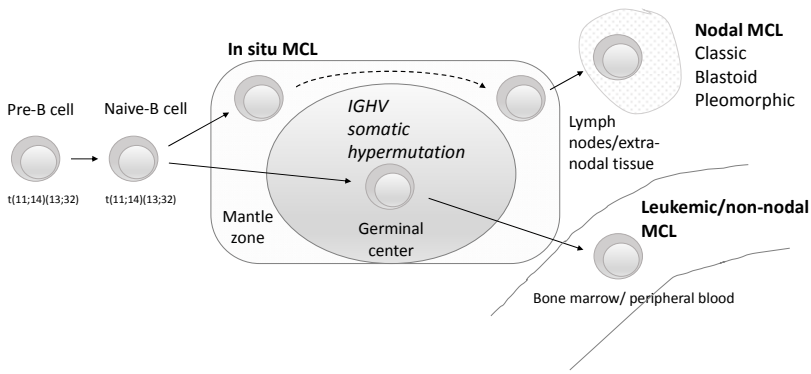
Pathobiology of MCL

Cell of origin

In 2012, a cell of origin concept of MCL was published in 2012 by Jares et al. suggesting two main subtypes of MCL based on presence of somatic *IGHV* mutations, which was recently adopted in the 2016 update on WHO classification [1, 26].

As illustrated in Figure 1, precursor B cells, carrying t(11;14)(q13;q32), migrate to lymph nodes and undergo development into naïve B cells. The majority of MCL are thought to arise from when a naïve B cell colonizes either the mantle or the marginal zone of the follicle, with development of in situ mantle neoplasia or MCL, characterized by limited somatic *IGHV* mutations, SOX11-positivity and genetic instability.

Figure 1. Cell of origin concept in MCL



Adopted from Jares et al [26].

Another subtype is thought to evolve from naïve B cells that enter into the germinal center and undergo IGVH somatic hypermutations. These cells are initially genetic stable and more frequently SOX11-negative, albeit acquired mutations, i.e. of TP53 may change these properties and develop into lymphomas with genomic instability and give rise to aggressive disease.

Genetic alterations

A genetic alterations refers to when the genome is affected by changes in DNA. Large alterations include deletions, translocations and amplifications and cover a range of base pairs involving one or more genes. These changes do not necessary affect the properties of the encoded protein but rather change the level of expression. Smaller alterations, mutations, affect the sequence within a gene which may cause changes in amino acid sequence and thereby change the structure and functional properties of the encoded protein.

MCL is characterized by genetic instability and secondary chromosomal aberrations are highly frequent, especially in blastoid MCL, when compared to other non-Hodgkin lymphomas [27].

Molecular studies have revealed that the genetic abnormalities in MCL frequently affect areas coding for genes involved in two cellular regulatory systems; cell cycle regulation and DNA damage repair. Furthermore, pathways regulating cell survival, proliferation, differentiation and response to environmental factors may be affected by genetic alterations and contribute to the pathogenesis of MCL.

Cell cycle regulation

Cell cycle regulation is of major importance for the normal function of dividing cells. Two pathways with a key role in cell cycle regulation, are frequently altered in MCL; the INK4a/CDK4/Rb1 and the ARF/MDM/p53 pathways. (Figure 2)

Deletion of *CDKN2A* locus (9p21) which involves coding areas for the CDK inhibitor INK4A and ARF, thus connecting these two pathways, is found in up to 30% of MCL and has also been related to worse prognosis, mainly in univariate analysis [28-32]. Alternatively, gene amplification of *BMI1*, a transcriptional processor of *CDKN2A* locus, could affect the same pathway [33]. Loss of Rb1, either by inactivating mutations or deletions of 13q14 has been found in >40% of MCL [30, 34].

Loss of functional p53 may be either due to gene amplification of *MDM2* (12q21), thereby increasing the degradation of p53 or, more frequently, by genetic alteration of *TP53*, as discussed below [35].

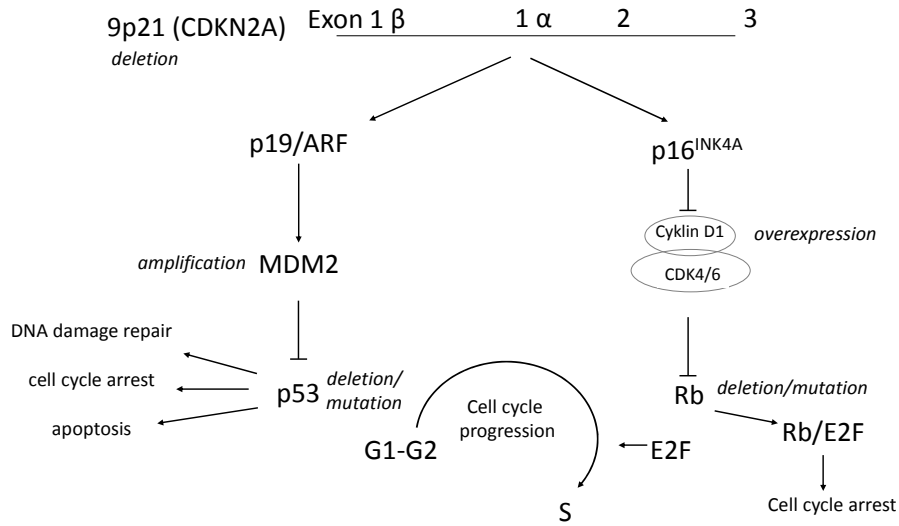


Figure 2. Cell cycle regulation via IK/ARF locus
Two major pathways of cell ycle regulation. P19/ARF and p16INK4A are encoded from the same locus by alternative reading frame, regulating two different pathways of cell cycle regulation, P19/ARF/MDM2/p53 and p16/CDK4/Rb1. Genetic alterations, frequently found in MCL are showed in italics which may cause altered levels of functional protein and dysregualtion of cell cycle progression.-

DNA damage response

TP53 alterations

The key regulator of DNA damage response is the tumor suppressor p53, orchestrating several response mechanisms including cell cycle arrest and apoptosis. The importance of a functional p53 in preventing malignancy is exemplified by the high risk of invasive tumors in patients with germline mutations in the encoding gene, *TP53,* (Li-Fraumeni sydome) and that alterations in *TP53* are found in a wide range of solid and hematological malignancies [36]. As discussed below, genetic alterations of *TP53* is found in a small subset of MCL and are associated with inferior outcome.

Other alterations

Ataxia-telangiectasia mutated gene (*ATM*), located in 11q22-23, encodes for ATM, another key player in the cellular response to DNA damage. Deletion of 11q22 as well as mutations in *ATM* are reported in > 40% of MCL patients [37-39].

Inactivated *ATM* is correlated with accumulation of chromosomal aberrations in MCL [40]. *In vitro* model on other solid tumors have shown that lower activity of *ATM*, is associated with increased effect of radiation, which might explain the radiosensitivity of MCL, as discussed by Ahmed et al. [41, 42].

The downstream activating enzymes CHK1 and CHK2, have been found to be downregulated in MCL, which seems to play an important role for predisposing cells to genomic instability [43, 44].

Cell survival and proliferation via membrane receptor pathways

Mutations in the genes encoding for the transmembrane transcription factors NOTCH1 and NOTCH2, are reported in 5-15% of MCL and have been associated with inferior survival [39, 45, 46]. Inhibition of the NOTCH1 pathway *in vitro*, reduced proliferation and induced apoptosis in cell lines [45].

Proliferation signals via the B cell receptor (BCR), Toll-like receptor (TLR) and CD40 belong to NF-kB activating pathways. In MCL, several genetic alterations have been detected with associated increased signaling in these systems, either by deletion or inactivating mutations of negative suppressors; (% detected) *BIRC3* (del 11q22 11-57% or mutation 6-10% respectively), *TRAF2* (7%), *NFKB1I* (5%) and *TNFAIP3* (19-37%), or by activating alterations of *CARD11* (3-15%) and *MAPK3K14* (2-3%), as summarized by Rosenquist et al. [47].

Apoptosis

Among regulators of apoptosis, translocation involving *MYC*, is perhaps the most described alteration in B cell lymphomas, as defining Burkitt lymphoma and a few other subgroups of aggressive B cell lymphomas. A few cases of *MYC* translocation have been described for MCL, all with blastoid morphology and associated with very short survival [48]. Although *MYC* has shown to be overexpressed in several cases of MCL, no other genetic alterations have been reported. Furthermore, *BCL2*, which is overexpressed in a majority of MCL may be dysregulated by gain or amplification of *BCL2* in 18q21, and deletions of the genes encoding pro-apoptotic factors, *BCL2L11* (BIM) and *FBXO25* have been reported [49-51].

Epigenetic modification

Epigenetic alterations refer to heritable changes of gene activity that do not involve the genome and include mechanisms like DNA methylation and/or modification of the histone complex by changes in chromatin structure, which thereby regulate binding of transcription factors to DNA and transcription.

In MCL, profiling of methylation/de novo methylation has showed different pattern in MCL compared to normal cells as well as compared to other B cell malignancies like CLL. Although epigenetic profiling has been suggested to be able to define indolent MCL vs conventional MCL the number of methylated genes seems to be heterogeneously distributed within the MCL population and the genes with altered methylation did not differ between high and low proliferative disease [52-54].

Among functional groups of genes affected by hypermethylation in MCL are homeobox transcription factors, regulating transcription, and Wnt inhibitor genes, thereby with possible constitutional activating of the Wnt signaling pathway and enhanced transcription of pro-survival and proliferation factors [53, 54]. Furthermore, mutations in methyltransferase genes *KMT2D* (*MLL2*) have been found in 14-20% of MCL, predominantly silencing mutations, as well as in *KMT2C* (*MLL3*). Other frequently mutated genes involved in epigenetic modification are the chromatin remodeler *SMARCA4*, and histone methyltransferase *H3K36* [39, 55, 56].

MicroRNA in MCL

Short single-stranded RNA molecules, recognized as micro-RNA (mi-R), with regulatory function on translation of mRNA, are encoded by short DNA sequences from introns or exons, in association with its host gene. Numerous mi-R have been described, with both tumor suppressive and oncogenic functions, and altered expression of these DNA sequences, have been characterized as pathogenic factors in different types of malignant diseases including B cell neoplasms.

In 2008, Schraders et al. showed that several genomic regions harboring mi-R were altered by either gain or loss of DNA in MCL [57]. Subsequent studies have confirmed that MCL can be distinguished from normal B cells and other B cell malignancies by mi-R expression profiling [58-60]. Among deregulated mi-R in MCL is the *miR16-92* family, which have been shown to be upregulated in a subset of MCL, and correlated with higher expression of genes of the proliferation signature [58, 60].

Two studies have found association between altered mi-R in combination with *MYC* overexpression and poor outcome; upregulation of *mi-R17*, possibly caused by copy number alteration of 13q31 and downregulation of *mi-34a*, which regulates *MYC* [58, 60, 61].

Another recurrent finding is down-regulation of *mi-R29*, which has been suggested a role as driver of proliferation in cyclin D1-overexpressed cases, by its affinity to mRNA of CDK6 [59, 62]. A lower expression of *mi-R29* would then increase CDK6 levels in the cell, causing formation of the CD1/CDK4/CDK6 complex with enhanced transition of cell cycle. This hypothesis was supported by *in vitro* models blocking *mi-R29* and by detection of high CDK6 levels in patients harboring downregulated *mi-R29*. Moreover, the level of *miR-29* family was associated with prognosis [59].

Micro-RNA as a prognostic marker was further evaluated by Husby et al. in the cohort of MCL patients within the Nordic MCL2 trial, showing higher expression levels of several mi-R in patients with short survival. One of them, *mi-R18b*, was incorporated with MIPI-B as a prognostic index, defining a high-risk group of patients with shorter progression-free survival and overall survival [63].

Several studies have tried to find patterns of m-R profile in relation to biologic variants of MCL, like *IGHV* mutational status, SOX11 expression, similarity with different stages of B cell maturation/differentiation and nodal vs non-nodal disease. So far, the results from these studies support the role of mi-R as a diagnostic and prognostic tool but the specificity is not high enough to be taken into the clinical setting for treatment stratification. [58, 60, 61, 64-66].

Alterations in gene expression

Since 1990s, the development of techniques for assessment of transcription of the genome has led to the introduction of gene expression profiling. Methods like micro-arrays or parallel gene expression analysis and real-time quantitative PCR (RT-qPCR) have made it possible to achieve quantitative data on transcription of multiple genes. By further analysis, patterns of expression can be used to create signatures that can define disease versus non-disease; subgroups within the same diagnosis/genetic group, response to treatment and serves as a novel approach for identifying possible targets for intervention/therapeutics.

By gene expression profiling, MCL can be distinguished from normal B cells or other B cell lymphomas as shown by several groups on both patient derived samples and on cell lines [67-71].

Several of the genes alternatively expressed belongs to the proliferation signature of B cells, as defined by Shaffer et al. (Shaffer et al. 2001) and may be related to outcome as first proposed by Rosenwald et al. 2003 [68, 72, 73].

G1-S transition via p16/p19-p53 pathway

One group of genes with altered expression in MCL is related to tumor suppressor proteins encoded by the INK4a/ARF locus on chromosome locus 9p21, p16 and p19, and the downstream targets, involved in regulation of cell cycle transition from G1-S phase via p53 (Figure 2). *MDM2*, a gene on 12q13, encoding a protein with capacity of degradation of p53 has shown to be upregulated in MCL without relation to altered copy number of the gene or presence of SNP variant and overexpression of *MDM2* has shown an independent impact on proliferation rate and survival [35, 73, 74] .

CDK4, also encoded by a gene on 12q13 region, is overexpressed in many cases of MCL [35, 70, 74]. In one study, all cases with overexpression and copy number alteration of the gene, were blastoid MCL and altered CDK4 expression was more frequently found in the presence of an inactivated *TP53* gene, and associated with poorer survival, suggesting a role in the pathogenesis of highly proliferative MCL [35].

Additionally, overexpression of Rb1 and low expression of E2F, was reported by Kienle et al.[73]. Overexpression of BMI1, a negative regulator of transcription of the INK4a/ARF locus was reported in a subset of MCL samples, although no relation to expression of p16/p19 could be detected [33].

Impact on cell survival and apoptotic signal pathways

Martinez et al. reported a 446-gene signature of homogeneously altered expression of genes in a study of 38 MCL cases. 137 of these genes could be associated with survival and by merging these, two clusters were identified that discriminated two risk groups with good and poor outcome respectively. In this series of samples, 23 genes, involved in cell survival and apoptosis, were altered in more than 50% of the cases. For example, Bcl-2 was 5.8 fold higher in expression, which also was reported from other cohorts [67, 75]. The pathways involving the NF-kB complex and regulation of transcription of both cell survival and apoptotic factors have been found to be constitutively activated in MCL [76, 77]. Furthermore, genes involved in cell survival signaling via cell membrane receptors via PI3K/AKT pathway, NF-kB and TGFβ receptor pathways have been shown to be overexpressed [67, 69, 78].

DNA damage repair/cell cycle arrest

Downregulation of *CHEK1* and *CHEK2*, encoding checkpoint inhibitors (Chk1/2) was reported from cases with high proliferative MCL, in spite of wild-type *ATM/TP53* and *CHEK* genes [43, 44].

In summary, gene expression profiling studies have revealed that MCL exhibits distinct pattern of altered expression of genes that may have a significant role in development of the disease as well as a prognostic impact. Although several score systems have been proposed including gene expression profile or expression levels of individual genes to predict prognosis, none has been implemented in clinical routine.

Selected altered protein expression in MCL

SOX11

SOX11 is a neural transcription factor, which belongs to the family of SOX proteins, all containing a DNA binding domain (a HMG box), and is exclusively expressed during embryogenesis [79]. SOX11 has been found to be overexpressed in MCL, but not in other non-Hodgkin lymphomas, except for some types of very aggressive lymphoid malignancies like Burkitt lymphoma and acute T-and B cell lymphoblastic leukemia [22, 80]. An increased expression of SOX11 has also been described in several other solid tumors including neuroblastoma, glioblastoma, epithelial ovarian tumors and subgroups of (basal-like/receptor-negative) breast cancer [81-84].

The role of SOX11 in pathogenesis in MCL may seem somewhat contradictory, describing both oncogenic and tumor suppressive properties.

SOX11 has been shown to block differentiating of B cells by affinity to PAX5 and to promote angiogenesis through the platelet-derived growth factor A (PDGFA) pathway [85, 86]. In contrast, studies have shown that SOX11 may prevent MCL growth by repressing Wnt/β-catenin signaling, by deregulation of Rb-E2F and by affection of the transforming growth factor β (TGFβ) pathway[87, 88]. Knockdown of SOX11 has been associated with both increased cell proliferation as shown by Gustavsson et al. and with reduced tumor growth in a mouse model [85, 88].

The prognostic role of SOX11 has been explored in several studies. Nygren et al. analyzed an unselected population-based cohort of 186 patients diagnosed in Stockholm 1998-2010. In this study, SOX11-negativity (7.5% of cases) could be

associated with superior survival compared to SOX11-postivity. However, SOX11-negativity could not be used to define patients with indolent course, lower proliferation or non-nodal disease [89]. In another study which evaluated *IGHV* mutational status in a heterogeneously treated cohort of MCL patients, cases with high number of *IGHV* mutations and SOX11-negativity were associated with better prognosis. Furthermore, a significant correlation between overall survival and SOX11-expression was detected in uni- and multivariate analysis, adjusted for age, nodal disease and *IGHV* mutational status [90].

Another correlation was found between level of SOX11 expression and survival in a cohort of 112 young patients within the Nordic trials MCL2 and 3, treated with immunochemotherapy including rituximab, cytarabine and HD-ASCT. In this study, high expression of SOX11 by immunohistochemistry was associated with superior event-free survival and overall survival compared to negative/low/intermediate SOX11 expression. Furthermore, blastoid morphology and high expression of p53 was more frequent in the low SOX11 expression group [91].

Cyclin D1

The hallmark of MCL is the t(11;14)(q13;q32) where the cyclin D1 gene *CCND1* (11q13) is transposed and comes under regulation of the gene for immunoglobulin heavy chain (IGH) (14q32) and becomes constitutively overexpressed.

Cyclin D1 regulates cell cycle transition from G1 to S phase by binding to cyclin-dependent kinases 4 and 6 (CDK4/6), generating an active complex. The cyclin-CDK complex further inhibits Rb, thereby allowing the E2F to promote transcription of factors, necessary for G1-S transition (reviewed by Diehl) [92]. (Figure 2)

Cyclin D1 has also been associated with other cellular functions, by its affinity for transcription factors, chromatin-remodeling enzymes and epigenetic modifiers and has been shown to be involved in response to DNA damage [93, 94].

Overexpression of cyclin D1 has been described in several other malignancies, either caused by gene transposition of amplification (reviewed by Casimiro et al.)[95].

Transcriptome analyses of mRNA have revealed that upregulation of *CCND1*, is related to proliferation rate in MCL as well as upregulation of other genes of the proliferation signature and correlated with survival. However, when adjusted for these genes, the impact of *CCND1* is lost, indicating that overexpression of *CCND1* does not have an independent role of inducing MCL or affect prognosis [68].

Shorter isoforms of the untranslated region, 3'UTR, of the *CCND1* gene, without the normal function of destabilizing elements during transcription, have been detected in MCL and related to higher levels of mRNA, higher protein levels of CD1, higher proliferation index and shorter survival [68, 73, 96].

Although the genetic hallmark of MCL is the t(11;14)(q13;q32), additional genetic alterations are required for the malignant transformation into MCL, as shown by detection of cells carrying t(11;14)(q13;q32) peripheral blood from healthy individuals[97]. Furthermore, cells with cyclin D1 overexpression have been observed in the mantle zone, either incidentally in reactive lymph nodes, or simultaneously with other lymphomas, which is now referred as in situ MCL [1, 98].

The role of microenvironmental factors in MCL

In recent years, attention has been paid to the important role of microenvironment of malignant cells, including stromal cells, adhesion molecules, T cells and different cytokines and chemokines. In MCL, the constitutively active BCR as well as overexpression of BCR-associated kinases, Syk and Bruton Tyrosine kinase (BTK), together indicate that external activation of the BCR is relevant in disease progression [99, 100].

High levels of macrophages in tissue has been found in MCL and correlated to blastoid morphology [14, 101]. Furthermore, Kurtova et al. has shown high expression of the chemokine receptors CXCR4 and CXCR5, as well as high affinity for bone marrow mesenchymal stromal cells (BMSC) in MCL cells which was associated with inhibition of the cytotoxic effects of the drugs fludarabine and cyclophosphamide on MCL cells adhesive to stromal cells [102].

Prognostic factors

A prognostic factor is a parameter associated with prognosis of the disease. It may be patient-related, such as age, sex, comorbidity, or related to disease presentation and the biological characteristics of the malignant cell or tumor. Prognostic factors can be used to evaluate outcome and may serve as a tool for selecting treatment strategy.

Ki-67

In MCL, the most established biological prognostic factor is the proliferation marker Ki-67, which is recommended for use in routine diagnostics. [25, 103]. Ki-67 denotes a protein that is expressed during all stages of cell cycle, expect for resting or senescent cells and is evaluated by immunohistochemistry as percentage of Ki-67 % -positive cells (reviewed by Scholzen)[104].

High Ki-67 (%) has been shown to be associated with inferior survival in population-based data as well as within clinical trials. [105-107]

Blastoid MCL

Blastoid variant was initially showed to be associated with inferior survival in univariate analysis but lost impact when adjusted for proliferation markers like Ki-67% or mitotic activity as used historically. [14, 105, 108]. Similar results were achieved in a study based on two randomized trials (MCL Younger and MCL-Elderly), including treatment with rituximab and, for MCL young, cytarabine and HD-ASCT [107]. MCL with blastoid or pleomorphic morphology were associated with higher Ki67% and higher score of the mantle cell lymphoma international prognostic index (MIPI), compared to non-blastoid cases. 5-year rates of progression-free survival (PFS) and overall survival (OS) were significantly lower in blastoid cases even after adjustment for MIPI but not after adjustment for high Ki-67%.

A retrospective analysis on patients treated with bendamustine at any relapse also demonstrated a significant correlation between blastoid morphology and inferior

outcome although multivariate analysis did not include proliferation marker or MIPI [109]. Furthermore, blastoid morphology was associated with lower response rate and shorter PFS and OS after treatment with ibrutinib in relapsed/refractory patients even after adjustment of other prognostic factors such as age, ECOG performance status, simplified MIPI and number of prior lines of treatment, although this multivariate analysis did not include proliferation rate of the tumor [110].

MIPI-mantle cell lymphoma international prognostic index

The MCL-International Prognostic index is a prognostic score, based on four clinical parameters with independent prognostic value; age, performance status (PS), normalized lactate dehydrogenase (LDH) level and white blood count (WBC). MIPI was developed from data collected from 455 patients with advanced stage disease, treated within three different randomized trials with chemotherapy with and without anti-CD20-antibody rituximab respectively [111]. Out of this work, MIPI discriminated patients into three risk groups (high risk, HR; intermediate risk, IMR; low risk, LR) in relation to overall survival. The prognostic value of MIPI has been validated after the introduction of anti-CD20 targeted therapy with rituximab and, for young patients, frontline high-dose chemotherapy with autologous stem cell support, both within clinical trials as well as in population-based studies [112-115].

Ki-67% was integrated into the index, initially as MIPI-b, defining two risk groups [111]. To improve the value, MIPI-c was introduced in 2015, based on one cut-off level of Ki-67% (30%), and discriminated four risk groups in relation to overall survival [107, 116].

Molecular prognostic factors

Molecular analysis of the tumor cells have revealed several characteristics that are associated with inferior outcome, of which mutations of/deletion of *TP53* and deletion of *CDKN2A* can be regarded as most important so far.

Based on population-based data, mutations in *TP53* was associated with inferior survival from the time period before introduction of rituximab as well as in patients treated with anti-CD20-antibody in combination with conventional chemotherapy [117, 118].

Two trials on patients treated with intensified regimens including rituximab, cytarabine and high-dose chemotherapy with autologous stem cell support (HD-ASCT), the European MCL Younger Trial and the Nordic Lymphoma Group MCL2 trial, showed that loss of p53 function and deletion of *CDKN2A*, was related to dismal outcome, irrespectively of MIPI risk group or Ki-67%. The worst prognosis

in both these studies was observed in patients with MCL harboring both deletions [31, 32].

In the Nordic study, *TP53* mutations defined a subgroup of patients with very poor outcome, with median OS of 1.8 years and median PFS 0.2 years compared to overall survival not reached and median PFS 10.6 years in non-mutated cases. Mutations of *TP53* was the only genetic aberration that retained its significant prognostic impact on survival when adjusted for MIPI-c, Ki-67% and blastoid variant [32].

In both these trials, several other genetic aberrations were found to be associated with impaired outcome in univariate analysis but lost its significance in multivariate analysis, why there is no evidence of an independent prognostic value.

Minimal residual disease

Minimal residual disease (MRD) refers to the amount of cancer cells that remain after a given treatment. It can be shown by detection of a tumor-specific immunophenotype or sequences of DNA/ RNA by flow cytometry or by RT-PCR. Assessment of MRD is performed on peripheral blood and/or bone marrow aspirates and has become a useful tool in hematological malignancies for response evaluation, for early detection of relapse and for treatment stratification.

In MCL, the standard marker for assessment of MRD is detection of clonal rearrangement of IGH gene by RQ-PCR. Another useful marker is detection of t(11;14) but is limited by a detection in < 50% of patients [119-121].

The prognostic value of MRD has been demonstrated on cohorts of both young and elderly patients. In young patients receiving HD-ASCT, MRD negativity pre-ASCT as well as post-ASCT have been associated with significantly longer PFS and OS in three large European trials, MCL Younger, MCL2 and MCL3 including induction regimens R-CHOP or R-CHOP/R-DHAP and R-CHOP/HD-cytarabine respectively[122-124].

In the European MCL Elderly trial, MRD negativity after induction was associated with prolonged response duration irrespectively of induction with R-CHOP or R-FC, with 77% of MRD-negative were in remission after two years vs 34% of MRD-positive patients. Furthermore, sustained or achieved MRD-negativity during the first year after induction was a strong predictor of higher complete remission rate after 2 years, 76% compared to 36 % in MRD positive patients [125].

Treatment of mantle cell lymphoma

Most patients with MCL receive systemic treatment at diagnosis due to symptoms and/or aggressive course of the disease and principles for management of these patients are presented in separate sections. Two subgroups of MCL show somewhat different clinical course and may be treated alternatively; limited stage disease and indolent or non-symptomatic disease.

Indolent MCL

As described previously, a small group of MCL cases is characterized by indolent course, presenting with non-nodal disease and detection of MCL in peripheral blood and bone marrow, i.e. as leukemic presentation, referred as indolent MCL (iMCL).

These patients do not always need therapy and can be followed by regular observation, referred to as "watch and wait" approach. A small fraction of these patients may later develop symptoms and require treatment. This group of patients show good prognosis with reported median OS of more than 5 years and, in some patients, up to 10 years [16, 17, 71, 126, 127]. After initiation of treatment, even with delayed treatment approach, these patients seem to have at least the same prognosis as when treatment is initiated at diagnosis.

Orchard et al. reported a comparison between patients with nodal and non-nodal disease based on 80 MCL cases with detection of t(11;14) in PB. In the non-nodal group of 18 patients, nine patients did not receive any treatment and nine patients received treatment after mean time to treatment of 29 months. Median overall survival was superior in the non-nodal group, 79 months compared to 30 months in the nodal group [16]. Further, a retrospective study on MCL cases 2000-2010 selected eight patients (3%) of leukemic MCL, of which six patients did never require treatment and were all alive at follow-up. In two cases, treatment was initiated, due [17].

Similar findings were made by Martin et al., who identified 97 patients where treatment were deferred more than 3 months and compared outcome with patients who started treatment within the first months after diagnosis and found that OS was superior in the group at diagnosis and when comparing outcome after time from start of treatment, OS did not differ between the groups, In this study, MIPI was not

predictable of need of treatment nor prognosis, indicating that it is of minor use for these patients, possibly due to with the fact that patients with leukemic MCL typically present with elevated WBC.

Tabel I.
Selected retrospective studies on outcome of derred therapy in MCL

Author	patients	n	com-ment	FU time	treatm required (%)	cause of treatm	time to treatm	overall survival
Orchard et al. [16]	MCL with t(11;14) in PB , non-nodal disease and no treatment at diagnosis	18 of 80		m 58 (8-175) m	9 (50%)	NR	m 29 (8-175) m	m OS 79 (22-136) all non-nodal cases
Ondrejka et al.[17]	non-nodal/ leukemic MCL 2000-2010	8		27 (5-109) m	2 (25%)	elevated WBC/B symptom	m 28 (26-30) m	7/8 alive at FU
Martin et al.[127]	deferred treatment at diagnosis 1997-2007 and no treat-ment < 3 months after diagnosis	31 of 181		md 55 m	28 (90%)	NR	md 12 (4-128) m	md not reached
Fernandez et al.[71]	leukemic non-nodal MCL 1994-2005, no treatment < 2 years after diagnosis	12	2 in situ MCL	md 6 (3-10)y	2 (17%)	Spleno-megaly/ progr to nodal disease	m 6 (5-7) y	NR
Nygren et al.[89]	MCL 1998-2010, no treatment < 2 years after diagnosis	17 of 186	3 leuk-emic MCL	28 (0-148)m *	NR	NR	NR	md 5.9 yrs

M=mean, md=median, NR=not reported

Molecular studies have shown that iMCL can be associated with non-complex karyotypes and hyper-mutated IGHV genes. By gene-expression profiling, iMCL may be distinguished from conventional disease by a signature of 13 genes, all overexpressed in classic MCL but not in iMCL, of which one was *SOX11* [71]. Expression of SOX11was then suggested as a specific marker for determination of iMCL, but as SOX11 negativity has shown correlation with both indolent course as well as with inferior outcome, it cannot be recommended as single tool for treatment stratification [71, 89]. Recently, expression of CD200 was shown to be associated

with non-nodal leukemic presentation of the disease, and follow-up studies may confirm its role. [128].

Local treatment in limited stage disease

Retrospective analysis of patients treated with radiotherapy (RT) have shown that RT is able to induce complete remission (CR) in patients with non-bulky limited stage disease (I-II) and may have additional affect when added to chemotherapy [129, 130]. These studies were performed in patients diagnosed 1984-2000 and 1990-2007 respectively, thus in the pre-rituximab era, why the need of consolidative RT should be confirmed with modern treatment.

Moreover, the same studies have shown that chemotherapy (CT) + RT in stage I-II is associated with long time survival with 5-y-OS 70% and median OS 6.4 respectively in the group of patients treated with a curative intent. A similar pattern was recently reported from a retrospective database cohort of patients diagnosed 1998-2012 by showing CT+RT to be superior to RT or CT alone with 3-y-OS 79.8 % for the combined treatment modality group [131]. Unfortunately, the study could not include data on rituximab or specific regimens.

Current guidelines suggest careful staging procedure, by examination with PET/CT to confirm limited stage and treatment with a shortened chemotherapy followed by consolidation with involved field radiotherapy, 30-36 Gy [18].

Systemic therapy

All patients with advanced stage disease with aggressive course and/or symptoms are treated with systemic therapy. Today, this consists of chemotherapy in combination with an anti-CD20-antibody (immunochemotherapy). Novel agents that not belong to the group of “conventional” chemotherapeutic agents including immune modulators and small molecule inhibitors are discussed separately. The choice of therapy depends highly on age and the physical status of the patients as well as if MCL is newly diagnosed or relapsed. Consequently, the different strategies of systemic therapy is discussed according to these clinical situations.

In treatment of hematological malignant disease, induction refers to first-line treatment given over a pre-decided time period with the intent of eradicate as much as possible of the tumor/leukemia burden.

To eradicate eventual remaining malignant cells and to prevent relapse, the induction may be reinforced by addition of high-dose chemotherapy with autologous stem cell support, which intends to consolidate the remission of the

disease. HD-ASCT is associated with higher toxicity profile and limited to young (mostly <65) and fit patients.

The induction and consolidation may be followed by a maintenance phase, where treatment is given continuously over a longer time period.

Treatment with anti-CD20 antibodies

The introduction of treatment with a monoclonal antibody against tumor-associated targets in treatment of malignancies and auto-immune disease has markedly increased disease control and survival in these patients. In B cell lymphoma, the first monoclonal anti CD20 antibody, later known as rituximab, was introduced in early 1990s by Reff et al [132].

Mechanism of action

CD20 is a transmembrane protein expressed on pre-B cells, mature B cells but not on plasma cells [133, 134]. The exact role of CD20 has not been fully understood but it is involved in calcium influx into the cell and possibly in cell cycle regulation [135, 136].

Targeting CD20 by anti-CD20 monoclonal antibodies (anti-CD20 mAb) is associated with several pharmacodynamics effects including direct cell death via apoptosis, complement-dependent cytotoxicity (CDC) via binding to C1q and antibody-dependent cellular cytotoxicity (ADCC) via binding to Fc receptors (FcR) on different effector cells including NK cells, macrophages, neutrophils and dendritic cells[132, 137-140].

NK-cell mediated ADCC

ADCC, mediated via Fc-FcR interaction on NK cells is thought to be of major importance of the response to antibody-coated target cells [141]. Several isoforms of FcR are described, expressed on different types of cells with both activating and inhibitory functions. NK cells express mainly the activating FcRIIIγ (also known as CD16), and upon binding to the Fc portion of the immunoglobulin, NK cells are activated by increased Ca^{2+} flow into the cell, followed by release of granzymes, perforins and other cytolytic enzymes, like IFNγ, which results in lysis of the target cell [142]. Furthermore, several intracellular pathways involved in proliferation and differentiation are activated [143].

The importance of FcγRIII is exemplified by the observed various response to rituximab by polymorphism of FcγRIII, as discussed below [140, 144, 145].

ADCC is enhanced by IL-2, as shown by increased proliferation and activation of NK cells and by increased effect on tumor cells by addition of IL-2 to anti-CD20 ab *in vitro* [146, 147].

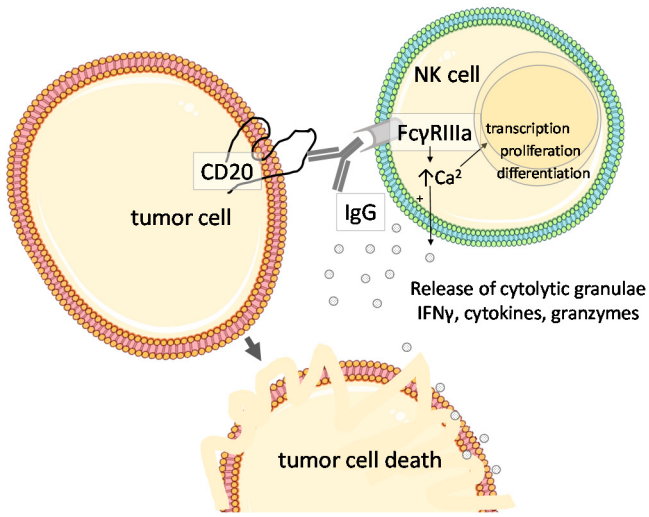


Figure 3. Principle of NK-cell mediated cell death via binding to FcαRIIIa (p16)
NK cell are activated by binding of Fc-portion of IgG to FcR which causes release of IFnγ, cytkines and granzyme into a cytolytic synapse. Upon NK cell activation, intracellular activation of transpriction factors promote differentiation and proliferation of NK cells. Empty cell models adapted from Servier Medical Art, creative commons, https://smart.servier.com/smart_image/cell/, and modified by addition of all other details by author.

Rituximab

Rituximab, the first introduced anti-CD20 mAb, is a chimeric anti-CD20 antibody that consists of human IgG1-kappa constant regions and variable regions from a murine monoclonal anti-CD20 antibody [132].Binding of rituximab induces aggregation of the CD20 molecule and translocation into lipid grafts [148].

The first results on humans was presented in 1994 by Maloney et al. from a phase I trial on R/R low grade lymphomas[149]. During the following decade, rituximab has been approved for all CD20-positive lymphomas and has improved disease control and survival rates in several B cell lymphomas, either as single therapy or in combination with chemotherapy and constitutes a backbone in treatment of CD20 positive lymphomas as reviewed by Engelhard [150].

Three randomized trials have investigated the addition of rituximab to conventional chemotherapy in MCL. Forstpointner et al. compared R-FCM (fludarabine, cyclophosphamide, mitoxantrone) with FCM on relapsed/refractory MCL and reported higher CRR (29% vs 0%) and higher 2-y-OS (90% vs 70%) in the R-FCM group [151].

In untreated MCL patients, R-CHOP was superior to CHOP in terms of higher CRR (34% vs 7%) and median time to treatment failure (TTF) (21 months vs 14 months), although no significant difference could be shown in PFS or OS[152].

Another randomized trial on untreated MCL reported higher ORR and OS by R-MCP (mitoxantron, chlorambucile, prednisone) compared to MCP [153] [154]. R-FC (fludarabine, cyclophosphamide) vs FC showed initially similar response rates but higher median PFS (29.8 vs 14.9 months) and OS at long-term FU (44.5 vs 37.0 months) in the group receiving rituximab [155].

Rituximab as monotherapy has shown potency to eradicate molecular relapse in patients previously treated with rituximab and chemotherapy [156-159].

Resistance to rituximab

Clinically, resistance to rituximab refers to refractory disease or early relapse (≤6 months) after rituximab treatment. Several mechanisms have been suggested as responsible for reduced sensitivity, including lower affinity to rituximab by FcγIIIR polymorphism, downregulation of CD20, loss of CD20 expression in subpopulations and trogocytosis or “shaving”, which can be described as capture and removal of CD20 from the cell surface [144, 160-163].

Second generation anti-CD20 antibodies

To improve efficacy of anti-CD20 mAb and to overcome resistance to rituximab, second generation antibodies have been developed. Anti-CD20 mAbs are now divided in two groups based on structural and CD20-binding properties. Rituximab is a type I antibody, characterized by aggregation of CD20 in lipid grafts and strong c1q binding.

Obinutuzumab

Obinutuzumab (Ga101) is a type II antibody, characterized by higher direct cell death/caspase-dependent apoptosis and higher ADCC, by higher affinity to the FcγIIIa receptor [164, 165].

The GAUGAIN trial investigated obinutuzumab (O) in patients with R/R aggressive B cell lymphoma. In the group of DLBCL + MCL, 63% were refractory to rituximab and of these, 16% (4 pts) responded to OBZ and 2 of 15 MCL patients achived CR/CRu [166]. The phase I trial OASIS trial (NCT02558816) is currently investigating obinutuzumab in combination with ibrutinib and in a second step with further addition of venetoclax [167].

Other anti-CD20 antibodies

Ofatumumab, a type I antibody with activity in CLL was evaluated in a phase II trial on 12 R/R MCL patients but was associated with partial remission only in one patient (8%)[168].

Ublituximab (TG-1101) is another type I antibody, with a capacity of inducing ADCC at lower concentrations and at low levels of CD20 expressions in CLL *in vitro* [169]. Ublituximab has been evaluated as single agent in 35 R/R NHL, of which 4 of 5 MCL patients achieved stable disease (SD) [170]. Ublituximab is currently explored in different combinations with the PI3K inhibitor umbralisib (U2), with and without ibrutinib or bendamustine (NCT02006485) and with lenalidomide (NCT02013128). So far, efficacy data is not yet reported, but the combination has shown acceptable tolerability from U2 with ibrutinib or bendamustine from preliminary analysis [171].

Primary treatment of young patients

Although several models have been explored and outcome has radically improved during the last two decades, a golden standard for primary treatment in young patients has not been defined. In the update on consensus guide lines from 2017, young fit patients with newly diagnosed MCL are recommended frontline intensified immunochemotherapy including cytarabine and rituximab followed by consolidative chemotherapy with autologous stem cell support. (HD-ASCT) [18]. A summary of selected trials are shown in table II.

What is the optimal induction in young patients?

Initially, MCL was treated similar as low grade lymphomas with agents or regimens like chlorambucile, CVP or CHOP. However, the response was not as good/remarkable as in low grade lymphoma or as in other aggressive B cell lymphomas such as diffuse large B cell lymphoma (DLBCL)[172 , 173-175]. During the last two decades, the integration of additional agents like cytarabine, methotrexate and cisplatin has shown improved outcome in MCL patients, besides the addition of rituximab and HD-ASCT.

In 2002, LeFrère et al. showed a distinct increase of complete remission rate (84%), and 3-y-OS (90%) by sequential addition of DHAP (cytarabine, cisplatin, prednisone) after CHOP prior to HD-ASCT [176]. This model was further evaluated in two French single arm trials, the GELA trial (CHOP x 3+R-DHAP x 3)and the LYSA trial (R-DHAP x 4 (+ R-CHOP x 4 if limited response) which demonstrated outcome of 4 year PFS and OS higher than 65% and 75% respectively [177, 178] (Table II). The randomized European MCL Younger trial, compared R-CHOP with alternating R-CHOP/R-DHAP and could finally confirm a superior efficacy of the addition of R-DHAP. [122, 179].

Hyper CVAD (fractionated cyclophosphamide, vincristine, doxorubicin, and dexamethasone altered with high dose cytarabine and methotrexate) without preceding to HD-ASCT showed high response rates in initial and subsequent trials but significant treatment-related toxicity, including high grade infections and new primary malignancies like MDS and AML [180-182]. A reduction of methotrexate

and cytarabine and etoposide as part of induction reduced toxicity with maintained response rate [183].

The benefit of adding cytarabine and rituximab to CHOP prior to ASCT in young patients can also be observed by an overview of the three Nordic Lymphoma group trials (MCL1-3). In the first trial (MCL1) evaluating maxi-CHOP followed by HD-ASCT, 11 of 41 (27%) patients achieved CR pre-transplant. Of these, 45% had failed at a median FU of 2.8 years and 4-y-OS of all patients was 51% [184]. The subsequent trial, MCL2, investigated alternating maxi-CHOP and high-dose cytarabine in combination with rituximab in 160 patients with significantly superior outcome with 3-year FFS and OS of 68% and 85% respectively compared to MCL1 (24% and 60% respectively) [185]. The Nordic MCL3 trial followed the same design as MCL2 but added Y^{90}-ibritumomab tiuxetan (zevalin) to patients not in CR pre-ASCT, which could not be associated with improved outcome compared to outcome in patients within MCL2. Both MCL2and MCL3 have showed comparable outcome with the other trials including cytarabine and rituximab in the pre-ASCT regimen with 4 years PFS and OS (MCL2/MCL3)73%/62% and OS 81%/78%,) [113, 123, 186].

Table II.
Selected trials oninduction regimens to young patients with untretaed MCL

phase III, randomized trials							
Author	**induction**	**n**	**ORR (CRR) pre-ASCT (%)**	**ORR (CRR) post-ASCT (%)**	**FU time**	**TTF/PFS**	**survival**
Hermine et al.[122]	R-CHOP/DHAP x 6 + ASCT	466	94 (55)	98 (83)	6.1 y	md PFS 9.1y	5-y 76%
	vs R-CHOP x 6 + ASCT		90 (39)	97 (76)		md PFS 4.3y	5-y 69%
Le Gouill et al.[178]	R-DHAP x 4 + ASCT+ maint rituximab	299	98 (86)	100 (94)	50 m after rand	4-y PFS 83%	4-y 89%
	vs R-DHAP x 4 + ASCT + observation		-	100 (92)		4-y PFS 64%	4-y 80%
phase III, randomized trials							
Author	**induction**	**n**	**ORR (CRR) pre-ASCT (%)**	**ORR (CRR) post-ASCT (%)**	**FU time**	**TTF/PFS**	**survival**
Damon et al.[183]	R-mtx-CHOP x 2 + EAR+ ASCT	78	45 (18)*	88 (69)	4.7 y	5-y PFS 56%	5-y 64%
Van t´Veer et al.[187]	R-CHOP x 3 + ara-C + ASCT	88	76(15)	70(64)	3.5 y	4-y PFS 44%	4-y 66%
Andersen et al.[159, 184]	maxi-CHOP x 4 + HD-ASCT	41	CR 27	31 (78)	2.8 y	3-y FFS 24%	3-y 60%
LeFrère et al.[176]	CHOP x 4 + DHAP x 3 (non-CR) + ASCT	28	92 (84)*	(86)/ (96)	37 m	3-y EFS 83%	3-y 90%
Delarue et al.[177]	R-CHOP x 3+ R-DHAP x 3 + ASCT	60	87 (57)	82 (78)	5.6 y	md PFS 7 y	5-y 75%
Eskelund et al. [46]	R-maxi-CHOP/ HD-ara-C x 6 + ASCT	160	96 (54)	96 (90)	11.4 y	md 8.5 y	md 12.7y
Kolstad et al. [123, 186]	R-maxi-CHOP/ HD-ara-C x 6+ zevalin- ASCT	160	97 (51)	90 (82)	4.4y	4-y 62%	4-y 78%
Romaguera et al. [180]	R-hyper-CVAD/ HD-mtx+ara-C x 6-8	97	97 (87)	-	40 m	3-y FFS 62%	3-y 82%
Merli et al. [181]	R-hyper-CVAD/ HD-mtx+ara-C x 4	63	83 /72)	-	46 m	5-y PFS 61%	5-y 73%
Bernstein et al.[182]	R-hyper-CVAD/ HD-mtx+ara-C x 6-8	49	86 (55)	-	4.8.y	md PFS 4.8 y	m 6.8 y

md=median

High-dose chemotherapy with autologous stem cell support

The support for HD-ASCT in MCL

To improve outcome in MCL patients, intensified approach including consolidation with high dose chemotherapy and total body irradiation with autologous stem cell support was introduced in MCL in the late 1990s.

Initial studies, including both single arm trials and retrospective register-based analysis, showed that the addition of an intensified approach including HD-ASCT increased complete remission rate, response duration and progression free survival in untreated MCL patients [184, 188-195]. Notably, some of the early studies were made before the introduction of rituximab and lacked a matched control group. Nevertheless, a pooled analysis including patients treated with rituximab supported a benefit in terms of superior OS in the subgroup receiving both rituximab and HD-ASCT [189, 195].

One randomized trial has evaluated ASCT in MCL, the European Young, by the European MCL Network, comparing HD-ASCT (with TBI) with two additional courses of chemotherapy and maintenance IFN-α after induction (predominantly (R)-CHOP) in 232 patients). At median FU 6.1 years, the experimental arm, receiving ASCT demonstrated a higher median PFS (39 vs 17 months) and OS (7.5 vs 5.4 months) in the group treated with HD-ASCT, why ASCT was incorporated in guidelines in 2012 [189, 196].

What is the optimal consolidation?

In MCL, there is no consensus of whether total body irradiation (TBI) should be a part of consolidation. An analysis of more than 400 patients treated with HD-ASCT 2000-2007, recorded from the European Society of Bone Marrow Transplant (EBMT) registry, compared HD-ASCT patients receiving TBI (37%) with non-TBI (of which >90% received BEAM/BEAC) and demonstrated a benefit from TBI in partial responders after induction but not in patients in complete remission.[197].

Similar results were achieved by analysis of combined data from three European trials that included both cytarabine and rituximab followed by HD-ASCT, either with TBI + melphalan (EU MCL Younger) or with non-TBI (BEAM/BEAC) (Nordic MCL2 and HOVON-45). Among patients in CR pre-ASCT, PFS did not differ between the groups but in patients in PR pre-ASCT, PFS was higher in the TBI-group compared to the non-TBI group [198].

Other efforts to improve consolidation includes radioimmunotherapy (RIT). Zevalin was explored after induction with R-CHOP by Eastern Cooperative Oncology Study Group and after R-Maxi-CHOP/R-HD cytarabine in patients with partial response in the Nordic MCL3 trial respectively but did not show any additive effect on survival [123, 199].

Similarly, the addition of rituximab to the conditioning regimen was explored by Gianni et al with OS 89% and EFS 79% at 54 months follow-up, but due to lack of inter-study comparison this is not included in consensus guidelines [18, 200].

Remission status prior to ASCT is prognostic

A recurrent finding from studies of HD-ASCT is that outcome is highly related to remission status prior to HD-ASCT, irrespectively of induction regimen and the use of rituximab. Patients in complete remission after induction (pre-HD-ASCT) show significant higher response duration and PFS compared to partial responders (PR) as shown by subgroup and/or multivariate analysis on data from homogenously treated cohorts as in clinical trials as well as from register-based studies [122, 184, 196, 197, 201].

Maintenance with rituximab after ASCT

The LyMA phase III trial randomized patients between two years of maintenance rituximab or watchful waiting after HD-ASCT. At median FU 50 months after randomization outcome in the experimental arm, receiving maintenance rituximab was associated with higher 4-years-event-free survival (79% vs 61%), PFS (83% vs 64%) and OS (89% vs 80%) compared to the control arm [178].

In summary, by the intensified approach in young fit patients with newly diagnosed MCL, more than 70% of patients survive five years or longer which is a remarkable improvement compared to twenty years ago and some patients may be cured. However, long-term follow-up shows no plateau curve and late relapses do occur, as exemplified in the prolonged update of the Nordic MCL2+3 trial [46]

Primary treatment of elderly patients

The majority of patients with MCL is older than 65 and do not qualify for an intensified treatment approach. During the last twenty years, different compounds have successfully been introduced for this patient group, including rituximab, as described in the previous chapter.

Induction with immunochemotherapy

Historically, several different approaches have been applied for treatment of elderly patients with MCL. An overview of selected regimens discussed below are shown in table III.

R-CHOP is a commonly used regimen, based on improved survival by the addition of rituximab and a trend of higher response compared to CVP [152, 175, 202].

Fludarabine and cyclophosphamide (FC) showed high response rates including 70% CRR in a single arm trial including both untreated and R/R MCL [203] and in a randomized trial comparing addition of rituximab to FCM (FC + mitoxantrone) in R/R FL and MCL, Forstpointier et al showed better outcome with R-FCM (CRR 29% vs 0%) in MCL patients. [151]. Consequently, R-FC was compared with R-CHOP in a large European randomized trial, European MCL Elderly on 560 patients with untreated MCL. However, R-FC was associated with inferior overall survival compared to R-CHOP (4-y-OS 47% vs 62%) and higher rate of persisting cytopenia and treatment-related deaths, were observed in the R-FC group [204].

Bendamustine, an alkylating agent, originally developed in the former German Democratic Republic, was re-introduced in early 2000 and approved for NHL in 2010 in Europe, as reviewed by Cheson [205]. Bendamustine and rituximab (BR) showed CRR of 50% in R/R MCL, including patients previously treated with R-CHOP [206]. Two randomized trials on indolent NHL (including MCL) have compared R-CHOP with BR; the German STIL trial and the American BRIGHT trial (BR vs R-CHOP/R-CVP) [202, 207]. Both trials demonstrated significant higher PFS and higher CRR in the MCL subgroup receiving BR at initial follow-up but no significant difference in overall survival has been detected at long term follow-up [208, 209]. A favorable toxicity profile of BR with less infections,

neuropathy and no alopecia in combination with a non-inferior outcome has made BR a first-line treatment option in consensus guidelines [18]. Recently, trials have reported benefit from expanding BR by addition of cytarabine, bortezomib or ibrutinib, which is discussed in a later section.

Similarly, the addition of bortezomib to R-CHOP without vincristine (VR-CAP) has been compared with R-CHOP (described in following chapter) with higher PFS in the VR-CAP group, albeit less preferable toxicity profile [210].

Table III.
Selected trials on primary treatment in patients not eligible for HD-ASCT

Phase III, randomized trials						
Author	**regimen**	**n**	**ORR % (CRR%)**	**FU time**	**TTF/PFS**	**survival**
Lenz et al.[152]	R-CHOP vs CHOP	122	94 (34) 75 (/)	18 m	md TTF 21m md TTF 14m	2-y OS 76% 2-y OS 76%
Kluin-Nelemann et al. **Hoster et al.** **[204, 211]**	R-FC vs R-CHOP	532	78 (40) 86 (34)	6.1 y	md FFS 31m md FFS 31m	5-y OS 42 % 5-y OS 58 %
	maintenance rituximab	274			5-y PFS 53%	4-y OS 79%
	vs IFNα				5-y PFS 23%	4-y OS 67%
	R-CHOP m rituximab				5-y PFS 51%	5-y OS 79%
	R-CHOP m IFNα				5-y PFS 22%	5-y-OS 59%
Rummel et al.[207, 209]	R-B vs R-CHOP	549 95MCL	NR in MCL	45 m	35 (29-55) 22 (15-34)	md OS 80 m*
Flinn et al.[202, 208]	R-B vs R-CHOP/ R-CVP	447 (74MCL)	94 (50) 85 (27)	5 y	md PFS 40 m md PFS 14m	5-y OS 82% 5-y OS 85%
Phase II, single arm trials						
Author	**regimen**	**n**	**ORR % (CRR%)**	**FU time**	**TTF/PFS**	**survival**
Gressin et al.[212]	RiBVD	74	80 (74)	52 m	2-y-PFS 70%	4-y-OS 87% MRD- 29% MRD+
Visco et al.[213]	R-BAC 500	57	-	35 m	2-y-PFS 81%	2-y-OS 86%
Ruan et al.[214, 215]	R+lenalidomide	36	87 (61)	58 m	4-y-PFS 70	4-y-OS 83%

md=median, NR=not reported

Maintenance with rituximab

Maintenance rituximab was also explored in the European MCL Elderly trial. A second randomization was performed after induction between maintenance treatment with rituximab and IFNα. At a median FU of 36 months, response duration was significantly improved by maintenance with rituximab compared to IFNα in all patients (median RD 26 vs 7 months). Furthermore, a significant benefit of maintenance rituximab by increased OS was observed in the R-CHOP arm but not in the FC-arm.

In 2016, Rummel et al. reported data from a randomized trial evaluating maintenance rituximab after BR but no significant improvement in PFS or OS could be demonstrated compared to BR without maintenance treatment [216]. Consequently, current guidelines recommend maintenance with rituximab only after R-CHOP [18].

Salvage treatment

Treatment of the relapsed or refractory patients is a highly relevant task in MCL since the majority of patients will relapse sooner or later, even after consolidation with HD-ASCT.

Current European guidelines basically include three recommendations: 1) consider a clinical trial, 2) include treatment with a non- cross-resistant agent or 3) aim for allogenic transplant in responding young fit patients. Furthermore, targeted therapy should be considered, of which ibrutinib have shown highest response rate so far [18].

Immunochemotherapy and novel agents

There are few randomized trials (RCT) performed on salvage therapy in MCL. A systematic review by Parrott et al. defined seven RCT between 1994 and 2016, all with different interventions and comparators [217]. Several of the trials were designed for indolent/non-aggressive NHL, and MCL constituted a small subgroup of the cohorts. Although inclusion criteria and patients characteristics vary between the trials, one can make following observations: longest median PFS and highest ORR were observed with BR (vs R-fludarabine), R-FCM (vs FCM), bortezomib (V) –CHOP (vs CHOP) and ibrutinib (vs temsirolimus), roughly varying between 14-18 months and 60-80 months respectively. The highest CRR was observed by BR and bortezomib-CHOP (V-CHOP) (35-38%). V-CHOP has not been taken further due to a more favorable toxicity profile by R-CHOP.

Two different novel agents were included in the review on RCT by Parrott et al, temsirolimus and ibrutinib. Although temsirolimus at high-dose was superior to investigator´s choice in an earlier trial, ibrutinib showed a clearly higher efficacy than temsirolimus [218, 219]. Furthermore, data suggests that ibrutinib may be more effective when given earlier, such as after first relapse [110].

Maintenance with rituximab in relapsed MCL

Maintenance with rituximab in R/R MCL has shown to improve the proportion of patients in remission for more than two years after R-FCM or FCM [151]. As described in previous sections, maintenance with rituximab after R-CHOP has been associated with better outcome in untreated patients but has not been evaluated in any randomized trial on R/R MCL, why the role of maintenance rituximab in these patients needs to be confirmed.

Is there a role for allogenic stem cell transplant in MCL?

Reduced-intensity allogenic stem cell transplant (RIST) have been applied in patients who relapse after ASCT. According to a retrospective analysis of EBMT data on >300 patients with MCL who underwent RIST during 2000-2008 in Europe, the observed disease-free survival was 30% at a median FU 72 months. In multivariate analysis, both disease-free and overall survival, non-relapse-mortality (24% at 1 year) and cumulative incidence rates at 1 and 5 years (25% and 40%) were strongly correlated with chemo-sensitivity prior to transplant, indicating that only patients in response to salvage treatment should be considered for allogenic SCT [220].

Novel agents

Targeting B cell receptor signaling

The B cell receptor is composed of a transmembrane immunoglobulin (IgA, D, E, G or M) and a transmembrane dimer of CD79ab harboring the enzymatic "active sites". Upon antigen binding, several kinases and proteins are recruited and activated, including BTK and PI3K. By Ca2+-influx into the cell, BTK and PI3K promote downstream signaling by the MAPK, PI3K/AKT/mTOR, NFkB and NFAT pathways. All these pathways display regulatory functions on intra-nuclear transcription factors involved in cell cycle, apoptosis, proliferation, differentiation and migration of cells/normal B cells (reviewed by Young et al.)[221].

In several lymphoid malignancies, B cell receptor signaling has been found to be upregulated, either by increased signaling in all pathways as in antigen-driven chronic "active" signaling as in ABC-DLBCL and MCL or by "tonic" increased activity of the PI3K/AKT/mTOR pathway as in Burkitt lymphoma.

The oncogenic role in chronic active BCR signaling has led to development of inhibitors of components/agents in these cellular systems and in MCL, inhibition of BTK, mTOR and PI3K have shown clinical activity.

Inhibition of Bruton´s tyrosine kinase

Ibrutinib, the first approved BTK-inhibitor, is an irreversible inhibitor of BTK, by covalent binding to a cysteine residue on BTK. Ibrutinib was initially investigated as single treatment in relapsed/refractory MCL with ORR 68% and CRR 21% in 111 patients of which a majority were pretreated with rituximab [222]. In a subsequent trial where ibrutinib was combined with rituximab in R/R MCL, ORR was somewhat higher (88%), but not CRR (22%), indicating that the addition of ibrutinib to rituximab does not affect remission rate in patient with R/R disease after previously treatment with rituximab[223]. Upfront addition of ibrutinib to rituximab and conventional chemotherapy is currently investigated in several MCL trials. The SHINE trial (NCT 01776840) will soon report outcome of addition of ibrutinib to BR vs BR. Furthermore two randomized trials, currently recruiting patients, are the British ENRICH (Eudra-CT Number 2015-000832-13), comparing R-ibrutinib vs

R-chemo to untreated elderly patients and the 3-armed European TRIANGLE (EudraCT Number 2014-001363-12), which will evaluate if the addition of ibrutinib to induction and to maintenance rituximab respectively has potency of consolidate the remission comparable to HD-ASCT, and thereby, potentially replace it [224, 225].

Resistance to ibrutinib

Although efficacy of ibrutinib in MCL is high, primary or acquired resistance to ibrutinib is observed in a substantial part of patients. Furthermore, patients who relapse or progress on ibrutinib seem to do very poor, as shown by two retrospective studies showing a median overall survival after ibrutinib cessation of 2.9 and 8.4 months respectively [127, 226].

The mechanism behind primary resistance is not fully clear, but DNA sequencing has revealed a couple of mutations (*CREBBP, PIM, ERBB4 kinase*) differentially present in primary refractory patients compared to sensitive cases [227]. In one study on CLL patients, those with altered *TP53*, either by deletion 17p, deletion or mutation of *TP53*, showed lower sensitivity to ibrutinib. Moreover, presence of a specific missense mutation in BTK (C481) has been associated with loss of irreversible binding capacity of ibrutinib as a mechanism of secondary resistance [228].

Off-target effects

Another limitation in the clinical use of ibrutinib is off-target binding to cysteine residues on other kinases, like epidermal growth factor receptor (EGFR), IL-2-inducable tyrosine kinase (ITK) and other members of the TEC family kinases, causing unwanted side-effects. Among the most common drug-specific adverse effects are diarrhea and skin rash, which may occur as a consequence of EGFR-inhibition, just like observed after treatment with EGFR-inhibition in other malignant diseases [229-231].

Atrial fibrillation has been reported in a higher frequency after treatment with ibrutinib. Although the mechanism is not fully understood, it might be due to inhibition of a certain isoform of PI3K (p110-α) in cardiac myocytes, but other host-related factors are probably needed making the patient more susceptible.

Bleeding is another, potentially, life threatening side-effect, observed in patients on ibrutinib and is thought to be caused by impaired platelet adhesion and aggregation via BTK and TEC-kinase inhibition [232, 233].

Furthermore, ibrutinib has shown to affect the NK-cell mediated response, probably via affinity for ITK. ITK is a kinase expressed by T cells and NK cells. By ligation with TCR, ITK is activated and displays a functional role activating T cells by increased Ca2+-influx and activation of NFAT and RAS/RAF/ERK pathway. ITK-deficient mice failed to respond to TCR activation, as shown by less phosphorylation of downstream enzymes, less interleukin-2 (IL-2) production and less Ca-release [234].

An inhibitory function of ibrutinib on ITK was demonstrated with IC_{50} level at 10.7 nM ibrutinib [235]. Confirming studies showed that ibrutinib was bound to ITK at a rate of 40-80% in CLL patients on ibrutinib and by cellular models, ibrutinib caused inhibition of ITK activation [236].

Furthermore functional *in vitro* studies on CLL cell lines showed that ibrutinib interfered with the NK-cell mediated response to anti-CD20 ab, as shown by decreased degranulation of NK cells and decreased cell death [237]. Similarly, Kohrt et al. demonstrated negative impact on NK cell activation and ADCC on cell lines and xenotransplant lymphoma mouse models [238].

Consequently, more selective BTK-inhibitors have been developed, of which some are discussed below.

Other BTK-inhibitors

Acalabrutinib (ACP-196), is a more selective BTK-inhibitor with minimal affinity for TEC, EGFR and ITK and without unwanted effect on platelets [239-241]. According to data from two trials on R/R CLL and MCL respectively, single agent ACP-196 was administered with manageable toxicity, although grade 3 headache seems to be more frequently reported than in previous trials with ibrutinib. The rate of grade 3 adverse events, including diarrhea was low and there was no reported case of atrial fibrillation. In the subgroup of MCL patients, ORR was 81% and CRR was 40% at median FU 15 months [241].

Zanubrutinib (BGB-3111), with less affinity for ITK and stronger inhibitory effect on BTK on MCL cell lines, was evaluated in 72 patients with DLBCL or MCL. No atrial fibrillation was reported and grade 3 adverse events included neutropenia, anemia, pneumonia (n=1) and three cases with grade 3 bleeding. In the subgroup of 32 MCL, ORR was 88% of which 25% received CR [242-244].

Spebrutinib (CC-292), is a BTK inhibitor, with capacity of inhibiting proliferation, cell migration and adhesion in MCL cell lines which are not dependent the alternative NFkB pathway, albeit some affinity for ITK and JAK3 [245] Cidal-Crespo et al. showed synergism of anti-proliferative effect by combination with

lenalidomide in MCL cell lines, and this combination is currently evaluated in a trial by Salles et al. (NCT01766583) [246]

Lenalidomide-an immune-modulating agent

Mechanism of action

Lenalidomide (CC-5013) (L) belongs to the group of thalidomide derivates, commonly called "immunomodulators". The antitumoral properties of lenalidomide can be ascribed with anti-proliferative and antiangiogenic effects on malignant cells as well as immunomodulatory actions increasing the host response.

Lenalidomide binds to cereblon, which together with two other molecules (DDP1 and CUL1) form the ubiquitation ligase complex, responsible for ubiquitation and proteasome degradation of proteins involved in cell proliferation, transcription and cell cycle regulation.

Treatment with lenalidomide has shown to enhance ubiquitation and degradation of the two transcription factors, Ikaros and Ailos (IKCZ1 and IKCZ3), and thereby alter expression of several genes involved in proliferation and activity of B and T cells, such as increased levels of p21, and reduced expression of MYC, SP1B and IRF4 [247, 248]. The degradation of IKCZ1 and IKCZ3 has also been shown to increase IL-2 secretion by T cells, and thereby enhance T cell activity as shown by increased levels of CD8+ cytotoxic T cells, decreased levels of T regulatory cells and enhanced immune response by recruitment and activation of NK cells [248, 249]. Furthermore, the reduced immunologic synapse formation between T cell and tumor cell, as observed in leukemic lymphoma, as a tumor-related immune evasion, was restored by lenalidomide in *in vitro* assays on FL and CLL [250, 251]. The anti-angiogenic properties, described as reduced micro vessel density, is probably by depletion of macrophages and monocytes involved in lymphoma-related angiogenesis [249, 252].

It is unclear which one of the pharmacodynamics mechanisms that is most important in MCL. Depletion/knockout of cereblon has induced cell death as well as resistance to lenalidomide in some studies on myeloma cells and ABC-DLBCL, indicating that this system is of importance for tumorigenesis as well as for sensitivity to lenalidomide [253, 254]. Contrary, a recent study on MCL cell lines and patient-derived samples suggests that the NK-cell mediated cytotoxicity may be of major importance for prognosis [255].

Sensitizing to antibody treatment

Based on the enhanced T cell activity, *in vitro* models investigated whether lenalidomide could increase ADCC induced by anti-CD20 ab. A first report in 2005 described a tendency of synergistic effect of CC-5013 (L) when combined with rituximab *in vitro*, albeit no synergistic effects were observed in their *in vivo* xenograft lymphoma model [256]. In 2008, Wu et al. showed synergistic effect on NK-cell mediated cell killing by pretreatment of NK cells with lenalidomide, with increased cytotoxicity of anti-CD20 coated lymphoma cells and increased release of IFNγ [257]. Similarly, the same group showed enhanced ADCC by lenalidomide in breast and colon cell lines coated with monoclonal antibodies trastuzumab and cetuximab respectively [258].

Lenalidomide in MCL

Single agent lenalidomide was initially evaluated in R/R MCL with overall response rates varying between 20 and 40%, complete response rate maximally 20% and median PFS 4-5.7 months [259-262]. One randomized trial was performed on R/R MCL to investigate PFS after lenalidomide vs investigator´s choice in 292 patients (2:1 lenalidomide: investigator´s choice). Objective response rate in the lenalidomide group was 40% and median PFS 8.7 months, compared to 11% and 5.2 months in the investigator´s choice group. Although median duration of response was longer in the lenalidomide group, no significant difference in overall survival between the groups could be demonstrated [263].

Given the enhanced immune activity by lenalidomide and synergism with anti-CD20 ab *in vitro*, the combination of rituximab and lenalidomide (R2) was taken into clinical trials [257, 258, 264, 265]. Wang et al. reported data from the initial phase I/II trial on R/R MCL patients, combining rituximab and lenalidomide, with somewhat higher response rates, ORR 57% and CRR 36% than previous data on single lenalidomide. All patients had previously received rituximab, of which eight patients as part of last regimen and nine patients as maintenance therapy.[266].

Furthermore, Chong et al. showed that lenalidomide had the potency of re-sensitizing to rituximab. Fifty patients with B cell lymphomas, previously defined as resistant/refractory to rituximab, received two cycles of pretreatment with lenalidomide followed by addition of rituximab, with observed doubling of ORR from 30% to 63%. Notably, in the subgroup of MCL patients (n=11), the overall response rate of 55% after single lenalidomide, did not increase by the addition of rituximab, albeit some more patients achieved CR [267].

L-R was investigated in untreated elderly patients with MCL by Ruan et al. as a “chemo-free” regimen. In the cohort, a majority of patients (68%) of patients were

scored low or intermediate MIPI risk group, no one had blastoid disease and 79 % had Ki-67% ≤ 30. Patients with high-risk MIPI were only included in case of ineligibility to tolerate chemotherapy. At recent report at median FU 58 months, 61% of evaluable patients were in complete remission, 4-y-PFS was 70 months and 4-y-OS was 83 months [214, 215].

Lenalidomide is currently being investigated in combination with established immunochemotherapy regimens as well as in combination with novel agents (Table IV).

Other small molecule inhibitors

PI3K inhibitors

PI3K exist in four isoforms α, β, γ and δ, each one expressed in different types of cells. Idelalisib, the first approved PI3K inhibitor, mainly targets δ isoforms, present in B cells, normally necessary for maturation and differentiation of the germinal center [268]. Idelalisib showed ORR 40% in 40 patients enrolled in the phase Ib trial on R/R MCL patients [269].

Idelalisib was further combined with rituximab and lenalidomide, with unexpected severe toxicity observed including sepsis, hypotension, rash and impaired liver function, which may be explained by enhanced immune response by affinity of idelalisib to other isoforms, like γ, active in T cells [270, 271].

Duvelisib (IPI-145) and copanlisib (BAY 80-6946), were constructed for dual targeting against γ+ δ and α+δ isoforms respectively for broader inhibitory effect. Duvelisib has shown activity in MCL cell lines resistant to BTK-inhibitor by BTK C481S mutations and is currently investigated in R/R NHL patients, albeit early data showed activity in 5 of 10 patients with MCL (ORR 50%)[272]. Recently, data on copanlisib (BAY 80-6946) in R/R indolent and aggressive NHL showed ORR 64% in subgroup of MCL patients which is markedly higher than for idelalisib and duvelisib [273, 274].

TGR-1202 (umbralisib) is another novel agent with dual inhibitory effect on both CK-1ε, a suppressor of regulatory T cells, and PI3K-δ but with no affinity for γ, thereby providing a potential for less toxic effects related to enhanced cytokine release and immune activation. Preclinical data has shown potency of reduced c-MYC expression and activity in MCL cell lines [275]. Recent data from phase I part of trials on single agent TGR-1202 or in combination with anti-CD20 antibody, ibrutinib or bendamustine showed acceptable tolerability in all subgroups [276].

mTOR inhibitors

The activated AKT/PI3K/mTOR pathway in MCL have made mTOR as a rational target for inhibition of proliferation signaling. Temsirolimus, the most studied mTOR inhibitor in MCL, showed response rates ~40% in R/R MCL patients in two initial phase II trials. After a European randomized trial demonstrating superior PFS compared to investigator´s choice, it was approved for use in R/R MCL patients [218]. Although mostly evaluated in previously heavily treated patients, temsirolimus is associated with a high rate of hematological toxicity which may be dose-limiting. Furthermore, a large randomized trial on nearly 300 patients showed a significant higher complete response rate and PFS by the use of ibrutinib in R/R MCL, and temsirolimus is not currently recommended as first choice in R/R disease [219].

Bcl-2 inhibitor

Bcl-2 protein is an anti-apoptotic member of the key regulator of apoptosis family of proteins, Bcl-2. Bcl-2 is encoded by the gene on chromosome 18 and has been found to be overexpressed in MCL by amplification 18p21 [277]. ABT-199 (venetoclax) is the first approved Bcl-2 inhibitor after showing activity in de17p CLL (reviewed by Davids 2017). In MCL, venetoclax has shown an ORR of 75% in R/R disease and preliminary data from the ongoing AIM trial on rituximab and ventecloax has reported CRR 63% and ORR 71% in R/R patients [171, 278].

Proteasome inhibitor

Bortezomib, the first approved proteasome inhibitor, has showed ORR 32% (4% CRR) in R/R MCL patients in the PINNACLE trial, leading to approval of the drug [279]. Subsequent trials combined bortezomib with rituximab with manageable toxicity, except from some neuropathy and ORR 29-50% in R/R MCL [280, 281]. In the LYM-3002 trial, untreated patients were randomized to receive either "VR-CAP", where vincristine in R-CHOP was replaced by bortezomib or regular R-CHOP. Although higher activity was observed in the experimental arm as shown by higher PFS and CRR, toxicity profile was detrimental to VR-CAP, with high rate of thrombocytopenia, neutropenia and infections [210]. A more tolerable regimen was bortezomib + BR (RiBVD), , showing ORR 74% in untreated elderly patients with MCL [212]. A synergy of bortezomib and cytarabine has been demonstrated *in vitro*, and in a trial on previously heavily treated MCL patients (5 of 8 > 4previous lines of treatment), 4 patients responded of which 2 (25% in total) achieved complete response after treatment with bortezomib, cytarabine and rituximab [282, 283]

Table IV Selected trials on novel agents in R/R MCL

target/ author	agent	trial	n(MCL)	ORR (CRR) (%)	DOR	FU time (median)	PFS	survival
BTK	**ibrutinib**							
Wang et al.[230]	ibrutinib	II	111	68 (21)	md 19.5m	27 m	md 13.9 m	md 22.5 m
Dreyling et al.[219]	ibrutinib	III, rand	141	72 (19)	18-m 58%	20 m	md 15 m	not reached
	vs temsirolimus		139	40 (1)	md 7 m		md 6 m	md 23m
Wang et al. [223]	Ibrutinib + rituximab	II	50	88 (44)	-	17 m	12-m 75%	12-m 86%
Wang et al.[241]	**acalabrutinib**	II	124	81 (40)	1-y 72%	15 m	1-y 67%	1-y-87%
Davids et al. [276]	**umbralisib+ ublituximab**	I/Ib	-	-	-	-	-	-
Bcl-2	**venetoclax**							
Davids et al. [171]	venetoclax	I/II	28	75 (-)		NR	md 14 m	1-y-82%
mTOR	**temsirolimus**							
Witzig et al. [284]	temsirolimus	II	35	38 (3)	md 6.9m	11 m	md-6.5 m	md 12 m
Hess et al. [218]	temsirolimus 175mg/25mg/125mg/25 mg vs invest´s choice	III, rand	108	22(-) (higher dose group)	3.6/7.1 m	NR	md-3.4/4.8 m	md 10/12.8
Proteasome complex	**bortezomib**							
Goy et al. [279]	bortezomib	II	141	32 (8)	md 9.2m	26 m	md 6.5m	md 23.5m
Agathocleus et al. [280]	bortezomib + rituximab	II	42 (19 MCL)	58 (16)	NR MCL	NR MCL	NR MCL	NR MCL
immunomodulator	**lenalidomide**							
Arcaini et al. [285]	lenalidomide	III, rand	170	68 (40)	md 16.1m	41 m	md 8.6m	md 5.4 m
	vs invest's choice		170	9 (11)	md 10.4 m	28m	md 21m	-
Goy et al. [262]	lenalidomide	II	134	28 (8)	md 17m	9.9 m	md 4m	md 19 m
Witzig et al. [286]	lenalidomide	II	57	42 (21)	-	5.7m		
Eve et al. [261]	lenalidomide	II	26	31 (8)	md 22m	3.9m	md 3.9m	md 10 m
Zaja et al. [287]	lenalidomide + dexamethasone		33	52 (24)	md 18m		md 12m	md 20 m
Wang et al.[266]	lenalidomide + rituximab	I+II	44	36 (56)	md 19m		md 11m	md 24 m
combinations								
Tam et al.[278]	venetoclax + ibrutinib	I/II	24	71 (63)	-	8 m	8-m 74 %	8-m 81%
Morrison et al. [288]	bortezomib + lenalidomide	II	53	40 (15)	NR		md 7 m	md 26 m
Jerkeman et al. [289]	lenalidomide + rituximab + ibrutinib	I/II	50	76 (56)	NR	17,8 m	md-16 m	md 22 m

Aims

The overall aim of this work was to investigate whether a combined analysis of population-based registry data, outcome of a clinical trial, and *in vitro* cellular models on drug interaction, could bring further insight into how novel agents should be taken into clinical use, to define reliable prognostic markers for survival and, most importantly, how outcome can be improved in patients with MCL.

The specific aims were:

- To study incidence and survival in patients with MCL in a population-based cohort from the Swedish and Danish Lymphoma registry during a ten year period. (paper I)
- To analyze outcome of MCL in a population-based cohort in relation to clinical prognostic factors and primary treatment. (Paper I)
- To investigate if lenalidomide can be combined with rituximab and bendamustine, in previously untreated elderly patients with MCL, by establishment of maximally tolerable dose. (Paper II)
- To evaluate the efficacy of lenalidomide, rituximab and bendamustine as primary treatment of elderly patients with MCL as measured by progression-free survival. (Paper II)
- To examine whether the combination lenalidomide, rituximab and bendamustine is active in the high-risk group of MCL, harboring *TP53* mutations. (Paper III)
- To establish an *in vitro* model for functional studies on NK-cell mediated cell death in MCL (Paper IV)
- To investigate whether a small molecule inhibitor like ibrutinib affects the immune-mediated response to a type I/II anti-CD20 monoclonal antibody, and if a negative impact can be restored by addition of lenalidomide. (Paper IV)

Material and Methods

Nordic Lymphoma Group

The Nordic Lymphoma Group (NLG) is a framework of clinicians and researchers with main purpose of conducting clinical trials and research on biology, treatment and epidemiology of lymphoma within the Nordic countries, as well as in collaboration with international groups. Paper I, II and III are made by collaboration within the NLG framework.

Paper I-an observational study on MCL

Hypothesis

In paper I, our hypothesis was that overall survival in patients with MCL in Sweden and Denmark had increased during the study time period, by integration of rituximab and, for young patients, HD-ASCT, in (routine) primary treatment, and that previously described prognostic factors for outcome, including MIPI, could be confirmed as predictors of outcome in our large unselected population-based cohort of patients. We also hypothesized that we could define the most effective regimen for patients >65 years in terms of overall survival.

Swedish and Danish lymphoma registries

Swedish lymphoma registry (SLR), is an expansion of the Swedish Cancer Registry (SCR). SCR is a national registry of cancer incidence, which has been in use since 1958. Information to the registry is made by a compulsory dual report system of the responsible pathologist and clinician. The registry does not include details on clinical parameters and all lymphomas are grouped together. In 2000, the Swedish Lymphoma Group initiated the SLR, with more precise data on lymphoma subtypes to facilitate output of data and to serve as a quality control for health care in Sweden. Initially, the data was limited to clinical parameters like diagnosis, patient

characteristics and disease presentation but since 2007, the registry includes data on primary treatment and response and since 2010, even relapse data. The regional cancer centers are responsible for administration of SLR and the coverage is estimated to be ~95% [290].

The Danish Lymphoma Registry (LyFo) was initiated by the Danish lymphoma group in 1983, originally covering only Western Denmark, and extended in 1999 to include all patients with lymphoma in Denmark. In the last years, the registry has reached a coverage of ≥ 95% [291].

Study design and patients

All patients with a registered diagnosis of MCL between January 1, 2000 and September 11, 2011 in SLR and between January 1, 2001 and December 31, 2010 in LyFo were included in the study. Data was extracted from the registries and, in Sweden, complemented by review of patients´ records for information of primary treatment. Data on survival was collected from the national population registry in Denmark and Sweden.

Paper II – a phase I/II trial investigating a new combination for elderly patients with untreated MCL

Hypothesis

Our hypothesis was that the addition of lenalidomide to R-B would be a tolerable combination in untreated elderly patients with MCL and that the regimen would increase disease control by increased/deeper response and prolonged progression-free survival compared to R-B, according to previous reported data.

Trial design

In 2009, the Nordic Lymphoma Group initiated a phase I/II trial, MCL4 (Lena-Berit), on lenalidomide, rituximab and bendamustine (LBR) as primary treatment to patients < 65 years with MCL. The trial was a prospective non-randomized, open-label multicenter trial, including patients from 19 centers in Denmark, Finland, Norway and Sweden.

Treatment

The regimen consisted of an induction phase of six cycles (c) of LBR (lenalidomide [by mouth, d1-14], rituximab [iv, 375mg/m^2, day 1], bendamustine [iv, 90 mg/m^2, days 1-2]), cycle duration 28 days, followed by a maintenance phase of seven cycles single agent lenalidomide day 1-21, cycle duration 28 days.

Phase I

Primary endpoint was to establish maximally tolerable dose (MTD) of lenalidomide in combination with BR.

The phase I portion followed a 3+3 design, with stepwise dose-escalation of lenalidomide, starting at 5 mg and increased by 5 mg I each step. In c7-14, the dose of L was 25 mg.

3+3 design is one of the most used methods for defining optimal treatment dose in phase I clinical trials and follows a predetermined sequential dose-escalating schedule [292, 293].

The first three patients entering the trial (no 1-3) receive treatment at a dose level, considered as safe (in this study 5 mg). If no dose-limiting toxicity (DLT) occurs in this cohort, the next cohort of three patients (no 4-6) starts at an escalated dose (in this study 10 mg). If one DLT occurs in the first cohort of three patients, this cohort will be expanded by the next patients (no 4-6) also receiving the starting dose. If no more DLT occurs in this expanded cohort (no 1-6), further dose-escalation will be performed for the following three patients (no 7-9). However, if two or more DLT occur in a cohort of up to six patients (≥33%) a de-escalation should be performed. MTD is defined as the maximal dose level where DLT occurs in less than 33% of subjects, in practice often the dose level below the one from which a de-escalation step was made.

In the MCL4 protocol, DLT was defined as any grade 3 to 5 non-hematologic adverse event (AE) within the first two cycles of LBR, except for thromboembolic events grade 3 to 4, non-persisting nausea, diarrhea or elevated transaminases, or events attributed to disease progression.

Phase II

Primary endpoint in phase II was progression-free survival (PFS).

Secondary endpoints included

- Overall response rate with and without PET
- Complete remission rate with and without PET
- Health-related quality of life
- Molecular remission rate by PCR
- Overall survival
- Safety
- Evaluation of biomarkers for efficacy

Patients

Inclusion criteria were >65 years, or ≤ 65 years unable to tolerate HD-ASCT, with newly diagnosed histologically confirmed MCL, stage II-IV, in need of treatment due to bulky disease, B symptoms, elevated serum LDH, symptomatic nodal or splenic enlargement, compressive syndrome, pleural/peritoneal effusion or cytopenia caused by bone marrow infiltration of lymphoma. Furthermore, patients should not have received any previous treatment for lymphoma except radiotherapy or one cycle of chemotherapy.

Evaluation of response and safety analysis

All patients underwent CT scan and examination of bone marrow and peripheral blood including samples for flow cytometry and MRD assessment at inclusion and after 3 and 6 cycles respectively as well as 1.5 months after completed therapy for evaluation of response. Response was assessed by using the international response criteria [294]. Evaluation of MRD was made centrally by using standard nesting PCR amplification of predefined patient specific primers, identified in diagnostic samples of BM and PB, either as specific clonal rearrangements of IGH or as chromosomal rearrangement of Bcl-1/IGVH (t11;14)(q13;q32).
Toxicity was evaluated according to the National Cancer Institute Common Terminology Criteria for Adverse Events Version 3.0 (NCI CTCAE). Lymphocyte population in peripheral blood was monitored at regular follow-up every six months until 36 months after completed therapy.

Paper III-mutational profile as prognostic marker

Hypothesis

Our hypothesis was that the addition of lenalidomide to BR, followed by six months of maintenance therapy with lenalidomide, would improve outcome, in terms of deeper remission and prolonged progression-free survival, in patients with MCL, harboring alterations in *TP53*.

Further, we hypothesized that the mutational profile of selected genes would show the same pattern in elderly untreated patients with symptomatic MCL as previously described in young patients.

Patients and genes selected for study

Patients with available DNA, extracted from fresh frozen PB and BM aspirates were included in the study. Data on response and survival was based on median FU of 46.5 months.

The panel of genes analyzed in the study was originally constructed on data from previous whole genome and whole exome sequencing studies on MCL and included *TP53* and *CDKN2A* for analysis of gene allele frequency and *ATM*, *BIRC3*, *CCND1*, *KMT2D*, NOTCH1, *NOTCH2*, *TP53* and *WHSC* for detection of mutations [39, 55].

NGS and ddPCR

For detection of deletion and mutations, two techniques, based on polymerase chain reaction (PCR) were used.

ddPCR for gene allele frequency

Gene allele frequency was investigated by Droplet Digital PCR (ddPCR), a technique first described in 1999 [295]. In principle, the sample is split into numerous fragments (10^3-10^6) and separated into droplets, containing either 0 or 1 copy of the target sequence, by an emulsifying process. After PCR on these droplets, the amplified number of target sequence is compared to a normal control. In our study, samples were compared with peripheral blood samples from healthy donors, and a copy number loss was defined by copy number (CN) < 1.95.

NGS for mutational profile

Next generation sequencing (NGS) refers to high throughput methods of gene sequencing that aloud multiple sequences to be analyzed simultaneously. In our study, we used an Ion torrent semiconductor-based technique. After amplification, the samples are read in micro-chip where a complementary DNA strand is built to the template, located in micro wells by fluiding of DNA polymerase and nucleotides. The binding of a specific nucleotide base pair causes a release in H+, which is detected by an alteration in current [296]. The panel of genes included coding regions, splice sites and untranslated regions (UTR) of respectively gene. Calling a variant was determined as a variant allele frequency (VAF) > 5% (>3% for *TP53*) and a coverage of 400X. Furthermore, mutations that did not give rise to amino acid change in the protein-coding region, common known single nucleotide polymorphisms (SNPs) and VAF 40-60% in combination with SNP database, thus regarded as rare SNPs, were excluded from the analysis.

Evaluation of ADCC on MCL cell lines (paper IV)

Hypothesis

Our hypothesis was that ibrutinib has a negative impact on NK cell activation, and thereby the potency of reducing cell death after treatment with anti-CD20 antibody in MCL (i). Further, we hypothesized that by addition of lenalidomide to anti-CD20 antibody and ibrutinib, the negative effect on cell death would be restored, by capacity of lenalidomide to sensitize the cellular response in NK cells to anti-CD20 antibody (ii). We also hypothesized that a type II anti-CD20 antibody would induce higher rate of ADCC on MCL cells compared to a type I anti-CD20 antibody (iii).

Assessment of ADCC by flow cytometry

A protocol for assessment of ADCC by flow cytometry was set up using the fraction of 7-AAD out of CFSE-positive cells as a marker for cell death in the target cell population. CFSE (carboxyfluorescein succinimidyl ester) is a fluorochrome which becomes fluorescent by intracellular hydrolysation and can be used for cell tracking by covalent binding to lysine residues in the nucleus [297]. 7-AAD (7-aminoactinpmycin D) is a fluorochrome, not permeable in viable cells but can enter cytoplasm and nucleus of non-viable cells where it binds to DNA and becomes fluorescent and is thus used for discrimination of non-viable cells [298].

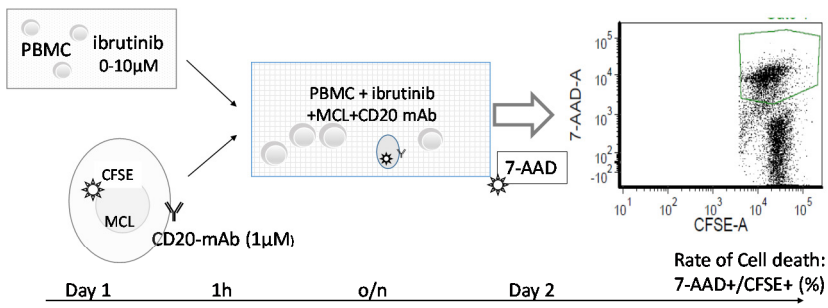
For evaluation of the inhibitory effect of ibrutinib on NK cell activation (i), PBMC were pretreated with ibrutinib (0.01-5µM) or DMSO. CFSE-stained target cells were incubated with 1µM type I (rituximab) or type II (obinutuzumab) anti-CD20 antibody and co-incubated with pretreated PBMC o/n followed by staining with 7-AAD for analysis of cell death by flow cytometry (Figure 5).

All experiments included two MCL cell lines, JeKo-1 and REC-1, two anti-CD20 abs and relevant controls without PBMC, anti-CD20 antibody and ibrutinib respectively. Samples were made in duplicates.

In the experiments including lenalidomide (ii), PBMC was pretreated with lenalidomide (0.01-10µM) or DMSO prior to ibrutinib (1 µM), followed by subsequent co-incubation with target cells and analysis as described above.

The gating procedure included identification of singlets by FSC-H and SSC-H (forward and side scatter height respectively). CFSE and 7-AAD positive cells were identified by channel BL-1 and BL-4 respectively on the instrument (iQue screener, Intellicyt) and by unstained samples as negative controls.

Figure 4. Assay for evaluation of ADCC in MCL cell lines by flow cytometry



Statistics

In paper I-III, estimates of survival, either as overall or progression-free survival, were calculated by the Kaplan-Meier method. Comparison of survival between subgroups were made by log-rank test. Progression-free survival (paper II-III) was defined as time from inclusion to first documented relapse, progression or death of any cause. In paper I, overall survival was defined as time from diagnosis to death of any cause and in paper II-III, overall survival was defined as time from trial inclusion to death of any cause.

Paper I

Analysis of incidence and relative survival was made by an additive model as previously described [299]. Cox regression was used for estimation of hazard ratios of prognostic factors in uni- and multivariate analysis, including base-line patient characteristics and treatment parameters. For evaluation of difference between frequency of parameters between groups, Pearson´s χ^2 and nonparametric tests were used.

Paper II

To determine sample size, a median PFS of 6 months longer than the reported median PFS of 30 months in MCL subgroup of German STIL trial was considered significant [300]. By calculation of a prolongation of PFS 6 months and exponentially distributed PFS, a 95% confidence interval of 23.1 months was achieved at 40 patients included, which was accepted. Sample size was determined as 60 patients with 20 patients in phase I and 40 patients in phase II. For analysis of frequency of adverse events between groups, Pearson´s χ^2 tests were used. The analysis on lymphocyte population in relation to incidence of infection was made by using Mann-Whitney U test.

Paper III

Cumulative incidence of relapse or progression was defined as the time from inclusion to first documented relapse or progression. Analyses on adverse events in relation to presence of specific gene alteration/mutation were made by using Fisher´s exact t-test.

Paper IV

For comparison of immune-mediated cell death, we defined *cell death (%)* as the mean value of (7-AAD+/CFSE+) ratio of duplicates with reagent (i) (ibrutinib) and (ii) (ibrutinib and lenalidomide), compared to mean value of control duplicates without reagents. In the analysis comparing a type I and a type II anti-CD20 antibody (iii), *cell death* was defined as the mean 7-AAD+/CFSE+- ratio of duplicates with anti-CD20 mAb compared to samples without anti-CD20 mAb from experiments with three different donors.

Differences were evaluated by Student's unpaired t test was performed to identify significance level. A p-value < 0.05 was considered significant.

Results

Paper I

Between 2000 and 2011, 1389 patients (895 from Sweden and 494 from Denmark) were diagnosed with MCL. The incidence increased in both countries during the time period, and the age-standardized incidence increased for males but not for females in Denmark and Sweden. The relative risk of MCL was higher in Denmark compared to Sweden, albeit only significant for females after analysis by gender.

Data on primary treatment was available in 1197 (86.2%) patients. This group showed a lower median age, 70 (range 28-95) vs 72.5 (range 34-96) years (p= 0.011), and higher 3-y-OS (57.8% vs 45.4%) compared to patients without data on treatment.

1066 patients received systemic treatment, 54 patients received radiotherapy, of which 43 with curative intent and 11 as palliative first-line treatment. 67 patients did not receive any treatment, of which 29 patients were recorded as "watch-and-wait" (WaW). Furthermore, 47 patients were deferred treatment due to poor performance status or comorbidity. The two groups treated by WaW and RT with curative intent, showed 3-y-OS 79% and 93% respectively.

Besides the factors included in MIPI, male gender was identified as a negative prognostic factor for survival. Consequently, MIPI and gender was used in subsequent multivariate models on prognosis in relation to treatment.

Overall survival was found to increase during the time period, by a significant higher 3-y-OS in patients diagnosed 2006-2011 (61%) compared to patients diagnosed 2000-2005 (51%).

During these years, treatment with rituximab increased from 52% during 2000-2005 to 77% during 2006-2011. Patients who received rituximab as part of primary treatment showed a higher 3-y-OS and by multivariate analysis, treatment with rituximab retained a prognostic impact on survival irrespectively of gender, MIPI, chemotherapy regimen and ASCT. Together, the data indicates that the use of rituximab contributed to improved survival in the cohort.

Next, the impact of ASCT was examined. Of all patients with data on ASCT (n=1143), 276 received ASCT, 97% within the MCL2 protocol. The group of patients who received ASCT was associated with superior 3-y-OS of 84% compared to 50% in the non-ASCT group. When adjusting for other prognostic factors like MIPI, gender, rituximab and chemotherapy regimen, survival outcome was still superior in the ASCT group (HR 0.55, 0.37-0.83, p=0.004).

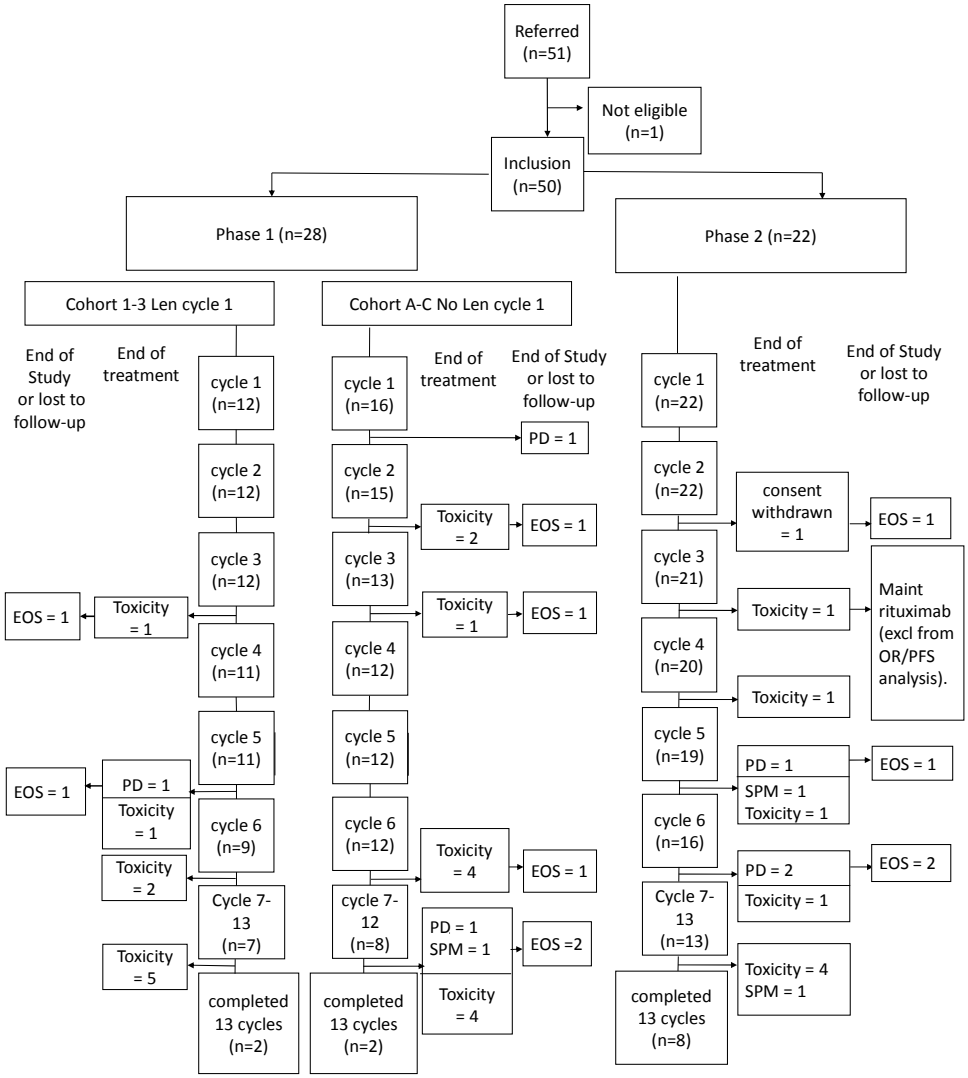
We also compared chemotherapy regimens that do not include ASCT. Among all regimens, CVP was associated with inferior survival, compared to CHOP, one of the most frequently used non-ASCT regimens. Next, we compared two regimens, commonly used in elderly frail patients, CVP and chlorambucile, either administered in combination with or without rituximab. OS was significantly inferior in patients receiving CVP compared to chlorambucile (HR=2.34; 85% CI: 1.32-4.14, p=0.003), after adjustment for treatment with rituximab, MIPI and gender.

The use of bendamustine increased in our cohort from the introduction in 2006 to represent >20% of all regimens in 2011. Altogether, 51(6.9%) patients, all >65 years, received bendamustine, mostly in combination with rituximab (88%). Regarding survival in patients > 65, 3-y-OS after bendamustine was longer than after all other regimens, except for MCL2. However, bendamustine could only be associated with superior survival to CVP but not to CHOP (HR 0.56; 95%CI 0.3-1.02, p=0.060) in multivariate analysis after adjustment for rituximab, MIPI and gender.

Paper II

51 patients were included in the NLG/MCL4 (Lena-Berit) trial between 2009 and 2013, of which 50 started treatment according to the protocol. Patients had predominantly stage IV disease and 88% had BM involvement at diagnosis. 2 of 41 were reported as blastoid MCL and Ki-67% was >30% in 9 of 38 reported cases. A consort diagram of all patients is shown in Figure 5.

Figure 5. CONSORT diagram of patients included in NLG/MCL4 trial



Phase I

In the early phase I portion, an unexpectedly high frequency of adverse events was reported from the first cohorts of patients, mainly immune-related rash and allergic reactions. Therefore, a protocol amendment was made where lenalidomide was excluded from cycle 1 and patients received corticosteroids in cycle 2. Hereby, phase I was expanded to include another three cohorts of patients. Phase I included 28 patients in total and the MTD of lenalidomide in combination with BR was established as 10 mg, given cycle 2-6, followed by 10 mg cycle 7-8 and 15 mg cycle 9-13.

Phase II

According to the outcome in patients of phase I+II, the addition of lenalidomide to BR showed median PFS 42 (95% CI 31-53) months at a median FU of 31 months. Median PFS was longer than reported data from STIL trial, showing a median PFS of 35 (29-55) months in the MCL subgroup, although the two confidence intervals of the estimated PFS are overlapping.

Safety analysis revealed that toxicity caused treatment discontinuation in 15 patients during the induction phase and in 9 patients during maintenance phase. The most common grade 3-5 adverse events (n=number of patients reported with AE grade 3-5 at any time) were grade 3-4 neutropenia (n=38), infection (n=21), grade 3-4 thrombocytopenia (n=10), rash (n=9) and allergic reaction (n=6). The majority of grade ≥3 infections occurred during induction phase (in 19 patients). Moreover, 2 patients died from severe infections and three patients were reported with opportunistic infections (pneumocystis jiroveci pneumonia, CMV retinitis). Together, these results show that the regimen causes a more profound bone marrow suppression with increased susceptibility to severe infectious complications.

In relation to bone marrow suppression and frequency of infections, we investigated lymphocyte populations during and after treatment. We found that CD4+ count was significantly lower already after 3 cycles compared to base-line levels and CD4+ count remained low until 13 months after completed therapy, as defined by a CD4+ count below lower reference limit. Furthermore, median CD4+ count was significant lower in patients who developed any grade infection during treatment.

Another finding was a high frequency of immune-related reactions, including rash and allergic reactions. Although grade 3 allergic reactions were prevented by the amended protocol, grade 3 cutaneous reactions were still reported in 5 of 37 (14%) patients that received the modified regimen.

Second primary malignancy (SPM) were reported in 8 (16%) patients at initial report (paper II) and in 11 (20%) patients after the prolonged update (paper III). The reported SPMs were three hematological; one CMML, one AML and one Hodgkin Lymphoma and eight cases with solid tumors; prostate cancer in two patients and invasive squamous skin cancer, squamous lung cancer, hepatocellular cancer, kidney cancer and endometrial cancer and benign pheochromocytoma, in one patient respectively. Three patients stopped treatment due to newly diagnosed tumor, one patient with benign onocytoma (not included in SPM analysis), one patient with squamous lung cancer and one patient with prostate cancer. SPM was reported as cause of death in three patients (AML, HL and squamous lung cancer). Three patients with solid tumor underwent local treatment with curative intent, one of the patient with prostate cancer received radiotherapy and the two patients with endometroid cancer and pheochromocytoma respectively underwent surgery. Furthermore, a number of non-invasive skin cancer were reported including basal cell cancer, noninvasive/in situ squamous cancer.

Response evaluation was made according to intention-to treat principle. At 6 months after start of treatment CRR was 64% and 1.5 months after completed treatment, CRR was 62%. Furthermore, 56% of evaluable patients were MRD negative in BM at 6 months and 64% after 1.5 months after completed treatment.

Paper III

In this study, we analyzed outcome of the NLG-MCL4 trial in relation to the presence of genetic alterations. Of all patients, 46 had available DNA, either from BM or PB, and were included in the study. An updated follow-up was also made and results were based on a median FU time of 46.5 months

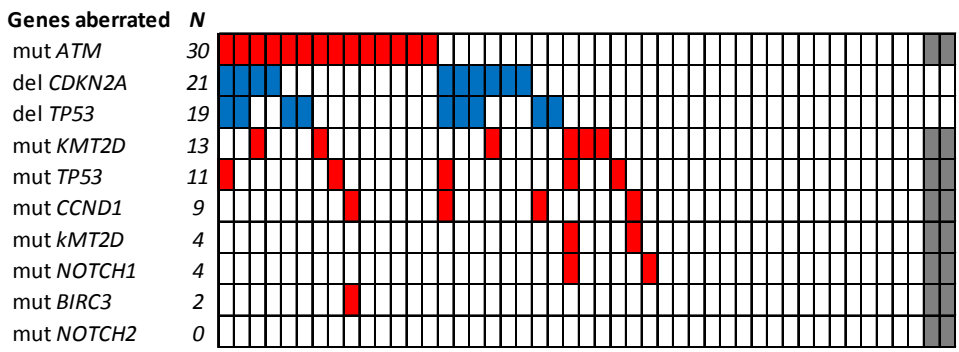


Figure 6. Genetic aberrations in the cohort of NLG/MCL4 patients.
Each column represents one patient. Deletions are showed in red and mutations in blue. Grey = missing data.

The pattern of genetic alterations of studied genes showed a similar pattern as previously described by Eskelund et al. [32]. (Figure 6) Any alteration was detected in 64% of the patients and of these, 32% had more than one (2-4) alteration. *TP53* alteration was detected in 12 (33%) patients, of which 9 had deletion and 6 had mutations and accordingly, 3 cases harbored both deletion and mutation.

The presence of *TP53* mutation was associated with significantly poorer outcome. At a median FU of 45 months, median OS in *TP53*-mutated cases was 25 months (95% CI: 7-43) compared to 69 (95% CI: 67-71) months in non-mutated cases and the median PFS was 10 months (95% CI: 0-23) compared to 42 months (95% CI: 22-62). None of patients in the *TP53*-mutated group achieved MRD negativity during treatment, although three were evaluated with clinical response after induction phase.

Both deletion of *TP53* or *CDKN2A* showed a trend of worse outcome but no other mutations showed prognostic impact.

Paper IV

In this study, we established an *in vitro* model for assessment of NK cell function, by measurement of cell death, induced by anti-CD20 antibody on MCL cell lines.

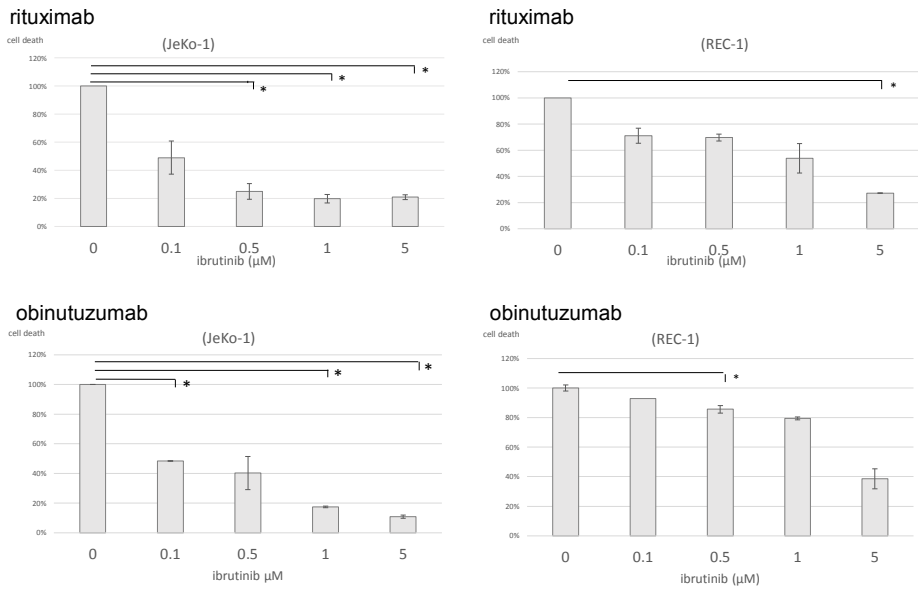
By initial investigations, we found that ibrutinib did not have a direct dose-dependent effect on viability of two cell lines, JeKo-1 and REC-1, at concentrations up to 1 μM (up to 10 μM for JeKo-1), why these cell lines were chosen for further experiments. Next, we identified 1 μM anti-CD20 ab as the optimal concentration for readout of ADCC and was chosen for further experiments.

Further, we showed that both rituximab and obinutuzumab induced significant higher cell death compared to samples without anti-CD20 ab (marked as ref in Figure 7) as well as in samples with PBMC compared to without PBMC, i.e. ADCC was represented in our model (data not reported).

Pretreatment of PBMC with ibrutinib was found to decrease cell death at treatment with either a type I or a type II antibody in both cell lines, as demonstrated in Figure 7. In this experiment, a tendency of dose-dependent inhibitory effect was observed in one of the cell lines, JeKo-1, showing a significant decrease in *cell death*, maximally to 21%, at an ibrutinib concentration of 0.5-5 μM in samples treated with rituximab and at 1 and 5 μM with obinutuzumab. A lower rate of cell death (~50%) was observed at 0.1 μM in both series but failed to be significant by p-value > 0.05 in samples treated with rituximab, mainly due to wider distribution among the samples. For REC-1, the difference in cell death was significant in a dose-dependent

manner, but the results may have been influenced by direct cytotoxic effects of ibrutinib on REC-1 at 1 and 5 μM.

Figure 7. Ibrutinib negatively affects cell death induced by anti-CD20 antibody on MCL cell lines



Cell death (%)± SD in MCL cell lines (JeKo -1, REC-1) opsonized with 1μM anti-CD20 mAb (rituximab, obinutuzumab) and co-cultured with PBMC pretreated with ibrutinib (0-5 μM).

Next, we added lenalidomide to investigate whether the inhibitory effect could be hampered by activation of NK cells (ii). We did not find any significant increase in cell death in samples treated with lenalidomide prior to ibrutinib.

Finally, we compared the potency of inducing ADCC, between a type I and a type II anti-CD20 antibody, by analyzing cell death induced by rituximab and obinutuzumab for the two cell lines. We found that obinutuzumab showed a higher mean cell death compared to rituximab in one of the cell lines, JeKo-1.

Discussion

In this work, we have studied outcome after primary treatment in MCL patients, with specific focus on a novel combination to elderly patients. We have also sought to establish an *in vitro* model for exploration of how novel agents can be combined with anti-CD20 targeted therapy.

The overall aim was to evaluate prognosis and outcome in MCL in relation to clinical and molecular factors, to primary treatment and, by integrating the results with the *in vitro* assay, give implications of how outcome in MCL can be improved.

MCL was defined as a specific entity in early 1990s and is recognized as intermediate grade B cell lymphoma that does neither fit into the group of indolent lymphomas nor the aggressive B cell lymphomas. MCL has long been associated with poor prognosis, partly due to an aggressive course and lack of response to anthracyclines in contrast to other aggressive lymphomas like DLBCL.

In the era of evidence-based medicine, randomized trials has achieved a superior position, constituting the primary basis of treatment recommendations. However, in MCL, the use and application of randomized trials is limited, mainly by the low incidence rate of the disease and a lack of a golden standard, especially in the elderly population. Many previous randomized trials have been designed for including either indolent lymphomas or aggressive lymphomas, and MCL patients have constituted a small subgroup within these cohorts. Consequently, the trials are not powered to the small fraction of MCL patients, why subgroup analysis from these trials could be unreliable.

With this in mind, both population-based registry data and *in vitro* models serve as important sources for evaluation of outcome and for possible strategies to be taken into clinical trials.

Understanding the improved outcome in MCL

In paper I we showed that overall survival of patients, diagnosed with MCL in Denmark and Sweden during 2000-2011 had improved. We could define at least two important factors related to the improved outcome; the use of anti-CD20 antibody rituximab and, within the younger patient population, HD-ASCT.

Population-based research has limitations, mainly due to lack of detailed information. In our study, data on primary treatment was missing in 14% and for this group, age, one of the strongest prognostic factors for outcome and survival, was significantly lower in the group with data available compared to the group without data on treatment. Furthermore, pathological review was not made centrally and the registry did not include details on morphology, comorbidity, or data on relapse/progression and second line treatment. Consequently, one cannot exclude that other parameters may be relevant and, if included i.e. as covariates in the multivariate analyses, would change the results.

Nevertheless, we could confirm the prognostic value of age, LDH, LPK and performance status, either as individual prognostic factors, or by incorporated in MIPI, in a large cohort of patients with MCL. In our study, male gender was found to be of negative prognostic value, which has not previously been demonstrated in MCL. Females were generally older at diagnosis and presented with high stage disease to a higher extent, why univariate analysis did not reveal a strong correlation. The observation that males do worse has previously been described in DLCL. The reason for this is unrevealed but different pharmacokinetic properties between males and females have been proposed, according to a study showing different distribution and elimination of rituximab in males corresponding to outcome. [301]. Obviously, this hypothesis could be applicable in MCL but needs to be confirmed.

Two registry-based studies, published in 2014, also demonstrated a benefit of rituximab, although associated with a smaller size and more heterogeneous in terms of study time period and geographic coverage [115, 302]. A randomized trial evaluating R-CHOP vs CHOP in MCL showed increased disease control in the experimental arm but failed to demonstrate superior OS by addition of rituximab to CHOP [152]. However, a subsequent randomized trial has showed a benefit in overall survival by maintenance rituximab in patients responding to R-CHOP compared to maintenance IFNα [204].

Concerning ASCT, improved survival has been demonstrated in retrospective unselected cohorts as well as in the randomized trial [189, 303]. However, it must be noted that 97% of patients who received ASCT in our cohort, were treated according to the MCL2 protocol and in this group, all received rituximab, why it is

not possible to evaluate the independent impact of ASCT by our model. Consequently, we made an additional multivariate analysis including adjustment of individual components included in MCL2 protocol; doxorubicin, cytarabine and cyclophosphamide besides MIPI, rituximab and ASCT, but none of them showed significant impact on outcome. Another limitation in the analysis of ASCT is caused by the lack of data on ASCT by intention-to treat, with a risk of overestimation of outcome in the ASCT-treated group compared to non-ASCT treated patients due to immortality bias.

A European randomized trial (EU MCL younger), compared induction with R-CHOP/R-DHAP vs R-CHOP followed by HD-ASCT in 497 patients with MCL. At a median FU time of 6.1 years, median PFS was superior in the R-CHOP/R-DHAP arm (9.1 years) compared to the control arm (3.9 years) [122]. Interestingly, MRD negativity prior to ASCT was a strong predictor of PFS in both arms but the clinical remission status after induction and ASCT failed to predict outcome in the R-CHOP/R-DHAP arm but did so in the control arm. Furthermore, the rate of patients with molecular remission was significantly higher after R-CHOP/R-DHAP but did not improve significantly after ASCT compared to the control arm. Together, these observations bring up the question whether all patients do benefit from ASCT after immunochemotherapy induction, or if some patients, as defined by MRD negativity/CR after induction could be saved from ASCT. The ongoing three-armed European TRIANGLE (EudraCT 2014-001363-12) trial and the American two-armed randomized trial ECOG-AGRIN (EA4151) will bring further insight into the role of ASCT and if it can be replaced by maintenance therapy with rituximab +/- ibrutinib in selected patients.

What is the optimal induction for elderly patient with MCL?

According to the analysis in paper I among patients >65 years who did not receive intensified treatment, CHOP, bendamustine and chlorambucile were all found to be superior to CVP by multivariate analysis. Furthermore, our data suggested a benefit of bendamustine, as shown by longer 3-y-OS than CHOP or cytarabine. A significant correlation was not achieved in multivariate analysis, either due to a small number of cases or by influence of other factors like rituximab or MIPI.

BR was introduced in clinical practice as primary treatment for MCL when Rummel et al. reported a superior PFS of R-B in combination with a favorable toxicity profile compared to R-CHOP in the subgroup of MCL patients included in the randomized STIL trial at initial FU [300]. The non-inferiority randomized BRIGHT trial demonstrated longer PFS (HR 0.40 (0.21-0.75; P= .0035)) after R-B in the MCL

subgroup, but no difference in OS compared to R-CHOP/R-CVP [208]. This trial has not reported outcome separated by R-CHOP or R-CVP for MCL patients. Based on the observed inferior survival of CVP compared to R-CHOP and R-B in paper I, one cannot exclude a negative influence of CVP in the R-CHOP/R-CVP arm, thus overestimating the superiority of R-B. Furthermore, treatment with maintenance rituximab was equally administered between the arms in the BRIGHT trial and bendamustine was allowed as second-line treatment which might have influenced the outcome analysis.

Nevertheless, both these randomized trials demonstrate significant less toxicity in the R-B arm compared to R-CHOP with lower frequency of hematological grade 3/4 adverse events, neuropathy, nausea and alopecia, which favor its use in the older patient population but there is no evidence of a benefit of R-B in terms of PFS or OS in MCL patients.

Can LBR be recommended to elderly untreated patients with MCL?

The Nordic Lymphoma Group wanted to improve the efficacy of BR by addition of a third agent, lenalidomide, in the NLG/MCL4 trial. The most important finding from this trial was the high grade of treatment-limiting toxicity, of which the infections related to immunosuppression, the allergic reactions and the incidence of second primary malignancies are of major concern.

Is LBR a feasible regimen?

MTD of lenalidomide

The MTD of lenalidomide in our study was similar to the results of the SAKK phase I/II trial 38/08 with LBR in patients with relapsed/refractory aggressive B-cell lymphomas although they used a dose of bendamustine of 70 mg/m^2 [304]. Another phase I/II trial investigated the combination of LBR in relapsing/refractory aggressive lymphomas by escalating lenalidomide stepwise up to 20 mg before adding rituximab without any observed DLT during dose-escalating [305]. Notably, only three patients received the complete LBR regimen and all of them stopped in advance due to PD, why it is difficult to make a comparison. In our study, a dose-reduction of bendamustine did not seem to prevent the risk of high-grade toxicity, why the dose of LEN seems to be of more importance for safety control.

Hematological toxicity and infections

In MCL4, grade 3-5 infections occurred in 42% of the patients in phase I+II, including two deaths related to treatment; one pneumocystis jiroveci pneumonia and one unspecified infection with neutropenia. In spite of mandatory administration of G-CSF to all 50 patients during LBR cycles, 78% were observed with grade 3-4 neutropenia and 19 (38%) with grade ≥3 infection. Hematological toxicity/neutropenia and infections were causes for treatment discontinuation in 8 and 5 patients respectively and three patients were diagnosed with opportunistic infections, two with pneumocystis pneumonia and one patient with CMV-retinitis.

Two trials have investigated LBR in previously treated MCL, the Swiss SAKK-38/08 and the Italian trial FIL-R2-B. Both reported high frequency of grade 3/4 neutropenia, 46 % and 71% respectively, and in the Italian trial, neutropenia remained during consolidation with lenalidomide. However, they did report lower frequency of infections, 4 of 13 (31%) and 3 of 42 (7%) patients in the SAKK/38-08 and FiL trial respectively [306, 307].

Bendamustine has been shown to suppress CD4+ counts when combined with erlotinib or rituximab and occurrence of opportunistic infections have been observed, including CMV, pneumocystis jiroveci and reactivation of hepatitis B virus even in previously untreated patients [308-310] [311-313]. In MCL4, we observed three cases of opportunistic infections, two pneumocystis jiroveci, of which one with lethal outcome, and one CMV retinitis. A rate of 10% of opportunistic infections was reported in the R-B group compared to 7% in the R-CHOP/R-CVP arm in the BRIGHT trial, although using a broader definition than in MCL4. A tendency of higher incidence of grade 3/4 infections after R-B compared to R-CHOP was observed in the GALLIUM trial, originally designed for comparison of rituximab and obinutuzumab (O) in combination with B/CHOP/CVP in untreated FL. The safety analysis of 1200 patients included in the trial demonstrates that CHOP and bendamustine-treated patients showed the same frequency of grade 3/4 infections, although neutropenia was half as less frequent in the bendamustine-treated group, (>50% compared to 20-30%). Furthermore, late onset infections were more frequent in the arms treated with bendamustine compared to CHOP, either during maintenance with anti-CD20 (>10% vs <10%)or during the observational FU period 2.3-9.3% R/O-B vs 1.4-1.6% R/O-CHOP)[314].

Lenalidomide, either as single agent or in combination with rituximab, may also induce lymphocytopenia in a substantial portion of patients, as reported from trials on previously treated as well as treatment-naive patients with MCL or iNHL. [215, 286, 315, 316]. The rates of neutropenia and infections are similar in all trials; grade3/4 neutropenia in 40-50% but grade 3 infections/febrile neutropenia in <10% of patients.

In summary, the addition of lenalidomide to bendamustine and rituximab may contribute to a more profound impact on immune system which may persist after end of treatment, why adequate prophylaxis and careful surveillance is highly recommended to prevent severe infectious complications.

Allergic and cutaneous reactions

The allergic reactions that occurred in the phase I cohorts were prevented by omitting lenalidomide from cycle 1 and by addition of corticosteroids in cycle 2. None of the other trials on LBR in relapsed MCL or aggressive lymphoma has reported similar reactions, even they did not include corticosteroids to the same extent as in Lena-Berit [306, 307].

Rash was observed in 27 patients (54%) in the Lena-Berit trial, of which nine (18%) were reported as grade ≥ 3, which is higher than after R-B, reported in 18% in German STIL trial and in 14% after RBL in previously treated patients with R/R disease in the SAKK38/08 trial[207, 306].

Grade 3-4 rash was reported in 29% of untreated patients, receiving lenalidomide in combination with rituximab, but did not persist during single lenalidomide [215]. Studies on R-lenalidomide in relapsing/refractory NHL or CLL patients have reported a lower incidence of grade 3-4 rash with rates of below 10% [266, 317, 318].When lenalidomide was combined with R-CHOP in untreated DLBCL, almost no (2%) rash was observed, possibly due to the administrated corticosteroids within the CHOP regimen[319]. A higher incidence (32%) was observed when R-L was combined with bortezomib [320, 321].

The addition of bortezomib to R-B was associated with rash in 12% and allergic reactions in 10% of the patients respectively. Similarly, a high degree of cutaneous was observed when combining R-B with ibrutinib, where grade 3 rash was observed in 12 (25%) of 48 patients of which 3 patients had to stop treatment definitely [322].

Altogether, these data indicates that previously untreated patients may be more susceptible upon treatment with lenalidomide in combination with rituximab and the addition of bendamustine to R-L, as in MCL4, increases the risk even further. Although the addition of corticosteroids made the LBR regimen more tolerable, it did not prevent cutaneous reactions in the treatment-naïve patients. Moreover, the risk of rash seems to increase when other novel agents like bortezomib or ibrutinib are combined with either R-B or R-LEN.

Second primary malignancies

In the MCL4 trial, 22% of patients were reported with SPM at the updated follow-up (paper III).

Bendamustine is an alkylating agent with capacity of inducing DNA damage, thus providing a rational for the potency of inducing de novo malignant clones [323]. A retrospective analysis to investigate long-term safety of patients treated with bendamustine has been performed by Martin et al [324]. Based on data from three trials on bendamustine (+ rituximab in one trial) to R/R NHL patients, 23 of 149 patients had been diagnosed with any new cancer after trial entry at a median FU of 9 years, of which 8 were MDS/AML and 7 were reported as cause of death. Altogether, a cumulative incidence rate of 6.2% was calculated after adjustment of death of any cause.

The German STIL trials did not report a significant higher incidence of SPM in the R-B arm compared to R-CHOP [209]. Second primary malignancy was reported in 19% in the R-B arm in the BRIGHT trial, higher than 11% R-CHOP/R-CVP arm, although when excluding non-melanoma skin cancers, the difference was not significant [208].

Lenalidomide is also associated with increased risk of SPM, albeit most data is based on multiple myeloma, both from randomized trials and by comparing with age-adjusted incidence towards registry data [325-327]. The frequency in cohorts treated with lenalidomide after ASCT were 5.5-6.5% vs 1-2.5% and a standardized incidence rate in one trial without ASCT (MM 015) was 4.5% compared to SEER database. Ruan et al. reported SPMs in 6 of 38 (16%) patients receiving R-lenalidomide upfront [214].

Lenalidomide targets cereblon, involved in cell cycle regulation via p21, CDK/cyclin complex and p53 as well as via IKZF1+3 ubiquitation mechanisms [248, 328]. One can speculate whether the cellular repair systems for DNA damage induced by an alkylating agent are inhibited by lenalidomide and thereby put the cell less sensitive to stress and genetic alterations.

In our study, the median age was 70 compared to 60-64 in the previously mentioned trials, why an age-adjusted analysis would have been useful for comparison before one conclude that patients receiving this combination are associated with a higher risk for development of new cancers.

Is LBR an active regimen for untreated patients with MCL?

At a median follow-up time of 31 months, the median PFS was 42 months (CI 95% 31-53), about six months longer than the reported PFS of 35 months (CI 95% 29-55) in the R-B arm of MCL patients in the German study[207]. Although the improvement in median PFS was higher than 6 months, which was the pre-determined clinically significant improvement, the two confidence intervals are

overlapping why it still remains unclear whether there really is a benefit of adding lenalidomide to BR.

After induction with LBR, CR/CRu was achieved in 64% of evaluated patients and CRR was 62% after maintenance with lenalidomide. Thus, LBR seems to be induce a higher CRR than R-B alone, as showed by CRR 50% in the R-B arm of the BRIGHT trial in the MCL subgroup [202]. Ruan et al. reported a CRR 61% with L-R at a median follow-up time of 30 months and a 4-year PFS of 70% [214, 215]. Notably, this regimen included 12 months of L+R followed by 3 years maintenance with R+L and high-risk MCL eligible for chemotherapy were excluded from the trial, yielding a somewhat skew distribution of MIPI risk-groups in the study population with one third low-risk patients (34%) compared to the MCL4 where 10 % was scored as low risk.

Does the addition of lenalidomide overcome poor prognosis in *TP53*-mutated MCL?

Being one of the most described tumor suppressor genes, somatic alterations in *TP53* has been recognized as a poor prognosticator in MCL for at least two decades, even after the introduction of modern principles of treatment like anti-CD20 antibody, high-dose chemotherapy and ASCTHD-ASCT as shown from studies on young patients [31, 32, 96, 117, 118, 329]. To date, most molecular studies on MCL have been performed on unselected cohorts and do not include detailed data on treatment. Moreover, clinical trials seldom include molecular profiling at baseline why outcome in in relation to *TP53* alterations have not been evaluated. Accordingly, the genetic analysis of the NLG/MCL4 patients rendered a possibility to describe the genetic profile of elderly untreated patients with MCL and to evaluate the activity of lenalidomide, rituximab and bendamustine in relation to baseline genetic alterations.

Although the small number in the trial, our analysis shows that elderly patients with MCL show a similar pattern of somatic mutations/genetic alterations as previous cohorts of young ASCT-eligible Nordic patients [32].

Deletions of *TP53* and *CDKN2A* was found in 9 and 10 cases respectively, representing roughly 20% of cases. Although previous studies have shown influence on survival after intensified treatment, these alterations merely showed a trend but did not reach significance in our cohort, possibly due to a small number of cases [31]. Due to the same reason, we were not able to perform multivariate analysis to adjust for other prognostic factors, which would have increased the power of our study. Furthermore, the rate of treatment discontinuation was high in the cohort due

to toxicity. It should be emphasized that none of the five patients with *TP53* mutations stopped treatment due to toxicity and three patients entered the maintenance phase, why the poor outcome in these patients cannot be explained of reduced treatment intensity.

Bendamustine and lenalidomide have shown activity in *TP53* mutated *in vitro* models on CLL and MCL cell lines [328, 330]. Lenalidomide has shown to be active in previously responding CLL with unmutated *IGHV* or *TP53* alterations, although a relatively short FU time (1.8 years) [331]. Nevertheless, based on the results in MCL4, we could not argue for LBR as an active regimen in *TP53*-mutated patients with MCL.

Out of the results from paper I-III, one can conclude that there are two groups of patients with MCL in great need for improved primary treatment; the elderly patients and those with MCL, harboring *TP53* mutations.

What is the optimal partner for R-B and R-L?

Apart from lenalidomide, R-B has been combined with other agents in phase I/II trials on untreated patients, including cytarabine (R-BAC), bortezomib (RiBVD) and ibrutinib (RBi) (table III).

RiBVD (R-B + bortezomib 1.3 mg/m^2 day 1,4,8,11) showed response rates of 74% after 6 cycles in a study population similar to MCL4 with median age 72 years and two thirds of the patients with high-risk MIPI score [321]. A similar outcome was shown by R-BAC (R-B + cytarabine 500mg/m^2), with CRR 70%, 2-y-PFS 81% and MRD response in 62% of evaluable patients after 6 cycles [213].

The addition of ibrutinib to R-B was investigated in 48 mainly previously treated patients with aggressive B cell lymphoma and MCL. Grade ≥3 infection or febrile neutropenia were reported in 7 (14%) patients of which one patient died in ARDS and grade 3 rash was reported in 12 (25%). Of the 17 MCL (5 untreated) patients, CRR was 76% but published data does not report PFS and OS for the MCL subgroup [322]. Besides the low number of treatment-naïve patients, there was a higher proportion of low-risk MIPI (41%) in the MCL group and the untreated patients were younger (62-72 years) in comparison to our study.

In summary, LBR is active in untreated MCL and has potency of inducing molecular remission, although treatment is limited by risk of toxicity. By addition of another partner to R-B, like bortezomib or cytarabine, a higher disease control may be achieved, albeit still with some hematological toxicity. The concept of adding a BTK-inhibitor to R-B has been taken further in trials on untreated MCL patients >65, including the large phase II trial SHINE (R-B with or without ibrutinib,

NCT01776840) and ACE-LY 308, (R-B with or without acalabrutinib, NCT02972840) and outcome analysis will clarify how the R-B regimen optimally can be expanded.

Alternatively, by exclusion of the cytostatic compound, a third partner to R-L may be an option, with regard to the favorable outcome and toxicity profile, as showed with R-L [215]. One promising regimen is R-L in combination with ibrutinib, as demonstrated by Jerkeman et al. in R/R MCL patients in the NLG/MCL6 trial [289]. One trial on R-L-ibrutinib to untreated patients with MCL is registered (NCT 03232307) and the results will prove the role of this combination upfront.

How can *in vitro* models be used in design of regimens?

In paper IV, we sought to build a model for exploration of how novel agents impair the immune-mediated response to anti-CD20 antibody in MCL.

Our results, demonstrating a negative impact of ibrutinib on ADCC, have not previously been shown in MCL but confirm previous data on other NHL and CLL *in vitro* models. [237, 238]. Besides that BTK is expressed on NK cells and required for NK cell activation, off-target binding of ibrutinib to ITK may explain the reduced response to anti-CD20 antibody [236, 332]. Ibrutinib has also been associated with reduced CD20-expression *in vitro* as well as in peripheral blood and bone marrow tissue of CLL patients during treatment with ibrutinib with functional impairment of response to anti-20 antibody [333, 334]. However, the observed release of CD20-positive cells to peripheral blood and the promising outcome of combining ibrutinib with anti-CD20 targeted treatment in CLL and MCL favor this combination, although there might be room for improvement with respect to the observed interactions in our study.

One way to overcome the reduced activity of NK cells may be to minimize concomitant use of the two agents, as supported by results from a mouse lymphoma model where sequential administration of ibrutinib and CD20 antibody was superior to simultaneous exposition for the drugs in terms of anti-tumor efficacy [238]. A sequential schedule was applied in a phase I trial on R/R patients with SLL/CLL/Richter´s transformation patients and the highest ORR and PFS were observed in the group with ibrutinib prior to addition of ofatumumab. Of note, this trial was designed for reducing early infusion-related events and all patients eventually received concomitant use of the two agents [335].

We observed no influence of ibrutinib on ADCC in REC-1, the cell line in which ibrutinib was associated with cytotoxic effects on target cells at concentrations ≥ 1 μM. One possible mechanism is that a negative impact on ADCC may be sheltered

by a high direct cytotoxic effect on target cells. In that case, a possible negative impact on ADCC might not be of high relevance in overall response to the combination. However, we know that acquired ibrutinib resistance is a recurrent phenomenon, and associated with dismal outcome [336]. In case of development of a resistant clone during ibrutinib treatment, a potent ADCC, as induced by anti-CD20 targeted treatment, still may have a role to suppress clinical progression, thus indicating that both pharmacodynamics effects are of value.

Our results suggest that obinutuzumab is a stronger inducer of ADCC in MCL, as shown by increased PBMC-mediated cell death in one of the cell lines but not in the other due to wide distribution of the estimates. As mentioned previously, obinutuzumab may have activity in rituximab-refractory disease. There are no reported randomized trials comparing rituximab with obinutuzumab, primarily designed for MCL and most published clinical trials include CLL, FL or DLBCL patients. However, the GALEN study (NCT01582776) evaluating obinutuzumab and lenalidomide in untreated FL and R/R DLBCL or MCL as well as the previously mentioned OASIS trial will prove the role of obinutuzumab in combination with novel agents.

Furthermore, the sensitizing effect of lenalidomide was not sufficient to overcome the inhibitory effect on NK cell mediated cell death in our model. With respect to a benefit of adding lenalidomide to rituximab in previous *in vitro* models and the very promising outcome by the combination of LRI in the MCL6 (Philemon) trial studies on NK cell function and phenotype in patient-derived samples would bring further insight how immune-mediated response is affected in patients receiving this combination.

Our assay was designed for functional studies of NK cell activation, as measured by death of target cells, and not quantitative measurement as i.e. CD107 mobilization of NK cells. The study does have limitations, mainly by representing an isolated part of physiological mechanisms by neither including all components of immune system nor microenvironmental factors.

Further work, including more selective BTK-inhibitors and exploration of sequential administration would probably give further insight of how these agents optimally should be combined for maximal anti-tumoral activity.

Future perspectives

MRD as a prognostic and treatment-stratifying marker in MCL

As discussed in previous sections, MRD is a strong predictor of outcome after treatment with immunochemotherapy in MCL. The prognostic impact of MRD in "chemo-free" regimens has to be proved, although it is rational to expect a similar correlation. To date, the MCL6 trial has demonstrated a significant longer PFS and OS in patients with molecular remission in peripheral blood after 6 months with LRI treatment [289].

Consequently, patients with MCL will certainly benefit from a wider use of MRD. First, clinical trials on novel agents/regimens should include response evaluation of MRD as being a stronger predictor of long-term outcome compared to traditionally used response evaluation with computer tomography and less sensitive assessment of PB and BM. Next, the idea of a MRD-driven approach appears as a relevant strategy by the potency to save patients from high-dose consolidation and ASCT. Furthermore, MRD may be used as a tool for stratification of maintenance treatment, either with MRD positivity as an indicator for pre-emptive intervention to prevent a clinical relapse or by using MRD negativity as a criteria for end of treatment. A wider use of MRD-driven treatment stratification would then lead to a more economic use of treatment and thus both reduce the risk of treatment-related harm and long-term side effects in the patient in combination of an economic benefit which should not be neglected.

Few trials have incorporated these concepts. In the Nordic MCL2 trial, patients with isolated molecular relapse during follow-up after ASCT, were treated with rituximab x 4 weekly, which resulted in MRD negativity in 92% of the patients, remaining during 18 months in at least 50% of the patients [159]. The LYMA-101 trial (NCT02896582) will investigate pre-emptive treatment with obinutuzumab vs observation in MRD positive patients after two years of maintenance with obinutuzumab following immunochemotherapy with HD-ASCT. As mentioned previously, the TRIANGLE (EudraCT 2014-001363-12) is will bring further insight into if addition of novel agents can replace ASCT.

Likewise, one can speculate whether the traditional response evaluation can be supplemented or replaced by assessment of MRD. With respect to limited resources

and availability of the routine, future trials including MRD assessment could possibly define subgroups where MRD assessment could be more valuable.

Is there any hope for TP53-mutated MCL?

The presence of *TP53*-mutations constitutes one of the major challenges in treatment of MCL. So far, most work is made on retrospective cohorts and on patients receiving immunochemotherapy within clinical trials as the European Younger/Elderly and the Nordic MCL2/3. Few studies have investigated outcome in relation to specific compounds like anti-CD20 antibody, cytarabine, bendamustine or novel inhibitors in MCL patients, and there is no data on the outcome after receiving allogenic transplant.

Resistance to chemotherapy in cells harboring *TP53* mutations is a well-known phenomenon. It has been explained by loss of normal response to DNA damage, by overexpression of mutant non-functional p53 which overrides the normal p53 by forming dysfunctional tetra dimers with wild-type p53, thus exerting "dominant function". Furthermore, mutation-related acquired functions "gain of function" of the protein may contribute to proliferation and resistance (review by Oren)[337]. The DNA damage induced by traditional cytostatic compounds like antimetabolites and alkylating agents would then be less active.

Analogous to the situation in CLL, "chemo-free regimens" could be more efficient in these patients, by targeting proliferating and anti-apoptotic pathways not dependent on a functional p53. In CLL, presence of del17p or *TP53* mutations are treated differently upfront, by omitting chemotherapeutic agents in favor of a small molecule inhibitor, currently with ibrutinib as first-line [338]. Other agents with activity in *TP53*-mutated patients are idelalisib in CLL and the methylating agent azacitidine in myelodysplastic syndrome [339, 340].

Very promising results were demonstrated with rituximab, lenalidomide and rituximab, in R/R MCL patients within the Nordic MCL6 (Philemon) trial, where 6 of 11 (64%) patients with *TP53* mutated disease were in complete remission at a median FU of 18 months of which 2 of 4 were MRD negative in BM at evaluation after 12 months from start of treatment [289]. Furthermore, survival in *TP53* mutated patients was not significantly inferior to non-mutated cases.

Altogether, the *TP53*-mutated MCL cases define a specific subgroup of patients which should be treated differently from current guidelines upfront. Whether it should be a combination of immunochemotherapy and a small molecular inhibitor or a chemo-free regimen needs to be proved by trials which include determination of *TP53* and other genetic alterations at base-line. Furthermore, incorporation of data on genetic alteration in the routine diagnostic work-up will also make it

possible to evaluate outcome on a population-based level, to improve prognostic scores and, as treatment options for this group emerge, improve outcome.

Chemo-free strategies in MCL

So far, one can conclude that immunochemotherapy may induce durable remission in a substantial portion of patients even without maintenance treatment. Moreover, several novel agents regarded as non-chemotherapy are active in MCL with the potency of inducing complete remission and MRD negativity, although most data is based on patients with relapse after immunochemotherapy. Despite lack of sufficient data on biology, i.e. from randomized trials, it is possible that some patients would do better with chemotherapy than small molecule inhibitors and vice versa. As observed by the promising outcome with RLI, *TP53* mutations probably would define a group where a combination of anti-CD20 targeted therapy and small molecule inhibitors like ibrutinib is preferable. Similarly, it has been suggested that blastoid cases of MCL may need induction with cytostatic compounds, based on a proved weaker efficacy of ibrutinib and temsirolimus in these patients [110, 341].

Another important question concerns toxicity profile. The word, "chemo-free" may in many ears reflect a loss of, or a relief from something potentially problematic, but it should be emphasized that chemo-free is not equal to no side-effects. Although the risk of some adverse events can be more balanced in poly-drug regimens by substituting a part of, or all cytostatic compounds with targeted agents, these novel regimens are currently used over a longer induction period like 12 months compared to traditional induction with chemotherapy, followed by maintenance treatment during a couple of years or "until progression". To date, we know that acute-onset adverse events do occur, as for example bleeding and atrial fibrillation with BTK-inhibitors as well as long-term effects such as increased risk of SPM by use of lenalidomide. Moreover, the long-term impact on host factors, such as off-target impact on immune system, as reflected by events like rash, allergic reactions and infections needs to be clarified in order to evaluate the toxicity profile of these agents.

Among novel agents, are the CDK4/6 inhibitor palbociclib (PD0332991), a rational target in MCL with regard to the overexpression of cyclin D1. Palbociclib has shown to be tolerable in combination with ibrutinib in a phase I trial (NCT02159755) demonstrating ORR and CRR of 67% and 44% in R/R MCL previously treatment-naive to any of the agents [342].

Buparlisib, a "pan-PI3K-inhibitor" is combined with ibrutinib in a phase I trial, showing acceptable tolerability and induced response in all 6 R/R MCL included, of which 4 achieved CR [343].

In the future, more subgroups of MCL will probably be defined with distinct molecular and pathological features that, in combination with patient-specific factors like age, can be used for treatment stratification. An integrated platform of data from clinical trials, population-based registry together with translational studies will be crucial in in order to establish these subgroups.

Possibilities with *in vitro* models

The importance of optimizing combinations with novel agents cannot be enough emphasized, with respect to activity and toxicity. For this purpose, preclinical models do have a role by the possibility of defining the maximum pharmacodynamic effects on target cells and to minimize possible interactions. Further, an economic use of drug, i.e. by sequential administration may reduce the risk of severe toxicity and harm to the patients, as observed in previous trials like in MCL4 and R-idelalisib and ibrutinib, and contribute to hamper the constantly increasing expenditures of novel agents in cancer health.

The high throughput flow cytometry concept applied in our study is one example of fast and broad exploration of several agents. Among other developing models are 3D *in vitro* models which, by including micro-environmental counterparts, such as stroma cells, enable studies on migration and adhesion, angiogenesis host-response and ischemia, as reviewed by Katt et al. [344]. It may be a valuable complement for drug screening as well as studies for drug resistance as shown by models on leukemia cells [345]. Although few models on MCL are reported so far [346], it is an interesting concept, with respect to the early dissemination of malignant clones, i.e. to gastrointestinal tract, bone marrow and peripheral blood, and the occurrence or acquired resistance to cytotoxic compounds including anthracyclines, alkylating agents and small molecule inhibitors like ibrutinib.

Evolving strategies in clinical research

To date, evidence-based medicine is based on clinical trials, often distinctly separated from population-based data-sets and patients records. In practice, clinical trials, especially the randomized ones, are highly limited by being designed for a selected patient populations and evaluating single or very few interventions, such as a comparison of the addition of a novel agent to an established regimen. Moreover, they are powered for estimating average outcome within the cohort, why retrospective subgroup analysis are restricted to be descriptive. The population-based studies are thus regarded as valuable complements allowing long-term follow-up on unselected cohorts and comparison of multiple factors, albeit often being limited by lack of details on molecular biology patient characteristics, disease

course and treatment. Hence, ideas of integrating these elements have appeared to minimize the gap between clinical trials and population-based registries. One example is the registry-based clinical trial, where the randomization is based on registry data, as discussed by James et al [347].

Another important issue concerns the narrow window of conclusion which can be drawn by randomized clinical trials designed for comparison between two or three interventions on an intention-to-treat basis. Consequently, the idea of "platform trials" has been presented which may include multiple interventions in different subgroups and adaptive approaches as discussed by Berry et al [348]. Thereby, these trials aloud broader enrolment and prospective controlled observation in relation to different treatment approaches. So far, there is no registered study on MCL but the concept seems highly applicable with respect to the heterogeneity of MCL in terms of disease presentation, molecular factors and patient characteristics as well as of outcome after given treatment.

Moreover, the term "big data" has evolved, representing platforms where electronic patient's records are embedded with molecular and genetic data to be used for faster and broader output. One application in relation to this principle would be pooled analysis of the small subset of patients with high-risk disease, such as *TP53* MCL within and outside clinical trials to evaluate i.e. outcome after ibrutinib.

In summary, multiple approaches have evolved during past years, some of them contributing to improved outcome in patients with MCL and novel strategies of treatment are continuously being developed. Future work will have to define how to make the best use of established and novel approaches. To do this, an integrated approach including molecular profiling for treatment stratification is needed in prospective trials. Furthermore, networks covering clinical trial units, population-based registry data and pre-clinical platforms are required to make practice of the idea of a tailored approach to the individual patient with MCL.

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Paper I

Regular Article

LYMPHOID NEOPLASIA

Real world data on primary treatment for mantle cell lymphoma: a Nordic Lymphoma Group observational study

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Key Points

- Rituximab and autologous stem cell transplantation are both independently associated with improved overall survival in mantle cell lymphoma.
- Male gender is an independent negative prognostic factor in mantle cell lymphoma.

There is consensus that young patients with mantle cell lymphoma (MCL) should receive intensive immunochemotherapy regimens, but optimal treatment of elderly patients as well for as patients with limited or indolent disease is not defined. Our aim was to evaluate and compare outcome in relation to prognostic factors and first-line treatment in patients with MCL in a population-based data set. Data were collected from the Swedish and Danish Lymphoma Registries from the period of 2000 to 2011. A total of 1389 patients were diagnosed with MCL. During this period, age-standardized incidence MCL increased, most prominently among males. Furthermore, male gender was associated with inferior overall survival (OS) in multivariate analysis (hazard ratio [HR] = 1.36; *P* = .002). Forty-three (3.6%) patients with stage I-II disease received radiotherapy with curative intent, showing a 3-year OS of 93%. Twenty-nine (2.4%) patients followed a watch-and-wait approach and showed a 3-year OS of 79.8%. Among patients receiving systemic treatment, rituximab (n = 766; HR = 0.66; *P* = .001) and autologous stem cell transplant (n = 273; HR = 0.55; *P* = .004) were independently associated with improved OS in multivariate analysis. Hence, by a population-based approach, we were able to provide novel data on prognostic factors and primary treatment of MCL, applicable to routine clinical practice. (*Blood*. 2014;124(8):1288-1295)

Introduction

Mantle cell lymphoma (MCL) represents 3% to 10% of all lymphomas and is associated with poor prognosis due to aggressive clinical course, low sensitivity to traditionally used chemotherapy, and high relapse rates.[1]

In previous population-based series, the median age at diagnosis was 70 years, with a male/female ratio of 2.3-2.5:1.[2-4] The majority of patients are diagnosed with stage IV disease, and the clinical presentation frequently includes lymphadenopathy and extra-nodal involvement, especially of the bone marrow and gastrointestinal tract.

Although some of the patients do show a highly aggressive course with a survival of <6 months, a minority (~8%) of patients present without symptoms, follow a more indolent course, and may survive more than 10 years even without any treatment.[1] For the small portion of patients with limited stage disease, optimal treatment is still not defined.

Although recent data demonstrate that the median survival of MCL has improved during the last decade,[2] the disease is still regarded as incurable, with a reported median overall survival (OS) of 3 to 4 years. One possible approach to this is the individualization of treatment according to predicted prognosis on standard therapy. Based on data from clinical trials, a specific MCL prognostic index (MIPI) was developed,[5] the prognostic impact of which has also been confirmed in a population-based setting.[6]

However, so far, the choice of treatment in MCL has largely been based on biological age. For young and fit patients, consolidation with total body irradiation and autologous stem cell transplantation (ASCT) was shown to improve survival in comparison with maintenance therapy with interferon-α.[7] The European MCL Network also recently showed that induction with rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone (R-CHOP) alternating with a cytarabine-containing regimen, DHAP (dexamethasone, cytarabine, cisplatin), before ASCT was shown to improve response in comparison with R-CHOP alone as well as improve progression-free survival.[8] A similar regimen is the Nordic MCL2 regimen, which combines rituximab with dose-intensified CHOP and high-dose cytarabine, followed by high-dose chemotherapy and ASCT.[9] This regimen has been shown to be associated with long-term remission and possibly cure in a substantial proportion of patients, most notably among patients with low and intermediate MIPI scores.[10]

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Because the majority of MCL patients are older and unable to tolerate ASCT, it remains a challenge to find effective treatments for this group. R-CHOP in comparison with CHOP alone was associated with a higher response rate and prolonged time to failure but not OS.[11] In comparison with rituximab, fludarabine, and cyclophosphamide, R-CHOP showed higher response rates as well as superior OS, if combined with rituximab maintenance therapy.[12] In contrast, the German Study Group Indolent Lymphomas have reported results from a randomized phase III trial comparing the combination of R-bendamustine with R-CHOP. Here, R-bendamustine was associated with significantly longer progression-free survival (PFS) in combination with less toxicity.[13]

The aims of this study were to determine the efficacy of different primary chemotherapy regimens in a population-based data set of MCL patients in Sweden and Denmark, including the impact of rituximab and ASCT, in terms of OS to evaluate the therapy options for older patients and to study the incidence over time of MCL as well as the prognosis of MCL in relation to clinical prognostic factors.

Materials and methods

Swedish and Danish lymphoma registries

The study was performed within the Nordic Lymphoma Group framework based on cooperation between the Swedish and Danish Lymphoma Group and their respective population-based registries. The Swedish Cancer Registry, established in 1958, is a dual compulsory report system where all pathological findings of malignancy as well as all patients with newly diagnosed cancer are reported by the responsible pathologist and clinician, respectively. In 2000, the Swedish Lymphoma Group initiated the Swedish Lymphoma Registry, including additional information such as treatment and prognostic factors. The Danish Lymphoma Registry was initiated in 1983 and extended in 1999 to include all patients with lymphoma in Denmark. The study was approved by the regional ethics committee of Lund, Sweden and conducted in accordance with the Declaration of Helsinki.

Study population

The study population includes all patients diagnosed with MCL in Sweden between January 1, 2000 and September 11, 2011 and in Denmark between January 1, 2001 and December 31, 2010. Data were extracted from the national lymphoma registries and in Sweden supplemented by review of patients' records in cases where treatment data were missing. Data on survival status were obtained from the Swedish and the Danish Population Registry.

Statistical analysis

Survival curves were estimated according to the Kaplan Meier method and compared by log-rank tests. Hazard ratios (HRs) for the variables were calculated at both univariate and multivariate levels by Cox regression. For frequency tabulation, the Pearson χ-square and nonparametric tests were used. Values were regarded as statistically significant if $P < .05$. Statistics were performed using SPSS version 20.0. In the analysis of incidence, an additive relative survival model for the computation of P values was used.[14]

Results

Patient characteristics

Patient characteristics are shown in Table 1. A total of 1389 patients (895 from Sweden and 494 from Denmark) were diagnosed with MCL between January 1, 2000 and September 11, 2011. The median age at diagnosis was 71 years with a male/female ratio of 2.5:1. Females showed a significant higher median age at diagnosis (72 years) compared with male patients (70 years) ($P < .01$), and 71% of all patients presented with stage IV disease. Median follow-up time of surviving patients was 107 months. At the time of the analysis, 766 (55%) patients had died.

Table 1. Patient characteristics

	Total		Data on treatment available	Data on treatment not available	*P* value
Number of patients	1389		1197	192	
Median age, years	71 (28-96)		70 (28-95)	72.5 (34-96)	.011
Age, years	N (%)	3-y OS (%)	N	N	
≤65	460 (33.1)	75.7	405	55	.156
>65	929 (66.9)	64.0	792	137	
Gender					
Male	996 (71.7)	55.8	851	145	.206
Female	393 (28.3)	56.7	346	47	
Ann Arbor stage					
I	84 (6.1)	79.5	68	16	.348
II	108 (7.8)	54.7	94	14	
III	167 (12.0)	71.0	149	18	
IV	985 (70.9)	53.4	851	134	
Missing data	45 (3.2)	—	1162	182	
MIPI					
Low risk	172 (12.4)	83.8	152	20	.341
Intermediate risk	323 (23.3)	78.6	297	26	
High risk	604 (43.5)	40.4	554	50	
Missing data	290 (20.9)	—			
LDH					
Normal	769 (55.4)	66.8	665	104	.946
Elevated	564 (40.6)	44.2	487	77	
Missing data	56 (4.0)	—			
WHO performance status					
0-1	1137 (81.9)	64.1	985	152	.375
2-4	238 (17.1)	18.5	201	37	
Missing data	14 (1.0)	—			

Data on first-line therapy was available in 1197 patients (86.2%). The median age in this group was 70 years (range: 28-95), lower than in the group without data on treatment (median age 72.5 years, range: 34-96; $P = .011$). The estimated 3-year survival in the group with data on treatment was 57.8% compared with 45.4% ($P < .001$) in the group without data on treatment.

Age-standardized incidence over time

The incidence of MCL was higher in Denmark during this period, 0.93/100 000 in 2001, increasing to 1.27/100 000 in 2010. Comparative figures for Sweden were 0.57/100 000 in 2001 and 1.09/100 000 in 2010. After adjustment for gender and age, the increased relative risk for MCL in Denmark compared with Sweden was 15.5% (3.2-29.3; $P = .012$). When analyzing the incidence of MCL for males and females separately, a significantly higher relative risk was observed in females in Denmark of 32.7% (7.6-63.7; $P = .008$) compared with Sweden between 2000 and 2010, but no significant difference was observed for males (Figure 1).

The age-standardized incidence changed during the period with an increase of the relative risk of 52.9% (26.2-85.2; $P < .001$). Over time, a significant increase of the relative risk was seen among males in Denmark compared with Sweden, with an increase of the relative risk of 58.9% (26.7-99.4; $P < .001$) during the period. There was no significant change in incidence over time for females (Figure 1).

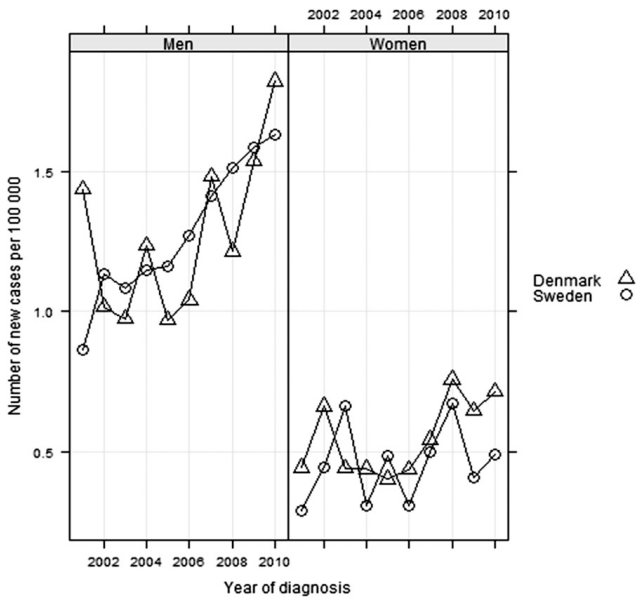


Figure 1. Age-standardized incidence of MCL in Sweden and Denmark.

Survival over time

The estimated 3-year survival for the patients diagnosed from 2000 to 2005 was 51% compared with 61% for patients diagnosed from 2006 to 2011 (Table 1). In univariate analysis, patients diagnosed from 2000 to 2005 were associated with a higher mortality with an HR of 1.3 (95% confidence interval [CI]: 1.1-1.5, $P < .01$) compared with those diagnosed from 2006 to 2011. However, when adjusting for chemotherapy regimen and rituximab, no significant difference in survival was seen between the groups.

Prognostic factors

All parameters included in MIPI (age, performance status, *S*-lactate dehydrogenase (LDH), and white blood cell count) were associated with impaired OS in univariate and multivariate analyses (Table 2). Data on Ki-67 expression were not available. Male sex was not associated with impaired OS in univariate analysis, but when adjusting for age or MIPI, male sex emerged as a negative prognostic factor.

There was no significant difference in survival between patients aged between 40 and 50 and between 50 and 60 years. For patients >60 years, a strong correlation was seen between more advanced age and inferior survival. Twelve patients (<1%) were <40 years at diagnosis. Except for one patient, all of these patients were alive at the time of analysis (supplemental Figure 3, available on the *Blood* Web site).

Treatment modalities

A total of 1066 patients were treated with systemic therapy, and 54 (4.5%) patients received radiotherapy as first-line treatment; 76 (6.3%) patients were given no therapy, of which 29 patients (2.4%) were recorded as active "watch-and-wait." OS for the different groups is shown in Figure 2.

Watch-and-wait

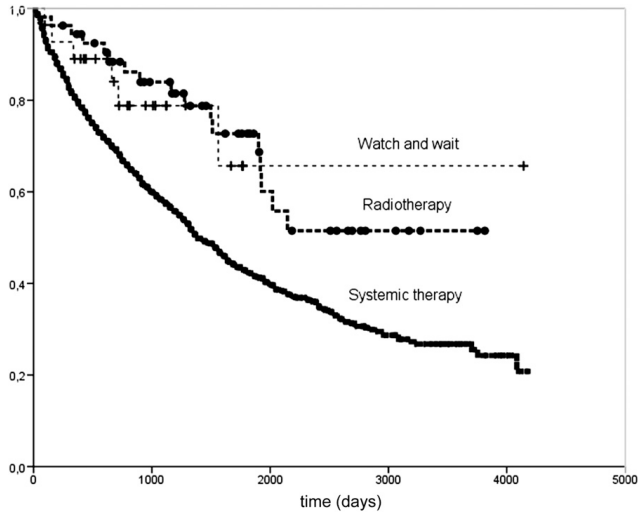
The watch-and-wait group was defined as patients without therapeutic indication for 2 years or more after diagnosis. In the Danish Lymphoma Registry, 16 patients were primarily treated as watch-and-wait, 2 patients in the years 2000 to 2005 and 14 in 2006 to 2012. The Swedish registry did not include specific data on watchful waiting, but after review of medical records, 13 patients were found with active follow-up without any treatment

Table 2. Prognostic factors in MCL

	Univariate analysis			Multivariate analysis		
Variable	**HR**	**95% CI**	***P* value**	**HR**	**95% CI**	***P* value**
Age (per year)	1.06*	1.05-1.07	<.001	1.06*	1.05-1.07	<.001
Male gender	1.04	0.89-1.22	.642	1.36*	1.12-1.64	.002
WHO performance status	1.92*	1.81-2.05	<.001	1.61*	1.47-1.76	<.001
Elevated LDH	1.93*	1.66-2.23	<.001	1.86*	1.55-2.22	<.001
Ann Arbor stage	1.26*	1.17-1.41	<.001	1.21*	1.07-1.35	.002
White blood cell count (per 1×10^9/L)	1.002*	1.001-1.003	.005	1.002*	1.001-1.004	.003

*$P < .01$.

Figure 2. OS according to type of treatment.



from the time of diagnosis until the record review in September 2012 and were classified as watch-and-wait subjects. All of these were diagnosed after 2006. The median follow-up time for these 29 patients was 29 months (3-138) and 3 year-OS was 79%. All patients, of whom 79% were older than 65 years, presented with ECOG Performance status (PS) 0-1 and stage IV disease. Twenty-three patients (79%) presented with normal LDH compared with 57% among the remaining patients ($P = .001$). Median white blood cell count was 11.0, not significantly different from 8.6 for other patients where data were available ($P = .212$).

Forty-seven (3.4%) patients did not receive any treatment due to other reasons such as comorbidities or poor performance status; 89% of these were older than 65 years, 53% presented with stage IV disease, and 58% with a PS of 2 to 4 at diagnosis. The estimated 3-year OS for this group was 21%.

Radiotherapy

Treatment intent was recorded for all patients. Forty-three patients (3.6%), all of whom presented with stage I-II disease, received radiotherapy as primary treatment with a curative intent and showed an estimated 3-year survival of 93%. Eleven patients (0.9%) were treated with radiotherapy as palliative first-line therapy, and the estimated 3-year OS for this group was 56%. Furthermore, 29 patients (2.4%) were given radiotherapy as complementary treatment to primary systemic therapy.

Systemic treatment: distribution and OS

The overall distribution of the most commonly used chemotherapy regimens is shown in Table 3. Of the patients ≤65 years, 375 patients (82%) were treated with systemic therapy, and the estimated 3-year OS for this group was 76% ($P < .001$). The majority of patients (259/404; 64%) received treatment according to the Nordic MCL2 protocol.

Of the 929 patients >65 years, 683 patients (73%) received systemic therapy, and 3-year OS for this group was 46% ($P < .001$). CHOP was the most frequently used regimen, given to 252 patients (37%), followed by chlorambucil, administered in 118 patients (17%). Sixty-five patients (8%) older than 65 years received the Nordic MCL2 (range: 28-83 years), and 20 were ≥ 70 years. In the latter group, the 3-year OS was 65%.

In both age groups, OS was highest for patients treated with the Nordic MCL2 protocol.

Analysis on the distribution of regimens over time showed that CHOP was the most frequently used combination in the first years, followed by Nordic MCL2 and chlorambucil. In later years, Nordic MCL2 emerged as the most commonly used regimen, followed by CHOP and CHOP/Cytarabine (supplemental Figure 2).

Rituximab

Data on rituximab were available for 1151 patients (82%), out of which 766 (67%) patients received this agent. The use of rituximab increased significantly between the period 2000 to 2005 and 2006 to 2011 from 52% to 77% ($P < .001$). The estimated 3-year survival was 57% in the rituximab group compared with 40% in the nonrituximab group ($P < .001$) (Figure 3A).

Rituximab showed a significant association with superior OS in univariate analysis as well as in multivariate analysis when adjusting for gender, MIPI, chemotherapy regimen, and ASCT (HR = 0.66; 95% CI: 0.51-0.85; $P = .001$) (Table 4).

High-dose chemotherapy with ASCT

Data on ASCT were available for 1143 patients (82%), out of which 273 patients (24%) underwent this procedure. The median age of the patients was 58 years (range: 28-70) and almost all (264, 97%) were treated according to the Nordic MCL2 protocol. The estimated 3-year survival was 84% compared with 50% of those who did not undergo ASCT (median age: 73 years). ASCT was associated with a significantly improved OS both in univariate analysis (HR = 0.32; 95% CI: 0.25-0.40; $P < .001$) (Figure 3B) and in multivariate analysis

Table 3. Distribution of different chemotherapy regimens and 3-y OS estimated by Cox regression analysis in MCL

	Nordic MCL2		CHOP		CHOP/cytarabine*		FC		Chlorambucil		Bendamustine		Other regimens†		Cytarabine		CVP	
N	324		310		84		43		132		51		57		30		35	
Median age, years	59		71		70		72		78		72		82		72		77	
3-y OS	79.7%		51.5%		59.5%		53.1%		39.3%		58.7%		28.4%		55.9%		22.9%	
	N (%)	3-y OS	N (%)	3-y OS	N (%)	3-y OS	N (%)	3-y OS	N (%)	3-y OS	N (%)	3-y OS	N (%)	3-y OS	N (%)	3-y OS	N (%)	3-y OS
Age, years																		
≤65	259 (79.9)	81.5	58 (18.7)	66.1	13 (15.5)	66.1	6 (14.0)	66.7	14 (10.6)	34.6	4 (7.8)	—	8 (14.3)	62.5	8 (26.7)	72.9	5 (14.3)	60.0
>65	65 (20.1)	72.0	252 (81.3)	48.1	71 (84.5)	57.8	37 (86.0)	50.9	118 (89.4)	39.7	47 (92.2)	62.3	48 (85.7)	24.5	22 (73.3)	50.8	30 (85.7)	16.7
Missing																		
WHO PS																		
0-1	297 (91.7)	82.2	257 (82.9)	58.5	71 (84.5)	65.0	33 (76.7)	66.2	97 (73.5)	47.2	44 (86.3)	59.3	44 (77.2)	39.5	22 (73.3)	66.8	24 (68.6)	25.0
2-4	26 (8.0)	52.6	52 (16.8)	15.5	13 (15.5)	35.9	10 (23.3)	10.0	30 (22.7)	14.7	7 (13.7)	57.1	13 (22.8)	—	8 (26.7)	25.0	11 (31.4)	18.2
Missing	1 (0.3)		1 (0.3)						5 (3.8)									
MIPI																		
Low	93 (28.7)	87.9	19 (6.1)	83.3	8 (9.5)	72.9	1 (2.3)	—	5 (3.8)	53.3	4 (7.8)	—	4 (7.0)	50.0	3 (10.0)	66.7	1 (2.9)	—
Intermediate	91 (28.1)	88.7	96 (30.9)	75.3	24 (28.6)	76.6	11 (25.6)	72.7	20 (15.2)	60.6	13 (25.5)	75.0	4 (7.0)	—	7 (23.3)	83.3	4 (11.4)	25.0
High	93 (28.7)	61.6	145 (46.8)	38.8	36 (42.9)	47.1	25 (58.1)	48.0	83 (62.9)	31.4	29 (56.9)	61.4	41 (71.9)	25.6	17 (56.7)	40.3	27 (77.1)	14.8
Missing	47 (14.5)		50 (16.2)		16 (19.0)		6 (13.9)		24 (18.2)		5 (9.8)		8 (14.0)		3 (10.0)		3 (8.6)	
Rituximab																		
No	0 (0)	—	96 (31.0)	36.7	3 (3.6)	33.3	9 (20.9)	40.0	109 (82.6)	40.0	6 (11.8)	—	27 (47.4)	19.6	5 (16.7)	—	13 (37.1)	23.1
Yes	324 (100)	79.7	195 (62.9)	59.4	81 (96.4)	60.5	34 (79.1)	55.9	19 (14.4)	45.0	45 (88.2)	68.2	24 (42.1)	47.2	24 (80.0)	62.3	19 (54.3)	26.3
Missing			19 (6.1)						4 (3.0)				6 (10.5)		1 (3.3)		3 (8.6)	
Years of diagnosis																		
2000-2005	117 (36.1)	81.2	181 (58.4)	49.1	21 (25.0)	47.6	19 (44.2)	73.7	77 (58.3)	31.2	0 (0)	—	32 (56.1)	25.0	8 (26.7)	50.0	13 (37.1)	23.1
2006-2011	207 (63.9)	78.8	129 (41.6)	55.4	63 (75.0)	66.4	24 55.8)	36.4	55 (41.7)	54.4	51 (100)	58.7	25 (43.9)	41.8	22 (73.3)	51.6	22 (62.9)	22.7

*In this regimen, cytarabine was given at the dose of 1 g/m^2 twice daily, days 1-2 per cycle.

†Other regimens: CVIP (cyclophosphamide, etoposide, idarubicine, prednisone), hyper-CVAD (rituximab + fractioned CHOP alternating with rituximab + high-dose methotrexate/cytarabine), "mini-BEAM" (carmustine, etoposide, cytarabine, melphalan), single rituximab, bortezomib, trofosfamide, vincristine, fludarabine, and cyclophosphamide.

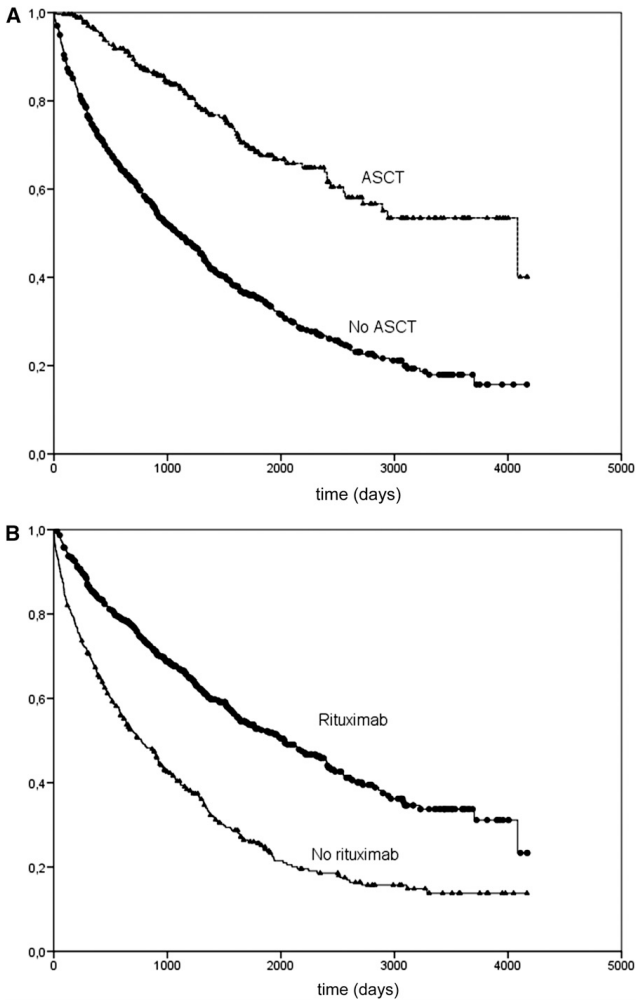


Figure 3. OS of mantle cell lymphoma in a population-based series. (A) OS with or without treatment including rituximab. (B) OS with or without treatment including ASCT.

when adjusting for chemotherapy regimen, rituximab, gender, and MIPI (HR = 0.55; 95% CI: 0.37-0.83; $P = .004$) (Table 4).

Comparison of individual regimens

When comparing the outcome of chemotherapy regimens, all patients with systemic therapy were initially included in the analysis, including adjustment for ASCT, gender, MIPI, and rituximab. Nordic MCL2 and female sex were used as reference categories. Nordic MCL was significantly superior to cyclophosphamide, vincristine, prednisone (CVP), but no other significant differences were seen (Table 4).

In a separate analysis, all regimens that did not involve high-dose chemotherapy were compared with CHOP adjusted for MIPI, gender, and rituximab. Also in this case, only CVP was found to be significantly inferior in terms of survival (HR = 2.23; 95% CI: 1.40-3.56).

CVP was then compared with chlorambucil in a separate analysis, as these regimens are frequently used in patients unable to tolerate CHOP or more intensive regimens. Of all patients, 132 received chlorambucil as first-line therapy and 32 patients were treated with CVP. Rituximab was added to 19 of the patients in each group. When adjusting for MIPI, gender, and rituximab in multivariate analysis, OS was significantly inferior in the group treated with CVP (HR = 2.34; 95% CI: 1.32-4.14; $P = .003$) (supplemental Figure 1). A

Table 4. Multivariate analysis on OS in patients receiving systemic therapy for MCL, adjusted for gender and MIPI

	HR	95% CI	*P* value
Chemotherapy regimen*			
Nordic MCL2	—	—	—
CHOP	1.080	.73-1.59	.698
CHOP/cytarabine	.900	.53-1.52	.692
FC	1.018	.61-1.70	.945
Chlorambucil	1.167	.73-1.85	.514
Bendamustine	1.032	.51-2.10	.930
Other regimens	1.613	.97-2.68	.065
Cytarabine	1.202	.62-2.33	.585
CVP	2.827	1.68-4.76	<.001
Rituximab	.660	.51-.85	.001
ASCT†	.553	.37-.83	.004

*The Nordic MCL2 protocol is used as a reference.
†High-dose chemotherapy with ASCT.

similar multivariate comparison was performed for chlorambucil and bendamustine, adjusted for MIPI, gender, and rituximab. However, chlorambucil was not significantly inferior (HR = 1.12; 95% CI: 0.45-2.8; $P = .80$).

Comparison of individual components

A multivariate analysis was performed to investigate the impact of individual regimen components. Doxorubicin, cytarabine, rituximab, and ASCT were analyzed and adjusted for MIPI and gender. Neither doxorubicin nor cytarabine showed a significant impact on survival, whereas ASCT (HR = 0.59; 95% CI: 0.42-0.82; $P = .002$) and rituximab (HR = 0.68; 95% CI: 0.54-0.85; $P = .001$) were strongly associated with improved survival.

Discussion

Treatment options of MCL have undergone a dramatic development during the last 2 decades. High-dose chemotherapy with autologous stem cell support, high-dose cytarabine, and the introduction of rituximab are important contributors to improved clinical outcome in MCL evolving it into a potentially curable disease, at least for the younger subset of patients. However, relapses do occur, and for elderly or unfit patients, optimal treatment still needs to be defined.

As this is a disease with a relatively low incidence, the use of real-world data is a valuable complement to randomized studies, enabling comparisons of outcome and long-time survival in a large number of patients.

In this series, we found an increased age-adjusted incidence for MCL in males as well as an improved OS for patients diagnosed from 2006 to 2011 compared with those who were diagnosed from 2000 to 2005. Our data confirm previous reports showing an upward trend in the incidence of MCL among men and ethnic whites during 1992 to 2009.[15]

Our results also confirm MIPI as a prognostic tool for MCL. In addition, we show that male gender is an independent negative prognostic factor, also in relation to treatment factors, including regimen, rituximab, and ASCT. In this data set, females were older at diagnosis and received ASCT to a lower extent, which explains why no significant difference was seen in univariate analysis. One possible explanation could be due to pharmacokinetics of rituximab, where a correlation between higher clearance in males and less benefit from rituximab in terms of PFS was observed in patients with diffuse large B-cell lymphoma.[16] However, the difference between males and females remains after adjustments for treatment components including rituximab and consequently needs further explanation.

Age, which is included in the MIPI score, was strongly associated with poor prognosis in patients older than 60 years. Not previously recognized, there is a small population of younger adults, <40 years, with MCL associated with an excellent prognosis, suggesting that this group constitutes a subgroup with distinct/different biological features.

The benefit from rituximab in terms of improved OS in MCL confirms the results of a previous observational study of older patients[17] but has not yet been proved in randomized studies. In this series, we found a significant association between rituximab and prolonged survival in all age groups even when adjusted for MIPI, gender, chemotherapy regimen, and ASCT. Survival in MCL has improved during recent years, and our results strongly indicate that this is related to a more frequent use of rituximab, as this difference is no longer detectable when adjusted for rituximab and chemotherapy regimen.

Among treatment components, ASCT was the factor strongest associated with improved survival independent of age. However, although we have corrected for all prognostic factors available, it cannot be excluded that patients receiving ASCT may have other favorable characteristics, including the lack of comorbidity. We could not show any significant impact on survival of any other individual components of chemotherapy regimens. However, almost all patients (97%) receiving ASCT did so as part of the Nordic MCL2 protocol, including cytarabine, rituximab, and doxorubicin.

Apart from rituximab and ASCT, we were unable to show any significant impact on survival of other components of chemotherapy regimens. This may be explained by the fact that almost all patients receiving ASCT did so as part of the Nordic MCL2 protocol, which includes both doxorubicin and cytarabine in addition to rituximab. Based on the recently presented European MCL data, all younger patients with MCL should receive these agents as part of their induction regimen pre-ASCT,[8] and our results do not contradict this.

Our results indicate that the Nordic MCL2 regimen is an effective treatment of patients with MCL even up to 70 years and that ASCT and rituximab are essential components of this regimen.[9]

For older patients, rituximab was also associated with improved OS and should be considered for all patients receiving systemic therapy. We found no major differences among therapeutic regimens, except that CVP was inferior to CHOP and chlorambucil when adjusted for rituximab and prognostic factors, indicating that this regimen is of limited value in MCL and that chlorambucil may be the preferred chemotherapy for frail patients.

MCL is a very radiosensitive malignancy. In this series, patients with low-stage disease were shown to have a favorable outcome when treated with radiotherapy with curative intent, with 9 of 10 patients surviving after 3 years. A retrospective study on radiotherapy as primary treatment, either in combination with systemic therapy or as single therapy, on stage I-II MCL was recently reported. For patients treated with curative intent, radiotherapy showed high rates of local control (95%) and high survival rates (5-year OS of 62%).[18] Our findings support that radiotherapy may be an effective treatment in localized disease, even when given without systemic therapy. However, we cannot rule out that these favorable results may partly be explained by the low tumor burden in these patients.

The use of the wait-and-watch approach was found to increase during the period of the study and was associated with a favorable 3-year OS of 79%. This increase is probably due to a higher awareness of the existence of indolent MCL.

Today, it is well established that indolent MCL exists as a specific subset with its own clinical and biological features. It is more commonly characterized by a leukemic presentation with no or limited lymphadenopathy, nonelevated LDH, and low proliferation index.[19,20] Our data confirms the important role of identifying these cases accurately to avoid overtreatment.

The strength of a population-based data set is the lack of selection bias, which is present in data from clinical trials. However, in this case, our dataset was not complete in terms of treatment data, especially prior to 2007. The missing cases constitute 14% and were significantly older and characterized by an inferior OS, although similar in terms of MIPI, indicating that there was a bias, likely excluding a population receiving less intensive or no therapy. Another limitation is the lack of pathology review, although the diagnosis of MCL is more reliable and reproducible than for other lymphomas due to the existence of specific markers [cyclin D1 and/or t(11;14)]. Furthermore, the registries do not include data on comorbidity, relapse, second-line therapy, or cause of death.

In summary, by this population-based approach, we are able to compare outcome and long-time OS on an unselected group of patients that would never be subjected to randomized trials. We could confirm that that radiotherapy is a valid option for localized MCL as well as the use of a watch-and-wait approach for nonsymptomatic MCL. In addition, we establish that both rituximab and ASCT are essential components of systemic therapy regimens in MCL associated with improved OS.

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Authorship

Contribution: M.J., P.N.B., and C.H.G. designed the study; A.A., S.B.-W., P.N.B., L.M.P., F.D., H.N.-E., P.J., M.P., and M.J. collected data; and A.A., A.A.-L., and M.J. analyzed the data and wrote the paper.

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Supplemental file

Figure 1S

Overall survival for patients with mantle cell lymphoma according to age

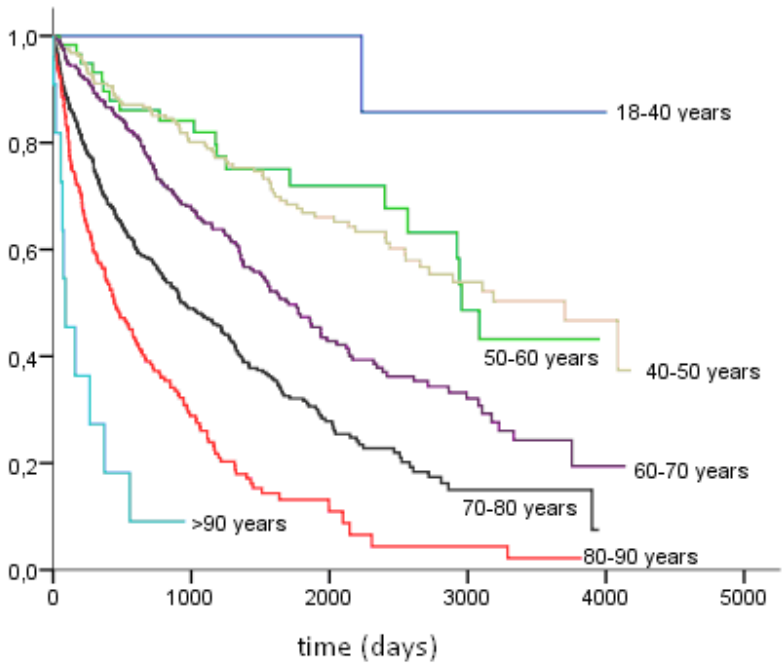


Figure 2S

Overall survival for patients treated with CVP or chlorambucil, with or without rituximab

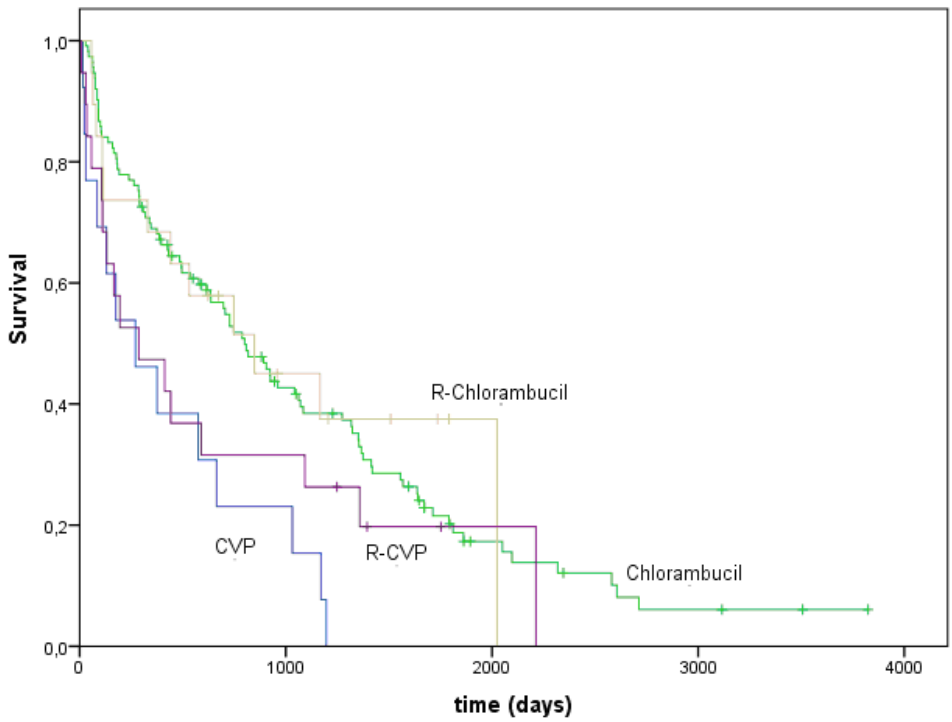
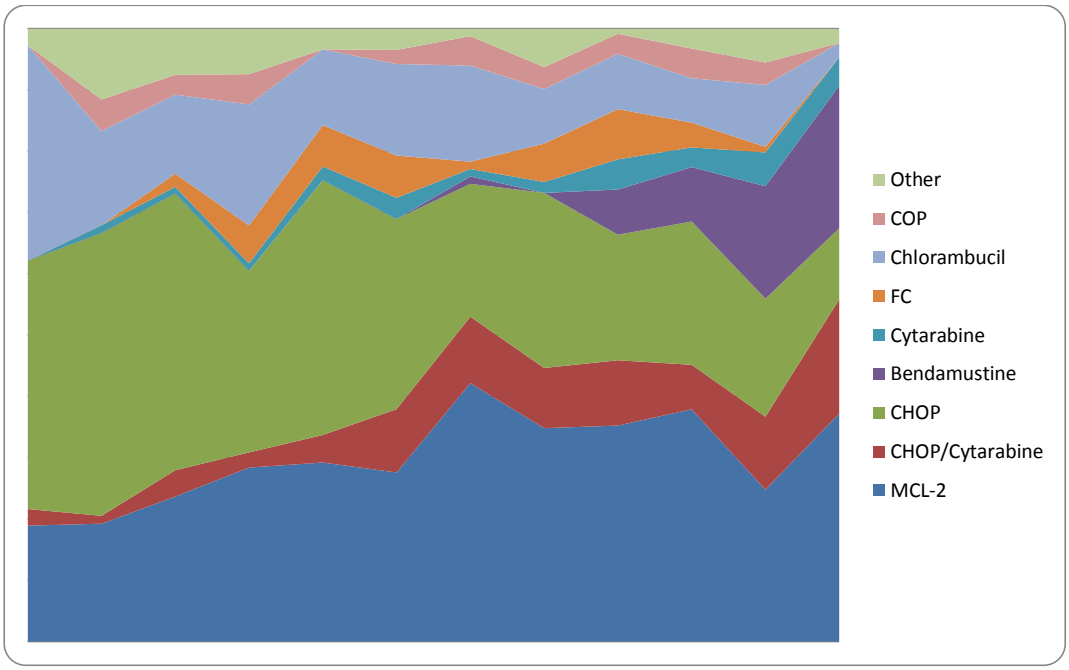


Figure 3S

Distribution of chemotherapy regimens for mantle cell lymphoma in Sweden and Denmark 2000-2011



Paper II

Regular Article

blood

CLINICAL TRIALS AND OBSERVATIONS

Lenalidomide-bendamustine-rituximab in patients older than 65 years with untreated mantle cell lymphoma

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Key Points

- Addition of lenalidomide to R-B is highly active in patients with untreated MCL, but associated with unexpected high rates of infections and SPMs.

For elderly patients with mantle cell lymphoma (MCL), there is no defined standard therapy. In this multicenter, open-label phase 1/2 trial, we evaluated the addition of lenalidomide (LEN) to rituximab-bendamustine (R-B) as first-line treatment for elderly patients with MCL. Patients >65 years with untreated MCL, stages II-IV were eligible for inclusion. Primary end points were maximally tolerable dose (MTD) of LEN and progression-free survival (PFS). Patients received 6 cycles every four weeks of L-B-R (L D1-14, B 90 mg/m^2 IV, days 1-2 and R 375 mg/m^2 IV, day 1) followed by single LEN (days 1-21, every four weeks, cycles 7-13). Fifty-one patients (median age 71 years) were enrolled from 2009 to 2013. In phase 1, the MTD of LEN was defined as 10 mg in cycles 2 through 6, and omitted in cycle 1. After 6 cycles, the complete remission rate (CRR) was 64%, and 36% were MRD negative. At a median follow-up time of 31 months, median PFS was 42 months and 3-year overall survival was 73%. Infection was the most common nonhematologic grade 3 to 5 event and occurred in 21 (42%) patients. Opportunistic infections occurred in 3 patients: 2 *Pneumocystis carinii* pneumonia and 1 cytomegalovirus retinitis. Second primary malignancies (SPM) were observed in 8 patients (16%). LEN could safely be combined with R-B when added from the second cycle in patients with MCL, and was associated with a high rate of CR and molecular remission. However, we observed a high degree of severe infections and an unexpected high number of SPMs, which may limit its use. This trial is registered at www.Clinicaltrials.gov as #NCT00963534. (*Blood*. 2016;128(14):1814-1820)

Introduction

Mantle cell lymphoma (MCL) is associated with poor prognosis, with a reported median overall survival (OS) of 5 years.[1] The MCL International Prognostic Index (MIPI), which divides patients into 3 prognostic risk groups based on the parameters of age, performance status (PS), lactate dehydrogenase level, and white blood cell count, was proposed in 2008 and has been validated retrospectively as well as in a prospective randomized study.[2-5]

Survival rates of MCL have improved during the last decade, mainly because of the addition of rituximab (R) and, for the young patient population, frontline intensive treatment including cytarabine.[1,6-9] However, for the older patients, who constitute the majority of the MCL population, there is no defined standard therapy. For this group, R-CHOP followed by rituximab maintenance was associated with prolonged survival compared with R-FC.[10] The German STiL group compared R-bendamustine (R-B) and R-CHOP in a randomized trial and concluded that R-B was associated with higher PFS and less toxicity, making this regimen preferable.[11,12] Lenalidomide (LEN), an immunomodulating agent, has shown activity in relapsed/refractory MCL as well as in first line therapy.[13-15]

Consequently, the Nordic Lymphoma Group designed a trial to investigate efficacy and safety of LEN in combination with R-B as first-line treatment of patients >65 years with MCL.

Methods

This multicenter, open-label, nonrandomized phase 1/2 study was carried out in 19 centers in Sweden, Norway, Denmark, and Finland. The study was performed in agreement with the Declaration of Helsinki and subsequent updates until 2008, and was conducted according to the guidelines for Good Clinical Practice, issued by The International Conference on Harmonization (ICH). The protocol was

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The study has been presented in part at the 3th International Conference on Malignant Lymphoma (ICML) in Lugano, Switzerland, June 2015.

The online version of this article contains a data supplement.

There is an Inside *Blood* Commentary on this article in this issue.

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approved by all national Ethical Review Boards. All patients signed a written informed consent. The study was registered at www.ClinicalTrials.gov as #NCT00963534.

Study design/objectives

The primary end points were in the phase 1 part to determine the maximally tolerable dose (MTD) for LEN in combination with R-B, and in the phase 2 expansion cohort, progression-free survival (PFS). Secondary end points included overall response rate (ORR), complete remission rate (CRR) with and without positron emission tomography (PET), molecular remission rate measured by polymerase chain reaction, OS, and safety.

Treatment

The regimen consisted of an induction phase with 6 cycles of LBR (LEN [by mouth, days 1-14], bendamustine [90 mg/m^2 IV, days 1-2], rituximab [375 mg/m^2 IV, day 1]), cycle duration 28 days, followed by a maintenance phase with single-agent LEN (by mouth, days 1-21), cycle duration 28 days, up to a maximum of 7 cycles (total duration 52 weeks).

In phase 1, the treatment plan followed a sequential dose escalation according to a 3+3 design. The initial dose of LEN in cycles 1 to 6 was 5 mg, escalated by 5 mg in each step. In cycles 7 to 13, the dose of LEN was 25 mg.

Dose-limiting toxicity (DLT) was defined as any grade 3 to 5 non-hematologic adverse event (AE) within the first 2 cycles of LBR, with the exception of thromboembolic events grade 3 to 4, nonpersisting nausea, diarrhea, elevated transaminases, or events attributed to progressive disease. A recovery to absolute neutrophil count $\geq 1.0 \times 10^9$/L and platelet count $\geq 100 \times 10^9$/L was required before the next cycle was started.

Initially, the protocol included premedication with corticosteroids before rituximab infusion exclusively in cycle 1, but after protocol amendment (discussed later), corticosteroids were administered before every rituximab infusion, and in cycle 2, all patients received oral prednisone 20 mg days 1 to 14, followed by 1 week tapering of the dose. The use of granulocyte colony-stimulating factor was mandatory in cycles 1 to 6, because the addition of LEN was expected to augment hematologic toxicity.

Antibiotic prophylaxis was not initially recommended. After the first case of *Pneumocystis carinii* pneumonia (PCP), co-trimoxazole was prescribed to all patients.

All patients received allopurinol 300 mg per day by mouth, days 1 to 3 in cycle 1, but not thereafter because of the risk of cutaneous reactions in combination with bendamustine.

Thrombosis prophylaxis was recommended to all patients during the treatment phase, unless contraindicated (aspirin 75 mg/day, or low-molecular-weight heparin to patients with a history of a thromboembolic event and/or a known hypercoagulable state).

Eligibility criteria

Patients were eligible if they were >65 years or ≤65 years but unable to tolerate high-dose chemotherapy, with a confirmed diagnosis of MCL stage II to IV and World Health Organization Performance status 0-3, requiring treatment as a result of at least one of the following symptoms: bulky disease, nodal or extra nodal mass >7 cm, B– symptoms, elevated serum lactate dehydrogenase, involvement of ≥3 nodal sites (each with a diameter >3 cm), symptomatic splenic enlargement, compressive syndrome, or pleural/peritoneal effusion. Further, patients should not have received any previous treatment (1 cycle of chemotherapy and/or radiotherapy was accepted).

Assessment during study

At baseline, all patients underwent clinical examination, collection of blood samples, bone marrow (BM) biopsies and aspirates, and computed tomography (CT) of the neck, thorax, abdomen, and pelvis. BM and peripheral blood (PB) samples were sent for MRD analyses and a formalin-fixed tissue sample was collected for central review. During treatment, patients were assessed with clinical examination before each cycle and blood samples were obtained at days 1, 7, 14, and 21, respectively.

Response evaluation was performed after 3 and 6 cycles of LBR, as well as 6 weeks (1.5 months) after completion of therapy, and included CT and BM examination including samples for MRD assessment. PET scan was recommended (not mandatory) at baseline, and after 6 and 12 months. Patients were subsequently assessed with clinical examination, labs, and CT scan every 6 months until 36 months after end of treatment.

Response was evaluated according to the International Response Criteria of 2007.[16,17] Toxicity was evaluated according to the National Cancer Institute Common Terminology Criteria for Adverse Events Version 3.0 (NCI CTCAE).

Detection of MRD was performed as previously described.[8] Briefly, DNA was extracted, sequenced, and used as a template for patient-specific primer design and standard nested polymerase chain reaction amplification of clonally rearranged immunoglobulin heavy-chain (IGHV) genes and/or Bcl-1/IGHV rearrangement (translocation 11;14).

Statistical methods

A prolongation of PFS of 6 months compared with the reported median PFS of 30 months (at time of protocol design) in the R-B arm in the German STiL group trial was considered significant.[11] Based on exponentially distributed PFS, a 95% confidence interval was calculated to 23.1 months by 40 observations, the reason the total sample size was determined as 60 patients with 20 patients in phase 1 and 40 patients in phase 2.

Progression-free survival was defined as the interval between registration date and date of documented progression, lack of response, first relapse, or death of any cause. Overall survival was defined as time from registration to death from any cause. The Kaplan-Meier method was used to estimate survival curves for PFS and OS. Comparison of frequency of adverse events in different groups was based on χ^2 tests. Analysis on the incidence of infection in relation to lymphocyte subpopulations was conducted using the Mann-Whitney *U* test. For statistical analyses, SPSS v.22 was used. All analyses were based on data collected through February 27, 2015.

Results

Fifty-one patients were enrolled between October 12, 2009 and May 22, 2013, from 13 centers in 4 Nordic countries. The accrual was slower than expected and enrollment was stopped prematurely. One patient was excluded because of screen failure and was removed from all analyses. Baseline characteristics are shown in Table 1.

Treatment

Among all patients in phase 1+2, 37 patients (74%) completed the induction (cycles 1-6) and 12 patients (24%) completed the maintenance phase (cycles 1-13). Thirty-six patients (68%) received the established MTD dose of LEN 10 mg in combination with R-B. In summary, all 50 patients received 266 cycles of L-B-R and 28 patients received 131 cycles of single LEN. The causes for treatment discontinuation were, in descending order: toxicity (n = 28 [74%], 15 during the induction phase); progressive disease (n = 6 [16%], 5 during the induction phase); second primary malignancies (n = 3 [8%]); and consent withdrawn (n = 1). Among those who stopped treatment as a result of toxicity, 2 patients received treatment outside the study with rituximab maintenance and R-B, respectively. For CONSORT diagram of phase 1+2, see supplemental Figure 1 (available on the *Blood* Web site).

Safety

Phase 1. Dose escalation and AEs including DLT are showed in supplemental Table 1. The starting dose of LEN in cohort 1 (n = 3) was 5 mg. AE grade 3 or 4 occurred in 2 patients within the first 2 cycles. One patient had infection and 1 patient had cerebral infarction after

Table 1. Patients' characteristics

Characteristic	
Age median, (range)	71 (62-84)
Male/female	37/13 (73/27)
MIPI risk group, n (%)	
Low	5 (10)
Intermediate	19 (38)
High	26 (52)
Extra nodal sites (n)	
0	9
1	24
2	10
3	3
4	3
Missing data	2
Prior treatment (1 cycle), n (%)	4 (8)
1 R-CHOP	2 (4)
1 R-Bendamustine	1 (2)
1 R-ARA-C	1 (2)
WHO performance status, n (%)	
0	25 (50)
1	22 (44)
2	3 (6)
Ann Arbor stage, n (%)	
II	2 (4)
III	4 (8)
IV	44 (88)
Median leukocyte count, ($n \times 10^9/mm^3$)	8.4 (1.7-135.9)

MIPI, Mantle Cell Lymphoma International Prognostic Index.

cycle 1 and allergic reaction after cycle 2, reported as related to rituximab. These events were not considered related to study treatment by the data monitor committee and the next 3 patients (cohort 2) received the escalated dose of 10 mg. In cohort 2, AE grade 3 occurred in 2 patients: 1 patient developed allergic reaction and infection and 1 with rash and infection, none assessed as DLT. In cohort 3, one patient was reported with DLT, urticaria grade 3, and sensory neuropathy with edema and hypotension, and the cohort was expanded to include another 3 patients. Among these, 1 patient developed hypotension grade 3, also regarded as DLT. Further, 1 patient had urticaria grade 3 and received a lower dose of LEN in the following cycle.

As described, a high number of AEs were observed in the first 3 cohorts, including high rate of allergic and cutaneous reactions, predominantly in the first cycle. Combined with DLT in cohort 3 at 15 mg, the protocol was amended to exclude LEN from cycle 1. Further, to exclude a dose-dependent impact of bendamustine, the amended protocol included a de-escalation schedule of bendamustine (B) for the 3 following cohorts ("A-C") B 90 mg/m^2 + LEN 10 mg (cohort A, n = 6), B 70 mg/m^2 + LEN 10 mg (cohort B, n = 6), and B 70 mg/m^2 + 5 mg (cohort C, n = 4), respectively. Because of hematologic toxicity, the protocol amendment also included a reduction of the dose of LEN in the maintenance part: 10 mg in the first 2 cycles after induction (cycles 7-8), and 15 mg in cycles 9 to 13. All patients received corticosteroids and PCP prophylaxis after protocol amendment.

In these 3 cohorts (A-C) of 16 patients, grade 3 AEs occurred in 3 patients during cycle 1: rash (1), pneumonia (1), and tumor lysis syndrome (1), of which the pneumonia was recorded as DLT. After cycle 2, 4 patients were reported with DLT: 3 with rash (and mucositis grade 3 in 1 patient) and 1 with sepsis grade 4. Two patients had other AEs grade 3: 1 acute coronary syndrome and 1 infection grade 3.

At this point, the assessment was made that by excluding LEN from cycle 1 and by adding corticosteroids during the L-B-R cycles, LEN could be combined with R-B and a dose reduction of bendamustine did not affect the incidence of DLT. MTD of LEN was determined to be 10 mg, given in cycles 2 to 6 in combination with bendamustine 90 mg/m^2 and rituximab 375 mg/m^2. The dose of LEN during maintenance was 10 mg in cycles 7 to 8 followed by 15 mg in cycles 9 to 13.

Adverse events. The AEs, including those previously described in the phase 1 part of the study, are summarized in Table 2. In total, 29 grade 3 to 5 infections were reported in 21 (42%) patients. The infections occurred during the induction phase in 19 patients and during the maintenance phase in 2 patients. Opportunistic infections were diagnosed in 3 patients: 1 case of fatal PCP caused acute respiratory distress syndrome during induction and 1 PCP after cycle 13, as well as 1 case of cytomegalovirus retinitis.

When comparing the incidence of AEs (grades 3-5) in the first cohorts (92 cycles) with the subsequent cohorts of 37 patients where LEN was omitted from cycle 1 (299 cycles), all allergic reactions occurred in the first 3 cohorts (n = 5). Furthermore, 4 of 12 (33%) patients in the first cohorts receiving LEN in cycle 1 were reported with severe cutaneous reactions compared with 5 of 37 (14%) patients in the subsequent cohorts. Regarding other AEs, no difference could be clearly distinguished.

Nine second primary malignancies (SPMs) were found in 8 patients (16%) during follow-up, of which 7 were invasive malignancies: 1 chronic myelomonocytic leukemia, 1 Hodgkin lymphoma, 1 renal cancer, 1 squamous epithelial cancer of the skin, 1 squamous epithelial lung cancer in a heavy smoker, 1 hepatocellular carcinoma, and 1 prostate cancer. Two patients had noninvasive malignancies: 1 with basal cell carcinoma and 1 with squamous cell carcinoma in situ and basal cell carcinoma.

Deaths during study. Twelve deaths have been reported: 6 resulting from progressive disease, 3 resulting from infection during induction (of which 1 was reported to be caused by myelosuppression), and 2 resulting from SPM (lung cancer and chronic myelomonocytic leukemia). One patient with progressive disease died without a report of the cause of death.

Response

Response data are shown in Table 3. After 6 courses of LBR, ORR was 80% based on intention to treat. Seven patients were not evaluated for the following reasons: 2 deaths, 2 patients were withdrawn from study because of toxicity, 1 patient withdrew consent, 1 patient who did not undergo CT/BM (but was in CR based on PET, not included as CR), and 1 patient who had stopped treatment after 4 cycles and was evaluated as CR, recorded at the point of 1.5 months after completed therapy. At evaluation 1.5 months after completing therapy, ORR was 64%. Complete remission/Complete remission undefined (CR/CRu) was achieved in 64% (n = 32) of all patients after 6 months of LBR and in 62% (n = 31) 1.5 months after completing therapy. PET was not mandatory in the study protocol and was only performed in a minority of patients. After induction therapy, 16 of 20 evaluable patients were in complete remission (80%) and 1.5 months after completed therapy, 7 of 8 evaluated patients were in CR (88%).

MRD. A primer for assessment of MRD could be identified in 88% (43/49) of the patients before treatment, of which 42 of 43 (97%) patients were MRD-positive in BM and/or peripheral blood (PB). At 3 months, 18 of 36 (50%) analyzed patients (36% of all patients) were MRD-negative in BM, and at 6 months, 18 of 32 (56%) analyzed patients (36% of all patients) were MRD-negative in BM. At 1.5 months after completing therapy, molecular remission was achieved in 64% (16/25) of patients in BM (32% of all patients) (Table 3).

Table 2. Summary of adverse events in phase 1+2, reported as number of patients, the highest grade per patient

	G1	G2	G3	G4	G5
Hematologic					
Anemia	29	14	2	1	
Neutropenia	4		11	27	
Thrombocytopenia	15	8	9	1	
Nonhematologic					
Infection	2	6	13	6	2
Cutaneous					
Rash	10	8	9		
Immune system disorders					
Allergic reaction	1	6	6		
Cytokine release syndrome		1			
Gastrointestinal					
Abdominal pain	1				
Abdominal distention	1				
Constipation	3	4			
Diarrhea		5	2		
Hemorrhoids/rectal bleeding	4				
Mucositis/esophagitis	2	7	3		
Nausea/vomiting	9	4	2		
Respiratory tract					
Cough	1				
Dyspnea	2	1			
Cardiac					
Acute coronary syndrome				1	
Arrhythmia/conduction disorder	1	4	1		
Neurologic/psychiatric					
Cerebral infarction				1	
Confusion			1		
Dizziness	3				
Dysgeusia		1			
Headache		3			
Neuropathy	4		1		
Syncope	1				
Insomnia	1				
Musculoskeletal					
Gout		1			
Joint effusion		1			
Musculoskeletal pain	4	5	3		
Hepatobiliary disorders					
Cholecystitis		1			
Hepatic failure			1		
Hypoalbuminemia	1	2	0		
Alkaline phosphatase elevation	2	1	1		
Aminotransferase elevation	2				
γ-GT elevation	1		1		
Vascular					
Flushing		1			
Hypotension	1	1	2		
Phlebitis		2			
Thromboembolic event		3			
Renal and urinary					
Creatinine elevation	2				
Hematuria	2				
Urinary tract obstruction		1			
Other renal and urinary symptoms	4	3	1		
General					
Anorexia	4	2	3		
Chills		4			
Edema	2	3	1		
Fatigue	8	3	2		
Fever	5	6	1		
Weight loss	2	4		1	
Weight gain		1			
Hyperglycemia	1				
Sweating	1				
Visual disturbance	1				
Dry eyes	1				
Tumor lysis syndrome			2		

Progression-free survival and overall survival

At a median follow-up time of 31 months (range, 13-59), median PFS was 42 months (95% confidence interval [CI], 31-53), median OS 53 months and 3-year OS 73% (Figure 1A-B). A separate analysis was performed on PFS and OS in relation to MIPI risk group, or age groups (≥75 years or ≥71 years, respectively) but no significant correlation could be observed. In the MIPI low-risk group, all 4 patients were alive (supplemental Figure 2A-B).

Lymphocyte populations

A significant decrease in median level of all lymphocyte subpopulations could be detected after 3 cycles compared with baseline levels except for CD8 (supplemental Table 2). Median values of CD4 count (10^9/L) was 0.6 at baseline and 0.12 after 3 months ($P < .001$) and remained below the lower reference limit until 13 months after completed therapy (Figure 2). Patients with any infection during treatment had significantly lower median CD4 counts at baseline (0.52 [interquartile range (IQR)] 0.34] compared with patients with no infections (0.77 [IQR 0.45] ($P = .037$).

Discussion

Although the survival for patients with MCL has improved, the disease is still considered incurable. Bendamustine in combination with rituximab has become a commonly used regimen in first line for elderly patients, on the basis of a favorable safety profile and noninferiority when compared with anthracycline-based regimens.[7,12,18,19] Our results show that LEN can be combined with R-B in untreated patients when omitted in the first cycle and with the addition of corticosteroids in subsequent cycles. We identified the MTD of LEN as 10 mg for 14 days in a 28-day cycle in combination with standard doses of rituximab and bendamustine. This combination was associated with a high response rate as evaluated by CT, PET, and MRD in evaluated patients, although when based on intention to treat, the response rates are clearly lower, because a high proportion were not evaluable and/or patients were not able to complete therapy.

At a median follow-up time of 31 months, the median PFS was 42 months, which is longer than the reported PFS of 35 months in the R-B arm of MCL patients in the German STiL study according to the update published in 2013.[11] In this paper, data on MIPI are not reported, but the median age of the MCL patients in the German trial was similar to our patient population. Although the difference in PFS of 7 months was the predetermined improvement that would be considered clinically significant, the 2 confidence intervals are overlapping, and consequently we cannot conclude that there is a true difference. The lower number of included patients than the precalculated sample size makes the confidence interval wider, which is why a comparison is even more difficult to make.

In our study, CR/CRu was achieved in 64% after the induction phase and in 62% after maintenance with LEN, which is higher than the

Table 3. Response rates and MRD according to CT scan and bone marrow examination

CT	3 mo	6 mo	1.5 mo after completed therapy
ORR (%)	88.0	80.0	64.0
CR/CRU	24 (48%)	32 (64%)	31 (62%)
PR	20	8	1
PD	1	3	8
Not evaluated*	5	7	10
Total	50	50	50
MRD-negativity	**3 mo**	**6 mo**	**12 mo**
BM	18 (50%)	18 (56%)	16 (64%)
PB	23 (61%)	21 (68%)	19 (80%)
Evaluated BM/PB	36/38	32/31	25/24
MRD-negativity (based on intention to treat)	**3 mo**	**6 mo**	**12 mo**
BM	18 (36%)	18 (36%)	16 (32%)
PB	23 (46%)	21 (42%)	19 (38%)
Total	50	50	50

CR, complete remission; CRu, complete remission undetermined; ORR, overall response rate; PD, progressive disease; PR, partial remission.

*Not evaluated: death of any cause, consent withdrawn, end of study because of something other than PD, end of treatment owing to any cause and not evaluated at this time point, not done of other cause/missing data.

50% CRR in the MCL subgroup of the R-B arm in the BRIGHT trial, although the latter included PET as part of the response evaluation,[18] but was inferior to the CRR of 74% achieved after 6 cycles of R-B plus bortezomib (RiBVD) in untreated patients with similar patient characteristics as in our study population, as well as to the CRR of 93% to 95%, observed with R-B in combination with cytarabine (R-BAC) in the subgroup of untreated MCL patients after 4 to 6 cycles.[20-22]

Molecular remission (MR) after combined immunochemotherapy has been defined as an independent prognostic marker for long-term remission in MCL and is associated with higher PFS in younger patients.[23,24] Our data show that 36% of evaluated patients were MRD-negative in BM after induction with LBR, suggesting that molecular remission can be achieved with this regimen. However, the MR rate in BM is lower than what has been demonstrated in elderly untreated MCL patients after R-FC/R-CHOP (67%) and with RiBVD (74%).[22,24] R-B followed by R-high dose cytarabine in young patients showed an even higher MRD negativity already after 3 courses of R-B (77%) and almost complete negativity (97%) after R-B+R-Ara-C, although, mainly because of a different age distribution, this study population was associated with a significantly more favorable prognostic profile, with 70% low-risk MIPI patients.[25] Together, these results indicate that the addition of LEN to R-B does not increase the MR rate more than has been showed with established immunochemotherapy combinations including alkylating agents, nucleoside analogs, and anthracyclines.

In the phase 1 portion of this trial, we observed an unexpectedly high degree of severe AEs, of which almost half were allergic or cutaneous reactions. By omitting LEN from cycle 1 and by adding corticosteroids in cycle 2, the allergic reactions observed in the first cohorts could be prevented and the risk of severe cutaneous reactions was diminished, although not completely eradicated.

A major concern is the high incidence of grade 3 to 5 infections (42%), which caused treatment discontinuation in 5 (10%) patients. A similar rate of infection grade 3 to 4 was observed in the SAKK trial combining LBR.[26] The incidence of severe infections is higher in our study than what has been reported with R-B alone as well as with other combinations such as RiBVD and R-BAC, which demonstrated grade 3 to 4 infections in 16% and 12% of patients, respectively.[18,20,22,27]

Recently, results from a trial on L-R in first line to MCL patients were published by Ruan et al. This regimen was associated with a lower number of high-grade AEs, including 13% grade 3 to 4 infections in combination with high response rate with a reported CRR of 61% and superior median PFS and OS not reached at 30 months. Notably, the median age of patients in our study was higher (71 vs 65) with more high-risk MIPI patients (52% vs 32%) and fewer patients with low-risk score (10% vs 34%).[15]

Rash is a common side effect of both bendamustine and LEN.[11-16,28] R-B was associated with a higher degree of cutaneous toxicity when compared with R-CHOP or R-CVP.[12,18,29,30] Concerning front-line LEN + rituximab in MCL, Ruan et al reported grade 3 to 4 rash in 29% of patients, in contrast to <10% in relapsed/refractory non-Hodgkin lymphoma.[15,29,31] In line with our results, this indicates that fewer treated patients may be more susceptible to the immunosensitizing effect of LEN, perhaps because of a more intact immune system, and that corticosteroids may be required to prevent severe reactions.

Low CD4 counts after primary treatment with R-B have previously been described.[32] Here, we demonstrate that the L-B-R regimen

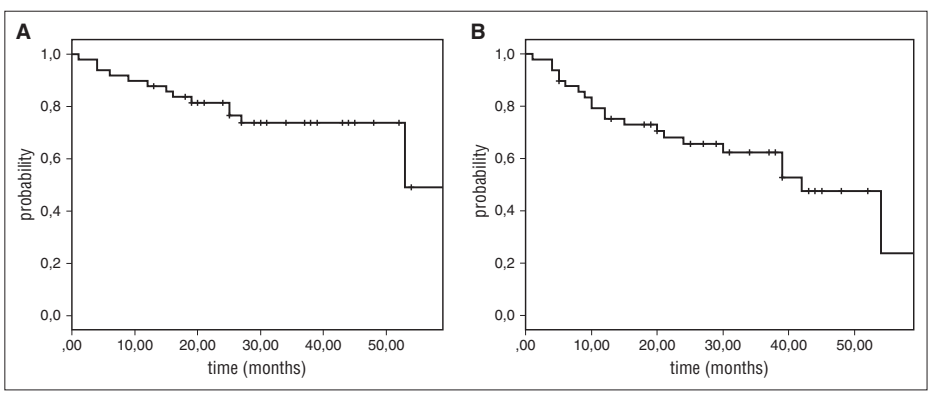

Figure 1. Overall survival and progression-free survival of patients enrolled in NLG/MCL2 (Lena-Berit) at a median follow-up time of 31 (13-59) months. (A) Overall survival; (B) progression-free survival.

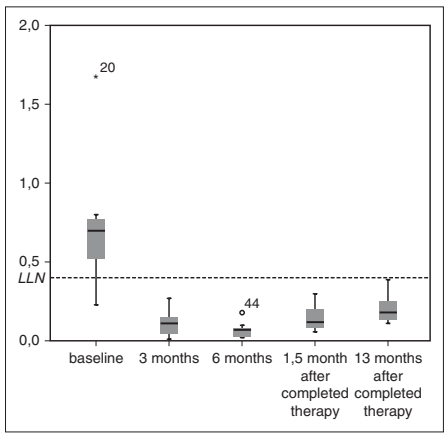

Figure 2. Boxplots of CD4-count (109/L) during treatment. LLN, lower limit of normal range.

induces a longstanding reduction of CD4 counts, which persists not only during the maintenance phase of single LEN but up to 1 year after completed treatment. Together with the incidence of opportunistic infections in 3 patients, of which 1 case of PCP occurred after 13 cycles, PCP prophylaxis is warranted when combining these agents. Possibly, the addition of prednisone during the induction may have contributed to the high incidence of opportunistic infections.

During the follow-up period, SPMs were recorded in 8 (16%) patients. A higher risk of developing SPM has previously been observed after treatment with LEN.[33] Studies on LEN/D in untreated MCL patients have reported SPMs in 5% of the patients and studies on L-R-CHOP in first-line have recorded SPMs around 5%.[34,35] These studies included somewhat younger patients at a median age of 56, 65, and 69 years, respectively, the reason age-adjusted incidence would be valuable for comparison.

In summary, the NLG/MCL4 trial shows that LEN in combination with R-B is an active regimen in untreated elderly patients with MCL and MR may be achieved but is associated with an unfavorable safety profile including a high infection rate as well as a notably high incidence of second primary malignancies. Despite the fact that all components are highly active in MCL, LEN may not be the optimal partner of R-B in untreated patients in favor of other combinations, including cytarabine or bortezomib. It is likely that the increased toxicity associated with LEN addition outweighs a possible benefit in efficacy. In this regard, nonchemotherapy combinations including LEN and rituximab, seem to be associated with a more favorable balance of activity and toxicity, and may also be given as a maintenance treatment after chemoimmunotherapy. Long-term data on these patients as well as results from ongoing trials on chemotherapy-free combinations and randomized trials will bring further insight on how to improve outcome in elderly patients with MCL.

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Authorship

Contribution: A.A.-L. collected and assembled data and wrote manuscript draft; A.K., A.L., R.R., C.G., and M.J. conceived the design and collected and assembled data; and K.G., J.S., L.B.P., E.R., M.-L.K.-L., C.S., and M.E. collected and assembled data.

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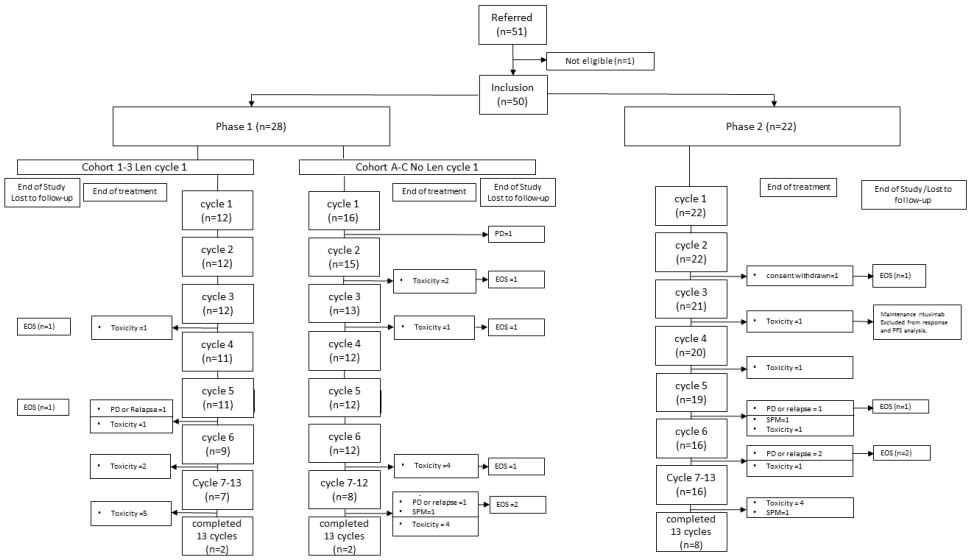
Supplemental Figure 1: CONSORT diagram for patients in phase 1+2



Supplemental Table 1

Cohort 1-3 and A-C in phase I. Adverse events and Dose-limiting toxicity

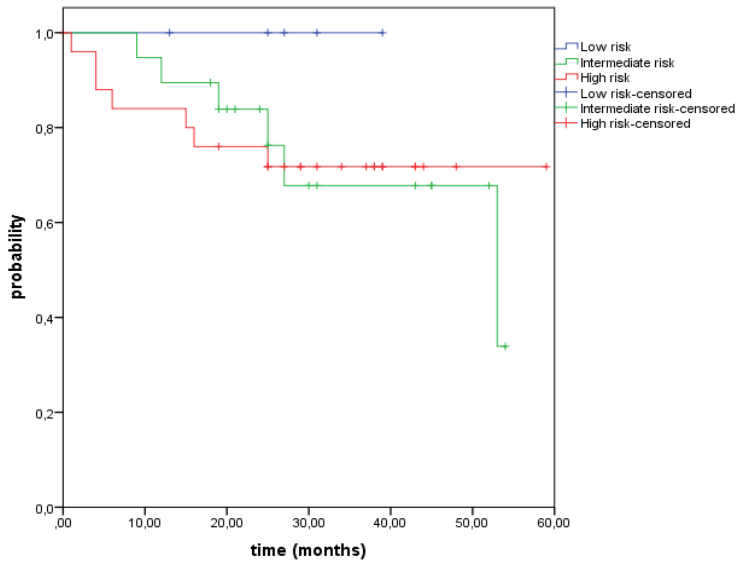
AE: adverse event, DLT (Dose-Limiting Toxicity): Any non-hematological grade 3-5 adverse event with relation to treatment according to the Data Monitoring Committee.

		Cycle 1				Cycle 2			
Cohort	**UPN**	Len(mg)	Bendamustine (mg/m^2)	Grade 3-5 AE (grade)	DLT	Len(mg)	Bendamustine (mg/m^2)	Grade 3-5 AE (grade)	DLT
1	**1**	5	90			5	90	0	
1	**2**	5	90	Cerebral infarction (4)	no DLT	5	90	Infection (3)	No DLT
1	**3**	5	90			5	90	Allergic reaction (3)*	No DLT
Cohort	**UPN**	Len(mg)	Bendamustine (mg/m^2)	Grade 3-5 AE (grade)	DLT	Len(mg)	Bendamustine (mg/m^2)	Grade 3-5 AE (grade)	
2	**4**	10	90			10	90	0	
2	**5**	10	90	Infection(3) + Allergic reaction (3)	no DLT	10	90	0	
2	**6**	10	90	Infection(3)+ Rash (3)	no DLT	10	90	0	
Cohort	**UPN**	Len(mg)	Bendamustine (mg/m^2)	Grade 3-5 AE (grade)	DLT	Len(mg)	Bendamustine (mg/m^2)	Grade 3-5 AE (grade)	
3	**7**	15	90	Urticaria(3)		10	90	0	
3	**8**	15	90			15	90	0	
3	**9**	15	90	Sensory neuropathy (3) + Hypotension (3)	DLT	0	90	0	
3	**10**	15		Allergic reaction (3) + Hypotension (3)	DLT	0		0	
3	**11**	15				15		fever (3)	no DLT
3	**12**	15				15		15	

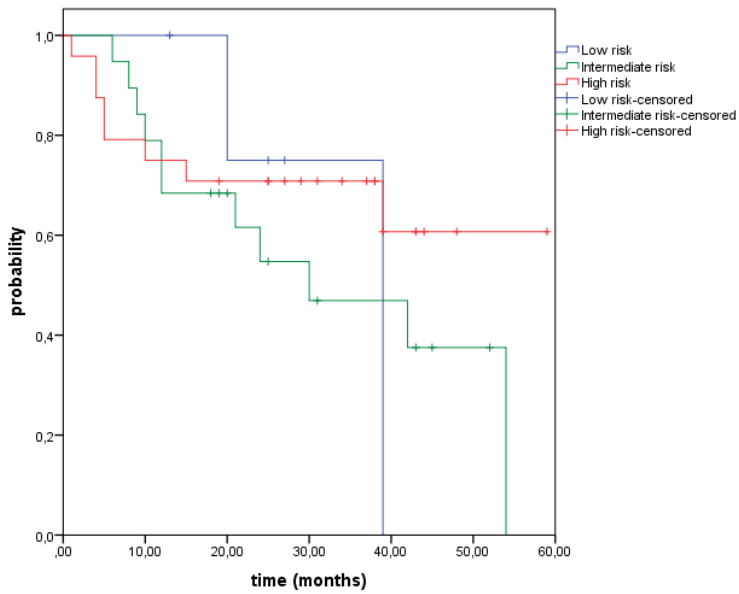
		Cycle 1				Cycle 2			
Cohort	**UPN**	Len(mg)	Bendamustine (mg/m^2)	Grade 3-5 AE (grade)	DLT	Len (mg)	Bendamustine (mg/m^2)	Grade 3-5 AE (grade)	DLT
A	**13**	0	90	0		10	90		
A	**14**	0	90	0		10	90		
A	**15**	0	90	0		10	90		
A	**16**	0	90	0		10	90		
A	**17**	0	90	rash with pruritus (3)†		10	90		
A	**18**	0	90	0		10	90		
Cohort	**UPN**	Len(mg)	Bendamustine (mg/m^2)	Grade 3-5 AE (grade)	DLT	Len (mg)	Bendamustine (mg/m^2)	Grade 3-5 AE (grade)	DLT
B	**19**	0	70	tumor lysis syndrome (3)		10	70	Acute coronary syndrome + atrial fibrillation (4)	no DLT
B	**20**	0	70			10	70	rash with pruritus (3)	DLT
B	**21**	0	70			10	70		
B	**22**	0	70			10	70		
B	**23**	0	70			10	70	rash (3)	DLT
B	**24**	0	70			10	70	rash(3)+ mucositis (3)	DLT
Cohort	**UPN**	Len(mg)	Bendamustine (mg/m^2)	Grade 3-5 AE (grade)	DLT	Len (mg)	Bendamustine (mg/m^2)	Grade 3-5 AE (grade)	DLT
C	**25**	0	70			5	70	infection (3)	no DLT
C	**26**	0	70			5	70		
C	**27**	0	70	infection (3)		5	70	infection (4)	DLT
C	**28**	0	70	PD (End of study)		0	0		

*Assessed related to rituximab.
†remaining in cycle 2

Supplemental Figure 2 a): Overall survival according to MIPI risk group



Supplemental Figure 2 b): Progression-free survival according to MIPI risk group



Supplemental Table 2

Median levels (IQR) of lymphocyte subpopulations and immunoglobulins

CD: cluster of differentiation, IQR: interquartile range.

Reference values: CD counts (10^9/L): CD3: 0,55-2,0 (58 – 82%); CD3+/CD4: 0,37-1,45 (32 – 59%); CD3+/CD8+:0,12-1,07 (12 – 44%); CD19: 0,06-0,52 (5,9 – 21%); CD16+CD56: 0,02-0,55 (2,4 – 22%); CD4/CD8 ratio: 0,84-3,8; Immunoglobulins (g/L) IgG: 6,7 - 14,5; IgA: 0,88 - 4,5; IgM: 0,27 – 2,10

CD counts (10^9/L)	Baseline	3 months	6 months	1.5 months after completed therapy	13 months after completed therapy
CD3+	1.14 (0.76)	0.43 (0.71)	0.37 (0.41)	0.42 (0.56)	0.51 (0.64)
CD3+/CD4+	0.60 (0.34)	0.12 (0.09)	0.08 (0.06)	0.11 (0.07)	0.17 (0.14)
CD3+/CD8 +	0.54 (0.63)	0.31 (0.90)	0.26 (0.36)	0.26 (0.44)	0.35 (0.25)
CD19+	0.58 (4.09)	0.00 (0.00)	0.00 (0.00)	0.04 (0.07)	0.19 (0.22)
CD16+/CD56+	0.26 (0.39)	0.11 (0.14)	0.10 (0.11)	0.15 (0.12)	0.23 (0.21)
CD4/CD8 ratio	1.08 (1.05)	0.30 (0.43)	0.34 (0.53)	0.40 (0.49)	0.60 (0.52)
Immunoglobulins (g/L)					
IgG	11.2 (5.60)	7.80 (5.98)	8.30 (5.70)	8.06 (5.00)	9.60 (6.00)
IgA	2.08 (2.40)	0.98 (1.38)	0.90 (1.20)	1.00 (1.44)	1.58 (1.20)
IgM	0.85 (1.20)	0.29 (0.39)	0.28 (0.24)	0.40 (0.33)	0.60 (0.37)

Paper III

Lenalidomide plus bendamustine-rituximab does not overcome the adverse impact of *TP53* mutations in mantle cell lymphoma

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Summary

Novel treatment strategies are needed for patients with *TP53*-mutated mantle cell lymphoma. We explored the outcome of 46 patients from the Nordic MCL4 trial, (bendamustin-rituximab and lenalidomide) in relation to genetic aberrations. After a median FU of 45 months, *TP53* mutations were significantly associated with shorter median OS (25 vs 69 months, $p<0.0001$) and PFS (10 vs 42 months, $p=0.001$). None of *TP53*-mutated patients achieved MRD negativity. The results confirm the poor prognostic impact of *TP53* mutations, despite the addition of lenalidomide to bendamustine-rituximab. Trials evaluating novel agents in relation to *TP53*-status are warranted to improve outcome in these patients.

Manuscript

Introduction

The outcome of mantle cell lymphoma (MCL) has been improved markedly during the past decades; however, the course of the disease remains highly heterogeneous.(Eskelund et al, 2016; Hermine et al, 2016; Abrahamsson et al, 2014) Several biomarkers have been proposed to stratify patients at diagnosis, i.e. morphologic subtype, proliferation index (Ki67) and the MCL international prognostic index (MIPI), but so far none have been systematically implemented in treatment stratification.(Hoster et al, 2016; Dreyling et al, 2014)

TP53 aberrations are associated with more aggressive disease and poorer outcome.(Delfau-Larue et al, 2015; Greiner et al, 2006; Halldórsdóttir et al, 2011) In a recently published study by the Nordic lymphoma group (NLG) of younger patients receiving intensive, cytarabine-containing therapy and autologous stem-cell transplantation (ASCT), we showed that *TP53* mutations signified a subgroup of patients with exceedingly poor outcome, overruling all other known prognostic markers.(Eskelund et al, 2017) In addition, Aukema et al recently reported similar findings based on p53 protein expression by immunohistochemistry.(Aukema et al, 2017) Thus, alternative therapeutic strategies are highly warranted in this subset of patients.

In CLL, lenalidomide has shown promising response rates in high-risk patients, including patients with *TP53*-aberrations.(Fink et al, 2017) The Nordic MCL4 trial investigated the additive effect of lenalidomide to bendamustin-rituximab (LBR) in elderly/frail patients.(Albertsson-Lindblad et al, 2016) In general, this regimen was associated with an unexpected high frequency of toxic events, especially infections,

cutaneous events and secondary malignancies; however, the patient cohort may serve to investigate the efficacy of lenalidomide plus chemo-immunotherapy in *TP53*-mutated MCL patients.

With this study we show that *TP53* mutation retain poor prognostic impact even after addition of lenalidomide to bendamustin-rituximab.

Methods

Treatment: Fifty patients were treated in the MCL4 trial (Lena-Berit), which included untreated patients >65 years or ≤65 years considered unfit for high-dose chemotherapy. An induction phase (weeks 1-24) of six cycles of LBR was followed by a maintenance phase of lenalidomide (weeks 25-56).(Albertsson-Lindblad et al, 2016) (Figure S1, supplementary data)

Patient samples: Pre-treatment DNA samples (39 bone marrow (BM) and 7 peripheral blood (PB)) were selected by availability. MCL was detected in all samples by either flow cytometry or by a positive MRD marker. (Supplementary data)

Genetic analyses: Mutational analysis with Targeted NGS was performed of eight MCL-related genes: *ATM, KMT2D, CCND1, TP53, WHSC1, BIRC3, NOTCH1* and *NOTCH2*. Median coverage was 3100X and cut-off for calling a variant was 5% in general, and 3% for *TP53*, as described previously.(Eskelund et al, 2017) Droplet digital polymerase chain reaction (ddPCR) was used to identify two commonly deleted regions, chr17p13 (*TP53*) and chr9p21 (*CDKN2A*), and cut-off for calling a deletion was set to copy number (CN)<1,95, likewise described previously. (Supplementary data)

Statistics: Overall survival (OS), progression-free survival (PFS) and cumulative incidence of relapses/progression (CIR) were used as patient- and disease-specific endpoints, all with starting point at date of trial inclusion. OS was measured until date of death of any cause, PFS until date of documented relapse/progression or death of any cause, and CIR until date of documented relapse/progression while MCL-unrelated deaths censored. (Supplementary data)

Results

The aim of this present study was to investigate the efficacy of a lenalidomide-containing regimen on *TP53* mutated MCL in an updated version of the Nordic MCL4 trial.

Patient characteristics and survival

Fifty patients, >65 years or ≤65 years and unfit for ASCT, were enrolled between 2009 and 2013.(Albertsson-Lindblad et al, 2016) Patient characteristics are shown in Table S1. After a median follow-up of 45 months (range 1-96 months) (31 months in our previous report), median OS and PFS were 69 months (95% CI 60.4-77.5; events=23) and 42 months (95% CI 28.5-55.5; events=30), respectively (Fig 1A-B). Median time to progression/relapse was 53 months (95% CI 34.1-71.9; events=24) (Fig 1C). None of the curves showed any sign of a plateau. At the current update, three additional cases of second primary malignancies (SPM, non-invasive skin cancers excluded) have been reported, making the total number of patients with SPM 9 (18%) (2 prostate cancers, 1 Hodgkin's lymphoma, 1 acute myeloid leukemia, 1 chronic myelomonocytic leukemia, 1 hepatocellular cancer, 1 squamous cell lung cancer, 1 endometrial cancer and 1 invasive non-melanoma skin cancer).

Genetic aberrations

Baseline DNA samples were available for 46 out of the 50 patients included in the trial (39 BM and 7 PB samples). Two samples did not reach sufficient quality for sequencing, and were only included in the deletion analyses. *TP53* deletions were detected in 9 (20%) patients and *CDKN2A* deletions in 10 (22%) patients. Five (11%) patients harboured both deletions. The most frequently mutated genes were *ATM*, detected in 15 (34%) patients, *KMT2D* in 8 (18%) and *TP53* in 6 (14%) patients (Fig 2, table S2). We detected >0 genetic aberration in 28 (64%) patients, and >1 (2-4) aberration was detected in 14 (32%).

Genetic aberrations' impact on outcome

Median OS for the *TP53* mutated and unmutated patients were 25 months (95% CI: 6.6-43.4) and 69 months (95% CI: 67.0-70.7), respectively ($p<0.0001$), median PFS were 10 months (95% CI: 0-22.9) and 42 months (95% CI: 21.8-62.2), respectively ($p=0.001$), and median CIR were 10 months (95% CI: 0-22.9) and 58 months (95% CI: 35.7-80.3), respectively ($p<0.0001$) (Fig 1D-F). One of the *TP53* mutated patients withdrew consent at day 28 and did not provide permission for further follow-up and was hence censored at this time point. Of the remaining five patients, three discontinued treatment due to progressive disease (PD) while still on therapy. Two withdrew due to adverse events (AE) after receiving 7 and 11 cycles of lenalidomide, respectively (of the planned 13 cycles); however, both were MRD positive in BM and PB at time of withdrawal, and one experienced PD only one month after discontinuation. All *TP53* mutated patients had available MRD markers, but none achieved MRD negativity in both BM and PB at any time during follow-up evaluation.

Deletions of *TP53* and *CDKN2A* both showed trends towards inferior outcomes (Figure S2). None of the other mutations showed any impact on outcome in our analyses (data not shown).

A total of 12 (27%) patients had a mutation and/or deletion of *TP53*, and they displayed significantly poorer outcome, with a median OS of 25 months (95% CI: 0-57.4, p=0.065), PFS of 12 months (95% CI: 6.6-17, p=0.016) and 50% of the patients had progressed/relapsed at 34 months (95% CI: 0.2-67, p=0.031) (Fig 1G-I).

Discussion

Collectively, despite the small cohort size, we show that *TP53* mutations retain very poor prognostic value despite the addition of lenalidomide to chemo-immunotherapy. Our findings are in contrast to preclinical models on lenalidomide, showing activity in CLL cell lines, independent of functional status of p53.(Fecteau et al, 2017) Furthermore, a clinical study in CLL has suggested activity of lenalidomide maintenance in *TP53*-aberrated (mutations AND deletions) patients, albeit only reported for PFS and not OS so far.(Fink et al, 2017) Ruan et al showed promising response rates of L-R in MCL; however, they did not include data on *TP53* status.(Ruan et al, 2015)

A limitation to our study is the high number of treatment terminations related to toxicity. However, among the five *TP53* mutated patients available for follow-up, only two patients withdrew due to adverse events (after receiving 7 and 11 cycles of lenalidomide, respectively, and while still being MRD positive), whereas the other three patients withdrew due to PD. Thus, we believe that our results do reflect the actual lack of efficacy of lenalidomide in these patients. Obviously, another draw-back is the small cohort size, and thus the results will need validation in a larger cohort. Nonetheless, the results still heavily argues against lenalidomide as the solution to the adverse impact of *TP53* mutations.

Interestingly, the only *TP53* mutated patient who had a long-lasting response (41 moths) harboured a splice-site mutation which is rare for *TP53* (2,4% according to the IARC *TP53* Database).(Bouaoun et al, 2016) This sort of mutation of possibly larger structural effect may cause only loss-of-function effect, rather than dominant negative and oncogenic effects.(Vries et al, 2001)

Thus, the combined results are similar to the findings in our recent report on younger MCL patients (Eskelund et al, 2017) both in terms of prevalence and impact of *TP53* mutations on survival. The two deleted regions showed significance in univariable models in our previous report, but only borderline significance in this present study. Most likely, this is only a reflection of the smaller patient cohort of this study, rather than a diminished biologic effect.

In conclusion, our study shows that the addition of lenalidomide to rituximab-bendamustine does not overcome the negative impact of *TP53* mutations. Thus, *TP53* mutated MCL remains a major challenge, and our results underline the importance of molecular profiling, including *TP53* status, in future trials exploring novel agents.

Acknowledgements

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Authorship

Contribution: CWE performed genetic analyses. AA-L collected and analysed the clinical data. CWE, AA-L, MJ and KG designed the genetic study and wrote the manuscript draft. AK, AL, RR, CHG and MJ conceived the clinical design. LBP purified DNA and performed MRD analyses. All co-authors critically read and approved the final version of the manuscript.

Conflict-of-interest: KG is on Celgene and Janssen advisory boards. MJ has received grants, personal fees and non-financial support from Janssen, grants and non-financial support from Celgene, during the conduct of the study; grants and non-financial support from Abbvie, grants, personal fees and non-financial support from Gilead, outside the submitted work. CG has received personal fees from Janssen. AK has received grants and personal fees from Nordic Nanovector, and grants from Roche and Merck. None of the other co-authors has any conflicts of interest to report.

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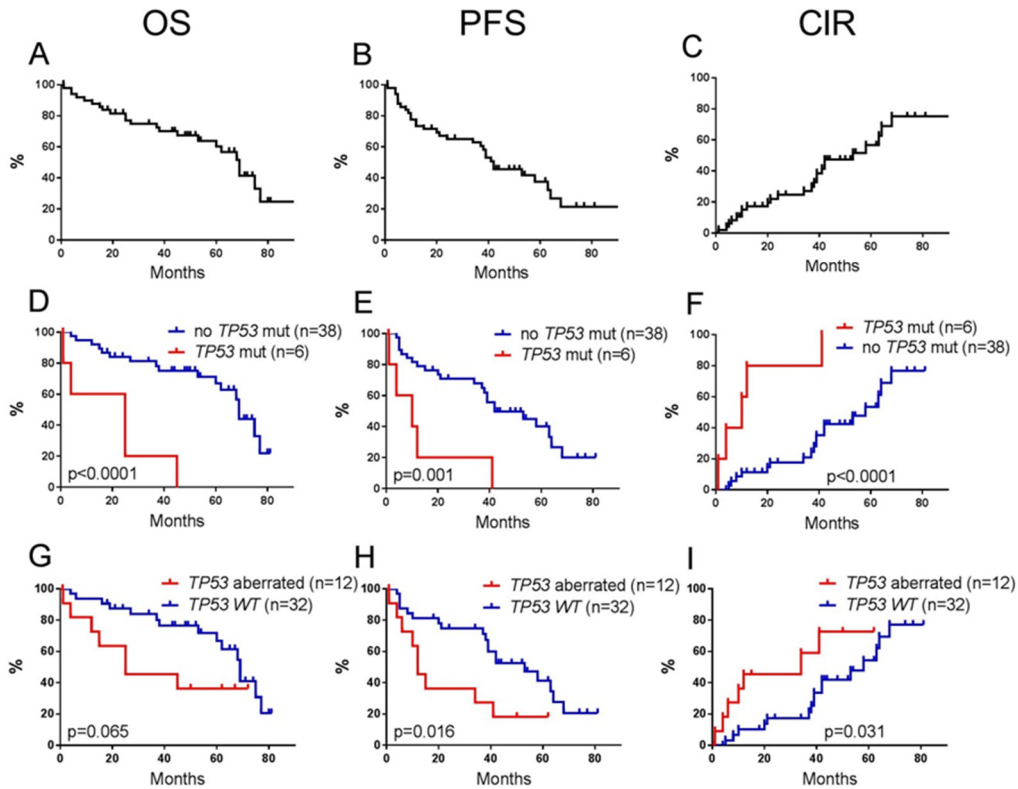
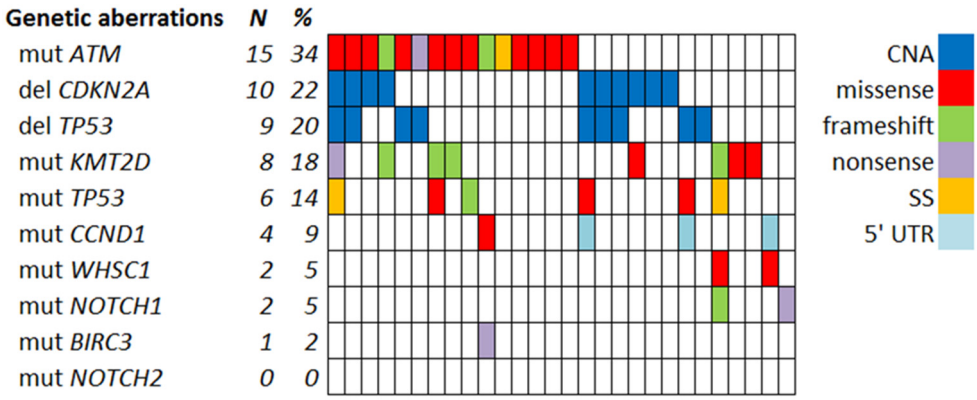
Figure 1: Kaplan-Meier estimates patients in the MCL4 trial.



Kaplan-Meier plots for (A-C) all patients by intention-to-treat (n=50), (D-F) all patients with available DNA according to presence or absence of TP53 mutations, and (G-I) all patients with available DNA according to TP53 aberrations (mutations and deletions) or TP53 wildtype (WT). OS=overall survival, PFS=progression-free survival, CIR=cumulative incidence of relapsing or progressive disease.

Figure 2: Overview of genetic aberrations



Overview of genetic landscape for all patients with detected genetic aberrations. Each row represents a gene, and each column represents a patient. Colour coding: Dark blue: Copy number alteration, (CNA); Red: Missense mutations; Green: Frameshift indels; Violet: Nonsense mutations; Orange: Splice-site mutations; Light blue: Mutations in the 5' untranslated region (UTR).

Supplementary material

Methods

Patients

Patients older than 65 years, or ≤65 years and considered unfit for high-dose chemotherapy, with previously untreated, stage II-IV, histologically confirmed, diagnosis of MCL were included in the Nordic Lymphoma group phase I/II trial MCL4 (#NCT00963534).1 Treatment consisted of an induction phase with six cycles of LBR (lenalidomide [days 1-14, cycles 1-6], bendamustine [90 mg/m2 IV, days 1-2], rituximab [375 mg/m2 IV, day 1]), cycle duration 28 days, followed by a maintenance phase with single-agent lenalidomide ([days 1-21], cycle 7-13, cycle duration 28 days). In the early phase I portion (after 12 patients included), the protocol was amended due to unexpected high portion of treatment-related toxicity. Lenalidomide was omitted from cycle 1 and included in cycles 2-6. Details on the regimen are found in supplement figure 1.

The diagnosis of MCL was confirmed by central pathology/histology review board according to WHO criteria by detection of t(11;14) or overexpression of cyclin D1.

The study was performed in agreement with the Declaration of Helsinki and was conducted according to the guidelines for Good Clinical Practice, issued by The International Conference on Harmonization (ICH). The protocol was approved by all national Ethical Review Boards. All patients signed a written informed consent to participate and to donate/provide samples from peripheral blood, bone marrow and tissue for biologic studies. The study was registered at www.ClinicalTrials.gov as #NCT00963534.

Patient samples

Bone marrow (BM) and peripheral blood (PB) samples were collected centrally for MRD measurements, and DNA was purified from unsorted specimens by Qiaprep Miniprep (Qiagen, Valencia, CA). Inclusion criteria in this study were available pre-treatment BM or PB sample with measurable MCL by flow cytometry or positive minimal residual disease (MRD) marker. BM samples were available from 39 patients, and PB samples from another 7 patients. Two of the PB samples did not reach sufficient quality for next generation sequencing (NGS) analyses, and were thus only included in deletion analyses, both described below.

Mutational analysis with Next Generation Sequencing

Targeted NGS was performed of selected coding regions, splice sites and untranslated regions (UTRs) of eight recurrently mutated genes in MCL: ATM,

KMT2D, CCND1, TP53, WHSC1, BIRC3, NOTCH1 and NOTCH2, as previously described.2 Libraries were constructed based on the Ion Ampliseq technology (Thermo Fischer Scientific, Waltham, MA), and quantitative polymerase chain reaction (qPCR) measurements performed using the TaqMan Ion library quantification kit. Template preparation was carried out on the Ion Chef instrument and sequencing was performed on the Ion PGM System, using Hi-Q view technology and reagents. All steps were carried out according to manufacturer's instructions, and reagents and equipment were manufactured by ThermoFisher Scientific. Median coverage of all runs was >3000X.

Cut-off for calling a variant was variant allele frequency (VAF) of ≥5% and coverage of ≥400X. For TP53, the lower limit for calling a variant was 3%, as described previously.2 Variants were carefully reviewed in the IGV software (Broad Institute). All known common single nucleotide polymorphisms (SNPs) (>1% in the SNP database, dbSNP) were excluded prior to analyses, and only variants giving rise to amino acid changes were reported, unless in splice sites or UTR regions. Variants with a VAF 40-60% and a SNP database (dbSNP) reference were considered rare SNPs and excluded. If both dbSNP and COSMIC references were present, the variant was reported here, including both references (supplemental table 2).

Deletion analysis by Droplet Digital PCR

Deletion analyses for the TP53 gene and CDKN2A locus were performed by Droplet Digital PCR (ddPCR) using the QX200 system (Bio-Rad Laboratories, Hercules, CA). RPP30 was used as a reference gene. All samples were run at least twice. QuantaSoft software was used for data analyses, and copy number (CN) below 1.95 was interpreted as a deletion, as previously described.2

Statistics

Overall survival (OS), progression-free survival (PFS) and cumulative incidence of relapses or progression (CIR) were used as patient and disease-specific endpoints with starting point at date of inclusion in the trial. OS was measured until date of death of any cause, PFS until date of documented progression, lack of response, first relapse, or death of any cause and CIR until date of documented relapsing or progressive disease. The Kaplan-Meier method was used to estimate survival curves for PFS, OS and CIR and subgroup analyses by specific gene alterations or mutations were compared by log-rank test. Analyses on adverse events (grade 3-5 infections, cutaneous reactions and incidence of SPM) in relation to presence of specific gene alterations or mutations were made by using Fisher's exact t-test. All analyses were made by using SPSS v.22.

Supplementary table I. patient varavhteristics

	n patients (%)
Male/Female	37/13
Median age (range)	71 (62-84)
Ann Arbor stage	
II	2 (4%)
III	4 (8%)
IV	44 (88%)
Performance score	
0	25 (50%)
1	22 (44%)
2	3 (6%)
Elevated LDH	31 (62%)
LPK, median ($x10^9/mm^3$)	8.4 (1.7-136.9)
Bone marrow involvement	44 (88%)
MIPI	
median (range)	6,3 (5,2-7,5)
Low	5 (10)
Intermediate	19 (38)
High	26 (52)
Morphological subtype	
Classic*	39 (76%)
Blastoid	2 (4%)
Ki-67%**	
<30%	29 (58%)
≥ 30%	9 (18%)
Prior treatment (1 cycle)	4 (8%)
R-CHOP	2 (4%)
R-Bendamustine	1 (2%)
R-Ara-C	1 (2%)
Clinical outcome	median (95% CI)
Overall survival	69 (58-50)
Progression-free survival	42(29-55)
Cumulative incidence of relapse	53 (34-72)

* Diffuse, nodal or mantle zone growth pattern

** MIB-1 in a few cases, values converted 1:

Supplemental figure 1:

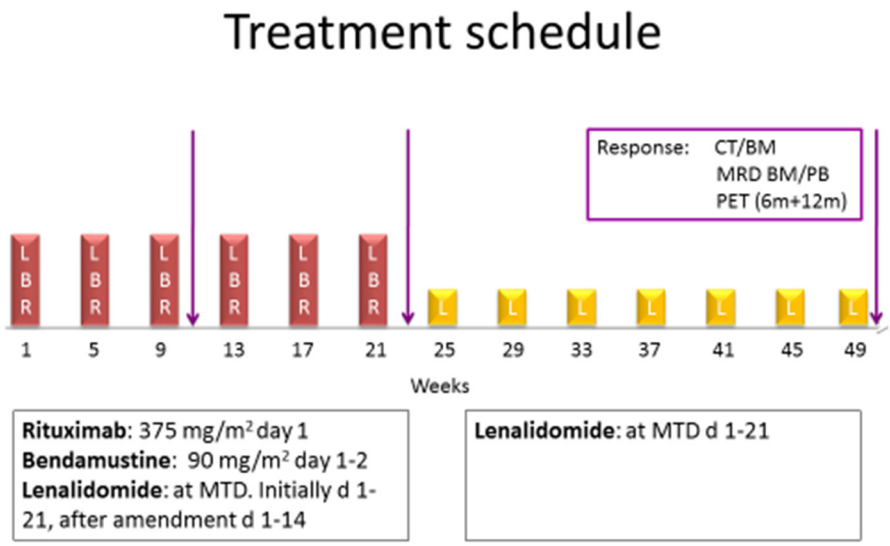


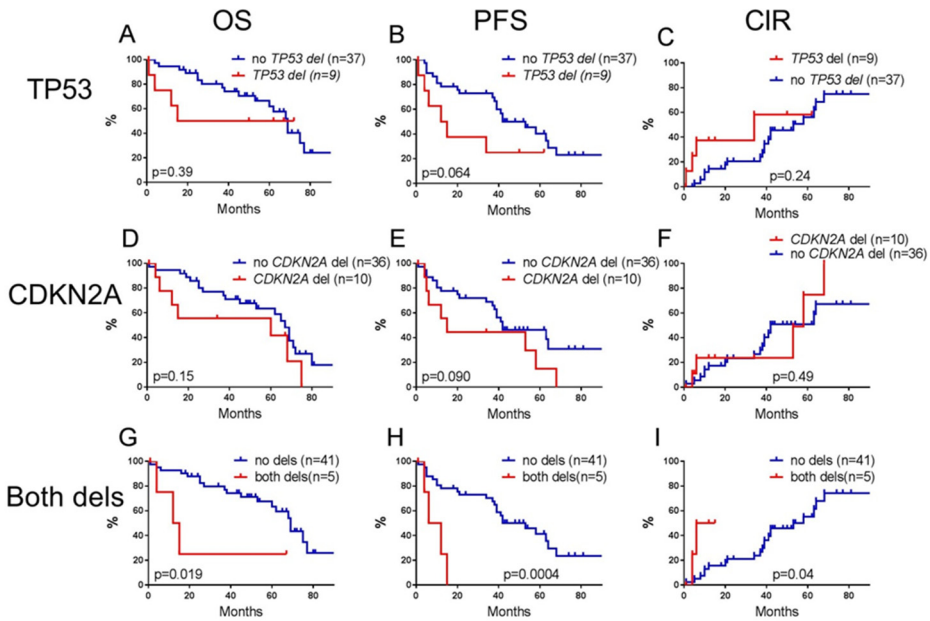
Table II. Dosing schedule of lenaldiomide in phase I

	cohort	c 1-6 (d1-21)		c7-13 (d1-21)	
Phase I	**1**	5		25	
	2	10		25	
	3	**15**		**25**	
after amendment		c1	c 2-6 (d1-14)	c 7-8 (d1-21)	c9-13 (d1-21)
	A	0	10 mg	10 mg	15 mg
	B		10 mg	10 mg	15 mg
	C		**5**	**10 mg**	**15 mg**
Phase II		c1	c 2-6 (d1-14)	c 7-8 (d1-21)	c9-13 (d1-21)
MTD lenalidomide	**0**	**10 mg d1-14 cycle 2-6**	**10 mg**	**15 mg**	

UPN	NM	Gene	Exon	Genotype Variant(ref/mut)	Chromosome	c.	p.	consequence	VAF, %	dbSNP/COSMIC ref *
2	NM_000051.3	ATM	37	A/T	chr11:108175463	c.5558A>T	p.Asp1853Val	missense	47,01	rs1801673 / COSP17291
3	NM_000051.3	ATM	17	CG/AT	chr11:108137925	c.2494_2495delCGinsAT	p.Arg832Ile	missense	48,27	rs199875915 / COSP25858
4	NM_000051.3	ATM	34	A/C	chr11:108170506	c.5071A>C	p.Ser1691Arg	missense	45,6	rs1800059 / COSP42470
6	NM_000051.3	ATM	50	G/C	chr11:108200993	c.7360G>C	p.Ala2454Pro	missense	5,31	
8	NM_000051.3	ATM	49	T/C	chr11:108199935	c.7277T>C	p.Leu2426Pro	missense	25,8	
13	NM_000051.3	ATM	25	AATTTTTGGACTTTTTTTCCAAGGCT/A	chr11:108114806	c.627_651delTTTGGACTTTTTTTCCAAGGCTATT	p.Leu210fs	deletion-frameshift	18,1	
19	NM_000051.3	ATM	56	A/T	chr11:108206594	c.8174A>T	p.Asp2725Val	missense	48,9	
19	NM_000051.3	ATM	18	G/A	chr11:108139336	c.2838G>A	p.Met946Ile	missense	46,2	
21	NM_000051.3	ATM	59	A/T	chr11:108218036	c.8615A>T	p.His2872Leu	missense	6,85	
22	NM_000051.3	ATM	42	G/A	chr11:108186757	c.6115G>A	p.Glu2039Lys	missense	30,59	
24	NM_000051.3	ATM	6	GAATAAT/G	chr11:108114749	c.567_572delAATAAT	p.Arg189_Ile191delinsSer	deletion-frameshift	5,93	
36	NM_000051.3	ATM	49	T/C	chr11:108199938	c.7280T>C	p.Leu2427Pro	missense	13,16	
42	NM_000051.3	ATM	17	C/T	chr11:108137985	c.2554C>T	p.Gln852Ter	nonsense	48,3	
43	NM_000051.3	ATM	50	G/A	chr11:108200975	c.7342G>A	p.Asp2448Asn	missense	6,38	
47	NM_000051.3	ATM	52	G/A	chr11:108202765			3' splice site	25,65	
47	NM_000051.3	ATM	63	C/T	chr11:108236086	c.9022C>T	p.Arg3008Cys	missense	23,02	
48	NM_000051.3	ATM	17	T/C	chr11:108138003	c.2572T>C	p.Phe858Leu	missense	51,4	rs1800056 / COSP37973
24	NM_182962.2	BIRC3	8	T/G	chr11:102206706	c.1334T>G	p.Leu445Ter	nonsense	6,51	
24	NM_053056.2	CCND1		A/ACC	chr11:69456042			5' UTR	11,32	
28	NM_053056.2	CCND1	1	CC/TG	chr11:69456209	c.128_129delCCinsTG	p.Ser43Leu	missense	29,16	
37	NM_053056.2	CCND1		C/T	chr11:69455955			5' UTR	5,24	
37	NM_053056.2	CCND1		C/T	chr11:69456037			5' UTR	5,24	
38	NM_053056.2	CCND1		G/A	chr11:69456049			5' UTR	33,82	
8	NM_003482.3	KMT2D	15	T/TCACACA	chr12:49440518	c.4291_4292insTGTGTG	p.Cys1430_Glu1431insValCys	insertion-frameshift	15,99	
13	NM_003482.3	KMT2D	39	TC/T	chr12:49425194	c.13293delG	p.Lys4432fs	deletion-frameshift	15,23	
14	NM_003482.3	KMT2D	48	T/C	chr12:49420120	c.15629A>G	p.Tyr5210Cys	missense	21,3	
22	NM_003482.3	KMT2D	42	TC/T	chr12:49424113	c.13948delG	p.Glu4650fs	deletion-frameshift	23,33	
29	NM_003482.3	KMT2D	10	C/CG	chr12:49445222	c.2243_2244insC	p.Glu748fs	insertion-frameshift	5,72	
40	NM_003482.3	KMT2D	31	C/T	chr12:49434759	c.6794G>A	p.Gly2265Glu	missense	55,56	
46	NM_003482.3	KMT2D	31	G/A	chr12:49434801	c.6752C>T	p.Ser2251Leu	missense	47,63	rs189199944 / COSU540
48	NM_003482.3	KMT2D	34	G/A	chr12:49432396	c.8743C>T	p.Arg2915Ter	nonsense	36,45	
29	NM_017617.4	NOTCH1	34	CAG/C	chr9:139390648	c.7541_7542delCT	p.Pro2514fs	deletion-frameshift	5,39	
49	NM_017617.4	NOTCH1	34	G/A	chr9:139390690	c.7501C>T	p.Gln2501Ter	nonsense	24,15	
4	NM_000546.5	TP53	7	AG/A	chr17:7577557	c.723delC	p.Cys242fs	deletion-frameshift	10,87	
4	NM_000546.5	TP53	5	T/TGAGG	chr17:7578539	c.390_391insCCTC	p.Asn131fs	insertion-frameshift	8,32	
8	NM_000546.5	TP53	8	GC/AA	chr17:7577121	c.816_817delGCinsTT	p.Arg273Cys	missense	21,52	
28	NM_000546.5	TP53	7	A/T	chr17:7577517	c.764T>A	p.Ile255Asn	missense	42,33	
29	NM_000546.5	TP53	9	C/T	chr17:7576852			3' splice site	24,14	
37	NM_000546.5	TP53	8	G/A	chr17:7577094	c.844C>T	p.Arg282Trp	missense	3,85	
48	NM_000546.5	TP53	5	C/A	chr17:7578370			3' splice site	60,89	
29	NM_001042424.2	WHSC1	18	G/A	chr4:1962801	c.3295G>A	p.Glu1099Lys	missense	20	
38	NM_001042424.2	WHSC1	18	G/A	chr4:1962801	c.3295G>A	p.Glu1099Lys	missense	15,6	

** Listed are mutations with VAF 40-60% and both a dbSNP and COSMIC reference.*

Supplemental figure 2: Prognostic impact of deletions of *TP53* and *CDKN2A*.



Kaplan-Meier estimates of OS, PFS and CIR by subgroups according to presence of deletion of *TP53* or not (A-C); deletion of *CDKN2A* or not (D-F) and both deletions (G-I) and compared by log-rank test.

Paper IV

Ibrutinib inhibits antibody dependent cellular cytotoxicity induced by rituximab or obinutuzumab in MCL cell lines, not overcome by addition of lenalidomide.

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Abstract

Background: The BTK[1]-inhibitor ibrutinib is highly active in MCL but may inhibit response to anti-CD20 mAb as suggested by previous studies on chronic lymphatic leukemia. We investigated how anti-CD20 mediated cell death was affected by treatment with ibrutinib and lenalidomide, a potential sensitizer to anti-CD20 activity, in MCL.

Methods: Anti-CD20 opsonized MCL cell lines were co-cultured with PBMC, pre-treated with ibrutinib ± lenalidomide, and analyzed by flow cytometry for evaluation of cell death.

Results: Cell death was reduced by ibrutinib at 0.5 (25%, p=0.0023) and 0.1 (48%, p=0.003) μM with rituximab and obinutuzumab, respectively, but not increased by addition of lenalidomide. Moreover, obinutuzumab was associated with higher rate of cell death compared to rituximab.

Conclusion: Our *in vitro* model on MCL shows that ibrutinib negatively affects anti-CD20 induced cell death, not reversed by lenalidomide. Explorations of sequential administration and selective BTK-inhibitors may reveal the optimal combination of novel agents in MCL.

[1]Bruton´s Tyrosine Kinase

Background

Mantle cell lymphoma (MCL), an aggressive B cell lymphoma, is regarded as an incurable disease and novel agents as well as new combinations are warranted to improve outcome in these patients.

Several novel agents with activity in lymphoma including MCL, are currently explored in clinical trials but we know little about how they ideally should be combined with regard to synergistic and/or antagonistic interactions. *In vitro* models enable broad exploration of multiple agents on a cellular level and may provide insight in how combinations could be explored in clinical trials.

Targeting CD20 with rituximab, the first approved anti-CD20 monoclonal antibody (mAb), in combination with chemotherapy, is associated with increased disease control and improved survival rates in non-Hodgkin lymphoma, including MCL, and constitutes the backbone in the treatment of these patients (1-4).

Anti-CD20 mAbs act through several mechanisms, including direct cell death via intracellular apoptotic signaling, complement-dependent cytotoxicity (CDC) via C1q as well as via binding to FcIIIRa on different effector cells including NK cells (antibody-mediated cellular cytotoxicity (ADCC)), macrophages (antibody-mediated cellular phagocytosis (ADCP)) and dendritic cells (5).

Decreased sensitivity or resistance to rituximab may be caused by reduced CD20-expression, “shaving trogocytosis” and polymorphism in the Fc region and is of major concern for treatment outcome and has led to development of second generation anti-CD20 mAbs (6-9). Obinutuzumab (Ga101) is a glycoengineered type II anti-CD20 mAb, with higher affinity for the FcγRIIIa-complex and lower grade of binding into lipid rafts compared to rituximab. *In vitro* data has shown that obinutuzumab induces ADCC and direct cell death to a higher extent and may improve outcome in follicular lymphoma patients when combined with conventional chemotherapy, compared to rituximab (10, 11).

Ibrutinib is an orally bioavailable irreversible inhibitor of Bruton´s Tyrosine Kinase (BTK) with activity in several B cell malignancies, including MCL (12). BTK is a key component in the signal transduction from the B cell receptor (BCR) to downstream activation of transcriptional factors like NF-κB, MAPK and AKT, thereby promoting cell survival, proliferation and differentiation (reviewed by Buggy et al.) (13). In recent years, studies on CLL by *in vitro* and *in vivo* xenograft models, have reported reduced effect on cell death, partly by reduced NK cell activation, when anti-CD20-mAbs were combined with ibrutinib (7, 14).

Lenalidomide, an immune modulatory agent, has shown promising response rates both as single therapy as well as in combination with rituximab in relapsed or refractory (R/R) and untreated MCL (15, 16). A synergistic effect of lenalidomide

and rituximab have been demonstrated *in vitro* and lenalidomide has shown capacity of re-sensitizing previous rituximab-resistant MCL (7, 17-20). Lenalidomide is currently evaluated in combination with obinutuzumab in R/R aggressive B cell lymphoma including MCL (21).

This study aims to investigate if (i) ibrutinib may affect the response to type I and type II anti-CD20 treatment in MCL *in vitro* models, if (ii) lenalidomide, an immune-stimulatory agent, could revert the effect of a tentative repressive role of ibrutinib on ADCC in MCL and if (iii) type I and type II anti-CD20 antibodies have similar potency to induce ADCC in MCL.

Material and methods

Human cells and cell lines

To investigate whether the addition of ibrutinib interferes with the capability of anti-CD20-mAb to induce cell death in MCL *in vitro*, we chose two MCL cell lines, JeKo-1 and REC-1, with low or intermediate sensitivity to ibrutinib itself, according to data from previous experiments (Suppl. figure 1).Cells were cultured in R10 (RPM1640 (HyClone Laboratories, Utah, USA) supplemented with 10 % fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), and 1 % L-glutamate (Invitrogen). Heat-inactivated FBS was used for the ADCC assays. Fresh whole blood was achieved from voluntary healthy donors at the Department of Transfusion Medicine (Skåne University Hospital, Lund, Sweden). Peripheral blood mononuclear cells (PBMC) were achieved from buffy coat by Ficoll-paque (GE Healthcare, Little Chalfont, UK) density gradient centrifugation, cryopreserved in 50% R10/40% FBS/10%DMSO) and stored in -80°C prior to usage within experiments. The study was conducted according to protocols approved by local institutional review board in accordance with the Declaration of Helsinki.

Antibodies and reagents

Ibrutinib (PCID-32765 Selleck Chemicals, Houston, TX, USA), aliquoted in DMSO as 10 mM. Lenalidomide (PCID-216326 Santa Cruz Biotechnology, USA), solved in DMSO to 100 mg/mL, aliquoted as 10 mM. Rituximab (1.3 mg/mL) and obinutuzumab (4 mg/mL) were obtained from Roche (Basel, Switzerland). All reagents were diluted in R10 into desired concentrations.

Assessment of immune mediated cell death

To allow separation of target cells from PBMC, cells were stained in carboxyfluorescein succinimidyl ester (CFSE) (Cell TraceTM Cell Proliferation Kit, Molecular Probes®, ref C34554) for 30 min, RT. Cryopreserved PBMC were thawed and seeded in 96-well-plate and treated with 0.1/0.5/1/5/10 µM ibrutinib for 1 h, 37°C, 5% CO2. CFSE-stained target cells were incubated with 1 µM anti-CD20-mAb (rituximab/obinutuzumab) for 20 min, 37°C, 5% CO2, and then co-cultured with PBMC o/n 37°C, 5% CO2. On day 2, cells were washed in PBS and stained with 5% 7-Amino-Actinomycin D (7-AAD) (BD Biosciences, ref. 559925), 5 min, RT, to allow identification of non-viable cells, followed by dilution in MACS buffer (PBS/0.5%BSA/2.5%EDTA) to a final well volume of 120 µl prior to flow cytometry.

The effector:target cell ratio was 100:1 for all experiments. Each experiment included both cell lines, both rituximab and obinutuzumab and was made with PBMC from four donors to ensure a biological representation of the results. All samples were made in duplicates ((i) and (iii)) or triplicates (ii).

As the stock solution of ibrutinib is solved in DMSO, we performed separate series to exclude a toxic effect of DMSO per se on cells, by using identical protocol as described previously, with ibrutinib replaced by DMSO in correlating concentrations. We observed a lower rate of cell death at 10 µM compared to lower concentrations, which could be explained by an unwanted toxic effect of DMSO on PBMC (data not shown). Consequently, final analysis included samples with ibrutinib up to 5 µM.

To investigate whether the addition of lenalidomide could overcome the inhibitory effect of ibrutinib on ADCC (iii), we performed extended experiments on JeKo-1 with pre-treatment of PBMC with lenalidomide 0/0.01/0.05/0.1/1 µM for 2 h, 37°C, 5% CO2, prior to the addition of 1 µM ibrutinib. Subsequent procedure was identical as previously described.

Flow cytometry

Flow cytometry was performed using iQue screener (Intellicyt, Albuquerque, NM, USA). The protocol for collection of cells was: sipping time 55s, up-time 5 s with shaking (800rpm) of plate before and after every 6 well during sampling.

Data analyses were performed in Forecyte® Standard Edition 5.2 (Intellicyt). Gating procedure was made after compensation, based on FSC-H and SSC-H, to identify singlets and live cells. Gates for CFSE- and 7-AAD-positive cells were made in channel BL-1 and BL-4 respectively with unstained samples as negative

controls. Samples with a count of 7-AAD-positive cells < 60 or of CFSE-positive cells < 230 were excluded from further analysis.

Calculation of immune mediated cell death and statistics

The fraction of 7-AAD-positive out of CFSE-positive cells, representing the ratio of non-viable target cells was calculated from results in flow cytometry analysis.

The level of immune-mediated cell death, hereafter named as cell death (%), was defined as mean value of (7-AAD+/CFSE+)-ratio of duplicates with reagents; (i) (ibrutinib) and (ii) (ibrutinib and lenalidomide), compared to mean value of control duplicates without reagents. For (iii), level of cell death was defined as the 7-AAD+/CFSE+- ratio of samples with anti-CD20 mAb compared to samples without anti-CD20 mAb. In (iii), statistical analysis was made on mean values from three individual experiments measuring cell death.

For statistical analysis, student's unpaired t test was performed to identify significant differences. Analyses were made in Microsoft, Excel 2013. A p-value < 0.05 was considered significant.

Results

Pre-treatment of PBMC with ibrutinib affects ADCC in MCL lines *in vitro*

We used two different MCL cell lines to study the effect of ibrutinib on type I and type II anti-CD20 mAb treatment, REC-1 and JeKo-1. The cell lines were selected by having low or intermediated sensitivity to ibrutinib, as established in separate experiments. Ibrutinib did not have a direct effect on cell death on JeKo-1 at 0.1-10 μM, and on REC-1 at 0.1-1 μM (Suppl. figure 1). Thus, in these concentration intervals, cell death caused by ADCC alone can be assessed.

To investigate whether ibrutinib affects the NK-cell mediated response to type I and type II anti-CD20 mAb treatment in MCL *in vitro* models, we performed *in vitro* experiments of ADCC. We found that ibrutinib inhibits ADCC in a concentration dependent manner starting at 0.1μM (Figure 1).

Figure 1 and Table I show results from both cell lines and both antibodies with one representative donor of PBMC. A lower level of cell death was observed in all samples with PBMC pre-treated with ibrutinib. In samples with rituximab, a significant lower cell death was observed from 0.5 μM and higher for JeKo-1, and

at 5 μM for REC-1. In samples with obinutuzumab, a significant lower cell death was observed at 0.1, 1 and 5 μM ibrutinib for JeKo-1 and at 0.5 and 5 μM ibrutinib for REC-1. Of note, at 5 μM ibrutinib, the ADCC can be overestimated due to a potential direct effect of ibrutinib. Thus, the ibrutinib-related inhibition is likely more pronounced than shown here.

Maximum significant reduction of cell death in samples treated with rituximab was observed at 1 μM (20%, p=0.005, JeKo-1) and 5 μM (27%, p=0.001, REC-1) ibrutinib. In samples treated with obinutuzumab, the lowest value of cell death was observed at 5 μM ibrutinib (11%, p=0.008, JeKo-1) (39%, p=0.056, REC-1).

The immune modulator lenalidomide does not overcome the inhibitory effect on PBMC induced by ibrutinib.

To investigate whether the immune modulator lenalidomide could revert the repressing effect of ibrutinib on PBMC, we performed experiments where PBMC were pre-treated with lenalidomide prior to addition of 1μM ibrutinib and MCL cells (JeKo-1), opsonized with 1μM anti-CD20 mAb (rituximab and obinutuzumab).

Results from experiments on JeKo-1 with one representative donor of PBMC is showed in suppl. figure 2. A significant reduced cell death was observed in samples with addition of ibrutinib to 34% or 45% for rituximab and obinutuzumab respectively. However, cell death was not significantly affected by pre-treatment of PBMC with lenalidomide at any concentration (Suppl figure 2 and suppl table I). Hence, lenalidomide failed to revert the inhibitory effect of ibrutinib in this experimental set-up.

Obinutuzumab was associated with higher rate of cell death compared to rituximab on MCL cell lines

To investigate the potency of type I versus type II anti-CD20-mediated ADCC on MCL cell lines, we used rituximab and obinutuzumab to induce cell death in two MCL cell lines (JeKo-1 and REC-1) and PBMC from four unique donors to capture tentative biological variation among donors/NK cells. Results are based on anti-CD20-antibody concentration of 1μM.

A significant increase of cell death was observed for rituximab (JeKo-1: 31%. p=0.02; REC-1: 145%, p=0.03) and obinutuzumab (JeKo-1: 149%, p=0.01; REC-1: 164%, p= 0.04) compared to controls for both cell lines (Table II). When comparing the two antibodies, we found a significant higher cell death after treatment with obinutuzumab compared to rituximab for JeKo-1, but not with REC-1, as shown in Figure 2 and Table II.

Discussion

In the present study, we report the results from an *in vitro* model for ADCC on MCL, where we show that ibrutinib negatively affects the response to rituximab and obinutuzumab, a type I and a type II anti-CD20 antibody. The repressive impact of ibrutinib could not be reverted by the addition of lenalidomide, a potential sensitizer to anti-CD20 mAb. Further, our results suggest that obinutuzumab has a higher potency of inducing ADCC than rituximab on MCL *in vitro*.

Although survival has increased in MCL, by the introduction of the anti-CD20-mAb rituximab (R) and high-dose chemotherapy with autologous stem cell support (HD-ASCT) to young fit patients, the disease is still regarded as incurable, and identifying efficient treatment with limited toxicity is warranted to improve outcome in MCL patients (22). Ibrutinib is a BTK-inhibitor which is highly active in MCL, even in previously heavily treated patients with relapsed/refractory disease and has a favorable toxicity profile (23). Current trials focus on whether ibrutinib may replace chemotherapeutic agents as in the ENRICH trial where elderly MCL patients are randomized between R-ibrutinib and conventional R-chemotherapy (bendamustine or CHOP) (Cancer Research UK trial number/CRUK/14/026). The randomized, three-armed, phase III trial, TRIANGLE (NCT02858258) for young fit patients with MCL will investigate if the addition of ibrutinib to induction with R- chemotherapy (R-CHOP/R-DHAP) may improve outcome after HD-ASCT and, if consolidation with R-ibrutinib may achieve disease control comparable to HD-ASCT, and thereby, potentially replace it.

Preclinical data has indicated that ibrutinib may interfere with anti-CD20-targeted therapy negatively due to reduced ADCC as shown on CLL *in vitro* by da Roit et al. (7). Although the addition of rituximab to ibrutinib has shown promising activity in R/R MCL patients, little is known on how these two, each highly potent agents, optimally should be combined in MCL (23).

Our study shows that ibrutinib interferes with the cytotoxic effect of rituximab and obinutuzumab, in two MCL cell lines. The inhibitory effect could be observed already at 0.1 μM, which is physiological relevant with respect to 0.160 μM, at which >95% of BTK was saturated, and the mean plasma concentration of 0.07-0.2 μM in patients on 420-840 mg ibrutinib (24, 25).

In this *in vitro* model, we used two cell lines with low and intermediate sensitivity to ibrutinib to minimize the dose-dependent toxic effect by ibrutinib per se on target cells. For one of the cell lines, REC-1, the direct cell effect by ibrutinib was significant at 5 μM. Although our results suggest that higher concentrations of ibrutinib seems to be required to inhibit the cytotoxic effect of obinutuzumab and rituximab on REC-1, compared to JeKo-1, one cannot exclude that the less

difference in cell death may be explained by a higher direct effect of ibrutinib on REC-1 target cells.

Our results are in line with previous data on *in vitro* models on cell lines derived from CLL and other non-Hodgkin lymphoma (NHL) whereas *in vivo* xenograft models on NHL, treated with rituximab and ibrutinib, have reported both less tumor control as well as no impact of combining rituximab and ibrutinib, compared to only rituximab (7, 14, 26).

The unwanted repressive effect of ibrutinib on immune mediated cell death, may be explained by the affinity for other kinases including ITK (interleukin-2-inducible T-cell kinase), which is expressed on T cells and NK cells as reviewed by Berglöf et al. (27). Upon activation, ITK promotes activation, proliferation and differentiation of NK cells and T cells, which may explain the reduced capacity of NK cells in exerting ADCC, as observed in our study. Another mechanism that might be relevant is the reduced CD20-expression after treatment with ibrutinib as reported from *in vitro* models on NHL cell lines as well as from CLL cells derived from patients on treatment with ibrutinib (28, 29). In our study, target cells were exposed to anti-CD20 mAb prior to coincubation with PBMC and ibrutinib, why ADCC should have been actuated irrespectively of secondary modulation of CD20-expression.

We used PBMC instead of purified NK cells, which required a higher ratio of effector: target cells (100:1) and thereby limited the number of events of target cells besides that more unspecific processes might have been captured. On the other hand, PBMC should be more alike the *in vivo* situation although not all compartments such as complement-derived and micro-environmental tissue-related factors are present.

A model on sequential administration of anti-CD20 mAb and BTK-inhibitor was reported by Kohrt et al. on ex-vivo CLL mouse model, showing a benefit in terms of greater tumor shrinking and increased survival with weekly sequential administration of ibrutinib and rituximab compared to concomitant administration (14). A similar study on MCL would reveal if the negative impact of ibrutinib could be moderated or even avoided to potentiate the two agents.

In the present study, the inhibitory effect of ibrutinib on ADCC, induced by anti-CD20 mAb, could not be overcome by pretreatment of PBMC with lenalidomide prior to ibrutinib.

Lenalidomide has shown to potentiate ADCC *in vitro* and the combination of lenalidomide and rituximab is comparable to i.e. rituximab-bendamustine as upfront regimen in elderly untreated patients (17, 30). In the NLG/MCL6 (Philemon) trial (NCT02460276), the combination of ibrutinib-lenalidomide-rituximab in patients with R/R MCL was associated with high efficacy even in some of patients with poor

prognostic features, including TP53 mutations (31). It would be of value to explore how immune mechanisms including NK cell activation status are affected during this combination and if an alternative sequence of administration would overcome possible counteracting effects.

Further, our study suggests that obinutuzumab may have a more pronounced capacity of inducing ADCC in MCL compared to rituximab, although significance was only reached for one of the cell lines.

A higher ADCC has been described for obinutuzumab than for rituximab, from *in vitro* assays on degranulation of NK cells and cell death in CLL and NHL cell lines. Further, obinutuzumab may be able to overcome resistance to rituximab as shown in xenograft *in vitro* models as well as in phase I/II trials in R/R NHL (7, 11, 32).

A superior effect of obinutuzumab compared to rituximab has been shown in untreated CLL and FL when combined with chemotherapy, but not in DLBCL, indicating that the importance of the different pharmacodynamic properties of therapeutic antibodies may vary between diagnosis (33-35). Ongoing clinical trials on the combination of obinutuzumab and ibrutinib are underway and will, together with further preclinical investigations, reveal which is the superior agent for targeting CD20 in MCL (36).

In summary, we have investigated how two novel agents, ibrutinib and lenalidomide, affect ADCC, induced by anti-CD20-mAb in MCL *in vitro*. We show that ibrutinib inhibits the potency of both type I and type II anti-CD20-mAb with markedly lower cell death at concentrations comparable to *in vivo* serum levels. This inhibitory effect of ibrutinib could not be overcome by addition of a potential immune sensitizer such as lenalidomide. Further, our *in vitro* model shows that that obinutuzumab may be a stronger inducer of ADCC than rituximab, and, may better withstand the inhibitory effect of a BTK-inhibitor like ibrutinib.

Future studies on sequential administration of ibrutinib and anti-CD20-mAbs in MCL as well as exploring of more selective BTK-inhibitors with less off-target binding may reveal how these optimally could be combined *in vivo*, with respect to efficacy, potential synergism and toxicity of each compound.

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Disclosure statement

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No potential conflict of interest was reported by the other authors.

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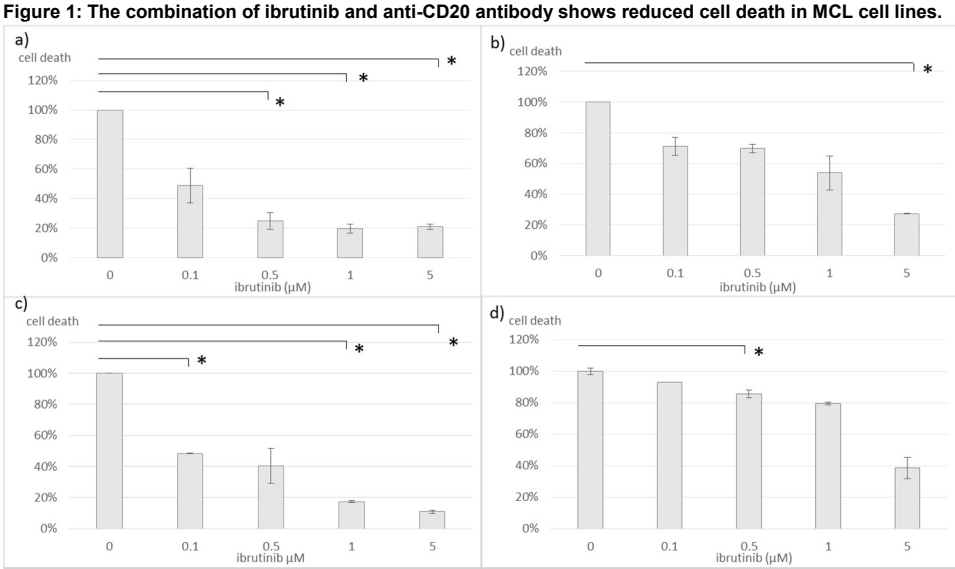
Tables and figures

Figure 1: The combination of ibrutinib and anti-CD20 antibody shows reduced cell death in MCL cell lines.



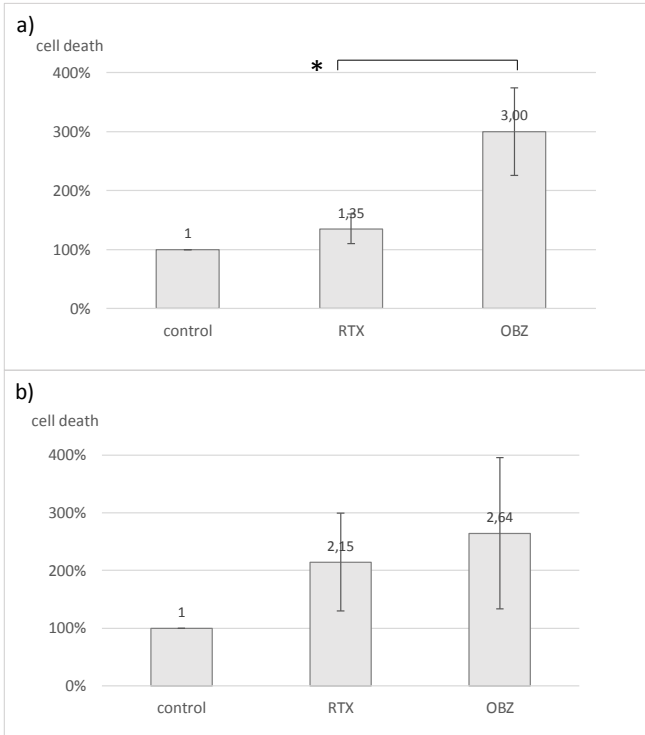
Cell death (%) ± standard deviation in MCL cell lines (JeKo -1, REC-1), opsonized with 1µM anti-CD20 mAb (rituximab, obinutuzumab) and co-cultured with PBMC pretreated with ibrutinib (0-5 µM). a) JeKo-1 + rituximab, b) REC-1 + rituximab, c) JeKo-1 + obinutuzumab, d) REC-1 + obinutuzumab. Results were compared by unpaired student's t-test. *=p<0.05,

Table I: Cell death (%) in anti-CD20 mAb-exposed MCL cell lines after incubation with PBMC pretreated with ibrutinib.

JeKo-1	rituximab (1μM)		obinutuzumab (1μM)	
Ibrutinib (μM)	cell death (%)(±SD)	p-value	cell death (%)(±SD)	p-value
0.1	48.98±11.79	0.145	48.43±0.26	0.003*
0.5	24.94±5.56	0.023*	40.25±11.19	0.118
1	19.84±3.03	0.024*	17.36±0.58	0.005*
5	20.99±1.65	0.012*	10.88±1.09	0.008*
REC-1	rituximab (1μM)		obinutuzumab (1μM)	
Ibrutinib (μM)	cell death (%)(±SD)	p-value	cell death (%)(±SD)	p-value
0.1	71.08±5.80	0.126	92.89±2.58	0.222
0.5	69.75±2.66	0.056	85.60±0.99	0.044*
1	53.79±11.27	0.152	79.49±6.82	0.204
5	27.33±0.27	0.001*	38.55±5.39	0.056

*=p<0.05, student´s t-test, compared to control.

Figure 2: Obinutuzumab induces higher rate of cell death compared to rituximab in MCL cell lines.



Cell death of MCL cell lines (JeKo -1, REC-1), treated with CD20-ab (rituximab, obinutuzumab (1μM)) and co-cultured with PBMC. Data shown are mean values ± standard deviation of cell death from three individual experiments compared to control (no anti-CD20 mAb). a) JeKo-1, b) REC-1. RTX=rituximab, OBZ=obinutuzumab. Results were compared with student t-test. *=p<0.05, **=p<0.001.

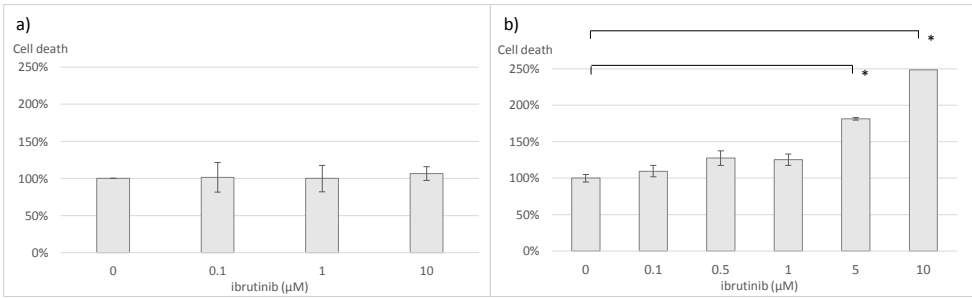
Table II. Comparison of ADCC between rituximab and obinutuzumab

	rituximab (1μM)		**obinutuzumab (1μM)**		**rituximab vs obinutuzumab**
	cell death (%)		cell death (%)		
JeKo-1	131.15± 25.38	p=0.024*	249.14± 102.02	p=0.012*	p=0.030*
REC-1	214.53 ± 84.56	p=0.029*	264.43± 130.98	p= 0.038*	p= 0.493

MCL cell lines (Jeko-1. REC) were stained with CFSE. treated with CD20-ab (rituximab. obinutuzumab (1uM)) and coincubated with PBMC from healthy donors (20 h). Ratio effector:target 100:1. After additional staining with 7-AAD. cells were analyzed in flow cytometry. The fraction of 7-AAD+ cells out of CFSE+ cells were calculated as a marker of target cell death/ADCC. Data shown are mean values ± st.dev of cell death from three individual experiments compared to control. Values were compared with student t-test. *= p-value < 0.05 A) Jeko-1: p=0.0091. B) REC: P= 0.4163

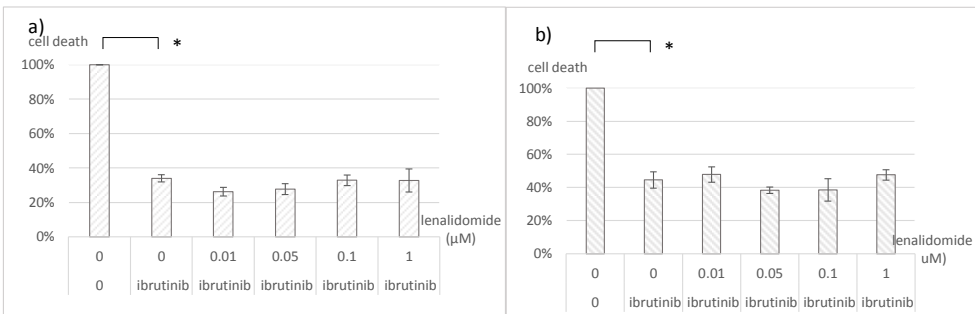
Supplementary figures and tables

Suppl figure 1. Ibrutinib does not have any dose-dependent direct effect MCL cell lines.



Cells were incubated with ibrutinib o/n and analyzed by flow cytometry. Mean values of cell death were compared with control by using unpaired student t-test. a) JeKo-1, b) REC-1: 5 µM, p=0.0286 and 10µM, p=0.0040 . *=p<0.05.

Suppl figure 2. Lenalidomide does not overcome the inhibitory effect on ADCC induced by anti-CD20-monoclonal antibody.



PBMC pretreated with lenalidomide (0-1 µM) 2 h, followed by addition of ibrutinib 1 µM/1 h and co-cultured with MCL cell line (JeKo-1) exposed for rituximab or obinutuzumab (1 µM). Data shown is mean values of triplicates ± standard deviation compared to control from experiment with one representative donor of PBMC. a) rituximab, b) obinutuzumab. The significantly lower cell death observed in samples treated with ibrutinib, compared to control, was not affected by addition of lenalidomide. Samples were compared by unpaired student t-test. *=p<0.05

Suppl table I) Lenalidomide does not overcome the inhibitory effect on ADCC induced by anti-CD20 monoclonal antibody.

	cell death (%) ±SD	p-value *	cell death (%) ±SD	p-value**	cell death (%) ±SD	p-value**	cell death (%) ±SD	p-value **	cell death (%) ±SD	p-value **
lenaliomide (µM)	0		0.01		0.05		0.1		1	
rituximab	34.02 ±2.11	0.001	26.30 ±2.54	0.031	27.72 ±3.08	0.084	32.89 ±3.05	0.692	32.80 ±6.70	0.826
Obinutu-zumab	44.59 ±4.92	0.004	47.83 ±4.65	0.536	38.30 ±1.99	0.205	38.60 ±6.73	0.372	47.62 ±3.19	0.513

Mean value of cell death from triplicates ± standard deviation compared to control from experiment with one representative donor of PBMC. *=p-value from comparison with control, **=p-value from comparison with samples with anti-CD20 mAb and ibrutinib.