Molecular approaches to the diagnosis and evaluation of follicular lymphoma

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Scientific environment

The research work started January 2008 and was initially combined with training for specialization in pathology at the Department of Pathology, Haukeland University Hospital. Since August 2012 I have been a full-time PhD candidate, financed by the Faculty of Medicine and Dentistry, University of Bergen, working at the Gade Laboratory for Pathology, Department of Clinical Medicine. The PhD project has also been funded by grants provided by the Western Norway Regional Health Authority and by the Dr. med F.G Gades legat.

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Bergen, October 2014 Ellen Berget

Abstract

Background: Molecular studies of *immunoglobulin (IG)* genes have provided insights into the pathogenesis of different lymphoid malignancies and allowed the development of useful diagnostic tools as well as powerful prognostic markers. This study concerns the role of molecular analysis of *IG* genes in the diagnosis and evaluation of follicular lymphoma (FL).

Aims: The aims of the present study were a) to investigate the application of PCR-based clonality analysis of *IG* genes on formalin-fixed, paraffin-embedded (FFPE) tumour samples, b) to determine the value of such analysis in bone marrow staging and c) to analyze the use of *IG heavy chain variable (IGHV)* genes and mutational status in relation to prognosis in FL patients.

Materials and methods: The thesis is based on three papers (**Paper I-III**). In **Paper I**, DNA from FFPE samples of 118 patients diagnosed with FL in the period 1998-2008 was used. PCR-based clonality was assessed by *IG heavy (IGH)*, *kappa (IGK)* and *lambda (IGL)* primers in multiplexed reactions, and by a PCR procedure that was optimized for FFPE tissue. In **Paper II**, DNA was obtained from fresh bone marrow aspirates of 96 FL patients and subjected to PCR-based clonality analysis. The PCR results were controlled by analysis of the primary tumour and related to morphological detection of bone marrow involvement. In 71 patients, the results were also compared with data from concurrent flow cytometric immunophenotyping. In **Paper III**, 106 patients with FL were included. *IGHV* gene sequences were determined using DNA from FFPE tumour samples and direct sequencing with forward and reverse primers.

Results: In **Paper I**, the highest clonality detection rates were reached when *IGH* and *IGK* assays were combined (94.9%). FFPE samples stored for 6-11 years did not perform significantly worse than those stored for 1-5 years with respect to clonality detection. In **Paper II,** bone marrow involvement by PCR-based clonality was found in 34.4% (33/96) of patients. PCR-positive patients had a significantly poorer

survival than PCR-negative patients (P=0.001, log-rank test). Thirteen patients positive by PCR but without morphological BM involvement, had significantly poorer survival than patients with negative morphology and negative PCR result (P=0.002). The poor survival associated with PCR-based bone marrow involvement was independent of high FLIPI score by multivariate analysis (P=0.007). Bone marrow involvement by morphology or flow cytometry did not show prognostic impact on survival. In **Paper III**, 104 productive rearranged *IGH* sequences were obtained from FFPE tumour samples of 99 patients. The *IGHV3, IGHV1* and *IGHV4* were the most frequently encountered subgroups while the *IGHV3-23* was the most frequently encountered gene. Patients with the *IGHV5* subgroup (P=0.013, log-rank) or more than one *IGHV* subgroups (P<0.001, log-rank) in their tumours showed significantly poorer survival than patients with other *IGHV* subgroups. *IGHV5/*>1 *IGHV* subgroup usage was of independent prognostic importance in multivariate analysis (P=0.005). Unmutated sequences, showing >98% homology to the closest *IGHV* gene, were detected in 15.2% of cases. Unmutated *IGHV* genes were associated with age >60 years at diagnosis, but not with survival.

Conclusions: An improved PCR protocol for detection of clonality in FFPE samples was presented, and a combination of *IGH* and *IGK* analyses was recommended for diagnostic purposes (**Paper I**). PCR-based clonality analysis significantly improved the prognostic value of bone marrow staging, and the inclusion of PCR-based analysis in bone marrow examination of FL patients was suggested (**Paper II**). The presence of the *IGHV5* subgroup or more than one *IGHV* subgroup identified patients with shorter survival, indicating that *IGHV* sequence analysis could aid in predicting prognosis for FL (**Paper III**).

Abbreviations

AID	Activation-induced cytidine deaminase
BCR	B-cell receptor
BL	Burkitt lymphoma
BM	Bone marrow
CDR	Complementary determining region
CHOP	Cyclophosphamide, doxorubicin, vincristine and prednisone
CLL/SLL	Chronic lymphocytic leukaemia/small lymphocytic lymphoma
DLBCL	Diffuse large B-cell lymphoma
EDTA	Ethylenediaminetetraacetic acid
FFPE	Formalin-fixed paraffin-embedded
FISH	Fluorescence *in situ* hybridization
FL	Follicular lymphoma
FLIPI	Follicular Lymphoma International Prognostic Index
FR	Framework region
HPF	High-power microscopic field
IG	Immunoglobulin
IGH	Immunoglobulin heavy
IGK	Immunoglobulin kappa
IGL	Immunoglobulin lambda
IGHV	Immunoglobulin heavy variable
IPI	International Prognostic Index
LDH	Lactate dehydrogenase
MALT lymphoma	Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue
MZL	Marginal zone lymphoma
MBR	Major breakpoint region
MCL	Mantle cell lymphoma
MCR	Minor cluster region
PCFL	Primary cutaneous follicle centre lymphoma
R	Rituximab
PCR	Polymerase chain reaction
RAG	Recombination activating gene
REAL	Revised European-American Classification of Lymphoid Neoplasms
RSS	Recombination signal sequence
SNP	Single nucleotide polymorphism
SMZL	Splenic marginal zone lymphoma
WHO	World Health Organization

List of publications

The thesis is based on the following papers, which will be referred to by their Roman numerals:

I. **Berget E**, Helgeland L, Molven A, Vintermyr OK. Detection of clonality in follicular lymphoma using formalin-fixed, paraffin-embedded tissue samples and BIOMED-2 immunoglobulin primers. *J Clin Pathol.* 2011;64(1):37-41.

II. **Berget E**, Helgeland L, Liseth K, Løkeland T, Molven A, Vintermyr, OK. Prognostic value of bone marrow involvement by clonal immunoglobulin rearrangements in follicular lymphoma. *J Clin Pathol.* 2014;67(12):1072-7.

III. **Berget E**, Molven A, T Løkeland, Helgeland L, Vintermyr OK. *IGHV* gene usage and mutational status in follicular lymphoma: correlations with prognosis and patient age. *A revised version is currently in press (Leukemia Research, online March, 2015).*

Contents

1. Introduction

Lymphoid malignancies or lymphomas are malignant neoplasms of cells native to the lymphoid tissue, and may originate in B-cells, T-cells, NK-cells, and histiocytes and their precursors and derivatives.

The current accepted diagnostic criteria of lymphomas are founded on the World Health Organization (WHO) classification of tumours of haematopoietic and lymphoid tissues, published in 2001 and updated in 2008.[1,2] The WHO classification is based on the principles initially defined in the Revised European-American Classification of Lymphoid Neoplasms (REAL),[3] and uses all available information, i.e. morphology, immunophenotype, genetic characteristics and clinical features, to define the disease. The latest classification lists over 80 different lymphoma entities and attempts to group the entities by cell type. The groups include precursor lymphoid neoplasms, mature B-cell neoplasms, mature T-cell and NK-cell neoplasms, Hodgkin lymphoma, histiocytic and dendritic cell neoplasms and post-transplant lymphoproliferative disorders. Accordingly, non-Hodgkin lymhomas is not recognized as a separate group. Hodgkin lymphomas are still considered separately within the WHO classification, although now generally accepted as being of B-cell lineage.[4,5]

Most lymphomas are mature B-cell neoplasms (Table 1), and diffuse large B-cell lymphoma, follicular lymphoma and marginal zone lymphoma dominate, accounting for more than 70% of the total.[6] Diagnostic work, classification and comprehension of B-cell lymphomas have been aided greatly by molecular studies of *IG* genes. Clonality assessment by analyses of *IG* gene rearrangements has become a valuable diagnostic tool for B-cell lymphomas, aiding in distinguishing lymphomas from the differential diagnosis of reactive hyperplasia.[7-9] Many B-cell lymphomas are also associated with specific *IG* chromosomal translocations that can be useful as diagnostic markers.[2] In recent years, *IG* sequence analysis and mutational status have significantly increased the understanding of the pathogenesis of particular B-cell lymphomas such as chronic lymphocytic leukaemia.[10,11] Furthermore, the increased

understanding has been translated into a biologically oriented assessment of prognosis that may assist the rational design of risk-adapted therapies. The literature search for the introduction to this thesis was performed prior to August 2014.

Table 1. Overview of mature B-cell neoplasms[2]

Chronic lymphocytic leukaemia/small lymphocytic lymphoma
B-cell prolymphocytic leukaemia
Splenic marginal zone lymphoma
Hairy cell leukaemia
Splenic B-cell lymphoma/leukaemia, unclassifiable
Splenic diffuse red pulp small B-cell lymphoma
Hairy cell leukaemia-variant
Lymphoplasmacytic lymphoma
Waldenström macroglobulinemia
Heavy chain disease
Alpha heavy chain disease
Gamma heavy chain disease
Mu heavy chain disease
Plasma cell neoplasms
Monoclonal gammopathy of undetermined significance (MGUS)
Plasma cell myeloma
Solitary plasmacytoma of bone
Extraosseous plasmacytoma
Monoclonal immunoglobulin deposition diseases
Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma)

Nodal marginal zone lymphoma

Pediatric nodal marginal zone lymphoma

Follicular lymphoma

Pediatric follicular lymphoma

Primary cutaneous follicle centre lymphoma

Mantle cell lymphoma

Diffuse large B-cell lymphoma (DLBCL), not otherwise specified

T-cell/histiocyte rich large B-cell lymphoma

Primary DLBCL of the CNS

Primary cutaneous DLBCL, leg type

EBV-positive DLBCL of the elderly

DLBCL associated with chronic inflammation

Lymphomatoid granlomatosis

Primary mediastinal (thymic) large B-cell lymphoma

Intravascular large B-cell lymphoma

ALK-positive large B-cell lymphoma

Plasmablastic lymphoma

Large B-cell lymphoma arising in HHV8-associated multicentric Castleman disease

Primary effusion lymphoma

Burkitt lymphoma

B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and Burkitt lymphoma

B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and classical Hodgkin lymphoma

The histologic types in italic are provisional entities.

1.1 Epidemiology

The incidence of lymphomas has increased considerably during the past decades. The geographical pattern shows the highest rates in more economically developed regions of the world.[6,12]

FL is a mature B-cell neoplasm that accounts for about 20% of all lymphoma cases.[2] Most subtypes of B-cell lymphoma present with a median age at diagnosis of more than 70 years, but FL tends to be diagnosed at younger ages, with a median age of 65 years. B-cell lymphomas are generally more common in men, whereas females tend to predominate in FL.[6]

Data on incidence and incidence rates specifically for FL are not available in the annual report of the Cancer Registry of Norway. In 2012, 1578 new lymphoma cases were registered in Norway.[13] They represented approximately 5% of new cancers registered that year. The average annual age-adjusted incidence rate for males has increased from 8.4 in 1953-1958 to 21.0 in 2008-2012 (Figure 1). For females the incidence rates have increased from 5.2 to 14.9 during the same time period.

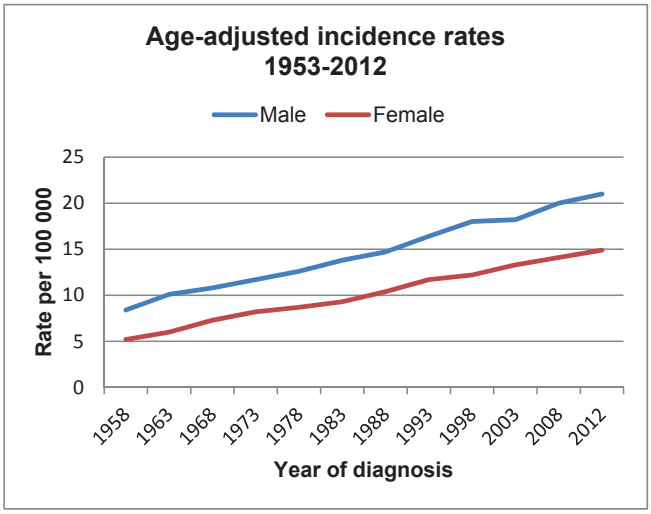

Figure 1. Age-adjusted (world standard) incidence rates per 100 000 person-years for lymphomas in Norway, 1953-2012.[13]

1.2 Etiology and risk factors

The etiology of lymphomas, as well as the rise in incidence, remains largely unexplained.[14] In the past, the identification of risk factors has been hindered by the heterogeneous nature of these diseases, lack of appropriate subtype classification or inconsistent application of diagnostic standards.[15]

It is nevertheless considered well-established that immunodeficiency syndromes constitute risk factors for lymphoma development.[16] Thus, infection with the human immunodeficiency virus (HIV),[17] iatrogenic immunosuppression to prevent allograft rejection or graft versus host disease (GVHD) [18] and primary immune deficiencies are commonly associated with an excess risk.[19] In addition to HIV, a number of other viruses have been suggested to impact on lymphoma risk. The most accepted viral associations are those with human T-cell leukemia virus type 1 (HTLV-1),[20] Epstein-Barr virus (EBV),[21], human herpesvirus-8 (HHV-8),[22] and hepatitis C virus.[23] Bacterial infections with subsequent chronic inflammation have also been consistently linked to increased lymphoma risk. One of the best known associations is that between *Helicobacter pylori* and extranodal marginal zone of mucosa-associated lymphoid tissue (MALT lymphoma).[24,25] Furthermore, autoimmune diseases are considered as established risk factors. Two of the most well-described associations are that between Hashimotos thyroiditis and thyroid lymphoma,[26] and that between Sjøgren's syndrome and salivary gland lymphoma.[27] Other consistent associations with lymphoma have been reported for rheumatoid arthritis and systemic lupus erythematosus,[28,29] and for gastrointestinal inflammatory conditions such as coeliac disease and Crohn's disease.[29,30]

There have been numerous epidemiologic studies of lymphoma in relation to other health-related states and to occupational or environmental exposures than those listed above. Consistent associations remain to be identified for factors such as obesity, smoking, alcohol consumption, and pesticide and ultraviolet radiation exposure.[14] A consistent finding is that of familial aggregation of lymphomas, implicating the potential etiologic role of genetic susceptibility.[31-34]

Very few studies have focused on specific risk factors associated with the development of FL. In a recent study, individuals with t(14;18) (q32;q21) translocation frequency reaching one in every 10 000 blood cells had a 23-fold greater risk of FL development.[35] Genetic variations at 6p21.33 and in the major histocompatibility complex (MHC) class II region have also previously been associated with FL susceptibility in genome-wide associations studies.[36,37] The risk of FL development was, however, only slightly increased among relatives of persons with FL.[31] To define healthy individuals at risk for FL development, a combination of risk factors identified by genomic, environmental and clinical investigations will probably be required.[35]

1.3 Antigen diversification reactions in normal B-cells

The diverse population of B-cell antibodies, which allows the immune system to recognize a wide variety of antigens, is generated through three genetic mechanisms. These mechanisms are *V(D)J* recombination, somatic hypermutation and class-switch recombination and they occur at different stages of B-cell differentiation. In many respects, B-cell lymphomas represent proliferation of cells that resemble different stages of B-cell development. In addition, the three genetic mechanisms mentioned above are all associated with double-stranded DNA breaks that predispose to chromosomal translocations, which play a pivotal role in lymphoma development. The three genetic mechanisms of antibody diversity will here be described and thereafter related to chromosomal translocations involved in pathogenesis of follicular lymphoma.

1.3.1 V(D)J recombination

The *IGH, IGK* and *IGL* genes are located on chromosome 14 (14q32.33), 2 (2p11.2) and 22 (22q11.2), respectively.[38] The *IGH* locus consists of variable (*V*), diversity (*D*), joining (*J*) and constant (C) gene segments, while the *IGK* and the *IGL* loci contain only *V, J* and *C* gene segments.

IG gene rearrangements are established during early B-cell differentiation by a stepwise combination of *V, D,* and *J* gene segments referred to as *V(D)J* recombination.[39] Almost all immature and mature B-cells[40] as well as neoplastic B-cells including FL cells have rearranged *IG* genes. The process of *V(D)J* recombination occurs in the bone marrow and is antigen-independent.

The initial steps of *V(D)J* recombination are mediated by recombination activating gene (RAG) enzymes. RAG enzymes associate with each other to recognize the recombination signal sequences (RSSs) that flank the regional gene segment and subsequently induce DNA cleavage.[41,42] *D* and then *V* gene segments of the heavy chain locus undergo sequence-specific recombination with the J region gene segments in order to produce a contiguous *VDJ* gene. This is followed by the transcription of a pre-messenger RNA containing the rearranged *VDJ* and a *C* gene (Figure 2). The *IGH* enhancer Eμ, lying downstream of the *J* segments, has been recognized as a regulatory element which activates the transcription.[43,44] A mature mu heavy chain is produced after splicing of the pre-messenger RNA, translation of the messenger RNA and elimination of the signal peptide. Light chain genes rearrange in a similar way, except that they lack a *D* segment. Assembly of the mu heavy chain and one of the light chains results in the formation of a membrane-bound antibody attached to the B-cell surface referred and to as the B-cell receptor (BCR).

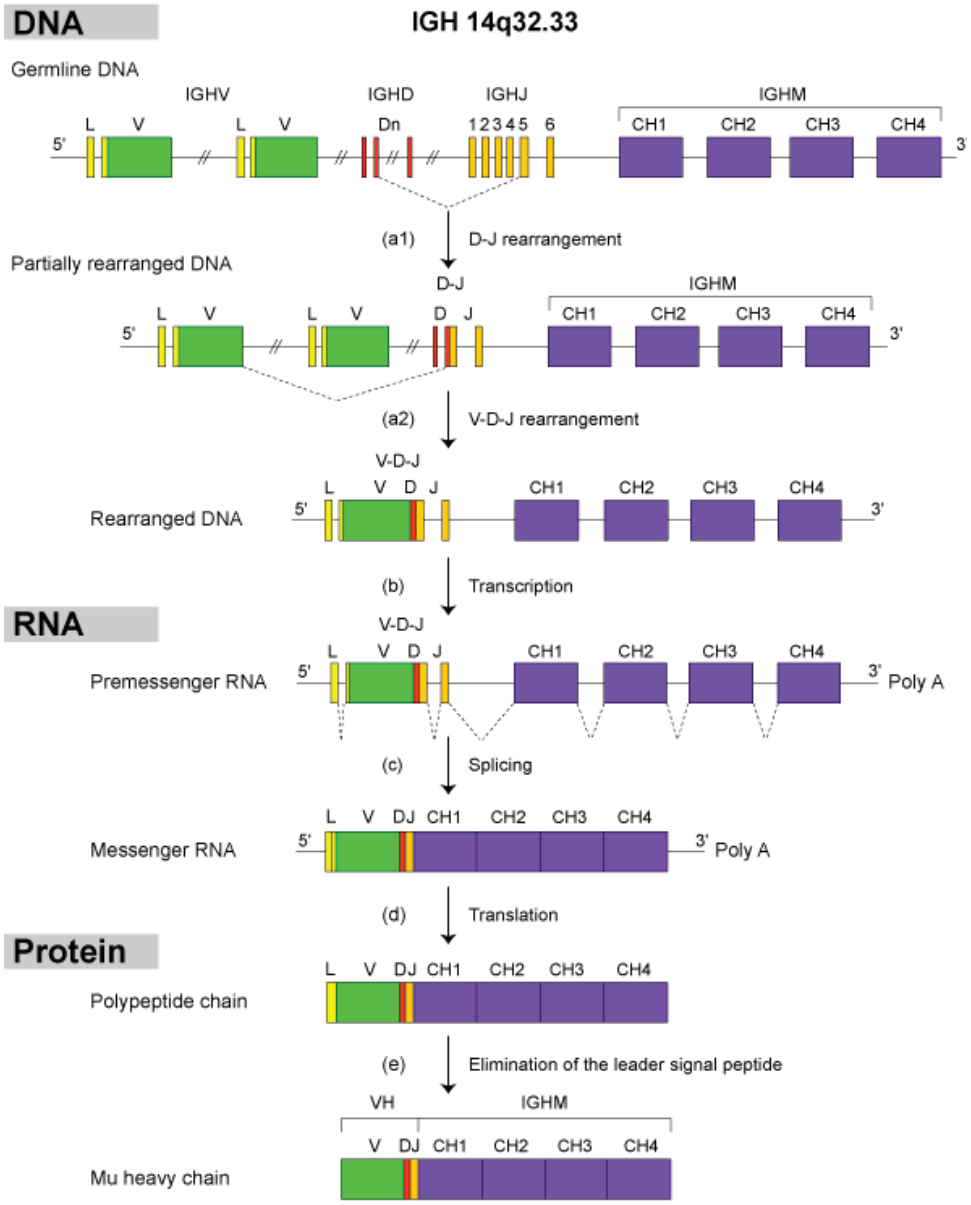

Figure 2. Synthesis of a mu heavy chain (http://www.imgt.org).[38]

A final point of interest concerning the recombination of *IG* genes is the process of allelic exclusion. As one allele in each B-cell successfully rearranges, the rearrangement of the other is prevented.[45-47] Accordingly, the productive genes are either those of the paternal chromosome or those of the maternal chromosome, and usually never the two together. If no successful rearrangement occurs on either chromosome, the cell dies.

Allelic exclusion of *IG* genes is the genetic basis of the concept "one B-cell – one antibody" or Burnet's clonal selection theory.[48] Although allelic exclusion has been investigated for more than 50 years, the mechanisms of allelic exclusion are still poorly understood.[45-47] Furthermore, it has been shown that this process can be subverted in some B-cell neoplasms including chronic lymphocytic leukaemia (CLL),[49] diffuse large B-cell lymphoma (DLBCL),[50] and mantle cell lymphoma (MCL).[51] The possible biological or clinical role of this observation is unknown.

1.3.2 Somatic hypermutation and class-switch recombination

The final differentiation stages of normal B-cells, from mature B-cells to plasma cells and memory B-cells, include two recombination processes called somatic hypermutation and class-switch recombination. These stages occur within the germinal centre of the secondary lymphoid organs (spleen and lymph nodes) following encounter with antigen.

Somatically mutated genes are therefore found in B-cell lymphomas of germinal centre origin like FLs, and also in B-cell lymphomas of post-germinal centre origin.[52] Somatic hypermutations cluster preferentially in the complementarity determining regions (CDRs), the antigen-binding sites of the *IGHV* genes, and represent a major mechanism for generating antibody diversity.[53,54] The somatic hypermutation process concerns single-nucleotide mutations or occasional insertions and deletions of nucleotides, and the transcriptional intronic enhancer of both heavy and light chains is necessary for somatic hypermutation to occur.[55] However, the

signals that initiate the mutation process and the reason why some regions are hot-spots for somatic hypermutations are incompletely understood.[56,57]

The class-switch recombination occurs shortly after somatic hypermutations and is frequently seen in follicular lymphomas.[58,59] In the switch recombination process, *C* gene segments are replaced resulting in switch of the IG isotype from IgM or IgD to either IgA, IgE, or IgG, that have defined roles in the immune system.[60] Class-switch recombination and somatic hypermutations probably have some common pathways as the enzyme activation-induced cytidine deaminase (AID) is required for both processes.[61] Expression of AID has also been found in FL.[62]

1.4 Pathogenesis of follicular lymphoma

A hallmark of many B-cell lymphomas, including FL, is the chromosomal translocations involving one of the *IG* loci and a proto-oncogene.[63] However, other factors than genetic alterations also have important roles in the development of lymphomas. Normal B-cells depend on B-cell receptor (BCR) expression for survival, and with few exceptions B-cell lymphomas are dependent on this expression. The tumour microenvironment might also contribute to the survival and proliferation of lymphoma cells.

1.4.1 Chromosomal translocations

The origin of IG chromosomal translocations in FL

The physiologic DNA damage introduced by somatic hypermutations and class-switch recombination places B-cells at risk for tumor-inducing mutations and translocations in the germinal centre, as alluded above. In accordance with this, most mature B-cell lymphomas are of germinal centre or post-germinal centre origin.

There is, however, evidence that the seminal translocation associated with FL, t(14;18)(q32;q21), occurs during *V(D)J* recombination in the bone marrow.[63,64] The t(14;18)(q32;q21) between the *IGH* locus and the *BCL2* gene have breakpoints at the *IGH* that are directly adjacent to the *J* gene segments, or that are adjacent to where the *D* gene segments join the *J* gene segments. The breakpoints often show loss of nucleotides at the end of the J or D gene segment and the addition of non-germline-encoded nucleotides, which also are typical features of *V(D)J* recombination. The breakpoints at the *BCL2* locus are clustered within the major breakpoint region (MBR), the minor cluster region (MCR) or in the intermediate cluster region. [65,66] It has been shown that the DNA in the MBR often acquires an altered structure that is cut by the RAG enzymes which mediate *V(D)J* recombination. Thus, the RAG enzymes may be responsible for the DNA breaks in both partner genes.[67]

The t(3;14)(q27;q32) between the *IGH* locus and the *BCL6* gene, also found in FL, involves breakpoints in the switch region, and most likely occurs owing to erroneous class-switch recombination.[68,69] Table 2 provides an overview of different chromosomal translocations found in some mature B-cell lymphomas and indicates the proposed underlying genetic mechanisms. The table is adapted from Bende et al.[70]

Table 2. Chromosomal translocations of mature B-cell neoplasms[70]

Lymphoma	Translocation	Genes involved	Mechanism	% of cases
MCL	t(11;14)(q13;q32)	*CCND1/IGH*	V(D)J	~95%
FL	t(14;18)(q32;q21)	*IGH/BCL2*	V(D)J	~90%
	t(3;14)(q27;q32)	*BCL6/IGH*	CSR	~5%
MZL	t(11;18)(q21;q21)	*API2/MALT*		25-40%
	t(1;14)(p22;q32)	*BCL10/IGH*	CSR	~5%
	t(14;18)(q32;q21)	*IGH/MALT*	CSR	10-25%
	t(3;14)(p14.1;q32)	*FOXP1/IGH*	CSR	ND
DLBCL	t(14;18)(q32;q21)	*IGH/BCL2*	V(D)J	15-30%
	t(3;14)(p14.1;q32)	*FOXP1/IGH*	CSR	ND
	t(3;14)(q27;q32)	*BCL6/IGH*	CSR	~35%
	t(3;various)(q27)	*BCL6/various*	SHM	~5%
	t(8;14)(q24;q32)	*C-MYC/IGH*	CSR/SHM	~10%
	t(8;22)(q24;q11)	*C-MYC/IGL*	SHM	~5%
BL	t(8;14)(q24;q32)	*C-MYC/IGH*	CSR/SHM	↓
	t(8;22)(q24;q11)	*C-MYC/IGL*	SHM	Together 100%
	t(2;8)(p11;q24)	*C-MYC/IGK*	SHM	↑
MM	t(11;14)(q13;q32)	*CCND1/IGH*	CSR	15-20%
	t(6;14)(p21;q32)	*CCND3/IGH*	CSR	~5%
	t(4;14)(p16;q32)	*FGFR3-MMSET/IGH*	CSR	~15%
	t(14;16)(q32;q23)	*C-MAF/IGH*	CSR	5-10%

MCL, mantle cell lymphoma; FL, follicular lymphoma; MZL, marginal zone lymphoma; DLBCL, diffuse large B-cell lymphoma; BL, Burkitt lymphoma; MM, multiple myeloma (plasma cell myeloma); CSR, class-switch recombination; SHM, somatic hypermutation; ND, not determined.

The BCL2 translocation

As a consequence of the juxtaposition of *BCL2* to *IGH* in FL, the *BCL2* gene is subjected to control of the *IGH* enhancer Eμ leading to the overexpression of BCL2 protein. The recognition of deregulated expression of the *BCL2* oncogene in FL[71] and its anti-apoptotic activity [72] have not only been essential in the understanding of FL development, but also elicited the investigations of apoptosis in cancer in general at the molecular level. Accordingly, resistance toward apoptosis is now considered a hallmark of most and perhaps all types of cancers.[73,74]

How the BCL2 protein family control apoptosis have been extensively investigated. [75-81] They may have either pro-apoptotic or anti-apoptotic function, and act in part by governing death signal through cytochrome C release. [75,78-80] BCL2 also regulates cell cycle progression by inhibiting G0 to G1 transition in the cell cycle.[76,77,81] This latter effect is in accordance with the lower proliferation index seen in FL as compared with normal germinal centres.

Many mouse models have been developed to provide insights on various oncogenes and tumour suppressor pathways involved in FL pathogenesis, but an appropriate FL model is still not available. In the Eμ-*BCL2* transgenic mice model, in which *BCL2* was driven by the *IGH* enhancer Eμ, the mice primarily developed follicular hyperplasia and not lymphomas. When coexpressed with a *MYC* oncogene, the *BCL2* gene was able to promote the formation of DLBCLs, but not FLs [82-85] In an alternative *BCL2* transgenic mouse model, in which *BCL2* was driven by the pan-haematopoiteic Vav-P promoter, the mice developed mature lymphomas reminiscent of FL, but still lacked the typical follicular architecture.[86]

The observation that BCL2 has pro-apoptotic functions and cell cycle inhibitory effects as well as that *BCL2* transgenic mice do not readily develop FL, highlight that additional secondary (genetic) alterations are necessary for FL development. The need of additional oncogenic events is further illustrated by the finding that t(14;18)(q32;q21) is commonly detected in healthy individuals.[87-89]

The BCL6 translocation

The *BCL-6* proto-oncogene at 3q27 was originally identified because of its common involvement in translocations affecting DLBCL patients. However, *BCL6* translocations are not specific for DLBCLs and are in addition detected in approximately 5% of FL cases.[90,91] The *BCL6* gene has been found not only juxtaposed to *IG* genes, but also to multiple non-*IG* genes including genes with possible oncogenic potential.[92,93] In contrast, almost all the proto-oncogenes in B-cell lymphomas such as *c-MYC* and *BCL2* translocate only to *IG* loci.[94] Similarly to the *IGHV* genes, the *BCL6* gene is affected by point mutations in B-cell lymphomas.[95,96] Experimental models have demonstrated that expression of *BCL6* is required for germinal centre formation and function.[97,98] The precise role of the *BCL6* translocation in oncogenesis of FL remains to be elucidated.[99,100]

1.4.2 Secondary genetic alterations

Apart from t(14;18)(q32;q21), the most common chromosomal abnormalities in FL are gains in 1q, 2p, 7, 8, 12q, 18q and X, and losses of 1p, 6q, 10q, 13q and 17p.[101] Mutations or alterations affecting *MLL2, EPAH7, TNFRS14,* and *EZH2* have also been reported in FL.[102] The sequence of acquisition of these alterations and how they contribute in FL pathogenesis is currently unknown.

1.4.3 Genetics of follicular lymphoma transformation

Histological progression of FL to DLBCL has recently been associated with genetic alterations deregulating cell-cycle progression and DNA damage responses (*CDKN2A*/*CDKN2B*, *MYC* and *TP53*), and with aberrant somatic hypermutations.[103]

Translocations deregulating the *BCL6* proto-oncogene and alterations involving chromosome 1p36 have also been associated with FL transformation previously.[104,105]

1.4.4 The B-cell receptor

B-cells produce antibodies first as membrane-bound B-cell receptors (BCRs) and then as secreted antibodies. Investigations of the BCRs and BCR signaling have been stimulated by studies of *IG* genes in B-cell lymphomas,[106] and BCR signaling is implicated as an essential pathway in lymphomagenesis.[107-110] Mechanisms of BCR activation include chronic antigenic drive by microbial or viral antigens, autostimulation of B-cells by self-antigens and activating mutations in intracellular components of the BCR pathway.[110] B-cell receptor signaling is currently a therapeutic target in various B-cell malignancies.[111]

The possible role of antigen selection in pathogenesis of FL has been studied recent years with conflicting results. It has been proposed that FL cells are selected on the basis of their BCRs, as the mutation patterns in the *IGHV* genes of FL are found to be comparable to normal antigen-experienced B-cells.[112-115] In particular, the analysis of replacement and silent mutation patterns has shown fewer replacement mutations in the framework regions (FRs) and more replacement mutations in the complement determining regions (CDRs) than expected by chance[112,115]. In contrast, other studies have revealed negative selection for replacement mutations only in the FRs, but no positive selection in the CDRs.[116-118] These results have been interpreted in favour of remaining structural BCR integrity in FL. However, the apparent lack of positive selection for replacement mutations in FL CDRs may not necessarily indicate a lack of antigen selection, and a recent study demonstrated that at least a subset of FL tumors are capable of recognizing self-antigens.[115]

Alterations in BCR signaling as compared to normal B-cells have been observed in FL.[119] Furthermore, it has been shown that FL samples contain a variable proportion of cells with impaired BCR signaling probably due to specific

suppression.[120] Increased numbers of impaired cells have been associated with tumour progression, implying that BCR activation may not be so relevant in the late stages of FL. It has also been proposed that N-glycosylation of the CDRs may be common in FL, and that creation of new N-glycosylation sites may provide growth advantage during FL development.[121-123] Antigen-independent BCR signaling or interactions with structures in the microenvironment may be enhanced by N-glycosylation.

1.4.5 Tumour microenvironment

Over the past decades, the view of a tumour as nothing more than a collection of relatively homogeneous cancer cells whose biology could be understood by elucidating the properties of these cells, has changed. The current view is that also the tumour microenvironment contributes in important ways to the pathogenesis of tumours.[74,124]

The role of the microenvironment is highlighted by the failure of FL cells to survive and grow autonomously *in vitro*.[102,125] In addition, proliferation of malignant FL cells occurs in close contact with non-malignant T-cells, macrophages, follicular dendritic cells and other stromal cell in an organization similar to their normal counterpart in lymph nodes.[126] The local enrichment of CD4-positive T-cells and stromal changes found in bone marrow infiltrates also suggest an impact of the microenvironment in supporting growth and persistence of tumour cells.[127,128]The central role of the tumour microenvironment has been further emphasized by revealing that several highly frequent genetic alterations are not oncogenic *per se* but favour the crosstalk of FL cells with neighboring cells, and by observing an impact on prognosis by molecular features of non-malignant cells.[129]

1.5 Clinico-pathological characteristics

1.5.1 Clinical features

Clinically, FL predominantly involves lymph nodes, but can also involve the spleen, bone marrow, peripheral blood and Waldeyer ring.[2] At time of diagnosis only 22-33% of patients present with early stage disease.[130,131] The bone marrow is involved in 40-70% of patients. Involvement of non-haematopoietic extranodal sites usually occurs during the course of the disease. FL may occasionally originate in extranodal sites including skin, duodenum, ocular adnexae, breast and testis.

Symptoms may include fever, night sweats and weight loss (B symptoms), fatigue, the local mass effect of lymph node enlargement, as well as those of bone marrow failure. However, FL patients are more often asymptomatic. Less than 10% of patients present with leukaemic phase,[132,133] and less than 20% of patients present with haemoglobin concentration <120 g/l or elevated serum lactate dehydrogenase (LDH).[130,131] FL has commonly been seen as a chronic relapsing, indolent tumour characterized by relatively long median survivals historically ranging from 6 to 10 years.[134-136] The risk of histologic transformation to high-grade lymphoma for patients with FL is around 20% at 5 years and 30% at 10 years.[137] The occurrence of transformation is associated with a more aggressive clinical course with rapid progression of lymphadenopathy, extranodal disease, B symptoms and elevated LDH.[137,138]

1.5.2 Morphology

Recognized in the 1920s, FL is a neoplasm composed of germinal (follicle) centre B-cells, typically both centrocytes and centroblasts.[139] Most cases show nodal effacement by uniformly-sized, closely-packed follicles that lack tangible body macrophages and possess attenuated or absent mantle zones (Figure 3A). The interfollicular neoplastic cells are often small centrocytes.[140] Some cases have a

mixture of follicular and diffuse areas, and rare cases show a completely diffuse pattern without identifiable follicular structures. Diffuse areas, often accompanied by sclerosis, are particularly seen in mesenteric or retroperitoneal disease.[2] The WHO classification identifies four architectural patterns of FL; follicular (>75% follicular), follicular and diffuse (25%-75% follicular), focally follicular (<25% follicular) or diffuse (0% follicular), although the clinical relevance of this is unclear.[141]

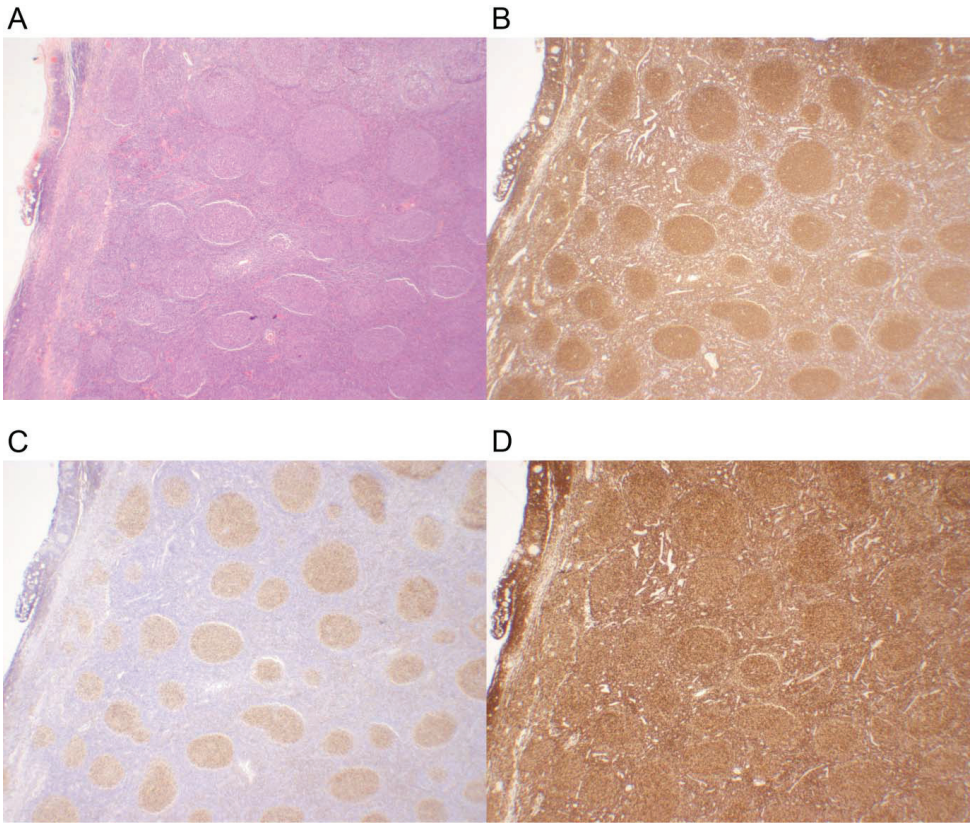

Figure 3. Microscopic images from a follicular lymphoma case. **A.** The typical follicular pattern (H&E) **B.** The follicles contain CD20-positive B-cells. CD20-positive B-cells are also seen in the interfollicular region, but to a lesser degree. **C.** CD10 is expressed by the follicles. **D.** The follicles, as well as the cells in the interfollicular area, are uniformly BCL2-positive. Magnification x25.

Grading of FL relies on counting the number of centroblasts in ten neoplastic follicles, expressed per 40x high-power microscopic field (HPF).[142] Three histological grades (grades 1 to 3) are recognized by the latest WHO classification.[2]

By definition, grade 1 has 0-5, grade 2 has 6-15, and grade 3 >15 centroblasts/HPF. Since grades 1 and 2 represent a continuum and are both clinically indolent, a distinction between them is not encouraged in pathology reports. Grade 3 FLs are subdivided according to the presence of centrocytes (grade 3A) and absence of centrocytes (grade 3B). Potentially, grade 3A FLs may belong within the spectrum of disease with FL grades 1 and 2. However, this issue remains controversial in the clinical setting.[90,91,143,144] Identifying areas of transformation to DLBCL may be more clinically relevant, and the WHO recommends reporting DLBCL as the primary diagnosis when transformation is present.[145]

The reproducibility of grading by the proportion of centroblasts has also been questioned.[146-148] The identification of centroblasts by histological examination may not be straightforward. Counting absolute number of centroblasts is time-consuming and errors may occur. Grading can be difficult where the histological features are not uniform and small needle core biopsies may not be representative. However, there has been insufficient evidence to recommend an alternative method or to recommend eliminating FL grading.[2,149]

Bone marrow involvement typically consists of paratrabecular infiltrates of lymphoid cells showing germinal centre cell morphology. Interstitial areas may be involved. A follicular growth pattern is rare but can be seen. Transforming cells on periphery of lymphoid cluster and foci of transformation resembling Reed-Sternberg cells with cleaved nuclei may also be seen.[150-152]

1.5.3 Immunophenotype

The tumour cells express B-cell associated antigens (CD19, CD20, CD22, CD79a) and germinal centre cell antigens (CD10, BCL6).[153] CD20 and CD10 expression in a follicular lymphoma case are shown in Figure 3B and 3C. The germinal centre cell antigens are downregulated or may be absent in the interfollicular areas and in the bone marrow.[140] Grade 3B FLs can lack CD10 expression, although BCL6 is often

retained.[90,144,154] In follicular areas, underlying networks of follicular dendritic cells are present, and can be demonstrated by CD21 or CD23.[155] In addition, numerous other non-neoplastic cells including follicular T-cells and varying number of histiocytes are found. Generally, the proliferation index in FL correlates with histologic grade.[90,156,157]

BCL2 protein is not expressed by normal or reactive germinal centre B-cells, but is expressed by a variable proportion of neoplastic cells in 85% of FL patients.[158] A follicular lymphoma sample with BCL2 positivity is shown in Figure 3D. BCL2 is more often negative in grade 3 FLs, than in low-grade FLs. BCL2 protein can be useful in distinguishing neoplastic from reactive follicles, although absence of it will not exclude the diagnosis. T-cells, primary follicles and mantle zones normally express this protein and can lead to erroneous interpretation.

In addition to reactive hyperplasia, the differential diagnosis of FL includes nodular lymphocyte predominant Hodgkin lymphoma, chronic lymphocytic lymphoma/small lymphocytic lymphoma (CLL/SLL), MCL and marginal zone lymphoma (MZL).[159] The nodules of nodular lymphocyte predominant Hodgkin lymphoma are usually larger than those present in FL and contain scattered large cells known as popcorn or lymphocyte predominant cells (LP cells). CLL/SLL shows a pseudofollicular pattern of small B-cells that are positive for CD5 and CD23 and are negative for CD10 and BCL6. MCLs usually express CD5 and cyclin D1. Both FL and extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) can form lymphoepithelial lesions and show proliferation of follicular dendritic cells,[160,161] but the tumour cells of MALT lymphoma are CD10- and BCL6-negative. Follicular colonization may be present in nodal marginal zone lymphoma, but the tumour cells are, as described for MALT lymphomas, CD10-and BCL6-negative.

In many cases, the distinction of FL from reactive hyperplasias and other B-cell lymphomas can reliably be made based on a combination of characteristic morphologic and immunophenotypic features. However, specialist review with

addition of molecular investigations is required at least in the cases displaying atypical clinical or pathological features.[159,162-164]

1.5.4 Molecular phenotype and investigations

Molecular tests have gained an established role in diagnosis of B-cell lymphomas.[2,164] The main methods are clonality assessment by analyses of *IG* gene rearrangements by polymerase chain reaction (PCR)[7,9] and fluorescence *in situ* hybridization (FISH) translocation analyses.[165,166] Other molecular strategies, such as gene expression profiling and array comparative genomic hybridization, have not been successfully integrated into standard practice.[167]

Clonality assessment

In principle, all cells of a malignancy have a common clonal origin. As B-cell lymphomas are derived from a single malignantly transformed B-cell, the tumour cells contain identically rearranged (clonal) *IG* genes. In contrast, normal lymphoid tissue and reactive lymphoproliferations contain cells with heterogeneity in their rearranged *IG* genes.[168,169] Thus, clonality assessment can aid in the differential diagnosis of a lymphoid malignancy versus a reactive condition.

PCR primers and protocols for detection of clonal *IG* rearrangements have been developed and standardized by a European consortium (BIOMED-2 Concerted Action BMH4-CT98-3936), and the BIOMED-2 strategy has become a world standard.[170-174] The BIOMED-2 approach include PCR primers that target multiple *IG* (*IGH*, *IGK* and *IGL)* gene rearrangements in eight multiplex PCR reactions and standardized methods for evaluation of PCR products, heteroduplex analysis and fragment analysis.[175-179] The majority of B-cell malignancies as well as FLs can be identified by the BIOMED-2 *IGH (VDJ)* and the *IGK* primer sets, when fresh or fresh frozen tumour material is used.[7-9]

Assessment of chromosomal translocations

Amongst the numerous B-cell lymphomas, only a few are associated with hallmark translocations. These include t(14;18)(q32;q21) in FL, t(11;14)(q32;q21) in MCL, and t(8;14)(q24;q32) in Burkitt lymphoma (BL).[167,180,181] Additionally, there are variable proportions of each of these lymphoma types that lack these hallmark translocations but still demonstrate other characteristic features that allow a specific diagnosis to be made. Thus, it is possible to diagnose FLs without t(14;18)(q32;q21), MCLs without t(11;14)(q32;q21), and BLs without t(8;14)(q24;q32) or a variant translocation. Furthermore, these hallmark translocations are not completely diagnostically specific, and t(14;18)(q32;q21) occurs in DLBCLs and t(11;14)(q32;q21) in plasma cell myelomas. Finally, the classical translocation t(8;14)(q24;q32) of BL, in which the *MYC* and *IGH* genes are involved, may be seen in DLBCL cases as well.

The t(14;18)(q32;q21) of FL can be detected by PCR, or by FISH using a break-apart probe for the *BCL2* gene. FISH seems to be the most reliable molecular method for diagnostic purposes.[165,166,181] The presence of t(14;18)(q32;q21) is related to grading of FL, and can be found in approximately 90% of the grade 1 and 2 FL, 60-70% of the grade 3A and 15 -30% of the grade 3B.[90] *BCL6* translocations in FL have previously been associated with grade 3B FLs, with or without a component of DLBCL.[91,143] This association was, however, not evident in recent study.[100] *BCL6* translocations can be detected by FISH analysis.[182]

Molecular investigations of t(14;18)(q32;q21) have been involved in identifying new entities and subtypes of FL. Primary cutaneous follicle centre lymphoma (PCFL) was previously denoted as a FL variant.[1] However, PCFL generally lacks t(14;18)(q32;q21) and is now recognized as a separate entity in the fourth edition of the WHO classification.[2] Some distinctive clinical and genetic FL subtypes are also recognized by the current WHO classification, such as primary duodenal (intestinal) FL and the paediatric type of FL. Although both subtypes

usually present with localized disease, primary duodenal FL frequently carries the t(14;18)(q32;q21), while paediatric FL frequently lacks t(14;18)(q32;q21) and *BCL6* translocations.[183-185] Thus, FL in children may differ from FL in adults. At present, the WHO working group does not recognize paediatric FL as a distinct disease.

These observations have been expanded on, and in particular several variants of FL lacking t(14;18)(q32;q21) appear to show some distinctive features.[149] A predominantly diffuse FL, clinically characterized by bulky disease mainly in the inguinal region and genetically characterized by a deletion in 1p36 has been reported.[186] Some t(14;18)(q32;q21)-negative FL show resemblance to late germinal centre B-cell stage by gene expression profiling,[187] whereas some exhibit intrafollicular plasma cell differentiation.[188] The current WHO classification retains also these negative cases within the broad group of FL, but the issue may be re-evaluated in the future as more clinical and genetic data are obtained.[149]

In addition, the WHO classification recognizes early neoplastic or pre-neoplastic proliferations, corresponding to the molecular phenotype of FL.[2,149] Intrafollicular neoplasia, or in situ FL, is characterized by the presence of t(14;18)(q32;q21) in CD10 and BCL-2 positive germinal centre B-cells of an otherwise reactive lymph node. Some of these patients are found to have FL elsewhere, but many patients remain without evidence of FL.[189,190] Thus, the significance of this phenomenon is unknown. The features of FL subtypes are summarized in Table 3. The table is based on an overview of FLs lacking t(14;18)(q32;q21) from Bagg et al.[167]

Table 3. Features of FL variants[167]

Variants	Features	t(14;18)
Primary duodenal (intestinal) FL	Duodenal FLs are predominantly found as multiple polyps in the second part of the duodenum. Most patients have localized disease and the prognosis is excellent.	Present
Paediatric FL	In contrast to adult FLs, paediatric FLs tend to be grade 3 and have large expansile follicles. They are usually localized and the prognosis appears to be good.	Absent
Diffuse inguinal FL	Typically bulky and localized disease with recurrent deletions of chromosome 1p36.	Absent
Testicular FL	FL can occur in almost any extranodal site. Testicular FLs are rare, and usually lack the translocation.	Absent
In situ FL	In situ FLs have preserved general architecture with germinal centres strongly positive for BCL2 and CD10. Involved follicles are often scattered, not confluent.	Present

1.6 Prognosis

1.6.1 Clinical factors

Prognostic scoring systems

Ann Arbor stage was the primary consideration in assessing prognosis of lymphoma patients. The Ann Arbor classification defines how widespread the disease is by four stages.[191] Stage I and II include disease in a single lymph node or lymph node region, or disease in two or more lymph node regions on same side of the diaphragm. Stage III and IV include disease in lymph node regions on both sides of the diaphragm and disseminated disease with involvement of extranodal sites such as the bone marrow. However, Ann Arbor stage was increasingly found to be an inadequate means of predicting survival. The International Prognostic Index (IPI) was therefore developed to aid in predicting prognosis for aggressive lymphomas. It identifies risk groups based on Ann Arbor stage, age, serum lactate dehydrogenase (LDH) level, performance status, and number of extranodal sites of disease.[192]

The IPI has previously been used in FL, but classifies only a small proportion of patients at high risk.[193] In 2004, an international collaborative study resulted in the publication of the Follicular Lymphoma International Prognostic Index (FLIPI).[130]. This index includes five adverse parameters: age >60 years, stage III-IV disease, haemoglobin concentration <120 g/l, elevated serum LDH, and number of nodal areas >4. Three risk groups were defined (low, intermediate, high) based on number of factors present (0-1, 2, ≥3) with 5-year overall survival 90.6%, 77.6% and 52.5%, respectively. The prognostic value of FLIPI has been confirmed in patients treated with immunochemotherapy,[194,195] and identifies patients with a higher risk of transformation.[196,197]

In 2009, the results from FLIPI2, another international collaborative study, was published.[131] In this prospective study, progression-free survival was chosen as principal end point. The FLIPI2 includes: age >60 years, bone marrow involvement,

haemoglobin <120 g/l, elevated serum β2-microglobulin, and lymph node size >6 cm. The FLIPI and FLIPI2 are useful prognostic scoring systems in clinical practice and valuable for stratification in clinical trials. However, marked variations in outcome remain within each risk group.

Biomarkers in blood

Other biomarkers in blood than those included in the FLIPI and FLIPI2 indices (haemoglobin, lactate dehydrogenase and β2-microglobulin) have also been correlated with outcome in FL, but none of these are well-established. Recently, presence of circulating FL cells in blood detected by cytologic smears and confirmed by flow cytometry was associated with shorter progression-free survival and overall survival.[133] Low levels of serum albumin, [130,198] elevated serum angiogenic factors including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and endostatin,[199,200] elevated serum tumor necrosis factor (TNF),[201] and soluble intercellular adhesion molecule-1 (sICAM-1)[202] have previously been associated with poor outcomes.

1.6.2 Histopathologic factors

Histologic grade

Many studies have suggested a correlation between histological grade and clinical outcome in FL.[156,203-205] It is clear that there is no difference in survival between patients with grade 1 and 2 FL. There are, however, still controversies about prognosis in relation to grade 3 FL. Several publications have suggested that grade 3B FL might behave similarly to de novo diffuse large B-cell lymphomas due to frequent lack of CD10 and BCL2 expression, and high frequency of *BCL6* rearrangements instead of *BCL2*.[90,143,154,206,207] Many of these studies actually included cases with diffuse areas, and these cases should have been classified as

diffuse large B-cell lymphomas according to the WHO third and fourth edition.[1,2] One report comprising 190 patients with FL grade 3 found no difference in survival outcome between FL grades 3A and 3B.[145] Furthermore, another study has found a gene signature in FL 3B that was closer to FL than to DLBCL of the germinal centre B-cell type.[208] Accordingly, these issues need to be revisited when more data are available.[149]

Proliferation index

Some studies have suggested that the proliferation index recognized by Ki67 immunostaining may aid or even may represent an alternative to the histological grading of FL.[156,157,209] Recently, a proliferation index level of 10% predicted poor progression-free survival and overall 5-year survival, and was an independent prognostic factor by multivariate analysis.[210] In another study, a proliferation index level of 30% was not associated with overall survival in grades 1 and 2 FL.[211] Determination of proliferation index is, similar to histological grading, subjective and can be influenced by staining technique. Thus, this issue needs to be further clinically validated.

1.6.3 Molecular factors

Cytogenetics

The majority of FL patients harbour a variable number of cytogenetic changes in addition to t(14;18)(q32;q21) at diagnosis. Previous studies suggested that a higher number of alterations was associated with an inferior survival.[212-215]Specifically, deletions of 1p, 6q, and 17p, and gains of 7 and 12q have been associated with poor prognosis and have been correlated with a higher risk of FL transformation.[216] In contrast, another large study of FL found no association between number or type of cytogenetic alterations and clinical outcome.[217]

Host constitutional genetics

The potential for host immune genetic signatures to predict FL response to treatment and prognosis has been investigated. Single nucleotide polymorphisms (SNPs) are changes in the DNA sequence affecting only one base pair. SNPs in the *FcγR* genes may alter the binding affinity between the Fc portion of rituximab and the Fc receptors of macrophages.[218,219] Recent studies have reported a correlation between the *FcRIIIA* and *IIA* genotype and response to rituximab monotherapy in untreated FL.[220-222] In contrast, other studies have not found a correlation between these polymorphisms and outcome in patients treated with cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) and rituximab.[223,224] Four immune response SNPs (*IL-8, IL-2, IL-12B* and *IL-1RN*) with prognostic impact have in addition been identified in FL patients not treated with rituximab.[225] Apparently, there have been no genome-wide association studies of FL prognosis.[226]

Microenvironment

Features of the microenvironment are likely to be important for the clinical course and biological aggressiveness in FL.[101] In 2004, a gene expression profiling study identified two survival-associated signatures, immune response 1 (IR-1) and immune response 2 (IR-2).[129] The IR-1 signature included genes expressed by T-cells and macrophages, and was associated with a more favourable prognosis. In contrast, the IR-2 signature comprised genes expressed by macrophages and dendritic cells, and was found to confer worse prognosis. Another gene expression profiling study however, did not confirm these findings.[227]

Numerous immunohistochemistry studies have tried to correlate the cellular microenvironment of FL with outcome.[228-235] However, the results of these different studies are highly inconsistent. The varying treatment modalities given,[233] small study cohorts and poor reproducibility of immunohistochemistry methodology might account for the differences.

T-cells comprise the majority of non-neoplastic cells in FL biopsies and could potentially play an important role in the microenvironment. A study from 2011 demonstrated that high levels of CD3, CD4, and CD8 T-cells in both tumours and blood led to superior treatment responses in FL patients treated with rituximab.[236]

In summary, the value of molecular factors in FL prognostication is currently limited. Additional molecular markers are probably needed to design further risk-adapted and targeted therapies for FL patients. Many biological correlates have been linked to FL prognosis, but the results are inconsistent and lack validation. The prognostic value of molecular staging by PCR-amplification of *IG* gene rearrangements has mainly been investigated in DLBCL and not in FL patients. Although *IGHV* mutational status has been established as a robust prognostic marker in CLL, *IGHV* mutational status has not been investigated in relation to FL prognosis

1.7 Treatment

The introduction of the anti-CD20 antibody rituximab has had a dramatic impact on how B-cell lymphomas are treated today.[237-239] Rituximab was first used in treatment of relapsed low-grade FLs,[240] then combined with cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) chemotherapy in treatment of DLBCLs.[241] Currently, rituximab is also indicated for the treatment of CLL in combination with fludarabine and cyclophosphamide (FC) chemotherapy, and for use in refractory rheumatoid arthritis (www.legemiddelhandboka.no). Furthermore, rituximab has been used in several immune-mediated diseases including granulomatosis with polyangiitis (Wegener's Granulomatosis), microscopic polyangiitis (MPA) and immune thrombocytopenic purpura (ITP) and as anti-rejection treatment of organ transplant recipients. It has been suggested that rituximab can help patients with chronic fatigue syndrome.[242]

1.7.1 Early stage disease

FLs are highly radiosensitive, and local radiotherapy is generally the treatment of choice for stage I and II disease.[159,243] The response rate of this treatment is high with 80% of patients having long-term disease control at 5 and 10 years.[244,245] It has been demonstrated no loss of efficacy with radiotherapy doses of 24 Gy as compared with the conventional doses of 40 Gy used in the past.[246] The majority of relapses occur outside the radiation field,[247] but there has not been demonstrated any additional benefit from adjuvant chemotherapy.[248] Observation may be an alternative approach when radiotherapy is thought to be undesirable, especially in patients with low-grade FL,[249] or in patients where the tumour has been fully excised at biopsy.[250] Recently, the paradigm that radiation therapy should be standard has been challenged. In a large prospective study, variable treatment approaches including the combination of chemotherapy and rituximab, radiation therapy, observation, and rituximab monotherapy, resulted in similar excellent outcomes.[251].

1.7.2 Advanced stage disease

The approach of watchful waiting in patients with asymptomatic stage III and IV disease has been supported by randomized prospective trials.[135,252] Patients who undergo watchful waiting do not have an increased risk of transformation to DLBCL,[253] and the initiation of systemic therapy can be deferred by 2-3 years.[135] An alternative option may be rituximab monotherapy.[254]

Standard first line treatment for patients with symptomatic advanced stage disease is the combination of chemotherapy and rituximab. The survival benefit of immunochemotherapy has been favoured by a Cochrane meta-analysis.[255] Five phase III trials have confirmed the efficacy of rituximab in combination with different chemotherapy regimens.[256-260] The chemotherapy regimens used in these studies were: CHOP;[256] bendamustine;[260] mitoxantrone, chlorambucil and prednisolone (MCP) with interferon;[258] cyclophosphamide, vincristine and prednisolone (CVP);[257]

and cyclophosphamide, doxorubicin, teniposide and prednisone (CHVP) with interferon.[259] It has been suggested that the duration of response in patients treated with anthracyclin-based therapies might be superior to other regimens utilizing alkylators. This has led to the widespread adoption of R-CHOP as first line therapy in FL. In a recently published phase III trial, R-Bendamustine had superior progression-free survival and less toxicity as compared to R-CHOP, thus R-Bendamustine could be considered as preferred first-line treatment approach.[261]

There is evidence from randomized phase III studies that rituximab maintenance significantly prolongs progression-free survival.[262,263] The chemotherapy regimens used in these studies were CHOP, CVP or fludarabine, cyclophosphamide and mitoxantrone (FCM). Rituximab maintenance is currently recommended by the British Committee for Standards in Heamatology in patients responding to first line rituximab-based chemotherapy.[159]

Radioimmunotherapy as the initial treatment has been evaluated in a limited number of FL patients or patients with limited follow-up.[264] As consolidation, radioimmunotherapy has been applied in several studies.[265,266] However, the potential benefits of combining rituximab-based induction with radioimmunotherapy as consolidation and/or extended rituximab maintenance therapy remain to be determined.

Previous studies have compared high-dose treatment and autologous stem cell rescue with conventional chemotherapy as first line treatment for FL.[267,268] No differences in survival were detected by these studies, a finding that could be related to an increased risk of secondary malignancies. Accordingly, autologous stem cell rescue has a limited role in first line therapy for FL.[159]

1.7.3 Relapsed or transformed disease

The combination of chemotherapy and rituximab has shown to improve outcomes of relapsed FL.[269-271] The optimal chemotherapy regimen at the point of relapse has not been determined. Options of chemotherapy include CHOP and FCM. [269-271] It appears that maintenance rituximab improves outcomes in relapsed FL.[270-272] Although rituximab has been used as monotherapy in the relapsed setting, the response rates are markedly improved with addition of chemotherapy. Palliative radiotherapy can be used in patients with localized, symptomatic disease.[273] Other treatment modalities that may be useful in relapsed FL are radioimmunotherapy and autologous or allogenic stem cell transplantation.[274-276]

Data regarding the optimal treatment for patients with transformed FL are limited.[277,278] They are generally treated with CHOP or CHOP-like regimens if they have not received them early in the course of the disease. Other chemotherapy regimens can be used as salvage therapy and include combinations of ifosfamide, carboplatin and etoposide (ICE), etopside, methylprednisolone, cytarabine and cisplatin (ESHAP), and cytarabine, cisplatin, and dexamethasone (DHAP). These regimens are usually co-administered with rituximab. High-dose treatment and autologous stem cell rescue may be a treatment option in patients responding to salvage therapy.[159,279]

2. Aims of the study

2.1 General aims

The general aims of this PhD project were to identify and evaluate molecular methods that could enable more accurate diagnosis, aid the identification of prognostic markers and provide insights into the pathogenesis of follicular lymphomas

2.2 Specific aims

1. In **Paper I**, the aim was to evaluate the application of molecular clonality analysis of *IG* genes on formalin-fixed, paraffin-embedded follicular lymphoma samples.

2. In **Paper II**, the aim was to determine the prognostic value of bone marrow involvement by molecular clonality analysis of *IG* genes in patients with follicular lymphoma.

3. In **Paper III**, the aim was to analyze the usage of *IGHV* genes and *IGHV* mutational status in relation to clinico-pathological factors and survival in patients with follicular lymphoma.

3. Materials and methods

3.1 Materials and patients

In **Paper I**, formalin-fixed, paraffin-embedded (FFPE) tumour biopsies from patients diagnosed between 1998 and 2008 with grade 1 to 3B FLs or FLs transformed into DLBCLs were obtained from the archives of the Department of Pathology, Haukeland University Hospital, Bergen, Norway and used for molecular studies (Table 4). In **Paper II**, all patients with grade 1-3B FL that had a bone marrow aspirate and a concurrent bone marrow biopsy obtained at diagnosis were included. These patients were diagnosed March 2003-July 2011. The bone marrow aspirates were used for molecular studies. In a subset of patients, FFPE tissue tumour biopsies were used for comparative analyses. The study cohort from **Paper II** was extended in **Paper III**. Patients with grade 1-3B FL in which a bone marrow aspirate was not obtained at diagnosis were also included. These patients were diagnosed February 2003-July 2011. In this study, FFPE tumour biopsies were used for molecular analyses.

Table 4. Study material

Inclusion period	No. of patients	Histology	Material	No. of samples	Method	Paper
1998-2008	118	FL 1-3B, DLBCL	FFPE	118	PCR-based clonality analyses	I
2003-2011	96	FL 1-3B	Fresh, FFPE	96 33	PCR-based clonality analyses	II'
2003-2011	106	FL 1-3B	FFPE	108	Sanger sequencing	III'

'Clinical information was included in paper II and III.

3.2 Clinico-pathological variables

Clinical variables including patient age at diagnosis, sex, date of primary diagnosis, clinical stage, Follicular Lymphoma International Prognostic Index (FLIPI) score, treatment data and survival were recorded from medical records in **Paper II** and **III**. Last date of follow-up (**Paper III**) was December 6, 2013, and median follow-up time for the patients was 61 months in **Paper II** and 75 months in **Paper III**.

FL diagnoses and grading in **Paper I-III** were based on morphological and immunohistochemical assessment of tumour biopsies from lymph nodes and extranodal sites, and were classified in accordance with the 2008 WHO Classification of tumours of haematopoietic and lymphoid tissues.[2] In **Paper II,** reports on lymphoma involvement of bone marrow biopsies were retrieved from pathology records. Routine assessment of bone marrow biopsies was based on H&E, CD20, CD3, IGK and IGL stains. Bone marrow biopsies are in diagnostic routine reported as positive (cytologic or architectural evidence of malignancy), negative (no or only a few well-circumscribed lymphoid aggregates), or indeterminate (increased number or size of aggregates without cytologic or architectural atypia). A review of the bone marrow biopsies of all cases with PCR-based clonality detected in the aspirate, but with negative pathology report, was performed according to these criteria in **Paper II**. The paratrabecular pattern predominant in FL showing lymphoid aggregates with broad bases aligned alongside bony trabeculae without intervening fat cells was recorded as well (Figure 4).

3.3 Flow cytometry

Results from immunophenotyping, of bone marrow aspirates were collected from laboratory records in **Paper II**. The flow cytometric analyses were performed at Department of Immunology and Transfusion Medicine, Haukeland University Hospital. All samples were tested for expression of CD5, CD10, CD11c, CD19,

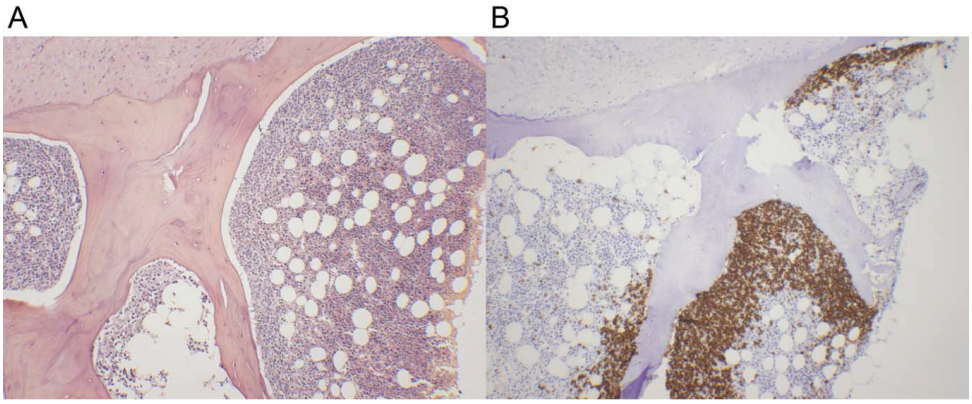

Figure 4. Bone marrow involvement in a case of follicular lymphoma. **A.** Paratrabecular lymphoid aggregates (H&E). **B.** The lymphoid cells are CD20-positive. Magnification x100.

CD20, CD22, CD23, CD45, IGK and IGL. Clonality was defined as light chain restriction with a IGK:IGL ratio of >3:1 or <0.3:1.

3.4 Molecular methods

3.4.1 DNA extraction

DNA extraction was performed by different methods. Isolation based on magnetic particles was employed in **Paper I** and **III**, while spin-columns were used in **Paper II**.

Two 10-µm sections from the FFPE tissue were deparaffinised with xylene and dehydrated in alcohol in **Paper I**. Deparaffinised tissue was digested overnight with proteinase K (20 mg/ml) before an automated workstation (BioRobot M48; Qiagen, Hilden, Germany) and the MagAttract DNA Mini M48 Kit (Qiagen) were used for DNA extraction.

In **Paper II**, DNA was extracted from fresh bone marrow aspirates and from FFPE tissue. The fresh aspirates were collected in EDTA (1.8 mg/ml) and

lymphocytes were isolated using Fiqoll-Paque PLUS medium (GE Healthcare, Little Chalfont, Buckinghamshire, UK). From FFPE tissue, two 10-µm sections were deparaffinised with xylene and dehydrated in alcohol. Isolated lymphocytes and deparaffinised tissue were digested overnight with proteinase K (20 mg/ml) and DNA was prepared manually using the E.Z.N.A. Tissue DNA kit (Omega Bio-Tek, Norcross, GA, USA).

In **Paper III**, two 10-µm sections from the FFPE tissue samples were deparaffinised with Deparaffinization Solution (Qiagen) and extraction was performed on a QIAsymphony SP automated workstation in combination with the QIAsymphony DSP DNA Mini Kit (both Qiagen).

3.4.2 PCR amplification

In **Paper I-II**, the PCR analyses were adapted for FFPE tissue by using the QIAGEN Multiplex PCR kit with a modified PCR protocol. PCR was carried out by mixing 2.5 µl primer mix (2 µM of each primer), 12.5 µl of the Multiplex buffer, and 2.5 µl DNA template to a final reaction volume of 25 µl by adding H_2O. PCR conditions were as follows: an initial denaturation/activation step (95ºC, 15 min), 38 cycles consisting of denaturation (95ºC, 45 sec), annealing of PCR primers (60ºC, 90 sec) and extension (72ºC, 90 sec), and a final extension step (72ºC for 10 min followed by cooling to 4ºC). All samples were run with undiluted and five-fold diluted template DNA. Polyclonal and non-template controls were always included. PCR amplification was performed according to the same protocol when DNA was obtained from the fresh bone marrow samples in **Paper II**. In **Paper I**, eight *IG* primer sets from the BIOMED-2 study were included.[7] In **Paper II,** the VH-FR2-JH, VH-FR3-JH, and Vκ-Jκ primer sets were included in most samples, whereas the VH-FR1-JH and V_K/intron-Kde primers were added in only nine. In both studies, reverse primers were labelled with fluorescent dye (6-FAM) and the PCR products were subjected to fragment analysis on an ABI 3100 capillary sequencer (Applied

Biosystems, Foster City, CA, USA). Data was analysed using the GeneMapper software (Applied Biosystems).

3.4.3 Sequencing analysis

Prior to DNA sequencing by Sanger method in **Paper III**, PCR was performed in six separate reactions with the *IGH* framework 1 primers combined with the JH consensus primer as designed for the BIOMED-2 study.[7] The PCR protocol was adapted for FFPE tissue as previously described. PCR products were analyzed on a 3% agarose gel, visualized with ethidium bromide staining, and PCR reactions that showed bands of appropriate size were identified. The Illustra™ ExoProStar™ 1-Step Kit (GE Healthcare, Little Chalfont Buckinghamshire, UK) was used in order to remove unincorporated primers and nucleotides, before sequencing was performed in both directions using the same primers as in the PCR amplification and the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing reactions were purified by the BigDye® XTerminator™ Purification Kit and subsequently analyzed on an ABI Prism 3130 Genetic Analyzer (both Applied Biosystems). Sequence data analysis was done using CLC Main Workbench (Qiagen) and DNA sequence reads were aligned to the germline *IGH* sequences derived from the IMGT/V-QUEST database (http://www.imgt.org/IMGT_vquest/vquest).

3.5 Statistical methods

The Chi-square test was used to compare frequencies in **Paper I-III**. Where applicable, Fisher's exact test was performed to compare two categorical variables. Agreement between clonality raters was measured by the Cohen's kappa coefficient in **Paper I**. Continuous variables not following the normal distribution were

compared between two or more groups using the Mann-Whitney *U* or Kruskal-Wallis tests in **Paper III**.

In **Paper II** and **III**, survival curves of time to death due to lymphoma were estimated using the product-limit procedure (Kaplan–Meier method) with date of histological diagnosis as the starting point. Differences between categories were estimated by the log-rank test. Patients who died of other causes than lymphoma were treated as censored observations. Median follow-up time was estimated by the reversed Kaplan-Meier method. Univariate and multivariate analyses of the influence on covariates on survival were performed with the Cox proportional hazards method. The variables were tested by log–log plot, and the proportionality assumption did not seen to be violated. Backward and forward selections of variables were performed to determine the variables' ability to be incorporated in multivariate models. Statistical analyses were carried out using the statistical package SPSS version 17.0 and 21.0 (SPSS Statistics, SPSS, Chicago, IL, USA). Results were considered significant if $P \leq 0.05$.

3.6 Ethical considerations

In this PhD project, human biological material has been used in all three studies, and personal health information was included in the two last papers. Approval of the study and dispensation from the requirement of patient consent was granted by the Regional Committee for Medical and Health Research Ethics (REC South East, 2013/211). In the discussion of this thesis, unpublished data are presented, and the approval and dispensation included these patients as well. The Norwegian Institute of Public Health was also contacted, and reservation against the use of diagnostic material for research purposes was not registered for any of the patients. All studies were performed in accordance with the Declaration of Helsinki.

4. Main results

In **Paper I**, we investigated the application of clonality analysis of *IG* genes on FFPE FL samples when the BIOMED-2 PCR approach targeting multiple *IG* genes were used. The clonality analyses were also adapted for archival tissue by using the Multiplex PCR kit with HotStarTaq DNA polymerase from Qiagen and with the number of PCR cycles increased from 35 to 38. By this modified PCR protocol we were able to amplify larger-sized PCR products. Clonality was detected in 94.9% of all FL samples when analyses for *IGH* and *IGK* gene rearrangements were combined. The clonality detection rate was somewhat lower than previously reported for fresh and frozen tissues, but was improved as compared to previous studies on FFPE tissue. With respect to performance of clonality detection, aged versus more recently stored FFPE samples did not appear significantly different.

In **Paper II**, we evaluated the contribution and prognostic value of bone marrow involvement by PCR-based clonality analysis of *IG* genes in patients with FL. Bone marrow involvement by PCR-based clonality was associated with advanced clinical stages, high Follicular Lymphoma International Prognostic Index (FLIPI) scores and the detection of bone marrow involvement by morphology or flow cytometry. PCR-positive patients had a significantly poorer survival than PCR-negative patients, whereas involvement by morphology showed no significant impact on survival. Thirteen patients positive by PCR but without morphologically detectable bone marrow involvement, had significantly poorer survival than patients with negative morphology and negative PCR result. The poor survival associated with PCR-based bone marrow involvement was independent of high FLIPI score. Flow cytometry analysis turned out to be the least sensitive method for detection of bone marrow involvement, and did not show a significant impact on survival.

In **Paper III**, sequence analysis of *IGHV* genes showed that *IGHV3, IGHV1* and *IGHV4* were the most frequently used subgroups in FL tumour samples and that *IGHV3-23* was the most frequently used gene. Patients with tumours using the *IGHV5* subgroup or more than one *IGHV* subgroup showed significantly poorer

survival than patients with other *IGHV* subgroups. The poor survival associated with such usages was of independent prognostic importance in Cox multivariate analysis. Unmutated sequences, showing >98% homology, were detected in 15.2% of cases. Unmutated *IGHV* genes were associated with older age at diagnosis, but not with survival.

5. Discussion

5.1 Methodological considerations

5.1.1 Patients

The selection of FL samples for the study of archival FFPE material in **Paper I** was based on a search in the electronic pathology record system of the time period 1998-2008 and the availability of biological material in the archives at the Department of Pathology. The electronic pathology record system was implemented in 1998, and no attempt to search for older material was done. Material older than 10 years is seldom needed for diagnostic purposes.

From January 2003 fresh bone marrow aspirates from patients diagnosed with lymphomas have been evaluated by molecular methods as a part of the staging procedures at the Haukeland University Hospital. All patients included in **Paper II** and **III** had a bone marrow aspirate obtained for molecular investigations prior to August 2011. In **Paper II**, only patients that had a bone marrow aspirate and a concurrent bone marrow biopsy at diagnosis were included. Although considered standard in initial investigations following a diagnosis of FL, bone marrow examination may not have been performed in all FL patients diagnosed at our hospital. There are, however, few contraindications to bone marrow examination, and it was likely that the procedure was performed in most FL patients. The patient characteristics were also in accordance with previous investigations of FL patients (Table 5). It is therefore reasonable to consider our patient series as representative for the FL patient group. Moreover, our relative long follow-up time contributes to a realistic picture of FL prognosis.

Table 5. Patient characteristics for paper II and III

Variables		FLIPI[130] % (n=1795)	FLIPI2[131] % (n=942)	**Paper II** % (n=96)	**Paper III** % (n=106)
Sex	Female	49	50	61	60
	Male	51	50	39	40
Age	≤60	63	57	44	43
	>60	37	43	56	57
Clinical stage	I-II	22	32	33	32
	III-IV	78	68	67	68
FLIPI score	0-2	73	76	71	71
	≥3	27	24	29	29
WHO grade	1-2	ND	72	68	73

FLIPI, Follicular Lymphoma International Prognostic Index; ND, not determined

5.1.2 Samples

FFPE tissue samples represent the most essential material for standard routine diagnostics. The advantages of FFPE include easy handling, high level of morphological preservation, suitability for immunohistochemical analyses, low cost of large-scale application, and inexpensive long-term storage.[280,281] The pathology archive of our hospital contains FFPE tissue blocks and original slides from the 1930s, and represents a valuable source for retrospective research of biomarkers in addition to its diagnostic value.[282,283]

However, fresh or fresh frozen tissues are the gold standard for molecular analyses as formalin fixation compromises analysis of biomolecules, in particular RNA and proteins. DNA is generally considered less altered.[284] In **Paper I-III**, DNA extracted from FFPE tissue samples from the archive was used for molecular studies. The challenge of a robust and reliable PCR amplification was recognized in all these three studies and more specifically addressed in **Paper I**. It is well accepted that DNA extracted from FFPE tissues frequently is degraded and contains mainly small fragments that may represent a poor substrate for PCR.[285,286] Furthermore, the extent of DNA degradation may be influenced by several factors including pre-fixation time, composition of fixative (formalin concentration, pH, salt concentration), tissue type, temperature and duration of storage.[284,287,288] Some variations in sample acquisitions, fixatives and tissue processing, and storage conditions most likely occurred during the study period, and accordingly may have affected the results in **Paper I-III**.

In addition, PCR amplification may have been inhibited by insufficient removal of paraffin or because of inhibition of the DNA polymerase by fragmented DNA from the FFPE tissue.[281] Formaldehyde also leads to the formation of DNA-protein crosslinks that may possibly reduce PCR performance and introduce artificial mutations when sequencing.[289]

In **Paper II**, also DNA extracted from fresh bone marrow aspirates was used for molecular studies. Fresh tissue samples may provide optimal quality DNA, but pose logistical considerations including handling of the specimens and storage.[290-292] Nevertheless, the growing collection of disease-oriented fresh samples and their derivatives (DNA/RNA) at the Department of Pathology also represents an important source for clinical and laboratory research.

5.1.3 Methods

DNA extraction

DNA extraction from FFPE tissue and fresh bone marrow aspirates was performed by extraction procedures that were routinely employed in the laboratory at the study time for each paper, and were then kept throughout each study. Accordingly, extraction with magnetic particles was used by an automated workstation (BioRobot M48) in **Paper I** and by another automated workstation (QIA Symphony SP) in **Paper III**, while manual spin-columns was used in **Paper II** as described in the methods section.

In the hospital laboratory, manual extraction by spin columns has been the preferred method because higher DNA yields were obtained as compared with automated DNA extraction methods. This is particularly true for FFPE samples where limited amount of tumour tissue is available. The use of the BioRobot system in **Paper I** and QIA Symphony system in **Paper III**, may thus possibly have affected the results. However, less hands-on time for DNA extraction is an important factor to consider when methods for use in routine molecular diagnostics are chosen. In addition, the methods and protocols for DNA extraction from FFPE tissues have improved.[293-295] Recently, magnetic particle technology produced optimum DNA concentration and quality, as measured by real-time PCR using the *GADPH* gene, from FFPE tissues.[295] This extraction technology has also successfully been used on FFPE tissues to investigate next generation sequencing (NGS) technologies in cancer genomics.[296]

In **Paper I-III**, whole tumour tissue sections were cut from the FFPE blocks. This procedure is easy to perform and less time-consuming as compared to micro-dissection which also requires expensive equipment. However, whole sections will contain a variable number of non-tumour cells due to tumour heterogeneity. In addition, small traces of foreign material can be introduced by the microtome blade during the sectioning, in the same manner as tissue contamination may occur during the process of paraffin embedding.[297,298] These aspects may have influenced the interpretation of results. Nevertheless, standard laboratory precautions, such as

changing microtome blades and gloves for different tumour samples, to avoid contaminations were undertaken in handling the FFPE blocks in **Paper I-III**.

Molecular clonality testing

Southern blot analysis has been the gold standard for molecular clonality testing for a long time. The Southern blot approach is highly reliable with a high specificity.[168] Southern blot analysis is, however, time-consuming and technically demanding. Another major disadvantage by this approach is the need of relatively large amounts of high-molecular weight DNA complicating analysis of small biopsies and FFPE material common in routine diagnostic practice. Thus, Southern blot has increasingly been replaced by PCR techniques. PCR techniques have several advantages, in particular speed. In addition, PCR techniques allow the use of limited amounts of medium quality DNA,[299] and are consequently more applicable in routine diagnostics. In **Paper I** and **II**, the chosen method for molecular clonality testing (PCR, BIOMED-2 primers and automated fluorescent fragment analysis) was up-to-date regarding to what is recently published[7-9] and what is recommended for use in diagnostic work.[2]

There are, however, technical and immunobiological pitfalls to consider when interpreting results from fluorescent fragment analysis (Table 6). The table is adapted from Langerak et al.[180] These difficulties in interpretation are also illustrated by the low interobserver agreement score observed for the Vκ-Jκ assay in **Paper I**. In principle, normal or reactive lesions show polyclonal PCR products with a Gaussian size distribution when electrophoretic data is analyzed (Figure 5A). The identification of one or two consistent peak(s), with or without a polyclonal background, within the expected size range is usually interpreted as positive for clonality and favours a lymphoma diagnosis (Figure 5B). Sometimes, however, a PCR product outside the expected size range can be obtained. The expected size ranges represent the 5 to 95 percentiles of the natural heterogeneity of the complementarity-determining region (CDR), and peaks just outside the expected size range can be interpreted as true

rearrangements products. In cases of considerably smaller (undersized) or larger (oversized) products, sequencing analysis is warranted for confirmation. Three FL cases in **Paper I** had undersized or oversized clonal products that accordingly were confirmed by sequencing.

Table 6. Pitfalls in *IG* clonality testing[180]

Pitfall	Possible explanation
Peaks just outside size range	Complementarity-determining region 3 regions/junctions outside 5-95% size range interval
Undersized or oversized peaks	Internal deletions (e.g. *V* segment) or extended amplification (e.g. somatic hypermutations in rearranged *J* segment)
Multiple clonal signals	Bi-allelic rearrangements or biclonality
Lack of clonal signal and lack of polyclonal Gaussian curve	Few B-cells in sample, poor DNA quality, and no detection of clonal signal due to somatic hypermutations
Selective amplification and pseudoclonality	Few B-cells in sample
Oligoclonal B-cell repertoire in peripheral blood of elderly individuals	Ageing of the immune system, might influence the B-cell repertoire as well as the T-cell repertoire
Oligo-monoclonality in histologically reactive lesions	Exaggerated immune response with dominant specificity, presence of large germinal centres

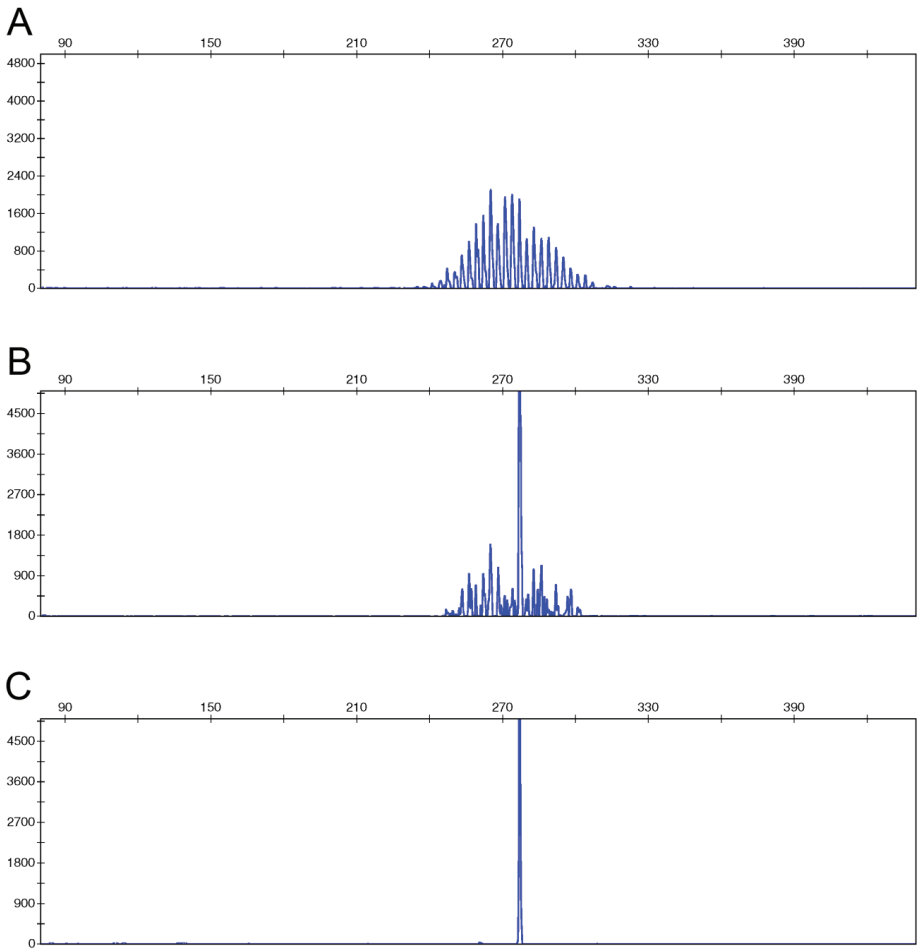

Figure 5. A typical polyclonal Gaussian curve generated by the *IGH* framework region 2 assay is shown (**A**). A typical clonal peak obtained in the bone marrow aspirate of a FL patient (**B**). An identical sized clonal peak was evident in the primary tumour of this patient (**C**). The expected size range of the PCR products obtained by the *IGH* framework region 2 assay is between 250 to 295 base pairs. The images have been edited for the purpose of clarity.

Pseudoclonality and oligoclonality may be more difficult to recognize in clonality testing. The PCR reactions are usually performed in multiplicates in order to avoid such pitfalls. In **Paper I** and **II**, all PCR reactions were accordingly run with undiluted and fivefold-diluted DNA template to compare patterns for consistency. In diagnostic routine at the hospital, PCR reactions are currently run with two undiluted and two fivefold-diluted DNA templates. PCR analysis may also be repeated using DNA from the same tissue, a second independent DNA isolation, and/or DNA from related tissue. Patterns may be compared with the primary process in case of staging, and a comparison with the primary tumour was performed for the positive bone marrow samples in **Paper II** (Figure 5C). Comparable peaks were obtained in most (94%) of the positive cases. Monoclonal, polyclonal or non-template controls may in addition facilitate the interpretation, and such controls have been included in **Paper I-III**.

It is also considered beneficial to confirm the presence of clonality using two independent PCR targets, whenever possible, and to interpret the data within the context of clinical, histological and immunohenotypic context.[7-9,180]

Sequencing analysis

In **Paper III,** direct DNA sequencing of the appropriate PCR reaction by the Sanger method was performed with forward and reverse primers as described in Section 3.4.3. The method is established in the hospital laboratory for routine analysis of mutational status in CLL patients, and is based on recently published literature,[170]as well as diagnostic guidelines.[11,300] The Sanger method provides accurate sequences with a reasonable read length. However, DNA sequencing technologies have made tremendous progress during the last decades.[301] The development of the next-generation sequencers that can sequence mixtures of up to millions of DNA molecules simultaneously, instead of individual clones separately will inevitably change the previously almost exclusive use of the Sanger method. Next-generation

sequencing technology is, however, not yet established in routine lymphoma diagnostics.

5.2 Comments on results

Progress in cancer research has been followed by improved treatment and survival of cancer patients. If patients are to receive appropriate treatment, early diagnosis of cancer with high accuracy is essential. Correct diagnosis is not only crucial for selection of treatment and for providing information on patient prognosis,[302] but also for inclusion of appropriate patients in clinical trials and cancer registries.

More effective treatments options have led to increased cure rates and improved survival for lymphoma patients. However, the diagnosis and classification of lymphomas is a well-recognized complex and difficult area in pathology. For most practicing pathologists, lymphomas are uncommonly seen, and lymphoma subtypes display considerable morphological overlap. This difficulty has previously been illustrated by high rates of pathology malpractice claims involving lymphoma misdiagnosis.[303] Multiple studies have also shown relatively high levels of disagreement regarding pathological diagnosis in this field, even between haematopathologists.[159,304-308]

Lymphoma diagnostics has become more consistent after the introduction of the WHO classification in 2001.[1] The WHO emphasized the use of methods such as immunohistochemistry and molecular techniques in addition to morphological features when assessing lymphomas. This approach was reinforced by the updated WHO classification in 2008.[2] Accordingly, a decline in the number of malpractice claims involving lymphomas in the period from 2004-2010 has been reported.[309] Recently, decreased discrepancies between referral and review diagnosis of lymphomas have also been observed.[159,308,310] This decrease may reflect the gradual adoption of the WHO classification, as well as the increased use of ancillary methods in routine practice. Furthermore, the implementation of expert panels of

haematopathologists involved in regular review and multidisciplinary discussion of lymphoma cases may have contributed to the observed decrease.

This PhD project is based on three publications (**Paper I-III**), and is focused on molecular analysis of *IG* genes in FLs. Molecular analysis of *IG* genes has an essential role in other B-cell lymphomas as well, and the results obtained in our study of FL are therefore discussed in that context.

5.2.1 Applications of *IG* clonality testing

Lymphoma versus reactive hyperplasia

The most important and valuable application of PCR-based clonality testing has been in the discrimination of lymphoma from reactive hyperplasia.[180] In **Paper I**, we provide information that could be important to laboratories employing diagnostic clonality testing as nearly all biopsies and surgical specimens are FFPE in the clinical routine practice. We present an improved PCR protocol for detection of clonality in FL FFPE samples, and the highest clonal detection rates were reached with the combined use of BIOMED-2 *IGH* and *IGK* assays. Our results have also recently been confirmed.[311,312] Similar to **Paper I**, Payne et al. [311] demonstrated increased clonality detection rate when the BIOMED-2 *IGK* assays were included in FFPE samples from 77 FL patients. Furthermore, they showed that this was probably due to fewer somatic hypermutations in the rearranged *IGK* locus than in the *IGH*. The sensitivity of clonality detection may accordingly be increased by the *IGK* assays in other B-cell lymphomas of germinal or post-germinal centre origin, such as DLBCLs, because of the high frequency of somatic hypermutations found in these lymphomas. At the hospital, we now include the *IGK* assays in addition to the three *IGH* framework region assays in routine clonality testing based on the findings in **Paper 1**.

In accordance with **Paper I**, Hartmann et al.[312] found DNA concentration to be an important factor for the interpretability of data. In their study, 1969 FFPE samples, including a broad spectrum of lymphoma and reactive cases, were analysed by the

BIOMED-2 primers. Samples were more likely to be evaluable when DNA concentration was above the threshold 50 ng/µl. Still, 53% of samples with DNA contents <10 ng/µl showed evaluable results by the *IGH* framework region 3 primers. Furthermore, samples with >500 lymphoid cells per section was critical for reliable PCR results. However, clonality was detected in several samples with a content of <500 lymphoid cells. In agreement with our study, a clonality test might thus always be worth trying, although only samples yielding control gene PCR products ≥300 base pairs by the BIOMED-2 specimen control reaction are usually recommended for diagnostic purposes.[7]

A major issue in molecular clonality testing concerns its meaning in diagnosing lesions with reactive hyperplasia. Indeed, reactive lesions can mimic lymphoma showing clonal peaks in fragment analysis.[180,312,313] A limitation of **Paper I** is therefore the inclusion of only 10 reactive FFPE samples as control tissue. However, the BIOMED-2 primers have been shown to be helpful in confirming the polyclonal character in the vast majority of reactive lesions in a previous study utilizing DNA from fresh tissue.[313] Recently, similar results have been obtained in FFPE tissue as well.[312] Interestingly, clonal *IGH* products were more often observed in reactive lesions from extranodal localizations such as the gastrointestinal or respiratory tract. Extensive exposure of antigens on mucosal surfaces might stimulate proliferation of specific B-cell clones,[299] and the results indicate that the localization of the diagnostic biopsy should be taken into account when clonality is assessed.[312]

Functionally rearranged *IG* genes result in surface membrane expression of IG molecules, thus the clonally rearranged *IG* genes of B-cell lymphomas might be detectable at the protein level. For many years, monotypic expression of IGK or IGL by immunohistochemical analysis has been used as a surrogate marker for clonality.[314,315] However, the reliable detection of single light-chain restriction in tissue sections is often difficult because of soluble IG molecules overlaying the cellular expression.

Flow-cytometric analysis of IG light chain expression has also been used for a long time to discriminate between reactive and malignant B-cells according to IGK/IGL ratios.[314,316] Significant technical advances have taken place in the field of flow cytometry, and standardized laboratory protocols and software tools for diagnostic screening by 8-colour, 12-marker flow-cytometric immunophenotyping of peripheral blood, bone marrow and lymphoid tissue specimens are available.[317-319] The main limitation of flow cytometry is the requirement of fresh samples. Additionally, the latest generation of cytometers may not be available for routine use or the availability of monoclonal antibodies may be limited. Another drawback is that the volume or cellularity of some samples may not allow thorough analysis.[320]

In many cases, standard histomorphology supplemented with immunohistochemistry or flow-cytometric immunophenotyping can discriminate between B-cell lymphomas and reactive hyperplasia. Establishing the diagnosis may, however, be more complicated in some cases. Previously, it has been estimated that molecular clonality assessment is warranted in 5-10% of lymphoma cases.[7] It has also been proposed to test more cases in order to increase the reliability of diagnosis, and that about 30% of cases in laboratories with limited specialization in haemapathology and about 10% of cases in specialized haematopathology centres will benefit from clonality testing.[9] Generally, the need for molecular *IG* analysis will vary according to the experience of the pathologists, and the estimate of how many cases to test will be debatable. As a guideline it can be stated, however, that every case with inconclusive diagnosis, all cases with unusual histological or immunophenotypical features, and cases in which pathological results contrast clinical findings need to be subjected to clonality assessment by molecular methods.[9,180]

Other applications

Several applications of *IG* clonality testing other than discriminating lymphoma from reactive hyperplasia were suggested almost 30 years ago (Table 7).[321]

Table 7. Applications of *IG* clonality testing[321]

Application	Value
Discrimination of tumour versus normal	++
Staging: evaluation of dissemination/localization	+/++
Evaluation of clonal relationship between multiple lesions at same time	++
Evaluation of clonal relationship between diagnosis and disease recurrence	++
Lineage determination (T versus B versus NK)	+
Monitoring/evaluation of treatment effectiveness	+

In **Paper II**, we investigated the application of PCR-based clonality testing in bone marrow (BM) staging of FL. The presence of limited versus disseminated disease, as characterized by BM involvement, may be crucial for therapeutic decisions and prognosis determination. Early-stage patients can be efficiently treated with local radiotherapy, and advanced stage (Ann Arbor stages III and IV) at diagnosis is an independent adverse prognostic factor. The current British Committee for Standards in Haematology guidelines on investigations and management of follicular lymphoma recommend aspirate, biopsy, immunohistochemistry and immunophenotyping by flow cytometry to be included in bone marrow examination.[159] Furthermore, the guidelines recommend that molecular detection of B-cell clonality in marrow or blood should not be considered standard but may form a part of studies directed specifically at the detection of minimal residual disease.

In our study, BM involvement by morphology or flow cytometry did not show any significant impact on survival, whereas BM involvement by PCR-based clonality analysis showed prognostic value, and was in addition an independent predictor of survival. Based on these results we suggest the inclusion of PCR in BM examination of patients with FL. Our study also showed the value of routinely incorporating PCR analysis rather than using PCR in histologically ambiguous cases only. Furthermore,

a positive PCR result could probably have therapeutic implications in an otherwise early-stage disease as cure cannot be expected with local radiotherapy alone.

We do not suggest that PCR can replace the two other investigations. Histologic examination of the BM is well-established in the staging of lymphomas, and the prognostic significance of morphological BM involvement has been demonstrated in large series of FLs.[130,131] The practice of performing flow cytometry as a part of BM staging may be more variable, and the clinical value in FL patients has not been clearly demonstrated so far.[322] Some of the general limitations of the flow cytometry technique have been addressed previously in this thesis. The cellularity may especially be a problem in BM aspirates as haemodilution might occur during the aspiration procedure. For FL in particular, the low sensitivity of the flow cytometry technique in detection of BM involvement may possibly be due to the characteristic paratrabecular infiltration, which might impede the ability to aspirate sufficient amount of neoplastic cells. However, the sensitivity of flow cytometry is likely to increase by the further development of the multicolour technique. Therefore, the three investigations will probably complement each other in BM staging of FLs in the future.

There are limitations of our study. It was small and retrospective, and a larger study may be required to confirm our results. Another issue concerns the generalizability of the results to other B-cell lymphomas. There are, however, few reports on the clinical value of ancillary investigations in BM staging of lymphomas in general.[323] [324-326] The clinical utility of flow cytometry analysis has been demonstrated in BM staging of DLBCLs in previous reports,[325,326] whereas PCR-based clonality has shown prognostic value in peripheral T-cell lymphomas (PTCL) [324] and DLBCLs.[323] In the study of 155 DLBCL patients by Mitterbauer-Hohendanner et al., [323] the presence of clonal *IG* rearrangements in peripheral blood or BM was significantly related to a poorer overall survival at 5-years ($P < 0.001$). Furthermore, they were able to define a group of patients with normal BM morphology with significantly poorer overall survival due to molecular detection of peripheral blood/BM involvement.

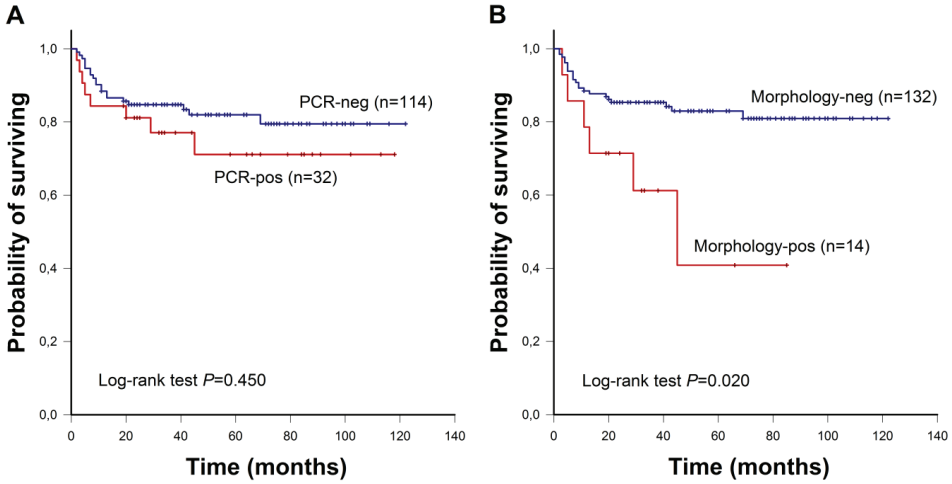

Figure 6. Kaplan-Meier survival curves of the 146 patients diagnosed with DLBCL at Haukeland University Hospital according to bone marrow involvement by PCR-based clonality (**A**) and morphology (**B**). Neg, negative; pos, positive.

We were, however unable to confirm the results of Mitterbauer-Hohendanner et al. in our unpublished data of 146 patients diagnosed with DLBCL in the same time period as the FL patients included in **Paper II**. The estimated survival at 60 months for patients with and without BM involvement by PCR was 71.1% (SE 9.1%) and 80.5% (SE 3.9%), respectively (Figure 6A). The difference in survival did not reach statistical significance by the log-rank test (P=0.450). The reason for the insignificant prognostic impact of PCR-based clonality in our DLBCL patients is unclear. It is possible that clones of large heavily somatically mutated B-cells could have remained undetected, even when multiple *IG* targets were analyzed,[7-9] However, BM involvement was detected by PCR in 21.9% (32/146) of patients, a finding that was in accordance with the previous report by Mitterbauer-Hohendanner et al.[323]

A limitation of PCR-based clonality in the BM examination of DLBCL patients may be related to the fact that discordant involvement (i.e. small cells) with the presence of a low-grade B-cell lymphoma is common, and that concordant BM involvement (i.e. large cells) is associated with a very poor prognosis (5-year overall survival, 10%).[327,328] Recently, in a study of 552 DLBCL cases, patients with discordant involvement actually had a prognosis similar to that of patients without BM involvement[329] Obviously, PCR is unsuitable in distinguishing large cells from

small. Thus, discordant involvement might have been overrepresented in our patients with BM involvement by PCR and this could partly explain the insignificant prognostic impact.

A comparison between the sizes of the clonal peaks obtained in the BM aspirates and the corresponding primary tumours were also performed in the 32 PCR-positive DLBCL cases. Identical clonal peaks were demonstrated in 20, whereas different clonal rearrangements were demonstrated in seven. In the remaining five cases, an oligoclonal pattern was obtained in the primary tumour of three and no material was available for molecular analysis in two. We were not able to show any significant prognostic impact of PCR when survival of the 20 patient with confirmed identical clonal peaks were compared to the other patients (P=0.240) It is possible that this insignificant result also could be related to the presence of discordant involvement, as identical clonal rearrangements with the primary tumour are present in discordant BM infiltrates as well.[330]

BM involvement by morphology, however, showed a significant impact on survival (P=0.020, Figure 6B) of our DLBCL patients. The estimated survival at 60 months for patients with and without BM involvement by morphology was 40.8% (SE 19.1%) and 81.7% (SE 3.5%), respectively. In the original pathology reports, morphological BM involvement was detected in 9.5% (14/146) of patients, and was somewhat lower than previously reported rates of involvement ranging from 11-27% in DLBCL patients.[151,327,328] The concordance or discordance of the BM involvement was not included in the original pathology reports, and it remains possible that many of our patients with positive BM morphology might have had concordant involvement. This is further supported by the fact that identification of large cells often is associated with extensive BM involvement.[327,328] It seems likely that extensive infiltration of large cells in a BM biopsy would not easily be missed in daily routine.

Our unpublished data on DLBCLs support the importance of the biopsy in BM examination, and reflect that the meaning of clonality can be dependent on the

pathologic context. Knowledge about technical and immunobiological pitfalls is of utmost importance in molecular clonality testing, but may not be sufficient in all cases. For appropriate interpretation it is essential to integrate molecular data with clinical data and the data obtained by immunohistochemistry, and preferably also with the results from flow-cytometric immunophenotyping.[180,331] An interactive interpretation model with regular contact with oncologists, pathologists, molecular biologists and immunologists, would probably aid in integrating all available data to achieve the most reliable diagnosis.

PCR-based clonality analysis was also performed on BM aspirates from 111 patients diagnosed with other B-cell lymphomas than FL and DLBCL, and related to morphological detection of BM involvement (unpublished data, Table 8). The two methods were comparable in assessment of BM involvement, but some discrepancies were observed. PCR was negative in only one patient positive by morphology. Interestingly, PCR was positive in 13 patients without morphologically detectable BM involvement. The results highlight that PCR-based clonality analysis of BM aspirates may serve as an additional diagnostic tool in the differential diagnosis of some lymphoma types. For example, the absence of BM involvement is a prerequisite to exclude a systemic DLBCL in cases of primary diffuse large B-cell lymphomas of the central nervous system (CNS DLBCLs) and primary mediastinal (thymic) large B-cell lymphomas (PMBLs). Furthermore, the results might indicate that PCR could be of importance in staging of other B-cell lymphomas.

Table 8. A comparison of morphology and PCR in the bone marrow examination of B-cell lymphomas

		Morphology +			Morphology -		
Lymphoma type	N	PCR+	PCR-	Total	PCR+	PCR-	Total
DLBCL	146	11	3	14	21	111	132
FL	96	20	9	29	13	54	67
CNS DLBCL	16				0	16	16
MALT lymphomas	15	1	0	1	2	12	14
MCL	15	8	0	8	5	2	7
CLL/SLL	14	12	1	13	0	1	1
PMBL	9				0	9	9
DLBCL/BL	8	3	0	3	1	4	5
BL	6	1	0	1	0	5	5
Others	28	13	0	13	5	10	15
Total	*353*	*69*	*13*	*82*	*47*	*224*	*271*

DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; CNS DLBCL, primary DLBCL of the central nervous system; MALT lymphoma, extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue; MCL, mantle cell lymphoma; CLL/SLL, chronic lymphocytic leukaemia/small lymphocytic lymphoma; PMBL, primary mediastinal (thymic) large B-cell lymphoma; DLBCL/BL, B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and BL; BL, Burkitt lymphoma.

An evaluation of the prognostic value of PCR-based clonality in BM staging could be of interest in patients with other B-cell lymphomas. We performed a survival analysis according to BM involvement of the patients with other B-cell lymphomas as well, excluding CNS DLBCLs and PMBLs. Thirty-six patients were diagnosed with aggressive B-cell lymphomas including Burkitt lymphoma (BL), B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and BL (DLBCL/BL), lymphomatoid granulomatosis (LYG), mantle cell lymphoma (MCL),

plasmablastic lymphoma (PBL), primary cutaneous DLBCL (PCBCL) and T-cell/histiocyte-rich large B-cell lymphoma (THRLBC). Significant poorer survival by log-rank test was observed in the 21 patients with BM involvement detected by PCR (P=0.027), but not for the 12 patients with morphological involvement (P=0.061).

Fifty patients were diagnosed with indolent B-cell lymphomas such as chronic lymphocytic leukaemia/small lymphocytic lymphoma (CLL/SLL), hairy cell leukaemia (HCL), extranodal marginal zone lymphoma of mucosa-associated tissue (MALT lymphoma), lymphoplamacytic lymphoma (LPL), nodal marginal zone lymphoma (NMZL), primary cutaneous follicle centre lymphoma (PCFCL), splenic marginal zone lymphoma (SMZL). Survival analysis revealed no significant survival difference neither for the 30 patients with BM involvement detected by PCR (P=0.305) nor for the 27 patients with morphological BM involvement (P=0.118).

A reliable evaluation of the prognostic value of PCR analysis would, however, need larger series to allow analysis by specific lymphoma entities as BM involvement may not correlate with worse prognosis in all B-cell lymphomas. In fact, better prognosis of MCL patients presenting with BM, peripheral blood and sometimes splenic involvement but without lymphadenopathy has been reported.[332] and no adverse prognosis has been observed for MALT lymphoma patients presenting with involvement of multiple extranodal sites or BM involvement.[333] The inclusion of other clinical, pathological and even molecular data in multivariate survival models would probably also be essential. For example, in MCL patients a high proportion of Ki67-positive cells, e.g. >40% or >60%,[334,335] SOX11 expression and *IGHV* mutational status with homology >97% have previously been independent prognostic factors,[336] and the prognostic effect of BM involvement could be dependent on such other factors.

A final point of interest concerning the detection of BM involvement by PCR-based clonality analysis is related to the high sensitivity of PCR. An erroneous detection of seemingly clonal or oligoclonal lymphoid cell populations is conceivable. The presence of a limited number of normal B-cells, as may well be the

case especially in BM samples, can produce pseudoclonality or selective amplification due to low level of specific *IG* template.[337,338] Furthermore, monoclonal B-cell populations in BM without evidence of B-cell lymphoma have been reported in certain immunologic conditions, such as infections and inflammations.[339-342] Hence, clonality can in some cases reflect an exaggerated immune response with a dominant immunospecificity, and not be specific for BM involvement by lymphoma.

To recognize oligoclonality or pseudoclonality may be difficult. Due to this difficulty and based on the findings in **Paper I**, the three *IGH* framework region assays and the *IGK* assays are now routinely included in the PCR-based clonality testing of BM aspirates in our hospital laboratory. The importance to include a DNA sample from the primary location for the purpose of comparison in case of disease staging has also been emphasized previously,[180] and a DNA sample from the primary location when assessing BM involvement by PCR is routinely included at the hospital. In **Paper II**, we were able to demonstrate identically sized clonal rearrangements in 31 of the 33 patients with positive PCR results in the BM aspirates. In two cases, the PCR products obtained from the primary tumour was insufficient for the detection of clonality. Although a confirmation of the presence of lymphoma by comparative analysis may be preferable in the diagnostic setting, it cannot always be achieved.

Similarly, five of the DLBCL patients and 15 of the patients with other B-cell lymphomas and a positive PCR result, had no or insufficient material available for comparative analysis in our unpublished data (Table 8). In addition, seven DLBCL patients and four patients with other B-cell lymphomas had differently sized clonal peaks obtained from the primary location. The interpretation of the positive PCR result of the bone marrow in these cases deserves some attention. These clonal peaks may reflect an exaggerated immune response as previously stated. However, the possibility of two clonally distinct, unrelated B-cell lymphomas presenting synchronously at different locations cannot be excluded.[330] Additional selected subclones of the original neoplastic clone may also take part in populating the BM, [112]

giving rise to additional clonal signals that could hamper interpretation when comparing profiles.

As mentioned in Table 7, PCR-based clonality can be useful in evaluation of the clonal relationship between multiple lesions at the same time, because the size of the clonal peaks can distinguish one lymphoid malignancy from another. Similarly, clonality testing may also aid in discriminating relapse from secondary malignancy in recurrence of disease. In **Paper I,** FFPE samples stored for more than 5 years did not perform significantly different from more recently stored samples, and provided information that could be particularly relevant for the diagnosis of relapsed disease. Our results in **Paper I** on FFPE samples stored for five years have also been confirmed recently in a study of 100 archival FFPE samples from patients with DLBCL.[343]

In contrast to the initial assumption, *IG* and *T-cell receptor (TCR)* gene rearrangements are not necessarily restricted to B- and T-cell lineages, respectively. Cross-lineage of *IG* gene rearrangements occurs in T-cell malignancies, and cross-lineage of *TCR* gene rearrangements occurs even more frequently in B-cell malignancies,[344,345] particularly in precursor B lymphoblastic leukaemia/lymphoma (B-ALL).[344] Monitoring of treatment effectiveness can be performed by clonality testing, but the value is influenced by the limited sensitivity level of the PCR assays.[7] More sensitive approaches like real-time quantitative PCR with specific primers and probes may be preferable in analysis of minimal residual disease.[346]

5.2.2 *IG* genes in FL prognosis and pathogenesis

The strongest clinical predictor of outcome to date in FL patients is the Follicular Lymphoma International Prognostic Index (FLIPI). Both FLIPI and the modified FLIPI2 index are useful in clinical practice and valuable for stratification in clinical trials. The current British Committee for Standards in Haematology guidelines on investigations and management of follicular lymphoma do recommend to record the

FLIPI and FLIPI2 at diagnosis.[159] Additionally, the histological grade and the proliferation index, recognized by Ki67 immunostaining, may also be useful markers for predicting outcome. Although there have been improvements in survival during the past decade, the prognosis of FL remains heterogeneous as does its treatments options. Given the variable clinical course and the complex biological nature of FL, the described clinical and pathological prognostic indices may not be sufficient. To tailor future therapeutic approaches, additional molecular and genetic markers are probably needed.[101]

In **Paper III**, we analysed the *IGHV* gene repertoire in FL, and showed that usage of different *IGHV* subgroups was associated with survival. Specifically, usage of the *IGHV5* subgroup and more than one *IGHV* subgroup were adverse prognostic factors. Similarly, the *IGHV3-21* gene has been correlated with poor clinical outcome in many studies of CLL.[347-350] Other *IGHV* genes or subgroups have, to our knowledge, neither been linked to adverse prognosis in CLL nor in other B-cell lymphomas. On the other hand, the presence of more than one productive rearrangement in one tumour sample has been observed in other B-cell lymphomas including CLL,[49] DLBCL,[50], and MCL.[51] This observation may be related to lack of allelic exclusion, the occurrence of numerical chromosomal abberations or reflect the existence of biclonal populations in the same patient.

There are limitations of our study. The study cohort was small, and it will therefore be important to address the prognostic value of *IGHV* sequence analysis in larger series of patients. In addition, a productive *IGHV* gene rearrangement was not obtained in 7% of cases. A multivariate survival analysis incorporating the results from **Paper II** could also be appropriate. Eighty-six of our patients had results from both *IGHV* sequence analysis and PCR-based clonality analysis of bone marrow aspirates. Usage of *IGHV5* or more than one *IGHV* subgroup (P=0.004) and bone marrow involvement by PCR (P=0.006) remained independent prognostic factors in a multivariate model when age >60 years, sex, and high FLIPI score was included (unpublished data). Thus, the inclusion of *IGHV* sequence analysis when assessing FL in diagnostic routine should be further evaluated.

Previous reports have shown that FL in general carries mutated *IGHV* genes.[116,351,352] In contrast, unmutated *IGHV* genes, showing more than 98% homology to the germline sequence, were detected in 15% of our FL patients with productive *IGHV* gene rearrangements in **Paper III**. A possible explanation for the discrepant result may be related to the small sizes of cohorts and selection of patients, as the largest previous series was of 36 low-grade FLs.[351] Considerable heterogeneity in mutational status of *IGHV* genes has been demonstrated in several other B-cell lymphomas, including CLLs,[353,354] DLBCLs,[50], SMZLs,[355-357] and MCLs.[51,332,336]

A finding in **Paper III** was that unmutated *IGHV* genes were not associated with poorer survival. We were not able to show any significant differences in survival when different homology cutoff values, >93%, >96%, and >99%, were assessed. However, we cannot exclude that such differences may be observed in larger series. The prognostic significance of *IGHV* gene mutational status has been demonstrated particularly in CLL, [353,354] but also in other B-cell lymphomas including SMZLs,[355-357] and MCLs.[332,336]

From 2004, *IGHV* gene analysis has been performed in routine assessment of prognosis in CLL patients at the hospital laboratory. Analysis using complementary DNA (cDNA) was initially performed. From 2007, genomic DNA (gDNA) was used as starting material. The sequences obtained were initially aligned to germline *IGH* sequences derived from the IgBlast database, and later on the IMGT database. An unpublished work-up of 144 CLL samples, mainly peripheral blood samples, analysed from June 2004 to December 2011, also confirmed the prognostic value of *IGHV* gene mutational status. The estimated overall survival at 60 months for patients with mutated and unmutated *IGHV* genes was 91.6% (SE 2.8%) and 75.4% (SE 7.3%), respectively (Figure 7). The difference in survival was significant by log-rank test (P <0.001). The *IGHV3-21* gene, however, was expressed a lower rate (7.6%) compared to previous reports,[347,358,359] and we were not able to show any significant adverse survival of the CLL patients with *IGHV3-21* gene expression.

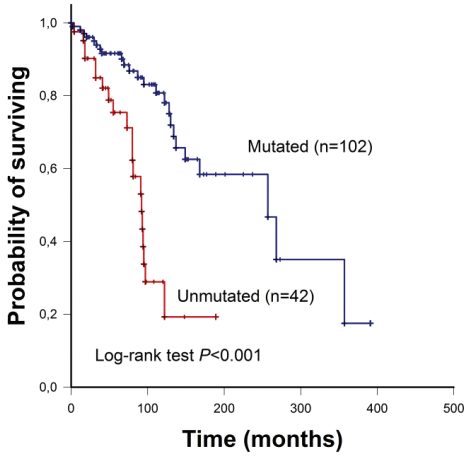

Figure 7. Kaplan-Meier survival curves of the 144 patients with chronic lymphocytic leukaemia (CLL) based on the presence (mutated) or absence (unmutated) of *IGHV* mutations.

Information on cause of death was not available in 33 of the CLL patients, because they were treated and followed at other hospitals in Norway. We therefore chose to estimate overall survival for all the patients in this work-up, and not to treat patients who died of other causes than lymphoma as censored observations. Additionally, exact time of diagnosis was not accessible for the 33 patients followed at other hospitals, and time to death were estimated with date of reporting results from sequencing analysis as starting point. The estimation of time to death might be somewhat imprecise for these patients. It is furthermore possible that these 33 patients might be a selected group of patients. Moreover, only 85 of the patients treated at Haukeland University Hospital had the sequence analysis performed at initial diagnosis according to medical records. Our patient series may thus not be entirely representative for the CLL patient group, and the survival analysis could possibly show somewhat biased results.

In the work-up, unmutated *IGHV* genes were detected in 29.2% (42/144) of cases, in contrast to previous studies reporting 50%.[353,354] However, recent population-based reports from Europe and USA have shown similar frequencies of unmutated *IGHV* genes in CLL patients.[360,361] In addition, the median survival of our patients with unmutated and mutated *IGHV* genes was 92 and 257 months,

respectively, and these median survivals were also in accordance with previous studies, showing median survivals ranging from 77 to 117 months of unmutated cases[353,354,362] and 152 to 293 months of mutated.[354,362] Despite the previously described limitations of our work-up, it seems likely that the frequency of unmutated *IGHV* genes detected and that the survival curves of our CLL patients could be regarded as representative for the patient group.

The discovery of two mutational subsets in CLL raised the question that CLL comprises two different diseases.[10,354,363,364] Mutated cases could be those that have been exposed to the somatic hypermutations of germinal centres and accordingly be tumours derived from memory B-cells. Correspondingly, unmutated cases could be those that have not been exposed to the mutation process of germinal centres and be tumours of more naïve B-cells, showing a more malignant disease. Although these two subtypes differ in many clinical and biological parameters, the cellular origin of the unmutated CLL cases is still unclear.[363]

The cellular origin of a subset of FL may also be debatable. A reasonable explanation for the demonstration of unmutated sequences in FLs in **Paper III** is that the tumour cells have not been exposed to the mutation process. However, the *IGHV* gene usage of the unmutated cases was biased with the *IGHV1* subgroup recording the highest number of unmutated sequences. Therefore, the possibility of antigenic exposure cannot be eliminated.

6. Conclusions

1. DNA from FFPE FL tumour samples can be used for PCR-based clonality testing. An improved PCR protocol using a commercial multiplex PCR kit and BIOMED-2 *IG* primers was developed (**Paper I**).

2. Highest clonality detection rates were reached with the combined use of *IGH* and *IGK* primers. Therefore, the combined use of analyses was recommended for FL diagnostic purposes (**Paper I**).

3. Aged FFPE samples stored for 6-11 years did not perform significantly differently from those stored 1-5 years with respect to clonality detection, allowing the use of archival material in PCR-based clonality testing, which is particularly relevant in the diagnosis of relapsed diseases (**Paper I**).

4. PCR-based clonality analysis of bone marrow aspirates contributed to the diagnosis of bone marrow involvement in FL (**Paper II**)

5. The prognostic significance of bone marrow staging was improved by PCR-based clonality analysis, and bone marrow involvement by PCR was also an adverse prognostic factor independent of high Follicular Lymphoma International Index (FLIPI) score. The inclusion of PCR-based clonality in routine bone marrow examination was therefore suggested (**Paper II**).

6. Sequence analysis of *IGHV* genes in FFPE FL tumour samples showed that the *IGHV3, IGHV1* and *IGHV4* were the most frequently used subgroups and the *IGHV3-23* was the most frequently used gene (**Paper III**).

7. The use of the *IGHV5* subgroup and more than one *IGHV* subgroup identified FL patients with shorter survival, and was also of independent prognostic importance. This indicated that sequence analysis of *IGHV* genes could aid in predicting prognosis for FL patients (**Paper III**).

8. The *IGHV* genes were commonly mutated, but unmutated *IGHV* genes were also detected, suggesting the possibility that some FL cases might be derived from naïve B-cells. (**Paper III**).

7. Concluding remarks and future perspectives

During the past decades molecular genetics has become integrated in lymphoma diagnostics and prognostication.[2,149,167,181] Currently standard technologies such as PCR-based clonality tests, fluorescence *in situ* hybridization (FISH) investigations for specific chromosomal translocations and sequencing analysis of *IGHV* genes are performed on routine basis in many pathology laboratories. To improve the quality and accuracy of diagnostic molecular work is an ongoing process, and this PhD project has been a part of that process. In line with the aims of the study, we have evaluated the application of PCR-based clonality in settings that are relevant in pathology routine. We have also identified molecular prognostic markers that may be relevant in FL, although larger series is needed to confirm our results.

The repertoire of newer genetic technologies is expanding rapidly, and the introduction of such methods to the routine diagnostic laboratory represents a major challenge for the future. Gene expression profiling and chromosomal array studies have confirmed the molecular and genetic identity of many diseases including lymphomas. In particular, these technologies have helped to discover and define molecular subtypes of DLBCL, the activated B-cell (ABC) and germinal centre B-cell (GCB) types.[365] In the near future, next generation of sequencing (NGS) technology, which implies the possibility of elucidating the whole genome and exome sequences of large number of tumours at an affordable cost, is likely to be implemented in the pathology laboratory. NGS already allows routine analysis of cancer gene panels in epithelial tumours, making the determination of somatic mutations with therapeutic implications more efficient. Similarly, the new molecular technologies promise new insights into the complex biology of lymphomas, enabling us to refine our clinical and diagnostic perspectives.

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