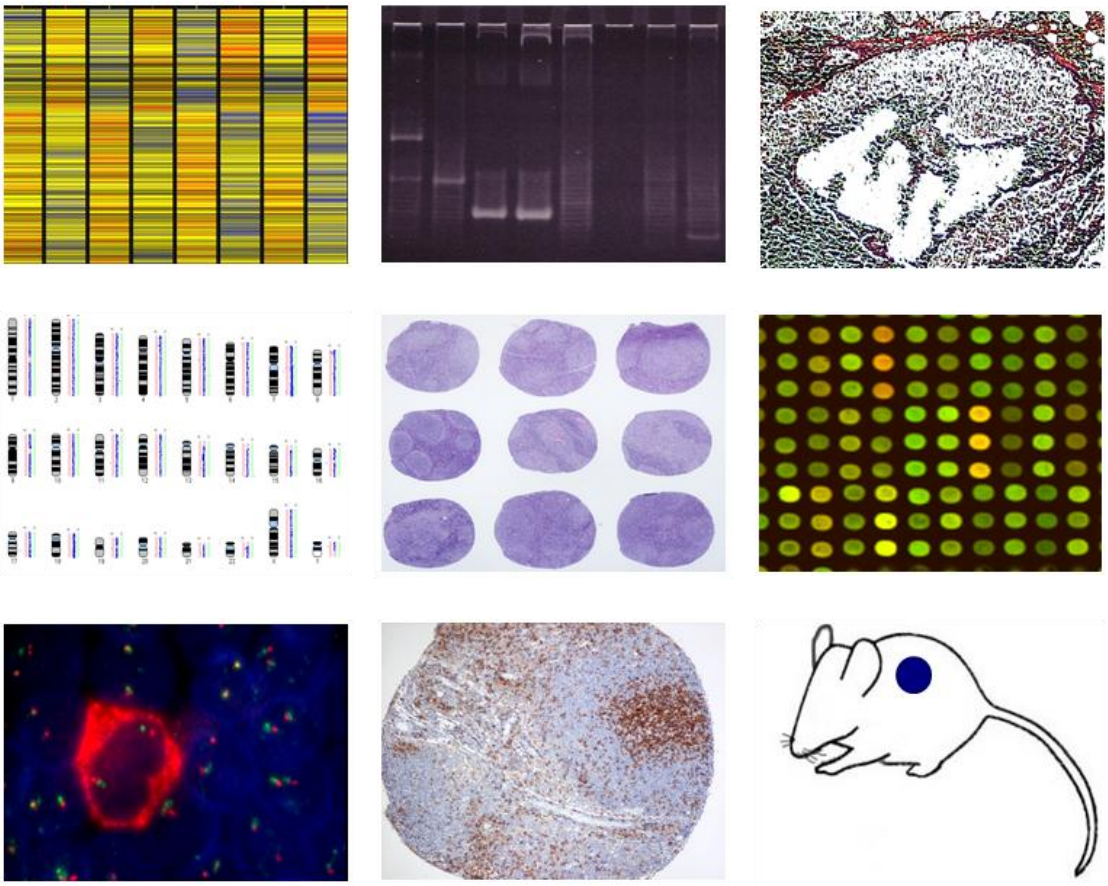
BIOLOGICAL PREDICTORS OF SURVIVAL IN LYMPHOMA AND MECHANISMS UNDERLYING FOLLICULAR LYMPHOMA TRANSFORMATION INTO DIFFUSE LARGE B CELL LYMPHOMA



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A PhD thesis in Medicine in the specialty of Anatomic Pathology

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Abstract

Cancer biomarkers provide an opportunity to identify those patients most at risk for disease recurrence, predict which tumours will respond to different therapeutic approaches and ultimately define candidate biomarkers that may serve as targets for personalized therapy. New biomarkers are especially needed in the management of lymphoid cancers. At present, these tumours are diagnosed using a combination of morphologic, phenotypic and molecular features but prognosis and overall survival are mostly dependent on clinical characteristics. In most lymphoma types, these imprecisely assess a significant proportion of patients, in particular, those with very poor outcomes. Follicular lymphoma (FL) is the second most common lymphoma subtype worldwide. It is typically an indolent disease with current median survivals in the range of 8-12 years, but is usually fatal when it transforms into an aggressive high-grade lymphoma, characteristically Diffuse Large B Cell Lymphoma (DLBCL). Morphologically and functionally it recapitulates the normal cells of the germinal center with its survival dependency on non-malignant immune and immune-related cells. Informative markers of transformation related to the intrinsic biology of FL progression are needed. Within this thesis two separate approaches to biomarker discovery were employed. The first was to study the global expression of genes ('genomics') obtained using high-throughput, whole-genome-wide approaches that offered the possibility for discovery of new genetic abnormalities that might represent the important biological mechanisms of transformation. Gene signatures associated with early events of transformation were found. Another approach relied on hypothesis-driven concepts focusing upon the microenvironment, rich in several non-malignant cell types. The immunoarchitectural studies of macrophages, regulatory T cells and microvessel density on diagnostic biopsies of uniformly treated FL patients significantly predicted clinical outcome and, importantly, also informed on the risk of transformation. Techniques that enabled the use of routine formalin fixed paraffin embedded diagnostic specimens from the pathology department archives were preferentially used in this thesis with the goal of fulfilling a rapid "bench-to-beside" translation for these new findings. Although FL was the main subject of the thesis the new findings and hypotheses allowed easy transition

into other lymphoma types. Several promising biomarkers were proposed and validated including the implication of several non-neoplastic immune cells as important contributors to lymphoma biology, opening new options for better treatment planning and eventually new therapeutic targets and candidate therapeutics.

Resumo

Os biomarcadores tumorais permitem identificar os doentes com maior risco de recorrência da doença, predizer a resposta tumoral à terapêutica e, finalmente, definir candidatos a novos alvos terapêuticos. Novos biomarcadores são especialmente necessários na abordagem clínica dos linfomas. Actualmente, esses tumores são diagnosticados através de uma combinação de características morfológicas, fenotípicas e moleculares, mas o prognóstico e o planeamento terapêutico estão quase exclusivamente dependentes de características clínicas. Estes factores clínicos são, na maioria dos linfomas, insuficientes numa proporção significativa dos doentes, em particular, aqueles com pior prognóstico. O linfoma folicular (LF) é, globalmente, o segundo subtipo mais comum de linfoma. É tipicamente uma doença indolente com uma sobrevida média entre os 8 e 12 anos, mas é geralmente fatal quando se transforma num linfoma agressivo de alto grau, habitualmente o linfoma difuso de grandes células B (LDGCB). Morfologicamente e funcionalmente, as células do LF recapitulam as células normais do centro germinativo na sua dependência de sobrevivência do microambiente não-tumoral, especialmente das células do sistema imunológico. Biomarcadores preditivos de transformação não existem pelo que um melhor conhecimento da biologia intrínseca de progressão do LF poderá revelar novos candidatos. Nesta tese descrevo duas abordagens distintas para a descoberta de novos biomarcadores. A primeira, o estudo da expressão global de genes ('genomics') obtidos por técnicas de alto rendimento que analisam todo o genoma humano sequenciado, permitindo identificar novas anomalias genéticas que possam representar mecanismos biológicos importantes de transformação. São descritos novos genes e alterações genómicas associados à transformação do LF, sendo especialmente relevantes as relacionadas com os eventos iniciais de transformação em LDGCB. A segunda, baseou-se em várias hipóteses centradas no microambiente do LF, rico em vários tipos de células não-malignas. Os estudos imunoarquitectural de macrófagos, células T regulatórias e densidade de microvasos efectuado em biopsias de diagnóstico de doentes com LF tratados uniformemente correlacionaram-se significativamente, e independentemente dos critérios clínicos, com a evolução clínica e, mais

importante, com o risco de transformação em LDGCB. Nesta tese, foram preferencialmente utilizadas (e optimizadas) técnicas que permitam o uso de amostras fixadas em parafina e formalina (FFPET). Estas são facilmente acessíveis a partir das biopsias de diagnóstico de rotina presentes nos arquivos de todos os departamentos de patologia, facilitando uma transição rápida dos novos marcadores para a prática clínica. Embora o FL fosse o tema principal da tese, os novos achados permitiram estender facilmente hipóteses semelhantes a outros subtipos de linfoma. Assim, são propostos e validados vários biomarcadores promissores e relacionados com o microambiente não-tumoral, sobretudo dependentes das células do sistema imunológico, como contribuintes importantes para a biologia dos linfomas. Estes sugerem novas opções para a abordagem clínica destas doenças e, eventualmente, novos alvos terapêuticos.

Table of Contents

List of abbreviations

Abbreviation	Definition
BCCA – CLC	British Columbia Cancer Agency Centre for Lymphoid Cancer
CTAG	Centre for Translational and Applied Genomics
CHLC	Centro Hospitalar Lisboa Central
IPO	Instituto Português de Oncologia
LLMPP	Leukemia/Lymphoma Molecular Profiling Project
NHL	Non-Hodgkin lymphomas
IPI	International Prognostic Index
FLIPI	Follicular Lymphoma Prognostic Index
LDH	Lactate dehydrogenase
IPS	International Prognostic Score
BCR	B cell Receptor
TCR	T cell receptor
SHM	Somatic hypermutation
CSR	Class-Switch Recombination
GEP	Gene Expression Profiling
FFPET	Formalin Fixed Paraffin Embedded Tissue
TMA	Tissue microarray
IHC	Immunohistochemistry
FISH	Fluorescence In-Situ Hybridization
FICTION	Fluorescence-Immunophenotyping and Interphase Cytogenetics as a Tool for Investigation Of Neoplasms
CGH	Comparative Genomic Hybridization
aCGH	Array Comparative Genomic Hybridization
BAC	Bacterial Artificial Chromosome
bp	Base pairs
CCD	Charge Coupled device
CpG	Cytosine-phosphate-guanine dinucleotide
CNV	Natural Copy Number Variation
DNA	Deoxyribose Nucleic Acid

cDNA	complimentary DNA
Kb	Kilobase pairs
FDC	Follicular Dendritic Cells
TAM	Tumour Associated Macrophages
LAM	Lymphoma Associated Macrophages
MVD	Microvessel Density
Tregs	Regulatory T cells
FOXP3	Forkhead box P3
EBV	Epstein-Barr virus
GCB	Germinal Centre B cell type
ABC	Activated B cell type
R	Rituximab
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
mRNA	messenger RNA
SMRT	Sub-Megabase Resolution Tiling-set
SNP	Single Nucleotide Polymorphism
SMLR	Sparse Multinomial Logistic Regression
NCBI	National Center for Biotechnology Information
TVD_{90}	Tumour to Vessel Distance that encompassed 90% of the tumour
WHO	World Health Organization

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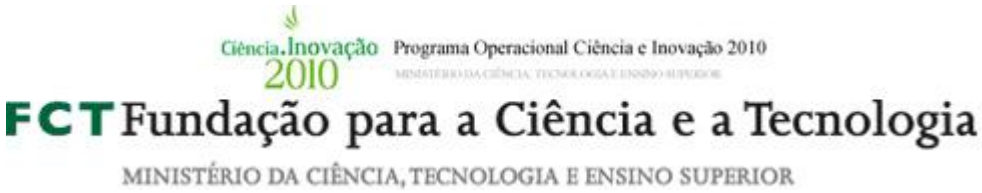

Co-Authorship Statement

Chapters 2, 3, 5, 6, 7 and 8 were co-authored as manuscripts for publication. The following author lists apply to each chapter. It is my honor to name and thank all the colleagues whose work is included in this manuscript.

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Dedication

To Susana my main mentor and co-author in life…

… and Maria do Rosário, Francisco, João, Miguel, Maria Leonor, Maria Teresa & Maria do Carmo.

Section 1: Introduction

1.1 LYMPHOMA

1.1.1. Definition

Lymphoid neoplasms are clonal tumours of mature and immature B, T or natural killer (NK) cells at various stages of differentiation.

Lymphomas represent approximately 4% of new cancers per year worldwide, are more common in the Western world and contrary to most other common neoplasias, have been increasing in incidence worldwide (1). Mature B cell non-Hodgkin lymphomas (NHL) comprise over 90% of these (2).

For the majority of lymphomas, tumour cells recapitulate the normal stages of differentiation so they can be classified according to their normal counterpart. However, some neoplasms do not have as yet a well-defined normal counterpart and within each lymphoma type there is morphologic, phenotypic and molecular heterogeneity.

B cell development takes place in distinct steps characterized by the structure of the B cell Receptor (BCR). Two pairs of identical heavy-chain and light-chain immunoglobulin (IG) peptides compose the BCR. After cross-linking, the BCR and associated membrane components transmit cytoplasm signals through several tyrosine kinases. Proliferation and differentiation of B cells depends on the recognition of antigens and on the activation of other modulator B cell surface receptors. Early B cell development occurs in the bone marrow where precursor cells rearrange IG heavy and light chain genes resulting in the expression of a functional surface antigen receptor on naïve B cells as they exit the bone marrow. These cells can join the immune response upon antigen binding to the BCR resulting in lymphoid follicle formation. They undergo clonal expansion in germinal centers (GC), where T cells and other accessory lymphoid cells enable naïve B cells to gain antigen specificity by modification of *IG* genes through somatic hypermutation (SHM) and class-switch recombination (CSR). Cells failing to express functional BCR or high affinity antigen specificity undergo apoptosis (3).

T cells arise from bone marrow precursors and mature in the thymus gland. Mature T cells are characterized by a rearranged antigen specific T cell receptor (TCR), either αβ or γδ, and associated with a fully expressed CD3 complex. They have a wide functional spectrum of both innate and adaptive immune responses. Mature T cells are functionally heterogeneous and quite plastic with differences difficult to characterize *in vivo* and *in vitro*, that are highly dependent on the particular techniques used to study them. These include cells of the innate immune system (γδ T cells), naïve, effector, regulatory, cytotoxic and memory T cells.

NK cells participate mostly in innate immune response, contain cytoplasmic cytotoxic granule proteins and do not have a complete TCR or a CD3 membrane complex.

Recently, much has been learned from new techniques of flow cell sorting and cell phenotyping as well as gene expression profiling (GEP). New techniques such as genome-wide platforms to study GEP, genomic copy number alterations (array comparative genomic hybridization or aCGH), genome methylation or genome sequencing had increased our understanding of lymphoid neoplasms.

1.1.2. Classification

The observation of BCR and TCR particular structure, the expression patterns of differentiation markers and the specific histological structures where it takes place was used to determine the origin of the various lymphomas. The rational used in the current classification of lymphoid neoplasms reflects the “frozen” state of the malignant cells in a particular differentiation stage, reflecting their cell of origin. For B cell tumours, most subtypes are derived from GC or immediate post-GC stage of differentiation. Classical Hodgkin lymphoma, considered historically as a separate disease, is in almost all cases a B cell lymphoma lacking a functional immunoglobulin.

For most lymphomas, morphology and immunophenotype have been correlated with the clinical features and together provide enough detail for an accurate diagnosis. Yet, no single antigenic or molecular marker is specific for any tumour and the correlation of clinical, morphological and a panel of antigen expression (detailed immunophenotype) or genomic mutations are needed for a

correct diagnosis. Thus, the current standard classification (2) uses all available information to define lymphoma types, including morphology, immunophenotype, genetic features and clinical features. The relative importance of each of the features varies among diseases, but there is no one gold standard for defining any lymphoma type.

Recent techniques such GEP have further characterized unrecognized differences within what were perceived to be specific subtypes. Moreover, while cell lineage defines most lymphoid malignancies, lineage plasticity within the hematopoietic system is increasingly recognized.

Finally, we have appreciated the common presence of small clonal populations of atypical lymphoid cells in otherwise asymptomatic patients that may or may not represent early involvement by a lymphoid neoplasm.

1.1.3. Therapy

Lymphoma can be treated in many ways from watch and wait to single or multiple agent chemotherapy, radiation, surgery, monoclonal antibodies, stem cell transplantation or a combination of these. Therapeutic decisions are mostly dependent on staging and clinical factors, but a key step in this process is dependent on the histopathological subtype of lymphoma. Thus, the most important step is determining whether a given patient will be offered treatment given with curative intent versus treatment given to relieve symptoms or to offer palliation. For most entities there are multiple treatment options for a single disease (4).

1.1.4. Prognosis

There are now more than a hundred well-defined lymphoma types in the WHO classification (2). Yet, even within each type, especially the most common lymphomas, such as Follicular Lymphoma (FL) or Diffuse Large B cell Lymphoma (DLBCL), these tumours are quite heterogeneous and often clinically similar diseases show different responses to the same treatment regimens. This variability in outcome underscores the need to gain further insight into the biology and clinical behavior of the disease to enable more precise individualized therapy. On the other hand, different drugs impact

differently in the tumoural cells, thus biomarkers need to be re-evaluated specifically and uniformly for each new therapeutic modality.

1.2 FOLLICULAR LYMPHOMA (FL)

1.2.1. Introduction

FL is the second most common form of indolent lymphoma in North America, comprising about 22% of all new cases of non-Hodgkin lymphomas (NHL), but 70% of the indolent lymphomas (5, 6). FL is a clinically heterogeneous disease with some patients progressing or transforming early and 15% dying within 2 years from diagnosis, whereas others remain alive without needing treatment after more than a decade. The median disease survival from diagnosis is now 8-12 years. FL may transform over time to a more aggressive lymphoma, mostly resembling DLBCL. This is a dominant clinical event that is frequently associated with a rapid clinical course, refractoriness to treatment and short survival. However, its incidence varies dramatically, with histological transformation being reported to affect between 10-70% of FL patients (7).

Prognosis and therapy of FL are currently based on clinical criteria, the International Prognostic Index (IPI) and more recently the Follicular Lymphoma Prognostic Index (FLIPI) 1 & 2 (8-10). The IPI, originally developed for aggressive lymphomas, identifies four different risk groups based on age, tumour stage, serum lactate dehydrogenase (LDH) concentration, performance status and number of extranodal sites of disease. The FLIPI published in 2004, is made up of five adverse prognostic factors including: age (>60 years vs. ≤ 60 years), stage (III-IV vs. I-II), anaemia (haemoglobin < 120 g/l vs. ≥120 g/l), number of involved nodal areas (>4 vs. ≤4 areas) and serum LDH (elevated vs. normal).

Besides these clinical features, many different biological markers have been proposed to impact prognosis in FL. However, most studies are retrospective using cohorts of patients treated with various protocols and thus consensus on what biomarkers to use in clinical practice and how these might impact treatment decisions is still lacking. Most prognostic markers were studied in the era of chemotherapy and require validation in the current era of chemo-

immunotherapy. The impact of host immune-genetics, microenvironment and therapy in FL are still not well elucidated (11).

Because there is no clear standard of care for FL treatment, a multitude of treatment options are available, ranging from simple observation in asymptomatic disease, single or multi-agent chemotherapy, monoclonal antibody therapy, with or without radioimmunoconjugates, to haematopoietic stem cell transplantation. There is no consensus approach for initial therapy. FL shows high sensitivity to initial therapy but becomes increasingly resistant following each successive treatment. The patients will invariably experience shorter remission durations and eventual relapses over time, in some cases leading to transformation. An association between increased FLIPI score and Risk of Transformation (RT) has been reported but not validated in other studies (12).

Despite the vast amount of biological data published on transformation very few biological markers have been shown to impact FL prognosis, most representing isolated reports and therefore none are used in routine clinical practice for clinical decision making. This is due in part to the low frequency of most of these markers as well as to the lack of data from cohorts with uniform treatment.

In BC, the BCCA lymphoma database represents a population-based electronic database that includes comprehensive information on diagnosis, treatment and outcome for virtually all patients with lymphoma in the province. Centralized data on diagnosis and treatment of FL patients is a powerful tool to assess incidence and outcome associated with this event (7).

1.2.2. Germinal Centre

FL is a B cell neoplasm characterized by a nodular histologic pattern and germinal centre origin. Its normal counterpart is a mature B cell with two important features: 1) it has a unique BCR that results from VDJ recombination of the *IGH* locus within the bone marrow, and 2) it is activated by recognising cognate antigen through an interaction with T cells and/or antigen presenting dendritic cells (DC), usually in the T cell zone of the lymph node. This activated mature B cell migrates into B cell follicles, up-regulates and express the hallmark and GC master gene *BCL6*, proliferates and differentiates into

centroblasts; thereby establishing secondary lymphoid follicles characterized by germinal centres and a surrounding mantle zone. The proliferating centroblasts activate the process of somatic hypermutation that generates mutations at a high rate in some genes including V-region genes of the immunoglobulin molecule resulting in the generation of antibody diversity (3). The different centroblasts are selected by the affinity of their BCR to the antigen that induced the formation of the follicle. Only those cells that have acquired high affinity mutations escape apoptosis. This process of differentiation terminates with a resting centrocyte having high affinity antibody on its surface. Some of these cells will then exit the follicle to become either short-lived plasma cells or long-lived memory B cells. The process requires the interaction of the B cells with the follicular T follicular helper cells and follicular dendritic cells (FDC) within the germinal centre. These cells provide the survival signals that rescue B cells from apoptosis. Furthermore, the FDC meshwork embodies the scaffolding structure of the follicle. Thus, FL is characterized by the development of neoplastic follicles, often morphological similar to reactive follicles, harbouring benign FDC meshwork, small blood vessels, macrophages and a variable percentage of non-neoplastic follicular T cells and benign B cells.

1.2.3. t(14,18) & Genomic Mutations

FLs are composed of neoplastic monoclonal B cells that in the majority of cases have a characteristic cytogenetic abnormality, the t(14;18)(q32;q21) or its variants t(18;22)(q21;q11) and t(2:18)(p11;q21). The translocation induces deregulated expression of the antiapoptotic *BCL2* gene, but additional cytogenetic alterations are required to establish a fully malignant phenotype (13). Although 15% of FLs are t(14;18)-negative, the translocation has been used as a genetic marker for FL. It has been postulated that it occurs as an error during VDJ rearrangement at the pre-B cell stage of differentiation in the bone marrow, and although necessary, is insufficient on its own to induce malignant transformation. The t(14;18) has been found in the blood of healthy blood donors as well as in germinal centers (“in situ” localization of FL) of patients who may not develop clinical disease or only develop FL several years later (14, 15). The favored hypothesis regarding the pathogenesis of FL suggests that B cells harboring a t(14;18) arise in the marrow and eventually

enter the circulation, home to the lymph node and seed the follicles. Here they undergo several rounds of replication within an environment where SHM and CSR are physiological processes that require double-strand DNA breaks. Eventually, a second and subsequent additional genetic hit occurs, some of which lead to the development of clinically evident FL. Like benign germinal centers, malignant follicles are further targeted by hypermutational events favoring accumulation of genomic abnormalities. One recently described and highly recurrent genetic event is inactivating mutations of *MLL2*, found in nearly 90% of FL biopsies (16). It remains possible that *MLL2* mutations and the subsequent changes in gene expression that follow loss of this H3K4 activating chromatin mark may represent the illusive 2nd hit that allows the fully malignant phenotype of FL to emerge.

Numerous structural chromosomal aberrations have been described, including gains, deletions, balanced and unbalanced translocations, all of which define the clonal evolution and disease progression of FL. Some of the most common events follow different pathways, which show clinical impact in the progression of FL (13). However, the majority of the breaks including those associated with transformation are thought to be stochastic, providing the lymphoma cells with a growth advantage and thus accelerate the clinical behaviour.

The dramatic advances in biology, specially the emergence of new technologies to assess gene expression and genomic abnormalities such as GEP array platforms, high-resolution comparative genomic hybridization (CGH) and multicolour fluorescence *in-situ* hybridization (M-FISH) have increased the number of genomic alterations, such as gene signatures and copy number deletions and amplifications characteristically found in FL (17).

1.2.4. Crosstalk Between Malignant & Non-malignant cells

Though a B cell neoplasm, FL lymphoma retains many of the morphological features of the normal follicle. Cellular interactions between tumoral B cells and their surrounding follicular T cells (helper, cytotoxic and regulatory), FDC and macrophages are maintained in FL. T cell ligands and specific cytokines, (such as CD40 ligand and IL4) play a major role in the growth of both non-neoplastic B cells and follicular lymphoma cells, indicating that responsiveness to paracrine factors is not a characteristic of the malignant phenotype. Important

survival and trophic signals from FDCs/macrophages and T cells reach the neoplastic B cells which are highly dependent on these, as suggested by the difficulty of establishing FL cell-lines *in vitro* (18).

The importance of the microenvironment and non-malignant cells within human tumours is not a recent finding. Rudolf Virchow first suggested it more than one hundred years ago (19, 20). Similarly the concept of tumoural immunosurveilance proposed by Paul Ehrlich represents a pivotal observation (21). Since then, tumoral immunity has been shown to evolve over time, characterized by crosstalk between tumour cells helping to shape their microenvironment and in turn being shaped by these surrounding non-neoplastic immune cells. Along with dampening the anti-tumoral response there are several different mechanisms by which the microenvironment promotes tumoral growth, so called cancer immunoediting, as the tumour sculpts its own immunity in favour of growth and proliferation signals (22).

Tumour associated macrophages (TAM) have been shown to promote not only neoplastic growth but also angiogenesis (23). Angiogenesis plays a vital role in growth and progression of both solid and hematological tumours. The "angiogenic switch" results from interactions of vessels with cancer cells as well as non-neoplastic cells in the microenvironment, including macrophages themselves (24). The abundance of macrophages, microvessel density (MVD) and recently of a subset of T cells, regulatory T cells (Tregs) associated with tumours, has been reported to correlate with poor prognosis in solid tumours. These cells are recruited and “educated” by tumours using a range of growth factors and chemokines, so that they adopt a “trophic” role that facilitates matrix breakdown, tumour-cell motility and angiogenesis (23). In FL, the role of cellular components of microenvironment in tumour progression and transformation is not well known with few studies published, many with conflicting results. In 2004, GEP studies highlighted the role of non-neoplastic immune cells in influencing overall survival of FL (25, 26). The Leukemia/Lymphoma Molecular Profiling Project (LLMPP) consortium study first revealed that non-neoplastic immune cells (not the malignant cells) were predominantly responsible for the GEP signatures most contributing to the outcome predictor. One signature, referred to as immune response-1, appeared to be derived from reactive T cells in the lymph node biopsies and conferred a favourable outcome. The other

signature, referred to as immune response-2, revealed a gene expression pattern most reminiscent of macrophages and/or FDCs and was associated with an inferior clinical outcome (25). However, the correlation of the gene signatures and the cellular components of the microenvironment and prognosis were not clear. These provocative data raised the question of non-neoplastic immune cells could be reproducibly identified and shown to be associated with survival and risk of transformation in FL. Could these cells be used to risk-stratify patients with FL and ultimately become the targets of novel future therapies?

The precise biological function of these non-malignant cells in FL remains poorly understood. Mouse models have shown great phenotypic heterogeneity amongst morphologically similar macrophages, but much less are known about human macrophages. The plasticity of macrophages is evident in the diverse range of functions of these cells, including inflammatory responses, immune reactions, tissue remodelling and morphogenesis. Macrophages can be functionally subdivided into two main classes, including “killer” or classically activated M1 macrophages or “healer”, alternatively activated M2 macrophages. These two types of macrophages differ in their receptor expression, cytokine and chemokine profiles as well in their metabolic pathways (27, 28). *In vivo* and within the tumoural microenvironment, macrophages of both kinds coexist with some cells showing overlapping or hybrid features. However, if markers representing the disparate ends of the macrophage spectrum are defined, these could be used as surrogate markers of anti or protumoral macrophages within routine FL biopsies.

Besides TAMs, there are other relevant non-neoplastic cell types with interesting features found within the tumour microenvironment in FL. Tregs have also been shown to promote tumour growth in mice models as well as to correlate with inferior survival in human tumours (29). In FLs, the role and significance of intra-tumoral T cells in the pathogenesis of the disease is still unclear. Cytotoxic CD8+ T cells and CD4+ helper T cells are usually present and classically have been considered as mediators of anti-tumour immunity (30). Tregs, a subset of CD4+ T cells with high expression of the IL2Rα chain (CD25), are crucial for the maintenance of self-tolerance (31). These cells represent less 1-3% of CD4+ T cells in humans (32). In addition to CD25, they

also express cytotoxic T lymphocyte-associated antigen 4 (CTLA4) and glucocorticoid-induced TNFR-related protein (GITR), which are associated with suppressive features of these cells (33). However, the critical regulator of the development and function of Treg cells and the most lineage-defining marker is forkhead box P3 (FOXP3) (33-35). Although the mechanisms of suppression by Tregs are not completely understood, they seem to require direct cell-to-cell contact between Tregs and target T cells (36). Recently, *in vitro* studies using FL samples demonstrated that Tregs are present in significantly increased numbers when compared with normal tissues and these cells suppress other intra-tumoral CD4+ and CD8+ T cell by inhibiting proliferation and decreasing perforin and granzyme B production (37, 38). Tregs are not only attracted to the lymphoma microenvironment by their CCR4 receptor ligand CCL22, produced by lymphoma cells, but there is also local up-regulation of FOXP3+ cells by malignant cells in part by CD27-CD70 signaling (39). These data suggests a suppressive and pro-lymphoma function of Tregs.

Other T cells resident in the follicle have been recently described and their biology studied in great depth, including CD4+ follicular T helper cells. These cells are vital to the normal physiology of the germinal center and usually express CD57 and/or Programmed Cell Death 1 (PD1). In lymphoma, these cells are the benign counterpart of (at least) angioimmunoblastic lymphoma (40) and perhaps HTLV-1 induced lymphomas (41). In FL, the role of benign follicular T helper cells is poorly understood.

FDCs are the supportive scaffolding of follicles and are responsible for helping to foster B cell maturation within the germinal center. In FL it has been shown that progression of disease is associated with less mature phenotype of these cells (42).

Finally, angiogenesis is considered crucial in carcinogenesis (24) and has also been studied, although in less depth in lymphoid neoplasms. In FL, data are controversial with some studies suggesting a positive correlation between vascularization and tumour grade (43), while others have shown increased microvessel density to correlate with improved clinical outcome (44). A direct link has been shown between angiogenesis and macrophages as monocytes may be recruited to sites of tissue injury or tumour microenvironment and directly contribute to angiogenesis, reaffirming the close relationships between

various cell types within the reticulo-endothelial system and even suggesting possible targets for anticancer treatments (45).

As indicated above, FL is characterized by the t(14;18)(q32;q21). *In vitro* studies have shown that precursor cells of the B cell lineage can differentiate into other cell types including tissue-based macrophages (46). Interestingly, in FL, a subpopulation of endothelial cells shows the characteristic t(14;18) translocation of FL (47), although this finding remains controversial. This finding may indicate (among other hypotheses) plasticity of a neoplastic precursor cell in the marrow that maintains the ability to differentiate into different mature cell types. Eventually, one can hypothesis that the pro-malignant t(14:18)+ clone could seed follicles not only with pre-malignant B cells but also with other types of "non-malignant" accessory cells that could provide the malignant cells with pro-survival signals.

Little is known about a possible role of the non-neoplastic cells in the microenvironment impacting the risk of FL transformation. Transformation is typically associated with the effacement of the follicular architecture, accompanied by loss and immaturity of the FDCs and a decrease in the admixed T cell content. These changes correlate with the accumulation of additional genetic abnormalities. However, in a GEP study cases that transformed more rapidly were characterized by an abundance of activated T cells (26).

1.2.5. Transformation

Several genetic alterations have been associated with histologic transformation of FL, including aberrations involving dominant oncogenes; loss of tumour suppressor genes and many as yet poorly characterized cytogenetic events. Well-known examples include rearrangements of the *MYC* gene, mutations or loss of the *TP53* or *TP16* genes, and rare somatic alterations of the *BCL6* and *BCL2* genes (48-55). However, these alterations are observed in limited subsets of transformed FLs, suggesting that the process of histological transformation can occur by multiple different mechanisms (56).

Studying transformation of FL requires the use of paired specimens comparing biopsy material from the same individual pre and post transformation, which explains the limited number and size of reported studies. Microarray CGH

studies have recently shown a heterogeneous pattern of genomic aberrations acquired upon transformation. These include gain/amplification of 1q21-q24, 2p16 (*REL/BCL11A*), 3q27-q29 (including the *BCL6* locus), 7q11.2-q22.1, 12pter-q12, 18q21 (including the *BCL2* locus) and Xq, and deletion of 6q22-q24, 13q14-q21 and 17p13 (*TP53* locus) that had been previously implicated in the FL & DLBCL pathogenesis (57). Additional changes include novel imbalances such as over-representation of 4p12-pter, 5p12-p15, 6p12.3-p21, 9p23, 9q13-q31, 16q, 17q21, and loss of the 1p36.3, 4p21-q23, 5q21-q23, 9q31-qter, 11q24-q25, and 15q23. None of these recently described changes were specific for transformation. More importantly, the specific genes involved at these sites are unknown (57).

These areas of amplification and deletion likely represent strong candidate loci to harbour genes important for cancer development and progression. Moreover, alterations of these loci likely influence gene expression secondary to copy number alterations. Knowledge of the key genes and the resultant aberrant expression patterns may also represent potential new targets for future therapies.

Previous GEP studies using cDNA microarray techniques have identified two groups of patients with distinct transformation profiles. One pattern demonstrated increased expression of *MYC* and its target genes and a second group demonstrated decreases in *MYC* and its downstream targets (58). However, many of the genes that were located in the abnormal chromosomal regions (found in some cases studied by low-resolution array-CGH) did not show significant alterations in expression. In addition, the majority of the genes that showed increased or decreased expression upon transformation were not located within these identified regions based on aCGH (e.g. the *MYC* gene).

More recently, Davies et al reported data on 20 paired fresh frozen specimens using Lymphochip cDNA microarray of transformed FL to have GEP similar to germinal centre B cell type (GCB) of DLBCL and none with the activated B cell type (ABC) DLBCL profile (56). Furthermore they suggested transformation could occur by at least two pathways, distinguished by cellular proliferation. One is characterized by increased proliferation, including recurrent higher expression of *MYC* and its targets as well as *TP53* mutations, *cREL* amplification and

CDNK2A loss. No characteristic features were recognized in the other subset of cases in which proliferation was not a dominant feature (56).

Despite the small number of studies, these current data suggest that FL transformation to DLBCL occurs by diverse genetic alterations. No single genetic event is responsible for all cases. The most common changes appear to affect different aspects of cell physiology (apoptosis, cell cycle control and specially proliferation) and are not specific for transformation, being present in non-transformed FL as well as in other lymphoma subtypes. Yet, early steps of the transformation event have proven difficult to study as has the role of FL microenvironment in transformation.

1.2.6. Prognostic Markers in FL

Many biomarkers associated with outcome have been proposed over the years, as is shown in Figure 1.1 (Prognostic markers in FL). For complete details see review (11). Yet, most studies are single reports, with very few validation studies reported and more importantly most studies were done retrospectively in cohorts of patients treated with inconsistent treatment protocols, the vast majority before the introduction of biological agents such as rituximab. Consensus on which biomarkers to use in clinical practice and how they might impact treatment decisions are still lacking. Currently, clinical characteristics included in robust indices such as FLIPI or FLIPI-2 (9, 10) are still the basis for clinical decision making. Inclusion of candidate biomarkers in prospective randomized clinical trials is mandatory to validate their future use and possible contribution to a combined clinical & biological index.

Finally, most of the aforementioned biomarkers with prognostic impact in FL patients used cohorts of patients administered heterogeneous treatment protocols. As there is no accepted standard of care treatment for FL, therapy varies from observation to high-dose chemotherapy and a bone marrow transplant. This variability undoubtedly impacts on overall survival as well as affecting the risk of transformation. Interesting clinical data supporting this were recently published by Al-Tourah et al, where a series of indolent B cell lymphomas treated with high-dose chemotherapy and involved field radiation showed a significant decrease in the risk of transformation when compared with lymphomas treated with a less aggressive regimen (7). Thus, it is of vital

importance that before comparisons are done of different clinical cohorts of FL that attention be paid to those reports that included uniform treatment. As therapy itself is an important prognostic variable, studies that include patients with vastly different therapy cannot easily be interpreted. As the treatments used are differentially visited upon both the malignant B cells and the immune cells in the microenvironment, the interpretation of prognostic biomarkers and those related to risk of transformation require that the treatment variable be held constant (11).

1.3 OTHER LYMPHOMAS

1.3.1. Diffuse Large B Cell Lymphoma

DLBCLs are a heterogeneous group of tumours consisting of large, transformed B cells, accounting for more than 30% of adult lymphomas in the western world. DLBCL occurs at any age, more commonly in the 6^{th} & 7^{th} decades of life. The disease typically presents as a nodal or extranodal mass with rapid tumour growth associated with systemic symptoms. Approximately 50 to 60 percent of patients will present with advanced stage. DLBCL includes an increasingly complex spectrum of lymphoid neoplasms with different morphologic, phenotypic, genotypic and clinical characteristics (2). Immunodeficiency is a risk factor for DLBCL, and about 5-10% of DLBCLs are EBV+, depending on the geographic population studied.

This disease is potentially curable with combination chemotherapy, such as cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP). Importantly, the addition of immunotherapy using an antibody against CD20, rituximab (R) to multi-agent chemotherapy has significantly improved outcome (59, 60). High-dose chemotherapy with autologous stem cell transplantation is effective in relapsed or refractory DLBCL. Yet, despite the improvements, 30 to 40% of the patients will not be cured of their DLBCL.

DLBCL can arise *de novo*, or as a result of a transformation event in a patient with previous low-grade lymphoma, including small lymphocytic lymphoma / chronic lymphocytic leukemia (Richter´s syndrome), follicular lymphoma and marginal zone lymphomas. These DLBCL represent a common final pathway of

histologic transformation of antecedent indolent lymphomas and are likely to be biological distinct from DLBCL cases that arise *de novo*.

1.3.2. Hodgkin Lymphoma

Hodgkin lymphoma (HL) was the first recognized lymphoma entity originally described in 1832 by Thomas Hodgkin (61-63). Called Hodgkin disease for more 150 years due to its unknown etiology, only in 1993 molecular studies on the microdissected pathognomonic Hodgkin and Reed-Sternberg (HRS) cells showed this to be a B cell tumour (64). The malignant HRS cells usually account for only 1% of cells in tumour tissue, whereas the majority of the cells in the biopsy represent non-neoplastic cells of the immune system. HL accounts for approximately 30% of all lymphomas with an incidence in the western world of 3 cases per 100.000 people per year. There are two distinct types of HL that show biological and clinical differences; classical HL (CHL) representing 95% of HL cases and nodular lymphocyte-predominant Hodgkin´s lymphoma accounting for the remaining 5%. Within classical HL four subtypes are distinguished including nodular sclerosis, mixed cellularity, lymphocyte-rich and lymphocyte-depleted with some differences in anatomic sites of involvement, clinical features, growth pattern, presence of background fibrosis and composition of the cellular infiltrate and frequency of EBV infection. In common to all CHL subtypes is the HRS immunophenotype, characterised by strong expression of CD30 and CD15 and no or weak expression of CD20, PAX5 and CD45. Nodular sclerosis accounts for 60-85% of HL (2).

Treatment is based on clinical, radiological imaging and pathological staging. A modified Ann Arbor staging system is used as follows: limited disease (stages I and IIA without constitutional symptoms) or advanced disease (stages IB and IIB with bulky disease [largest deposit, ≥10 cm in diameter] and stages III and IV either A or B [with or without constitutional symptoms]) (65, 66).

Dramatic therapeutic benefits where achieved in the last 2-3 decades. Currently, most patients receive at least four cycles of polychemotherapy and, if indicated, radiotherapy (67). Yet, at least 20% of patients are still not cured and a similar or greater proportion of patients maybe overtreated (68) Autologous hematopoietic stem-cell transplantation can rescue about 50% of patients in whom primary therapy has failed. Initial clinical decisions and risk stratification

for patients with Hodgkin's lymphoma are largely based on clinical variables that distinguish those who are at high risk from those at standard risk. The International Prognostic Score (IPS) (on a scale of 0 to 7, with higher scores indicating increased risk) is the standard that is used for risk stratification of advanced stage HL, but it does not apply to limited stages, and none of the published prognostic factor systems can reliably identify patients in whom treatment is likely to fail (69). Moreover, neither the IPS nor its individual clinical components are suitable for accurately predicting the outcome of autologous hematopoietic stem-cell transplantation in patients with HL. For these reasons, reliable biomarkers for predicting long-term survival at diagnosis are needed for such patients.

In HL, unlike most other cancers, the malignant HRS cells are outnumbered by non-neoplastic cells in the microenvironment of the tumour. The frequency and distribution of these cellular components and HRS cells vary considerably among individual patients and among subtypes of HL (2). Several studies have focused on the prediction of outcomes by means of markers expressed predominantly by HRS cells or the microenvironment (70-75). However, most of these markers require validation in independent cohorts.

1.3.3. Mantle Cell Lymphoma

Mantle cell lymphoma (MCL) accounts for roughly 6% of NHL and is characterized by the presence of the t(11;14) translocation that juxtaposes the *CYCLIN D1* (*CCND1*) gene downstream of the immunoglobulin heavy chain gene promoter resulting in deregulated G1/S phase transition leading to cellular proliferation (2). MCL usually shows widespread disease in most patients at the time of diagnosis with lymphadenopathy, hepatosplenomegaly and bone marrow involvement (76-78). The behaviour of MCL has largely been attributed to the intrinsic features of the tumour cells induced by the CCND1 overexpression, which is supported by studies relating mitotic rate and Ki67 expression, a marker of proliferation, with patient outcomes (79, 80). While MCL is essentially incurable, the clinical behaviour is heterogeneous with previous studies also identifying variations in mode of clinical presentation (76), tumour histology (81, 82), cytogenetic alterations (83, 84), immunoglobulin gene status

(85, 86), *TP53* gene mutations and TP53 protein overexpression (87, 88) and proliferation signature (89) as predictors of outcome.

The disease has a lower rate of complete remission, duration of response, and overall survival after conventional chemotherapy when compared with other lymphomas, with a rather short median survival of 5-7 years using aggressive, multidrug therapy, usually including cytarabine and R (90, 91). Consolidation therapy with autologous stem cell transplantation is also used. New chemotherapeutic agents and radioimmunotherapy may add further to advances in treatment.

1.3.4. Primary Mediastinal Large B Cell Lymphoma

Primary Mediastinal Large B Cell Lymphoma (PMBCL) is a distinct lymphoma entity arising in the mediastinum from putative thymic B cells (2). Biopsies of PMBCL are composed of medium to large-sized cells with abundant pale cytoplasm, ovoid nuclei and are characteristically associated with background of fine sclerosis. PMBCL accounts for 2-4% of NHL and occurs predominantly in young adults with a female predominance. It is locally aggressive, but less likely to extend beyond the anterior mediastinum. With similar therapy the outcomes are typically superior to nodal DLBCL in most series with a survival plateau after 2.5 years (92, 93). Yet, clinical criteria, such as IPI, imprecisely predict therapy response (94). PMBCL shares histological and gene expression features with both GCB subtype of DLBCL and classical Hodgkin lymphoma (95, 96).

1.4 GENOTYPE & PHENOTYPE ASSESSMENT IN CANCER

1.4.1. Gene Expression Profiling (GEP)

Microarray technology relies on the binding of a target molecule, either labeled RNA or cDNA fragments to an immobilized detecting reagent (oligonucleotides / cDNA) as a means to study the expression of thousands of molecules simultaneously (97). Developed since the 1950s, the technology benefited from widely disparate advances in a number of unrelated fields, such as the quantitative detection of complementary hybridization of nuclei acid; parallel

generation of gene fragments through PCR technology; laser and robotic application of multiple samples to small arrays; availability of large sets of cDNA clones, improvements in computing and software design, mathematics and bioinformatics and finally the completion of first reference human genome in early 2001. Both of the most common platforms; spotted arrays, in which measured amounts of specific cDNA or oligonucleotides are accurately spotted on microscope glass slides, and synthetic arrays, in which short oligonucleotides are synthesized *in situ* on the surface by photolithography (developed by Affymetrix®), rely on the hybridization of free, labeled nuclei acids derived from sample RNA to known gene sequences immobilized on glass (98). Upon hybridization, fluorescent signals are generated, captured and a ratio calculated that enables the quantification of the bounded probe. Data analysis and interpretation follows through quality assessment, filtering and normalization (98). Once these data has been processed, most researchers usually employ some form of clustering in order to identify and represent different patterns of expression hidden in the data. Differences and similarities between patterns (Class Discovery) and whether the distinctions can determine functional groups (Class Prediction) can be evaluated (99). Clustering can take two main forms, unsupervised and supervised. In unsupervised clustering, no previous information is assumed. Data are presented to an algorithm, such as the average-linkage hierarchical clustering algorithm (100), which organizes data based on similarities between either the levels of GE across samples or their expression patterns. It is hierarchical because it starts with one gene and progressively scores other genes for identity with the first gene, creating a branching tree-like structure in which the length of the branches represents the degree of relatedness between genes. This or other methods (101) result in grouping the samples with similar patterns of GE, with the assumption that similar transcriptional profiles indicate a close relationship between samples.

In supervised clustering, groups are defined previous based on expression profiles, clinical or biological distinctions and profiled. A learning algorithm, such as Nearest Neighbor Analysis, is applied to identify the smallest set of genes that can reliably distinguish between the predefined categories (99). The accuracy of the predictor is then validated on a separate set of data, or using different statistically approaches for cross-validation, often using Bayesian

mathematics to generate classifiers that include an element of probability in the value of each given gene within the classifier.

Microarray GEP of hematological malignancies has been instrumental in the early stages of this field (102). Reported studies have covered several aspects of molecular biology. Most have been basic profiling studies, primary descriptive, often trying to gain new insights of the origins or etiology of tumours. This is achieved by comparing the tumour gene signature with signatures of “normal” development counterparts (103). Likewise, divergent profiles from the nearest normal cell type may reveal insights into important pathways or mechanisms important in tumorigenesis. Other studies aim to identify the minimum set of genes required to identify a particular tumour subclassification, resulting in minimum diagnostic classifiers. Specific profiles can also be correlated with known clinical outcome of patients thereby creating prognostic classifiers.

One of the difficulties in microarray gene array studies is the comparison and validation of experiments. Despite the trend to uniformity in data storage and increased data availability for comparison, application of results to diagnostic and clinical decisions has been very slow to develop. For some diseases and often using similar technology, lists of genes for classification or outcome can vary widely. Yet, often the different gene sets are accurate in classifying and predicting tumours. Individual genes or set of genes may not be the most informative data, but the underlying disrupted intracellular pathways, often affecting large number of genes, can be of more value for classification purposes. Both technical and analytic issues are responsible for this variability. A wide range of factors are responsible for the technical discrepancies including, sample variability and archive conditions, RNA extraction methods, RNA amplification, which platform is used, when it is used and by whom, hybridization conditions, uniformity of samples and even the size of the sample (104). Analytic factors are the greatest source of variability as different algorithms and analysis method can produce different classifiers beginning with the same raw input data. The introduction of the minimum information about a microarray experiment (MIAME) protocol, which recommends all experiments to be similarly stored and make available a minimal set of data about the experiments, some of the issues have now been overcome (105). Yet, the issue

of lack of comparability is still present as evidenced by the lack of independent validation of many studies. The high cost of performing studies with enough samples to achieve statistical power, which usually requires series of hundreds, makes most of these experiments difficult to repeat or validate.

1.4.2. Genome-wide Scanning for Copy Number Alterations - Bacterial Artificial Chromosome (BAC) Array Comparative Genomic Hybridization (aCGH)

Using aCGH, an array of specific chromosome segments, in this study Bacterial Artificial Chromosomes (BAC), immobilized on a glass slide is used as the target (106). aCGH resolution is only dependent by the size and distance between each DNA segments spotted on the glass arrays. Figure 1.2 summarizes the principle of the method. Firstly, test tumour DNA and control normal DNA are differently labeled with fluorescent dyes and hybridized to each spot. The resulted ratio of fluorescence intensities is proportional to the copy number difference in that DNA segment. The utility of aCGH in detecting recurrent sites of DNA gain and loss in cancer is well established on the basis of numerous reports (107, 108). As technology has improved, several groups applied this method for high-resolution, genome-wide scanning of abnormalities. At the British Columbia Cancer Research Centre (BCCRC), an aCGH platform was built with a minimal tiling set of 32,433 BAC clones covering >95% of the human genome (109). The BAC library was fingerprinted using *Hind* III. Using a linear PCR method, the clones were then converted to Amplified Fragment Pools, representing segments of DNA with specific chromosome locations (110). These AFP were represented in triplicates in the array. Later the same group revised the platform to 27,000 BAC clones, having removed a number of redundant clones. The technical details of the array production, hybridization and imaging are published (109). To analyze the approximately 100,000 ratio signals (32,433 clones spotted in triplicate) and align on the human genome map, bioinformatic tools were developed by the same group (SeeCGH software) (111).

1.4.3. FICTION Technique

Simultaneous fluorescence immunophenotyping and fluorescence *in situ* hybridization (FISH), so called, fluorescence-immunophenotyping and interphase cytogenetics as a tool for investigation of neoplasms (FICTION) provides the advantage of both techniques and allows a simultaneous analysis of the phenotype and genotype of tumour cells at the single-cell level (112, 113). Using specific cell lineage and differentiation associated monoclonal antibodies for immunophenotyping as well as DNA probes specific for known chromosome alterations by FISH, this combined technique makes it possible to correlate particular gene aberrations directly with the differentiation capacity of tumour cells. This is especially important in cases where the cells of interest may be in the minority and morphological features are an important and defining criterion of the disease (113, 114). In formalin fixed paraffin embedded tissue (FFPET) sections, several early reports described difficulties in obtaining an efficient hybridization signal while retaining immunofluorescent staining (115). However, these problems can be overcome, and recent studies have shown simultaneous application of FISH and immunofluorescence on FFPET sections, including bone marrow samples (116).

1.4.4. Tissue Quality & Fixatives

Until recently, both GEP and aCGH studies have been done using frozen material because quality of RNA influences the reliability of the output data. Snap fresh frozen (FF) tissue from diagnostic biopsies is the preferred source of RNA for GE studies in oncology. Yet, often these biopsies are limited in size and provide barely enough for morphological procedures done using standard FFPET techniques. It is not uncommon that biopsies may be partially involved by disease and thus frozen samples may not be representative. Often morphologic and cytological features in FF sections are not helpful in distinguish different types of cells. Finally, this is not a routine procedure in many centres, thus limiting sample size and the number of successful cases with paired specimens to study lymphoma histologic transformation.

Ideally, a clonal relationship should be established between paired biopsies so as to exclude the possibility of a 2nd *de novo* lymphoma. Often this may not be demonstrable due to clonal evolution with loss of common abnormalities or

emergence of a different subclone from a precursor “FL stem cell”, which hampers interpretation of these data.

These are several different reasons why FFPET represent excellent materials to study lymphoma biomarkers, primarily because it is widely available as a routine source of biopsy material for more than 100 years. Biopsies kept in formalin can be retrospectively retrieved from most Anatomic Pathology Department archives; they are mandatory in the diagnosis of transformation in most cases and usually they are the most abundant and representative tissue of the tumour.

However, DNA and specially RNA quality extracted from FFPET has been an important issue in the past. Its consistent poorer quality is attributed to formalin fixation inducing the formation of methylene bridges between amino group in the DNA and proteins; cross-linking of RNA and proteins as well as monomethylol group addition to bases. These processes are helpful in preserving cellular composition and morphology of tissue, but unfortunately reduce the penetration of both antibodies, nuclei acid probes and importantly are known to interfere with reverse transcription and amplification reactions due to cross-linking of nucleic acids. Moreover, deficiencies in several steps of the routine acquisition, handling and processing of the samples can contribute to irreversible degradation of both DNA and RNA.

One important factor that determined the success of FISH and immunohistochemistry (IHC) in FFPET samples is adequate unmasking of the target nuclei acids before staining or hybridization. Thus, pre-treatment methods are vital to expose target genes or proteins to allow penetration and reaction of the probes with preservation of tissue morphology.

High-throughput microarray GEP analysis of FFPET samples has been infrequently reported. Yet, FFPET isolated RNA has been shown to be suitable in viral detection or quantitation of mRNA levels by PCR. Recently, several protocols have been reported to improve robust isolation and amplification of RNA from FFPET (117). Recent reports have shown successful results of both aCGH and GEP using FFPE samples (118-122)

Finally, reliable amplification has been a challenge not only in FFPET but also in FF samples. Due to technical biases of the methods, most GEP reports correlating pre and post amplification samples have shown important changes

in results precluding accurate and precise conclusions (123). FFPET consistently shows poorer profiles than FF. However, if amplification can be avoided and degradation measured and quantified, FFPET samples have shown consistent GEP results (117).

1.4.5. Tissue Microarray

As previously mentioned, Anatomic Pathology archives store large numbers of specimens of human tumours in various stages of development as well as normal tissue counterparts, and have become very precious since the completion of the first reference human genome. Thus, FFPET samples have gained importance in cancer research in view of the recent developments. The arrival of the array technology to morphologic pathology happened in 1998 as a high-throughput facility to use several (hundreds) tissue samples on a single slide (124).

The idea of embedding many pieces in a single block existed in the early days of anatomical pathology, but several embedding instruments for this purpose recently became popular, and technical refinements are under way. One well established brand of microarray instruments is Beecher Instruments (Beecher Instruments, Inc. Sun Prairie, WI, USA). Their models have 0.6 mm, 1 mm and 1,5 mm cylinders. There are pros and cons in regard to using the method. Yet, several validation studies in regard to possible sampling error when small core specimens are collected have been performed confirming its use. Very recently, donor blocks with different variations are available, enabling a tissue microarray (TMA) platform to be used for genomic, immunohistochemical and proteomics using imaging spectroscopy.

TMA technology rapidly became the preferred technique in the validation and clinical correlation of the GEP arrays. It facilitates the simultaneous analysis up to 1000 specimens in a single slide of numerous tissue samples based on their morphology, protein expression (IHC) and genomic mutations (FISH). It allows the multiple use of often limited biopsy material in a cost & time effective manner where samples are exposed to the same experimental conditions during analysis. Furthermore, arraying of multiple cores ensures the consistency of analyzed features diminishing technical errors when analyzing hundreds of samples (125). This led to improvements in translational research

by correlating proposed biological markers of disease with clinical outcome analysis.

Entire cohorts of patient´s biopsies can be stored in few blocks & slides enabling easy querying for phenotypic and genotypic abnormalities using immunohistochemistry or *in situ* hybridization. Using a combination of these methods facilitates identification of changes of protein expression or genomic locus in several hundreds of tissue samples at once. Libraries of antibodies that recognize almost all human proteins (such as the Human Protein Atlas project (http://www.proteinatlas.org) or of Bacterial Artificial Chromosomes (BAC) that cover all the genome enabling the construction of FISH probes that recognize any specific sequence of human DNA facilitates their use not only in research, but can also be used in routine diagnostic laboratories as a means of verifying a diagnosis, selecting subjects for particular molecularly targeted therapies, and for predicting recurrence. Similarly, FISH techniques can be performed as a means of validation, that is, to verify amplification data generated by other methodologies, such as by quantitative PCR, array based comparative genomic hybridization (aCGH), and single nucleotide polymorphism (SNP) arrays. These approaches can be further combined with other techniques such as immunofluorescence, including FICTION (Fluorescence Immunophenotyping and Interphase Cytogenetics) as a tool for the investigation of neoplasms.

When BAC clones are used for FISH studies in paraffin-embedded tissue sections, several steps must be carefully controlled including labelling and hybridizing to DNA. The BAC clone must be confirmed to map to the desired location using normal cells, because assignments of BAC clones often change to reflect the daily process of refining the human genome database. The information on exact location of each BAC probe according to the most recent annotation of the human reference genome is necessary (publically available in different sites, such as University of California Santa Cruz (UCSC) genome browser (http://genome.ucsc.edu/). Thus, all BAC probes must be tested to determine whether they are hybridized to the two corresponding sites (or two pairs of the signals on the sister chromatids) in the metaphase chromosome spread before they are applied to human tissues containing cancer cells.

In addition to the above-mentioned hurdles to obtaining the right BAC probes, there is another stumbling block to completion of a FISH study: the labelling

step. Several labelling methods are available, and some are commercially available, using protease treatment, microwave treatment, heating, and other treatments including various detergents. Since overlapping cells and cells whose nuclei are partially cut can cause miscounting of the numbers of signals, cut-off values must be set based on preliminary evaluation of the signals in several non-neoplastic tissue controls.

1.5 HYPOTHESES & SPECIFIC AIMS

Aim 1- Mechanisms Underlying Transformation of FL into DLBCL

The general aim of this study was to investigate how alterations in the genome impact transformation of FL into DLBCL. We hypothesized that structural genetic abnormalities found during transformation should impact on the transcriptome of important candidate genes responsible for transformation. As time and burden of disease may influence the spectrum of abnormalities detected or not detected, a proper study should focus not only on paired specimens of both disease components separated in time, but also in cases with both diseases showing early events in transformation. The correlations of alterations of GEP with copy number alterations may indicate important biological pathways of transformation. Differences of GEP in composite lymphomas may show features associated with early events of transformation and thus were a focus of our studies.

Aim 2 – Role of the Microenvironment in FL Transformation into DLBCL

The general aims of this study were to investigate how alterations in the microenvironment collaborate in the progression of FL into DLBCL, aiming to identify biomarkers heralding transformation and eventual future therapeutic targets. We hypothesized that non-neoplastic cells in FL are not just innocent bystanders. A symbiotic relationship exists between the neoplastic B cells, reactive T cells, FDCs, vessels and macrophages that may have an important impact in the clinical outcome of this disease and thus the risk of transformation. Tumour immunity may be a driving force in FL progression and transformation. The content and distribution of non-malignant cells may correlate with clinical outcome and risk of transformation and be used as biomarkers helping current

clinical indices in therapeutic decisions or prognostic information. Thus, we hypothesized that non-malignant cell content and distribution within FL may be a surrogate marker of protumoral immunoedited tumour immunity.

Aim 3 - Monocyte & Macrophage Interactions in Patients with FL Harboring a t(14;18): Is There a Clonal Relationship?

As indicated previously, some follicular lymphomas sculpt their microenvironment attracting and/or retaining predominantly M2-type macrophages. These reactive “non-malignant” cells may not be just “reactive” but eventually derived from FL clonally related precursors cells. A subset of these monocytes/macrophages could differentiate from the FL “stem cell pool” in the marrow and then be recruited to lymphoma sites. Once in the lymph node, these cells may provide the neoplastic B cells with a trophic environment in cooperation with regulatory T cells leading to an immunosuppressive intra-tumoral milieu. Identifying macrophages within the lymphoma microenvironment from patients with follicular lymphoma previously shown to have a t(14;18) might allow us to determine if a subset of these cells similarly harbour a t(14;18) and thus are clonally related.

Aim 4 – Identification of New Biological Predictors of Survival in Different Human Lymphomas Subtypes.

Expanding aim 2, the general aims of this study were to identify biomarkers with prognostic impact in different types of biologically and clinical relevant lymphomas and eventually inform on a list of future therapeutic targets. Similar to FL, we hypothesized that the microenvironment is not just an innocent bystander, but an active player in lymphomagenesis. The content and distribution of non-malignant cells may correlate with clinical outcome and be used as biomarkers helping current clinical indices in therapeutic decisions or providing prognostic information. Finally, special importance was given to the re-evaluation of previously studied biomarkers in non-Hodgkin lymphomas receiving recently introduced immunotherapy (monoclonal antibody anti-CD20, R).

1.6 FIGURES

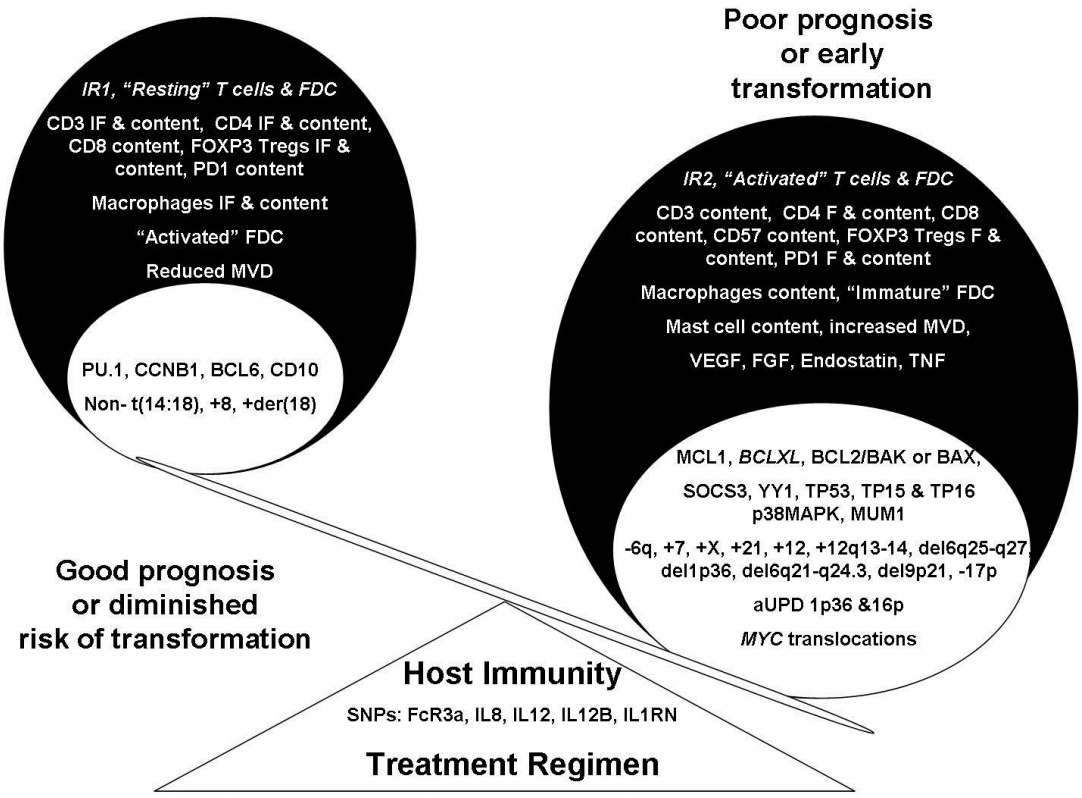

Figure 1.1 - Prognostic markers in FL. For complete details see review (11).

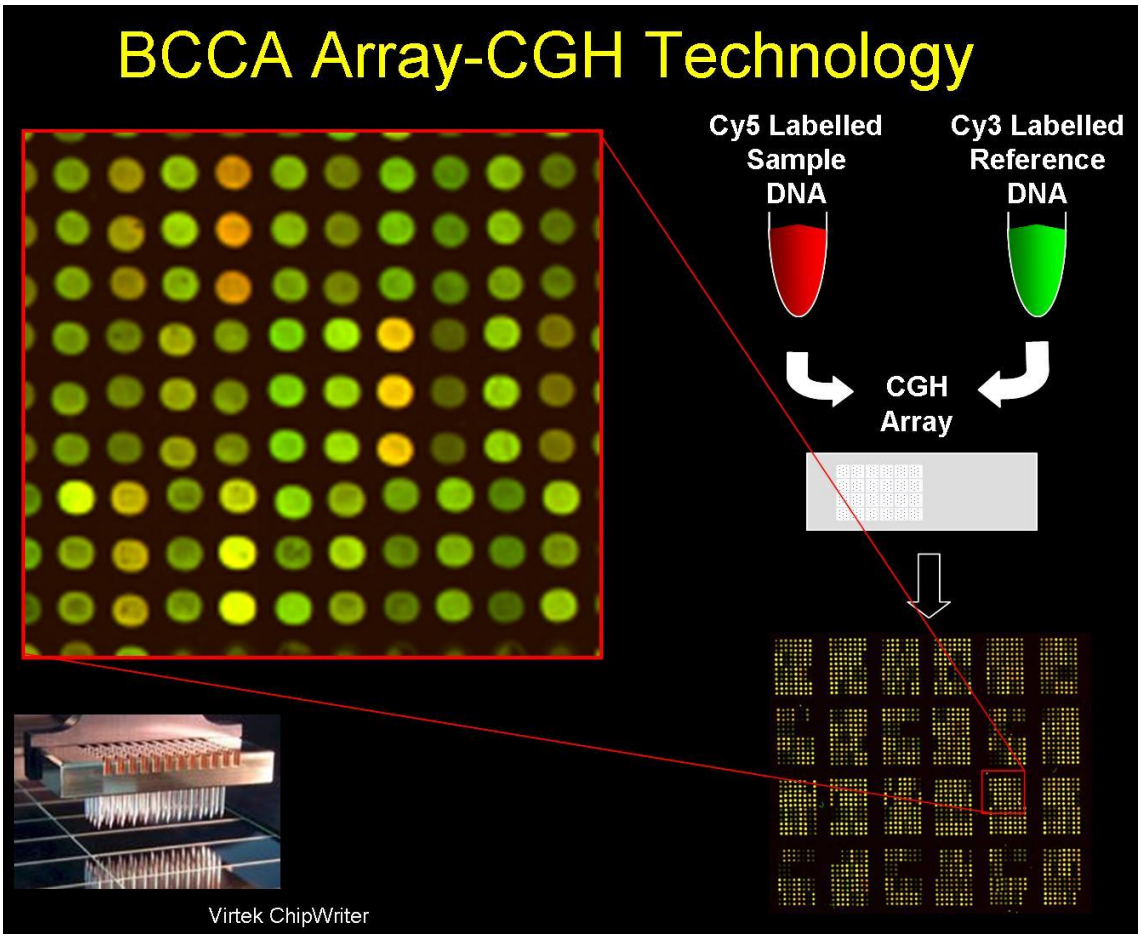

Figure 1.2 - Bacterial Artificial Chromosome (BAC) array comparative genomic hybridization (aCGH) – The British Columbia Cancer Research Centre built an aCGH platform was built with a minimal tiling set of 32,433 BAC clones covering >95% of the human genome. Using a linear PCR method, the clones were then converted to Amplified Fragment Pools, representing segments of DNA with specific chromosome locations. Test tumour DNA and control normal DNA are differently labeled with fluorescent dyes and hybridized to each spot. The resulted ratio of fluorescence intensities is proportional to the copy number difference in that DNA segment.

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Section 2: Mechanisms of Follicular Lymphoma Transformation into Diffuse Large B Cell Lymphoma

2.1 INTRODUCTION

This study was undertaken to further elucidate the biological mechanisms underlying the transformation of FL. The comparison of biopsy material from the same individual pre and post-transformation has been classically defined as the prerequisite for a clear understanding the molecular mechanisms driving the event. Until recently, most studies have revealed a number of recurring cytogenetic abnormalities (see Section 1) each one occurring in a small subset of cases, highlighting the molecular diversity of the transformation event. The combination of global gene expression analysis supplemented with genomic copy-numbered analysis may provide new insights into the mechanisms of FL transformation.

In the current study we performed two experiments using: (1) a series of paired fresh snap frozen (FF) lymph node biopsies material retrieved pre and post the transformation event and (2) a cohort of FFPET composite lymphomas with FL and DLBCL diagnosed in the some lymph node, most likely representing early transformation events. We used a combination of genome-wide microarray techniques including different commercially available expression array platforms (both Affymetrix® & Agilent® arrays) as well as the Vancouver BCCRC microarray-based CGH (array-CGH) using the submegabase resolution tiling arrays (see Section 1).

2.2 EXPERIMENT 1: Comparison of Gene Expression Profiling and Genomic Abnormalities Using Paired Fresh Frozen Samples

2.2.1. Material & Methods

2.2.1.1 Patients & Sample Selection

Twelve patients with sequential fresh frozen paired biopsies from pre- and post-transformation FL were identified from the Arhus University Hospital based on

histological transformation of FL to DLBCL (1) and fresh frozen tissue availability. Cases had a wide range of time to transformation (ranging from 1 to 10 years post FL diagnosis). In reviewing the available tissue samples at the BCCA, it was not possible to show definitive histological transformation into DLBCL in the available material in five cases and in two cases there was insufficient viable material. We next examined the clonal relation between biopsy pairs using extracted DNA and routine standard heteroduplex *IGH* and *BCL2* PCR methods based on established techniques at the BCCA pathology laboratory (http://www.bccancer.bc.ca/HPI/labservices/). Only four pairs were confirmed by PCR to be clonally related, harbouring both *IGH* & *BCL2* clonal rearrangements, which were identical size between the original FL and post-transformation tumors (Figure 2.1). The remaining three pairs failed due to either poor quality DNA or different *IGH* & *BCL2* clone rearrangement between the pairs, raising concerns about the possibility of completely unrelated lymphoid malignancies.

RNA was isolated from frozen tissue biopsies using standard protocols (http://www.bccancer.bc.ca/HPI/labservices/) and gene expression profiling (GEP) was performed using Affymetrix® Human Genome U133 Plus 2.0 Array (> 54,000 transcripts). All tumor samples were stored as tissue fragments in Optimal Cutting Temperature (OCT) medium. Total cellular RNA (Trizol) was isolated using standard protocols (http://www.bccrc.ca/). Extracted RNA was applied to the Affymetrix® array according to the manufacturer's instructions (http://www.affymetrix.com/).

2.2.1.2. Affymetrix U133 Plus 2.0 Array

The four samples were applied to the arrays following by reverse transcription using the manufacturer's standard protocol (http://www.affymetrix.com/). For each specimen, total RNA was converted into double-stranded cDNA using a cDNA Synthesis System kit. The cDNA resulting from this reaction was purified using the GeneChip Sample Clean-up Module (Affymetrix®). Labeled cRNA was generated using *in vitro* transcription labeling nucleotide mix (Affymetrix®). The resulting cRNA was then purified using the GeneChip Sample Clean-up Module (Affymetrix®) and quantified using the Nanodrop ND-1000 spectrophotometer (NanoDrop technologies®). Hybridization, washing, staining

and scanning protocols, respectively, were performed on Affymetrix GeneChip instruments as recommended.

The image files (DAT.) and cell intensity files (CEL files) were analyzed using Genespring 6.01 software package. Both supervised and unsupervised analyses were performed to determine differentially expressed genes using different cutpoints: 2x, 3x, 5x fold-change at a False Discovery Rate (FDR) <0.05. In the unsupervised analysis using 595 differentially expressed genes, the eight samples cluster by patient origin (Figure 2.2). In the supervised analysis of gene expression that compared directly between pairs of pre and post-transformation, we used $p<0.05$ and different multiple testing correction strategies, generating two gene lists with 273 (110 false-positive) and 18 (1 false-positive) genes, respectively (Figure 2.3). All GEP protocol and data analysis was done in collaboration with specifically trained staff from Genome Sciences Centre, Vancouver (http://www.bcgsc.bc.ca/), in particular Tarun Nayar and Alan Delaney.

2.2.1.3. CGH: BCCA Array

ArrayCGH was performed using formalin-fixed paraffin-embedded tissue (FFPET) and the same control of pooled male genomic DNA. In order to enrich the purity of malignant B cells, the FL samples were laser microdissected (Leica®) so that only malignant follicles were used in the comparison. In the setting of FL and its transformation to DLBCL histology, pure target material is particularly critical for follicular histology, where great care must be given to the retrieval of the tumor cell population devoid, as much as possible, of tumor-infiltrating non-neoplastic cells. A T cell marker (CD3) and/or follicular dendritic cell stain (CD21) were used to highlight the follicles (Figure 2.4). Standard protocols were used and an average of 10^6 malignant cells was used for each case.

Routine DNA extraction methods were used. 100-200ng of purified DNA from each sample was required per slide. Hybridization was successful in the 4 pairs and results are summarized in Table1. Total cellular DNA (using a proteinase K protocol) was isolated using standard protocols (http://www.bccrc.ca/). Extracted DNA was applied to BCCA aCGH tiling arrays according to the manufacturer's instruction. The sub-mega base resolution tiling array contains

26,819 BAC clones spotted in duplicate and covers >95% of the human genome (2). Array CGH was performed as previously described (3). The array slide was scanned using a charged-couple device (CCD) camera system to capture the cyanine-3 and cyanine-5 channels (Applied Precision®, Issiquah, WA). The images were then analyzed by SoftWoRx microarray analysis software (Applied Precision®), followed by a stepwise normalization procedure (4). Data were filtered based on both replicate standard deviation (data points with >0.1 standard deviation removed) and signal-to-noise ratio (data points with a signal to noise ratio <3 removed). Copy number alterations were visualized using the "SeeGH" software available at http://www.flintbox.ca/technology.asp?tech=FB312FB (5) – Figure 2.5. All aCGH protocol and data analysis were done in collaboration with W. Lam research group, in particular Brian Chi and Ronald deLeeuw, from BC Cancer Research Centre, Vancouver, who are responsible for this platform.

2.2.2. Results & Discussion

2.2.2.1. GEP

By hierarchical clustering using the 595 most differentially expressed genes, the 4 pairs clustered by patient and showed similar gene expression profiles upon transformation to DLBCL (Figure 2.2). When the gene expression changes of all FL cases transforming to DLBCL were analyzed, 18 genes showed a change in expression level that was highly significant (at least 5-fold change with standard deviation < 0.5 and multiple testing correction - Benjamin and Hochberg False Discovery Rate). These 18 genes were either up- (7genes) or down-regulated (11genes) upon transformation (Figure 2.3). These are poorly annotated genes have not been previously associated with transformation events in lymphoma (6-8).

Yet, some of the genes have potential associations of interest and might be relevant for the transformation event, including one up-regulated gene, ADP-ribosylation factor-like 4D (*ARF4L*), and four down-regulated ones, sirtuin 2 (*SIRT2*), chromodomain helicase DNA binding protein 6 (*CHD6*), cytochrome c oxidase subunit VIIa polypeptide 2-like (*COX7A2L*) or *EB1*(estrogen receptor binding CpG Island) and nucleosome assembly protein 1-like 4 (*NAP1L4*).

ARFL4 expression is controlled by the activated Akt/mTOR pathway, which is a downstream effect of the loss of *PTEN* function. Its increased expression follows the loss of the tumor suppressor *PTEN* (9). In gliomas, it is a Tumor Associated Antigen (TAA) that is regulated post-transcriptionally, at the level of translation, by the Akt/mTOR pathway. Although the exact function of ARF4L is unknown, evidence suggests that it participates in vesicle transport between the endoplasmic reticulum and Golgi complex. Peptide motifs from ARF4L were recently identified to induce HLA-A2–restricted and tumor-reactive cytotoxic T lymphocytes in peripheral blood mononuclear cells from patients with brain tumors (9). This may suggest ARFL4L as a candidate antigen to be used in cancer immunotherapy.

SIRT2 is a NAD^+-dependent deacetylase gene and is involved in the direct deacetylation of α-tubulin and histone H4. It has been reported that *SIRT2* down-regulation caused centrosome fragmentation in response to nocodazole, a microtubule inhibitor, prior to the alteration in spindle checkpoint function, implying not only a novel function of *SIRT2* for centrosome maintenance upon exposure to mitotic stress caused by microtubule inhibitors, but also its role in centrosome-mediated signaling pathway to sustain the spindle checkpoint (10). Moreover, SIRT2 interacts with p65 in the cytoplasm and deacetylates p65 *in vitro* and *in vivo* at Lys310. In $Sirt2^{-/-}$ cells, p65 is hyperacetylated at Lys310 after TNFα stimulation, which results in an increase in expression of a subset of p65 acetylation-dependent target genes. This provides evidence that p65 is deacetylated by SIRT2 in the cytoplasm to regulate the expression of specific nuclear factor kappa B (NFκB) dependent genes, an important pathway for lymphomagenesis (11).

CHD6 is thought to be a core member of one or more of the multisubunit protein complexes that remodel chromatin to allow patterns of cell type-specific gene expression. It colocalizes with both hypo- and hyper-phosphorlylated forms of RNA polymerase II (12). In human lymphoblastoid AHH-1 cells, silencing of CHD6 mediated by siRNA increased the growth rate and led to an increased radioesistance to radiation, highlighting a role of CHD6 in cell proliferation and radiosensitivity (13).

Finally, cytochrome c oxidase subunit VIIa polypeptide 2 like cytochrome c oxidase, COX7A2L or EB-1, the terminal component of the mitochondrial

respiratory chain, catalyzes the electron transfer from reduced cytochrome c to oxygen. This component is a heteromeric complex consisting of 3 catalytic subunits encoded by mitochondrial genes and multiple structural subunits encoded by nuclear genes. This gene is expressed in all tissues, and is up-regulated in a breast cancer cell line after estrogen treatment and is also a target gene of TP53 under stress conditions (14).

2.2.2.2. aCGH

Two sets of paired specimens submitted to GEP, were analysed using aCGH (Table 2.1). Common genomic alterations were present in both cases (80% in case 2 and 40% in case 4), which favors and confirms a clonal relationship. Examining global differences upon transformation, case 2 had more gains (4) than losses (1) while in case 4 the FL had many more gains (11) than the DLBCL (1) - Table 2.1. This latter finding suggests outgrowth of a less evolved subclone, as previously reported (15, 16). Classical karyotyping and recent CGH studies of paired specimens have shown that the transformed biopsy shares some, but not all, of the changes seen at FL diagnosis (8, 17). This suggests subclone selection and evolution from a common malignant initial clone in contrast to clonal evolution only resulting from stochastic genetic hits accumulated over time. It has been reported in several studies, as previously mentioned in chapter 1, that transformation of FL can occur over approximately 0-17 years after initial FL diagnosis with a constant rate of 3% per year (18, 19). One of these studies suggests that most FL patients may not be at risk to transform beyond 15 years. If clonal selection and evolution were the main and only drivers of the process, the transformation rate would likely have to change over time. The observed constant rate might be better explained if an ancestral pool of clonal lymphoma-originating-cells with limited proliferation capacity existed and is at risk for stochastic genetic events that may provide a growth advantage (15). Case 4 in this study supports this hypothesis as the transformed lymphoma cells had less overall genomic abnormalities than the initial FL suggesting outgrowth of a subclone closely related to the putative resting lymphoma-originating-cells.

2.2.2.3. Correlation GEP vs. aCGH

In the two pairs with available data, we looked for those genes differentially expressed that mapped to regions of genomic imbalance between the FL and subsequent transformed histology (19 gains and 4 losses) (Table 2.2). Five of the seven genomic imbalances acquired upon transformation have been described previously, including the common 2p15-q16 and 7q gains (8, 20-23). The “new” small deletion on 8q24.13-q24.21 followed by a gain 8q24.1-qter in case 2 suggests a *MYC* translocation with subsequent amplification (70x). This was confirmed by FISH analysis performed in the FFPET sample using a Vysis® breakapart probe (Figure 2.6).

Importantly, none of the 18 genes reveled by GEP were present in the genomic imbalances of cases 2 & 4. Moreover, the correlation between GEP and aCGH is complex, highlighted by the complete lack of correlation between gene expression and copy numbers alterations (Table 2.2). This is exemplified by the examples of the “top” three over expressed genes (>10 fold change) mapping to chromosome13 in case 2 (Figure 2.7). Two hypotheses can be put forward to explain these results. One is technical, as individual correlation of two whole genome platforms measuring different features of one case is challenging because significant biological characteristics cannot be clearly determined due to the complex bioinformatic analysis imposed by hundreds of thousands of variables in one sample. Despite the use of statistical tools most results called significant can simply be random changes reflecting the analysis method. Biological relevant variations will be more significant as we increase our sample size, but then many changes of individual samples will probably be non-significant. An alternative hypothesis is biological and relates to the many mechanisms that can explain the lack of correlation between gene expression changes and corresponding gene copy number alterations. These include epigenetic changes, RNA editing and microRNA modifications, all of which could preclude a direct correlation of copy number alterations and gene expression variations.

2.2.3. Conclusion

This pilot study, in agreement with the previous reports, suggested that mechanisms other than copy number alterations are responsible for deregulated gene expression during FL transformation. Furthermore, given this

heterogeneity and the complex relationship between genomic aberrations and GEP, a much large number of cases will need to be studied to fully appreciate the specific genetic events responsible for transformation, especially if using whole-genome analysis.

2.3 EXPERIMENT 2: Gene Expression Profiling of Composite Follicular and Diffuse Large B cell Lymphoma Using Formalin Fixed Paraffin Embedded Tissue Samples

2.3.1. Introduction

As previously discussed and shown in experiment 1 of the current study, suitable paired fresh frozen (FF) samples pre and post transformation from the same patient, a mandatory requisite for GEP transformation studies, are not easily available. Due to the wide range of time to transformation, the quality and quantity of the paired biopsies are often not ideal for study. And as shown in experiment 1, some of the pairs were not clonally related. Moreover, and as discussed previously, the interpretation of data obtained with whole-genome platforms needs a significant number of samples. The need for such a large amount of specimens makes it logistically impossible outside the scope of a multi-institutional consortium, as reported by Davies (24). Thus, due to the rarity of paired FF samples in a single institution we decided to focus on diagnostic biopsies of composite lymphomas showing in the same lymph node biopsy concurrent FL (grade 1 to 3a) and DLBCL (Figure 2.8).

All reported studies on FL transformation have used clinically and pathologically confirmed transformed disease, i.e. transformed lymphomas separated in time. What constitutes the early driving mutations in FL transformation remains mostly unknown. Although uncommon, composite FL and DLBCL are archived in routine FFPET blocks present in most departments of pathology. Thus, we chose to focus a study of composite histology FL cases in order to study early events in FL transformation. Moreover, GEP variability relates not only to the disease type under study, but also can be related to other variables such as tissue type, user variability, contamination of non-neoplastic cell types, the time from acquisition to sample preparation and tissue processing, etc. These biases

can be avoided using our study strategy and thus represents a significant advantage over the FF paired specimen experiments. Moreover, centralized hematopathology departments like BCCA do have FF samples of composite lymphomas but often the stored material is composed of just one (the predominant) of the components of the composite histology. Thus, composite lymphoma samples with enough tissue to be properly diagnosed and dissected are typically only available in the form of FFPET. Until recently, these tissues have been automatically excluded from RNA analyses, due to poor quality RNA. However, recently described new methods of nucleic acid extraction have improved and are reported to be suitable for low-density GEP (25).

Our next aim was to assess biological mechanisms underlying early events of FL transformation into DLBCL using GEP of routinely diagnosed lymph node biopsies with composite lymphomas comprising FL and areas of transformation to DLBCL.

2.3.2. Material & Methods

2.3.2.1. Patients & Sample Selection

We started this study by verifying the quality of RNA extracted from routine diagnostic lymph node samples, comparing the GEP of FF and FFPET from five reactive LN biopsies (cases recently diagnosed within less than one year old at the BCCA) using the 44K Whole-Genome Agilent® 60mer oligo microarray – Figure 2.9. In each experiment sample RNA was extracted using standard methodology Ambion® Optimum™ FFPET RNA Isolation Kit and was DNase-treated, precipitated and ran against a universal reference (UR) RNA derived from pool of cell lines (Stratagene®). Hybridization conditions were established using the universal reference RNA pool for optimal dynamic range, sensitivity and linearity in self-self hybridization: more than 99% of the data points fall in less than 2x ratio. For the 5 cases the log comparison of expression for all transcripts showed moderate correlations between FF and FFPET samples with ratio ranging from 0.46 to 0.65 (average 0.57). If we selected only the differentially expressed genes, the ratio of correlation increased to an average of 0.8 with expected less detection or more “loss” of gene expression in FFPET samples compared to FF. As an example, in Figure 2.10 are plotted some

lymphoid markers showing good correlation between samples. Finally, if we limit the analysis of the differentially expressed genes to a 2-fold change cut-off, the correlation is generally good, as the majority of the differentially expressed genes are still detected in both samples with more genes detected in FF samples and a smaller proportion detected in FFPET (in red).

In conclusion, in this pilot study of 5 recently diagnosed reactive LN biopsies we were able to successfully isolate RNA and generate array data from both FF and paired FFPET using standard extraction protocols and Agilent® Whole Genome microarrays. Despite some loss of signal in FFPET vs. FF, the majority of relevant gene expression changes were nonetheless detected in FFPET, including appropriate detection of pan-lymphocyte markers.

2.3.2.2. Composite FL and DLBCL

The study sample consisted of 14 lymph node biopsies with composite FL & DLBCL diagnosed at the BCCA. There were six females and eight males with a median age of 71 years (range 45-79). Histology was reviewed using the 2008 WHO Classification scheme (26). Both components were characterized morphologically, phenotypically and lymphoma cell purity (LCP) was separately determined for both diseases. By standard immunohistochemistry (Ventana®), immunostains for B (CD20 [clone L26]), T cells (CD3 [clone CD3 - Cell Marques/Novomix]), BCL2 (clone 124), CD10 (clone 56C6), BCL6 (clone PG-B6p) and IRF4 (clone MUM1) were performed to better characterize both components of the disease, determine DLBCL IHC subtype using the Hans algorithm (27) and help assessing LCP – Figure 2.11.

Distinct components were dissected separately and RNA extracted using the previously mentioned protocol Optimum™ FFPET RNA Isolation Kit. For each case RNA from each disease component was arrayed together in the same array slide - Figure 2.12.

2.3.2.3. Agilent® 44K Whole Human Genome-60mer Oligo Microarray

This microarray is comprised of approximately 41,000 (60-mer) oligonucleotide probes, which span conserved exons across the transcripts of the targeted full-length genes. The probes represent the whole human genome as known at the

time of its building using well-characterized full-length and partial genes from a number of major public data sources. The sequence and annotation information used in this microarray product is available through Agilent and publicly-available databases such as RefSeq, GoldenPath, Ensembl and more. Virtually all of the genes were mapped to the human reference genome and experimentally validated at Agilent´s laboratories (http://www.genomics.agilent.com).

2.3.2.4. Oligo Microarray Data Analysis

Microarray image files (DAT.) and cell intensity files (CEL files) were generated using default Agilent® software (http://www.genomics.agilent.com). Data preprocessing is described in the manufactures protocols. Data visualization and exploratory analysis, such as principal component analysis (PCA) and hierarchical clustering were performed with Genespring software (6.01). Network analysis was done using Ingenuity Knowledge Database software (www.ingenuity.com).

We performed paired Student t-test analysis of all patients including all probes and 2-fold change in at least 5 samples as well as global expression profiles obtained for both FL and DLBCL separately as well as combined FL & DLBCL and log-ratio (DLBCL vs. FL). Differential expression of DLBCL vs. FL in each sub-group and differential expression among sub-groups were done.

Publicly available GEP data from two previously reported studies of FL transformation (24) and DLBCL GEP classification (28) were used to query and cluster our data. This included predictor profiles of FL transformation as well as genes differentially expressed between DLBCL vs. FL used in Davies et al. and the different DLBCL gene signatures reported by Rosenwald et al: lymph node, MHCII, Germinal Center B Cell (GCB) and proliferation (28).

2.3.3. Results

Eleven cases were successfully arrayed for both components (Figure 2.12). Three cases were excluded due to poor RNA quality and/or insufficient RNA quantity to be arrayed. There were five females and six males, median age at diagnosis 69 years old. All specimens were nodal composite lymphomas;

including five FL grade one, three FL grade two and three FL grade 3A. As expected, the DLBCL component were all of the Germinal Center B Cell-like type (27) and had a higher concentration of malignant cells – Lymphoma Cell Purity - (median 93%, range 90-95%) than FL (median 76%, range 65-85%). Paired Student t-test of all cases including all probe profiles with a 2-fold change in at least 5 samples showed a small subset of 328 genes to be significantly differentially expressed between both components (FDR<0.1) – Figure 2.13. The vast majority of the transcripts are poorly annotated genes. Yet, like Davies et al report (24), among the up-regulated probes were genes involved in cell cycle and proliferation, growth factors and DNA binding and repair. The down-regulated genes included tumor suppressor genes, immune defense, T cells and macrophage associated genes – Figure 2.14.

Clustering the patients using global expression profiles of isolated DLBCL and FL components, combined FL & DLBCL and log-ratio (DLBCL vs. FL) showed three distinct clusters with two, four and five cases each (Figure 2.15). 6275 probes were differentially expressed between sub-groups (Standardized with mean 0, SD1, IQR>1.5, FDR<0.05). Cluster #1 included two high-grade FL3A, and both cluster #2 and #3 had cases with low-grade FLs (grades 1 and 2). The third FL3A was included in cluster #2. Neither grade nor LCP correlated significantly with GEP clustering, except for #1 including two FL grade 3A lymphomas [(#1 LCP: FL(80%) & DLBCL (95%); #2 LCP: FL(76%) & DLBCL (93%) and #3 LCP: FL(70%) & DLBCL (93%)] – Figure 2.16.

Not surprisingly, cluster 1, showed a predominance of up-regulated genes (1256) in comparison to down regulated genes (307) – Figure 2.17. Many of these genes mapped to network containing cell cycle, cell growth and proliferation MYC and 38pMAPK pathways (despite both genes, individually, were not differentially expressed) – Figure 2.18.

Profiles #2 and #3 were characterized by a lower number of significant genes and did not highlighted distinct pathways. In cluster 2 there were 34 up-regulated genes and 18 down-regulated ones (Figures 2.19 & 2.20). These mapped to different cell functions including up-regulation of cell transduction, cell cycle, apoptosis and DNA repair genes and down-regulation of genes related to transcription, protein-tyrosine kinases and macrophages genes. Within cluster 3 there were 20 up-regulated genes related to cell survival,

transcription and MAPkinase activity and 28 down-regulated genes related to protein transport, RNA binding, apoptosis and transcription (Figures 2.21 & 2.22).

Using the various signature gene profiles from the two published reports to query our data a similar three cluster structure is observed (Figures 2.23 & 2.24).

2.3.4. Discussion

This is the first study focusing in the early events of transformation using GEP composite FL and transformed disease present in the same FFPET diagnostic biopsy. In a pilot study using five FF vs. FFPE lymph node tissue, we demonstrate a reasonable correlation of GEP using the 44K Agilent® array between FF vs. FFPET. However, the results improved with a good correlation obtained in the differentially expressed genes instead of the whole genome.

In the study set of 14 composite lymphomas, eleven were successfully arrayed after dissection of disease components. Small subsets of genes were significantly differentially expressed between FL vs. DLBCL. As expected, among the up-regulated probe sets upon transformation were genes involved in cell cycle and proliferation, growth factors and DNA binding and repair. Among the down regulated genes, tumor suppressor genes, immune defense, T cells and macrophages-associated genes were enriched.

Three different clusters were observed with no correlation to either FL grade or non-malignant cell content evaluated by immunohistochemistry. Interestingly, a similar cluster structure was observed using various gene profiles characterized in reported studies of transformation or classification of DLBCL. Of note, is the identical three clustering obtained with the genes significantly differentially expressed in Davies et al (24), where the three clusters are separated by disease type (FLs and DLBCLs separated) and not by patient.

Cluster #1 showed relatively more consistent expression profile with a predominance of over-expression including genes involved in cell cycle, cell growth and proliferation, including several oncogenes and target genes mapped to MYC and MAPK pathway networks. This cluster is more distinguishable using the proliferation signature profile from Rosenwald *et. al*. and is in keeping with the results by Davies *et. al*., where 10 of the 20 paired specimens were

characterized by an increase of proliferation gene signature. Like Davies *et. al.*, proliferation signature clearly identified a distinct cluster of 2 out of 11 transformed FL.

In our study, profiles #2 and #3 were characterized by a lower number of significantly differentially expressed genes with a predominance of poorly annotated genes covering diverse cell functions. No significant network was highlighted in these two clusters. Yet, lowering the FDR to 0.1, we could map 385 significant probes for cluster #2 and 849 probes for cluster #3. As seen in Figures 2.20 & 2.22, different networks were obtained.

For cluster #2, three up-regulated cell transduction genes are highlighted: *GRB2*, *PTK2* and *RALA*.

Growth factor receptor-bound protein 2 (*GRB2*) is a ubiquitously expressed adaptor protein, which activates RAS and MAPkinases in growth factor receptor signaling that mediates B cell receptor (BCR) signaling pathways. B cell-specific Grb2-deficient mice showed impaired IgG and B cell memory responses, and impaired germinal center formation (29). It is associated with GCET2 adaptor protein in GC B cells in transducing signals from GC B cell membrane to the cytosol (30). Strong, cytoplasmic GRB2 expression has been reported in most NHLs including neoplastic cells of FL and DLBCL, while it is lost in Hodgkin lymphoma cells (31). Interestingly`, in T cells it is involved in TCR & CD28 signaling regulation including the transcription factor NFκB pathway in naïve and memory T cell responses (32).

PTK2, protein tyrosine kinase 2 or Focal Adhesion Kinase (FAK) is a protein tyrosine kinase essential for intracellular regulatory events, such as cell growth, differentiation, migration and tumor metastasis. Analysis of *FAK* promoter sequences revealed that *FAK* promoter harbors the NFκB and TP53 binding domains. It has been shown that bortezomib transcriptionally suppresses FAK expression by interrupting the NFκB pathway, which suggests that FAK could be a potential molecular target for bortezomib, a selective potent proteasome inhibitor that has been approved for treatment of non-Hodgkin lymphomas such as DLBCL (33). Studies of FAK promoter activity, real-time PCR, electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) assay revealed that bortezomib inhibits NFκB binding on the *FAK* promoter, thereby reducing FAK expression (34).

RALA, v-ral simian leukemia viral oncogene homolog A (ras related) belongs to the small GTPase superfamily, Ras family of proteins. RALA is a downstream effector of Ras function and are critical for both tumor growth and survival. The RAS/RAF1/RALA pathways converge to modulate NFκB activation and SF-mediated survival signaling (35).

In cluster #3, cell survival genes PPARG and PPARGC1A are key network genes. Peroxisome proliferator-activated receptor gamma (PPARG) and peroxisome proliferator-activated receptor gamma coactivator-1alpha (PGC-1alpha) are multifunctional transcription factor that regulates adipogenesis, immunity and inflammation. It has been shown that silencing PPARG expression in human lymphoma B cells increased basal and mitogen-induced proliferation and survival, which was accompanied by enhanced NFκB activity and increased expression of BCL2. These cells also had increased survival upon exposure to PPARG ligands and exhibited a less differentiated phenotype. In contrast, PPARG overexpression in lymphoma B cells inhibited cell growth and decreased their proliferative response to mitogenic stimuli. These cells were also more sensitive to PPARG-ligand induced growth arrest and displayed a more differentiated phenotype. Collectively, these findings support a regulatory role for PPARG in the proliferation, survival and differentiation of malignant B cells (36).

2.3.5. Conclusions

This is an exploratory study of a small clinical cohort in which we show that RNA can be extracted from FFPET diagnostic samples in reasonable quantity and quality for GEP studies. Based on our findings using composite lymphomas, we propose that there might be three potential different mechanisms responsible for early events in FL transformation. One distinct pattern is associated with proliferation as previously reported. Despite the small number of genes, distinctive gene sets characterizes the other two clusters with several good candidate genes for diagnostic and eventual therapeutic use. Further cases are needed to validate these results.

2.4 TABLES

Case	FL		DLBCL	
	Gain	Loss	Gain	Loss
2	2q37.3 11pter-qter 14q32.33-qter 17p13.2-p13.1 17p13.1-p11.2 18pter-p11.21 18cent-q21.33	2p11.2 4p16.1 4q21.21-q21.23 8pter-p12 9pter-p21.1 9p13.3-cent 9cent-21.11 15cent-q11.2 16p11.2-p11.1 17pter-p13.2 22q11.22	2q37.3 7pter-qter 8q24.1-qter 11pter-qter 13cent-qter 17p13.2-p13.1 18cent-q21.33 22q11.1-q11.22	2p11.2 4p16.1 4q21.21-q21.23 8pter-p12 8q24.13-q24.21 9pter-p21.1 9p13.3-cent 9cent-21.11 15cent-q11.2 16p11.2-p11.1 17pter-p13.2 22q11.22
4	2p14-p16.1 6pcent-pter 6qcent-q12 6q24.1-qter 8q23.1-qter 13q13.1-q13.3 16p11.2-pter 17pcent-p13.1 17qcent-qter 18pter-qter 19p13.11-pter 22q11.1-qter	2p13.3-p14 6q12-q24.1 9pcent-p13.1 13q13.3-q34 16pcent-p11.2 17p13.1-13.3 19pcent-p13.11	2p15-p16.1 6pcent-pter	2p14 5p14.3-p15.2 6q12-q24.1 9pcent-p13.1 16pcent-p11.2 17p13.1-13.3 19pcent-p13.11

Table 2.1 - ArrayCGH genomic copy number gains (red) & losses (green) alterations in pairs 2 & 4.

arrayCGH		GEP 10&(3)fold change	
		Genes↑	**Genes↓**
Gains	19	49(400)	25(163)
Losses	4	1(14)	2(20)

Table 2.2 - Correlation aCGH & GEP (cases 2 & 4). Regions of loss and gains are correlated with up and down regulated genes present in those areas with fold changes of 10x & 3x.

2.5 FIGURES

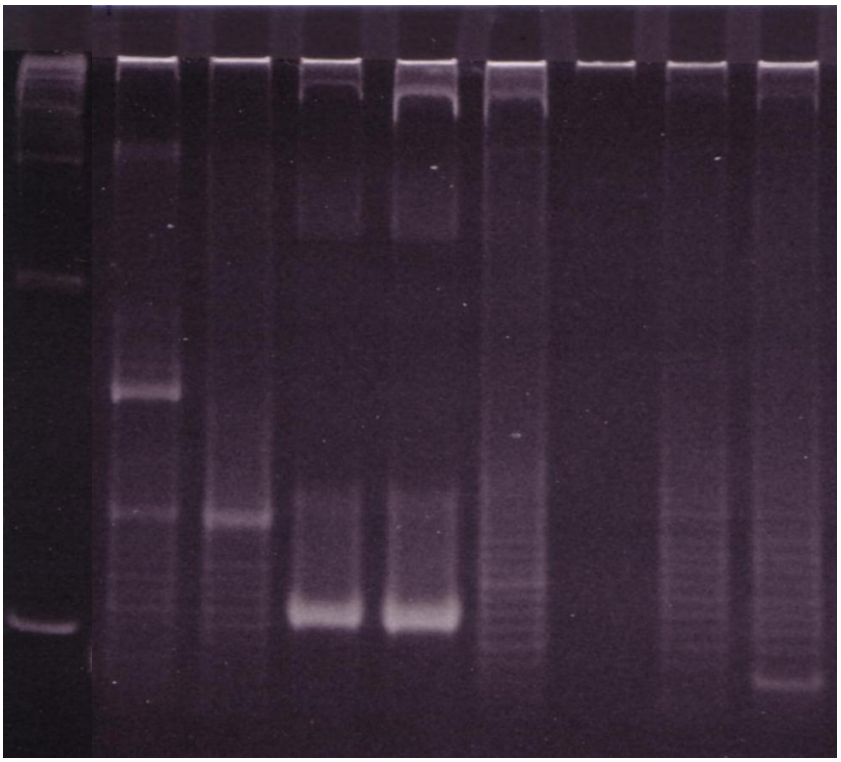


Figure 2.1 - Agarose PCR of four paired samples showing clonal relation.

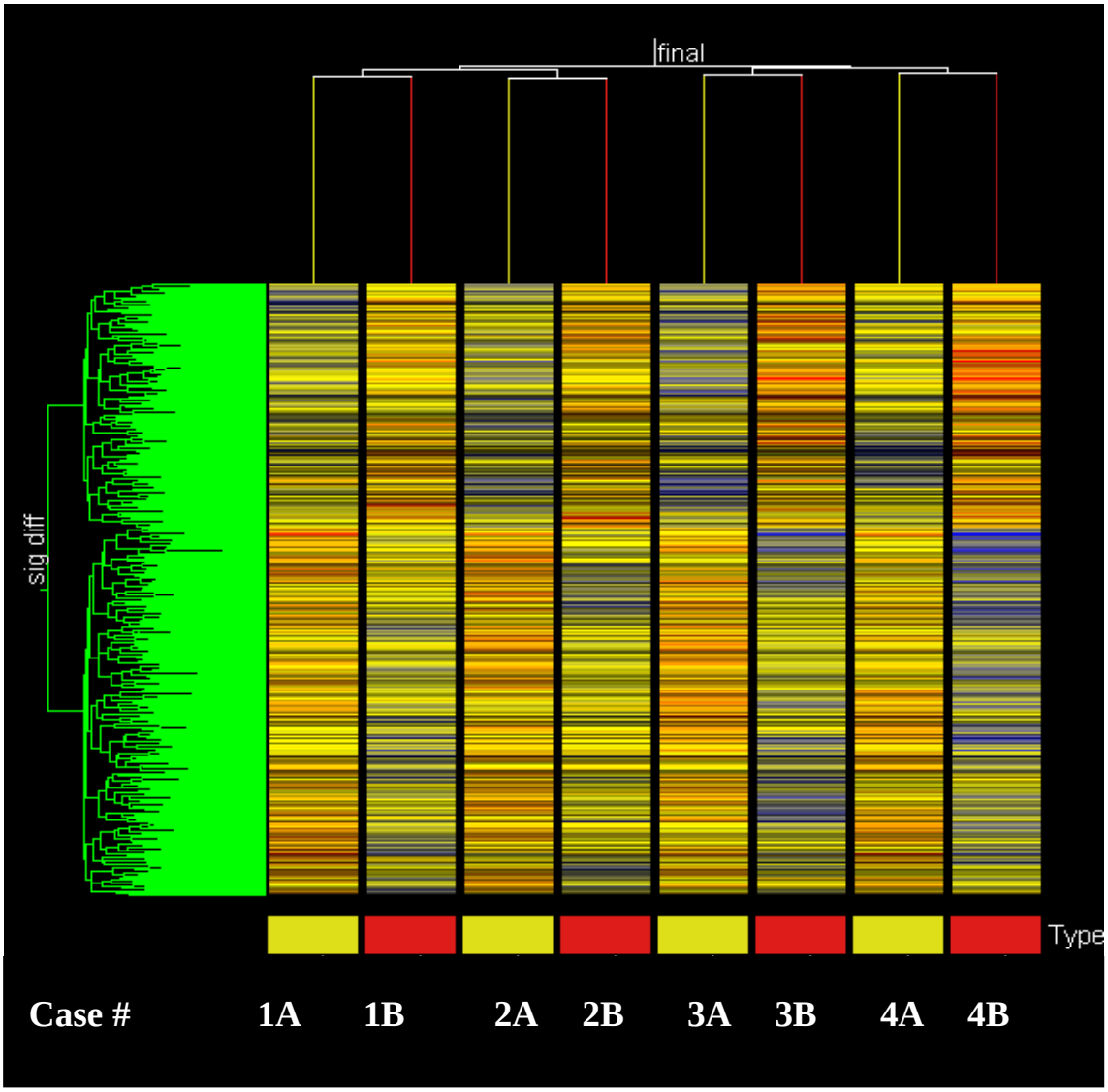


Figure 2.2 - Gene Expression Profile (cases 1 to 4). Unsupervised analysis using 595 differently expressed genes cluster the eight samples by patient origin.

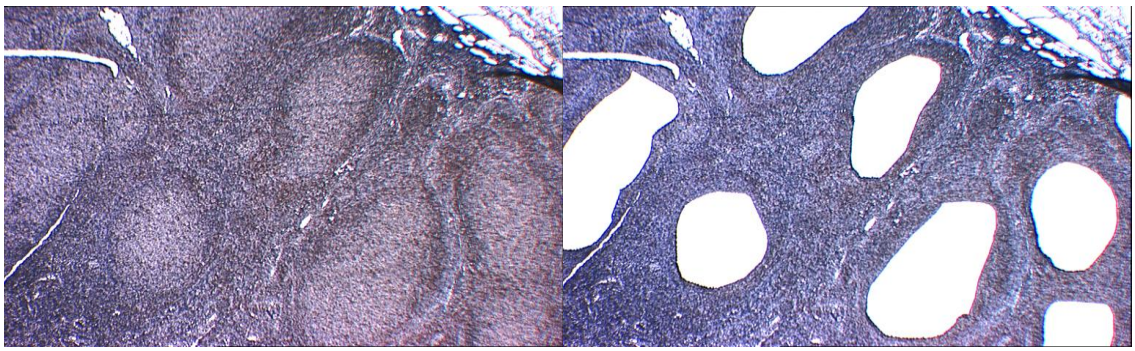
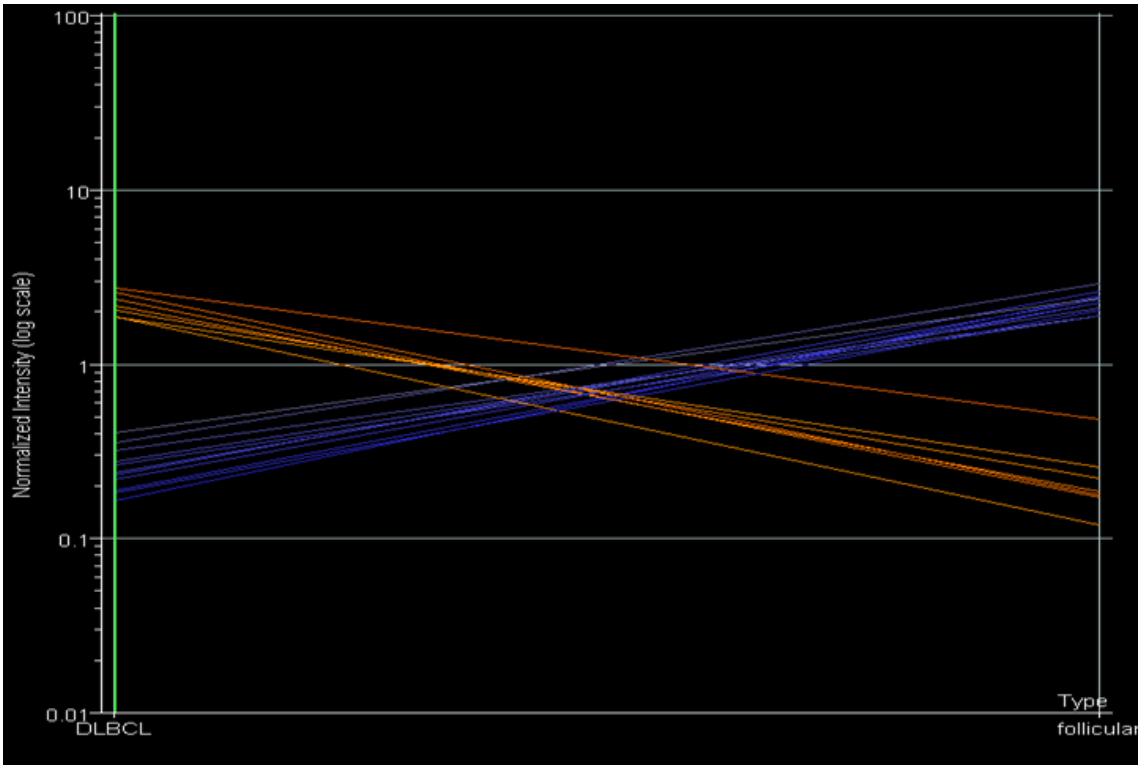


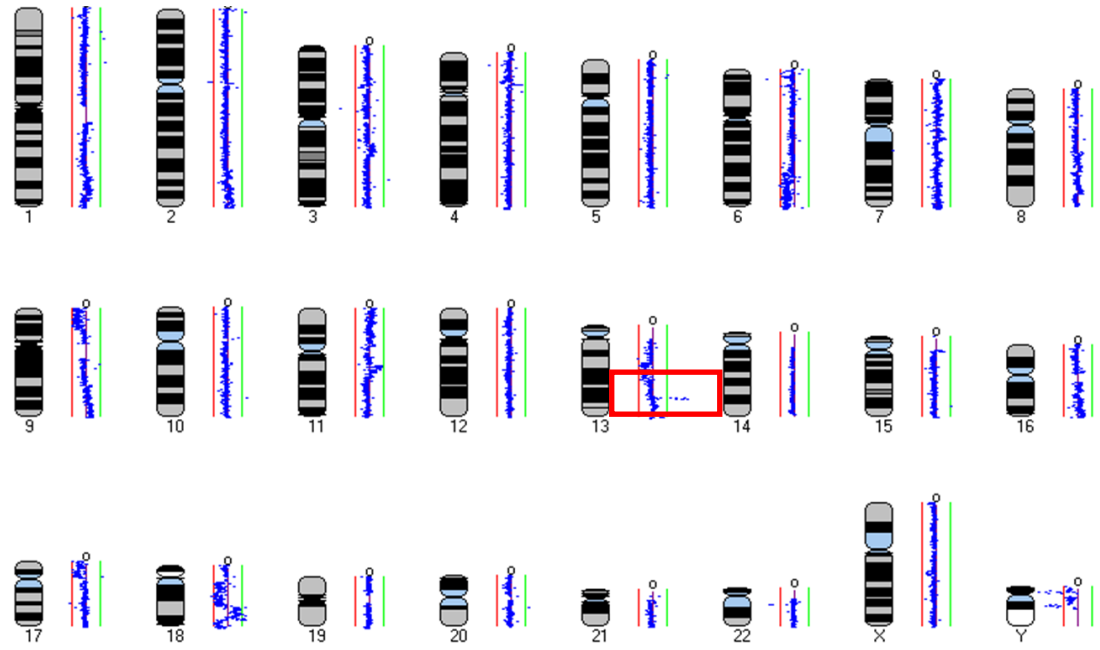
Figure 2.3 - Sequential sections of a microdissected FL case (Olympus BX40 / Nikon Elipse®; Digital Camera Dxm1200; 40x).



Gene	Chr
AIMIL	1p36
SCN11A	3p22
CALM3	10p15
CPN1	10q24
ARF4L	17q21

Gene	Chr
LOC118812	10q24
NAP1L4	11p15
EB1	12q23
SIRT2	19q13
CHD6	20q12

Figure 2.4 - The 18 genes (down regulated in blue & up-regulated in red) differently expressed upon transformation not previously reported in GEP studies. Small tables show the best characterized ones.



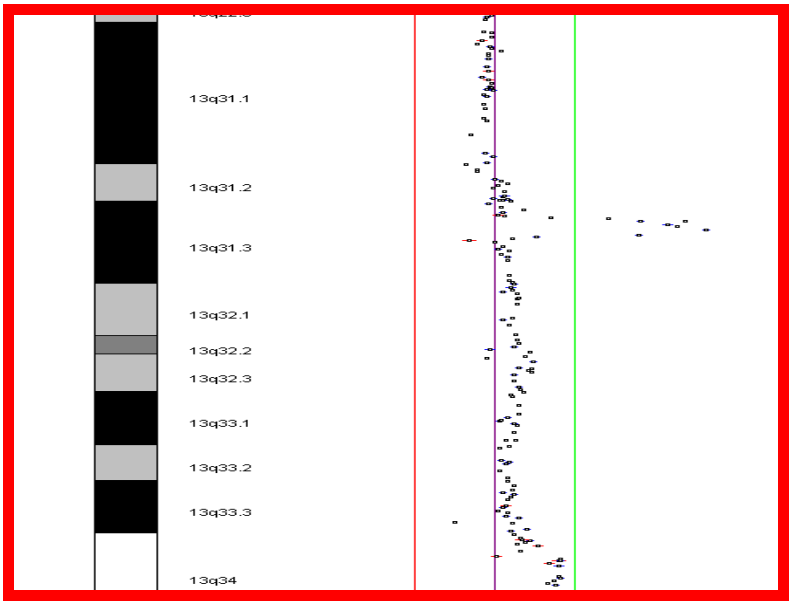


Figure 2.5 - ArrayCGH profile of case 2. Highlighted in the red box is a small region of amplification of chromosome 13q.

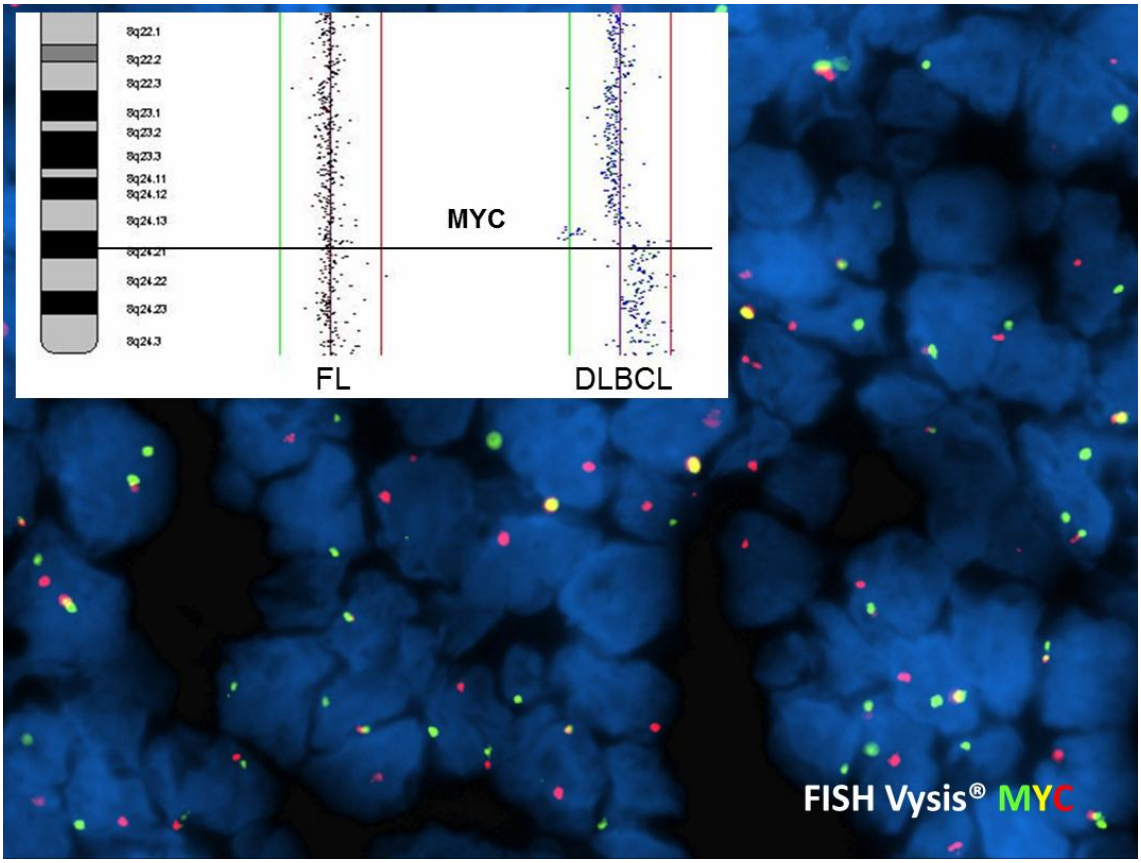


Figure 2.6 - Case 2 aCGH & FISH. A deletion on 8q24.13-q24.21 followed by a gain 8q24.1-qter is gained in case 2 upon transformation suggestive of *MYC* translocation. This was confirmed by FISH analysis performed in the FFPET sample using a Vysis® split apart probe. Zeiss & MetaSystems software; 1000x.

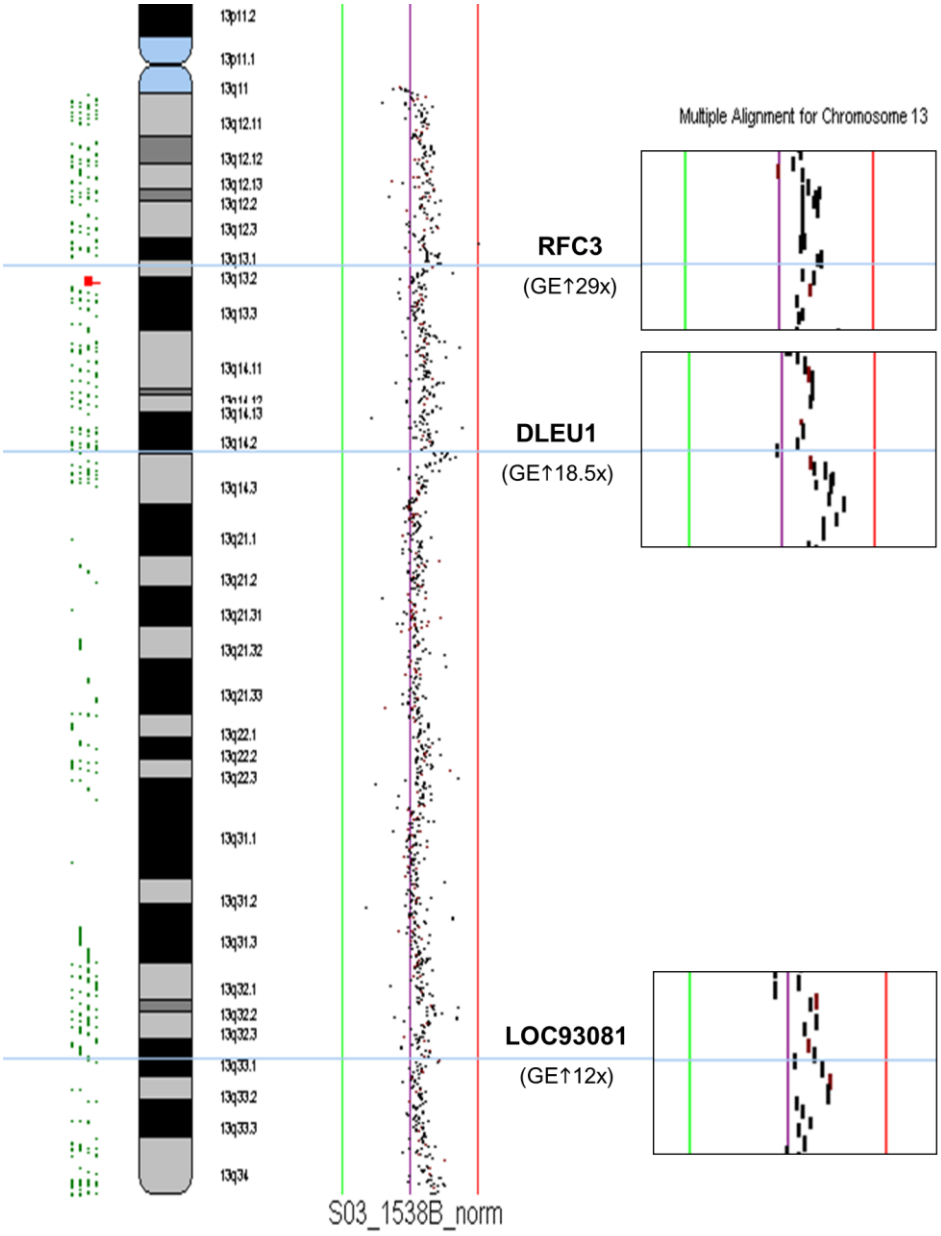


Figure 2.7 - ArrayCGH of chromosome 13 (case 2). Enlarged are the “top” three over expressed genes (>10 fold change) in case 2 showing lack of correlation between gene expression profiling and copy numbers alterations.

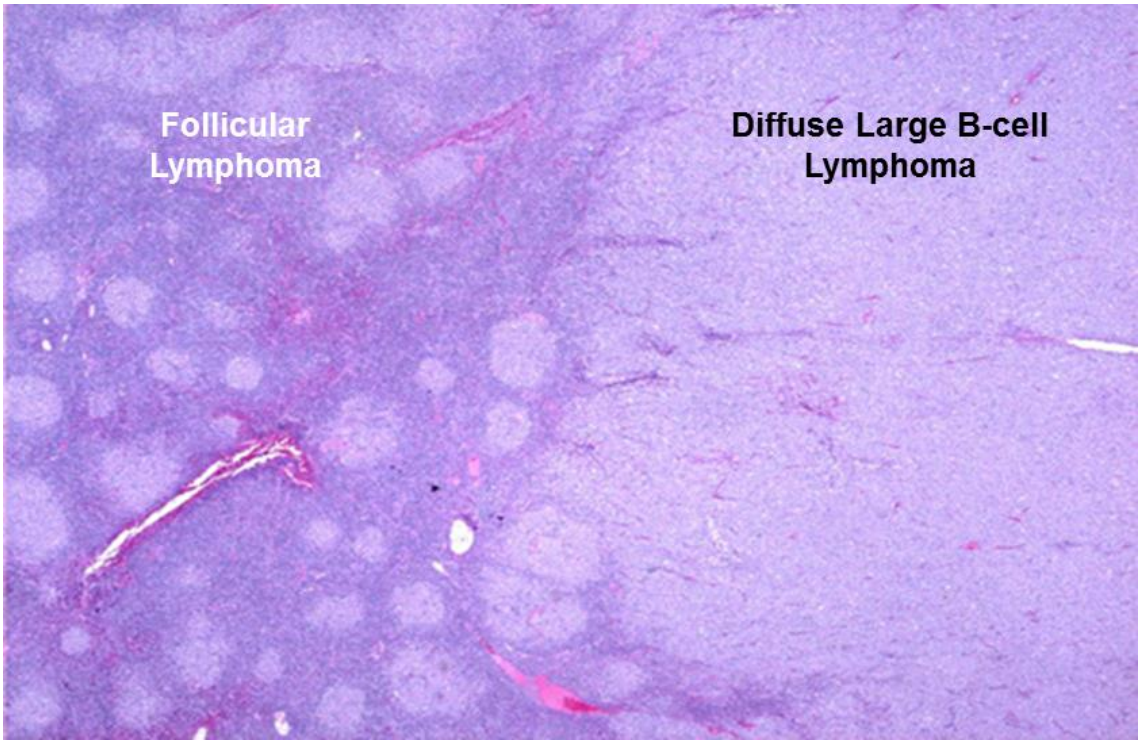


Figure 2.8 - Composite lymphoma with distinct follicular and diffuse large B cell components (H&E; Olympus BX40 / Nikon Elipse®; Digital Camera Dxm1200; 40x).

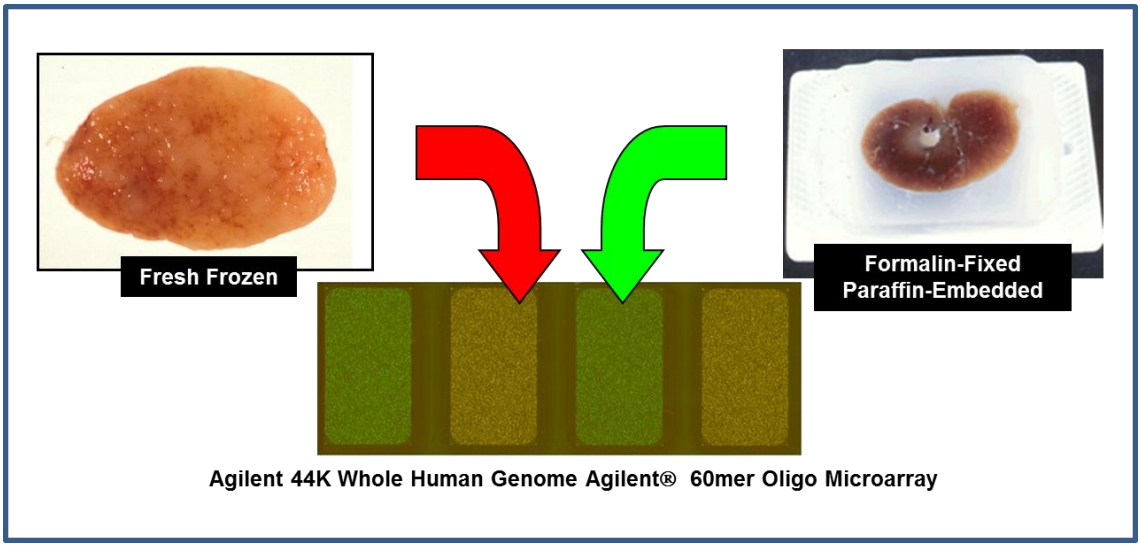


Figure 2.9 - Comparison GEP of FF and FFPET from the same reactive LN biopsies using the 44K Whole-Genome Agilent® 60mer oligo microarray.

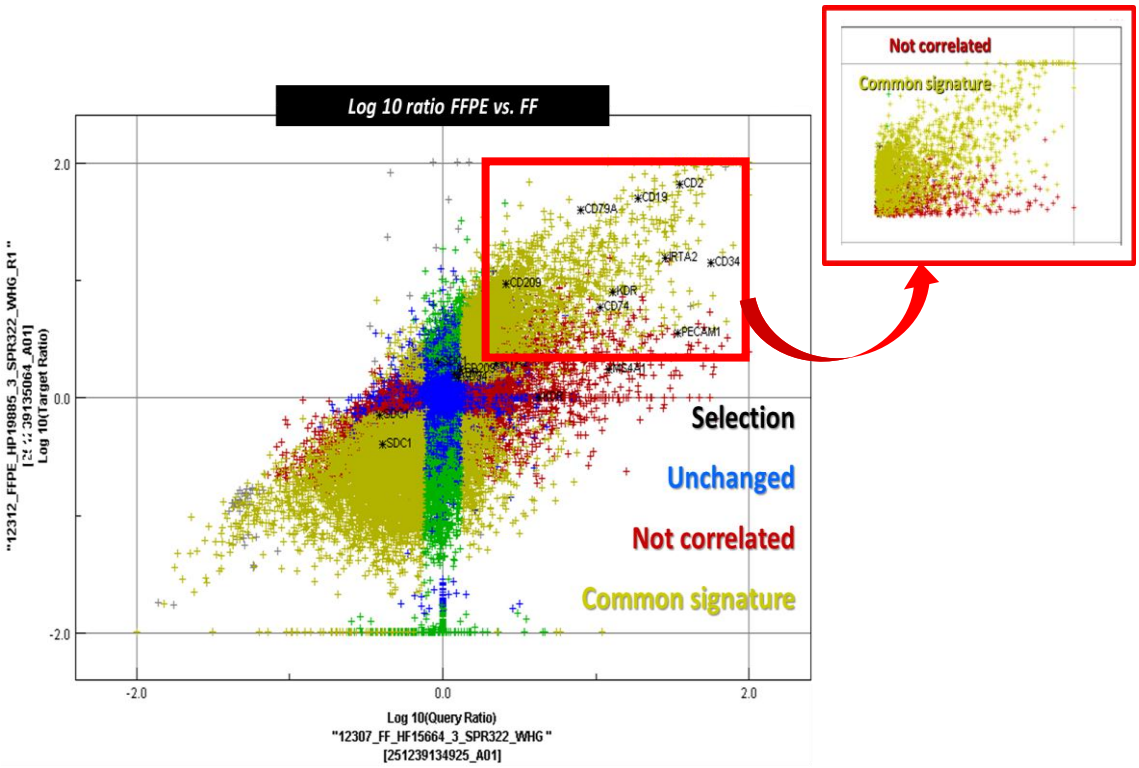


Figure 2.10 - Gene expression Log comparison of all Agilent® microarray transcripts between FF and FFPET samples from the same LN. If only the differentially expressed genes (not blue) are considered, the ratio correlation is approximately 0.8 with expected less detection or more “loss” of gene expression in FFPET. If the analysis of the differentially expressed genes is limited to a 2-fold change cut-off (highlighted in the red box), the correlation is improved, to around 0.9.

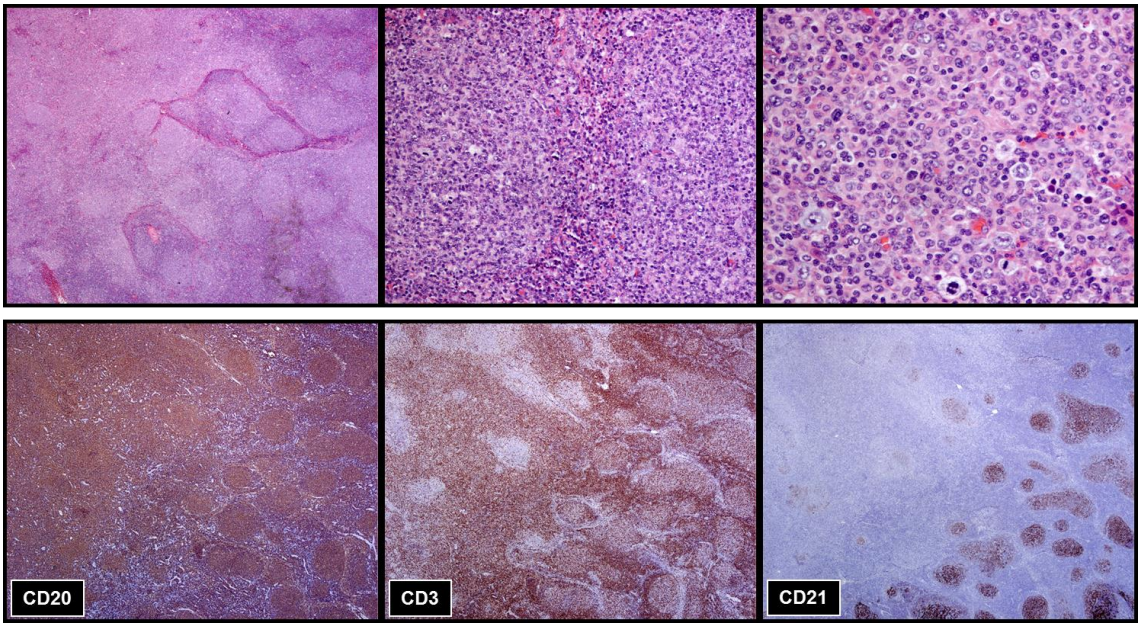


Figure 2.11 - Histology and immunohistochemistry of a composite lymphoma case - H&E (upper images) & IHC stains (lower images) (Olympus BX40 / Nikon Elipse®; Digital Camera Dxm1200; 40x; 100x & 400x).

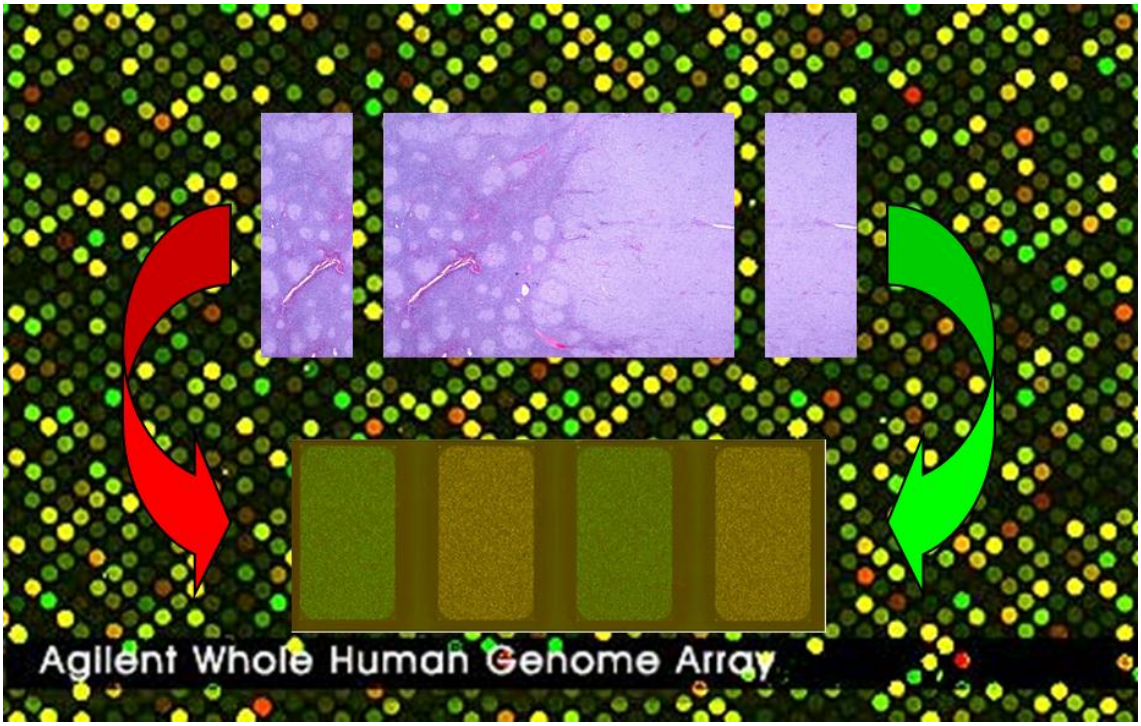


Figure 2.12 - Distinct components were dissected separately and arrayed together in the same slide.

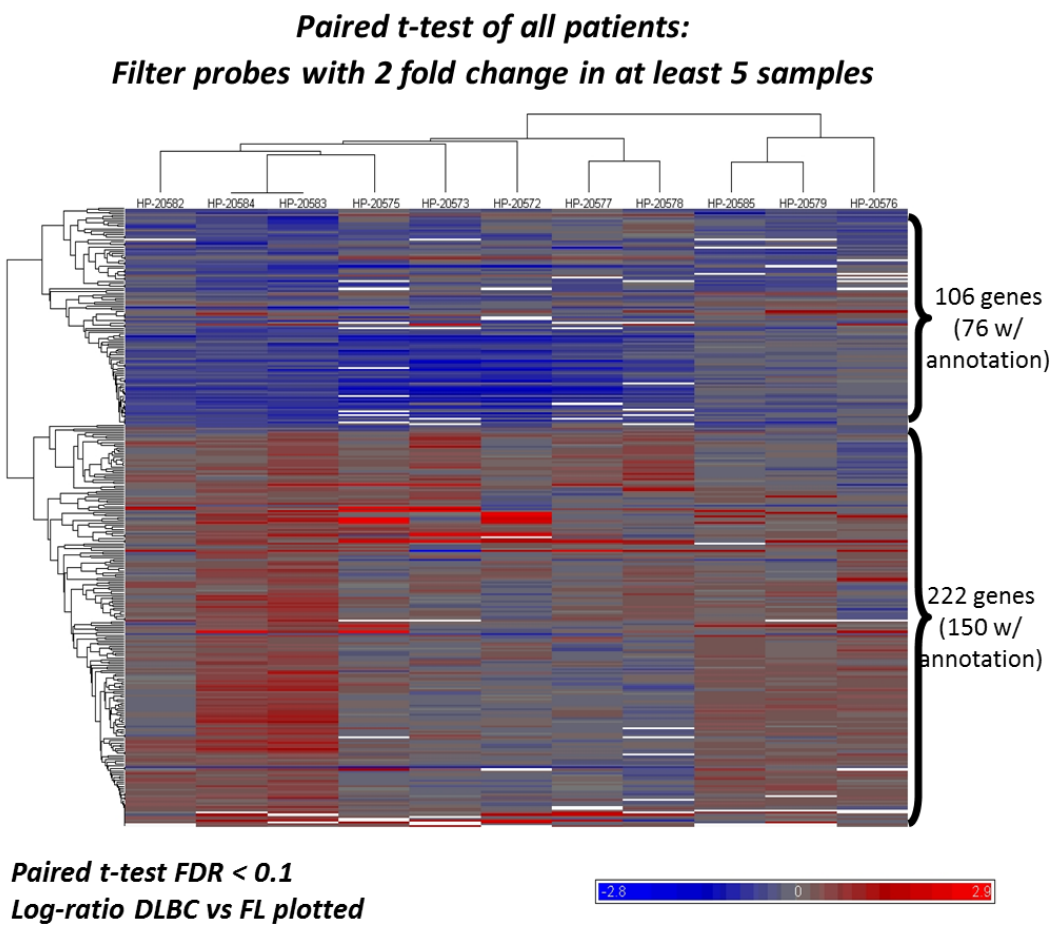


Figure 2.13 - GEP of 11 composite lymphomas. Paired Student t-test of all cases including all probes profiles with a 2-fold change in at least 5 samples showed a small subset of 328 genes to be significantly differentially expressed between both components (FDR<0.1).

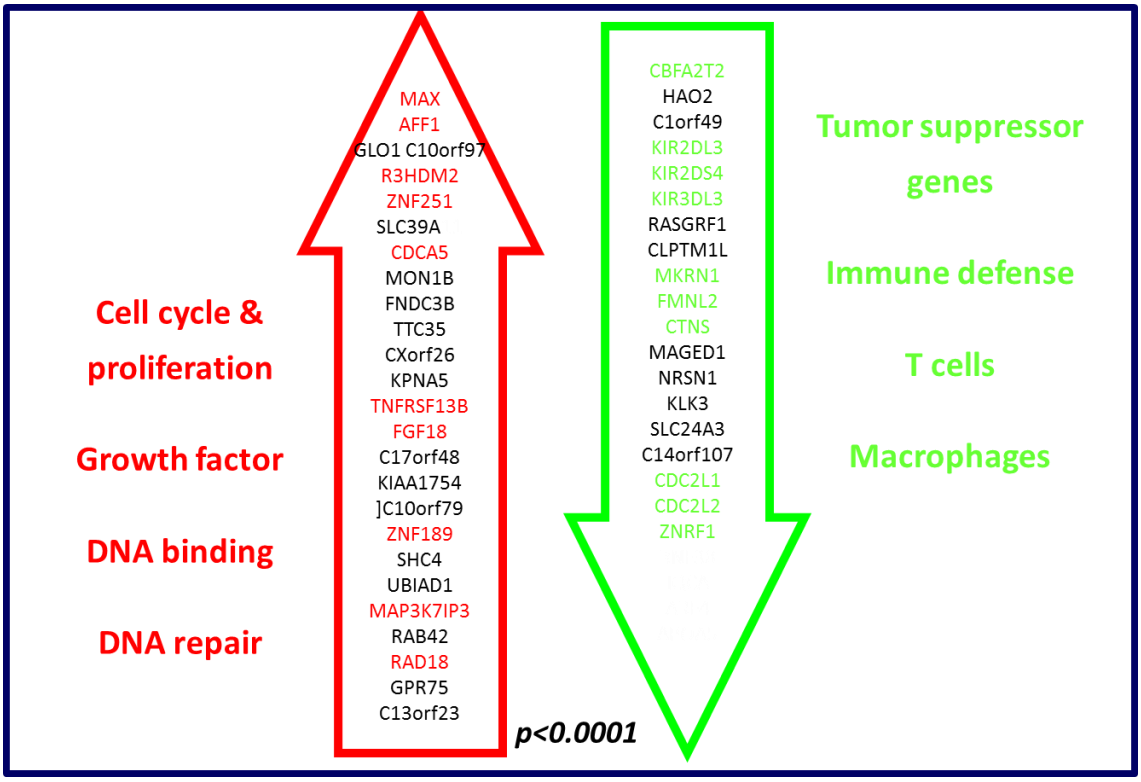


Figure 2.14 - Significant differently expressed genes & annotated gene functions. Among the up-regulated annotated probes predominated genes involved in cell cycle and proliferation, growth factors and DNA binding and repair. Among the down-regulated ones, predominated tumor suppressor genes and immune defense, T cells and macrophages-associated genes.

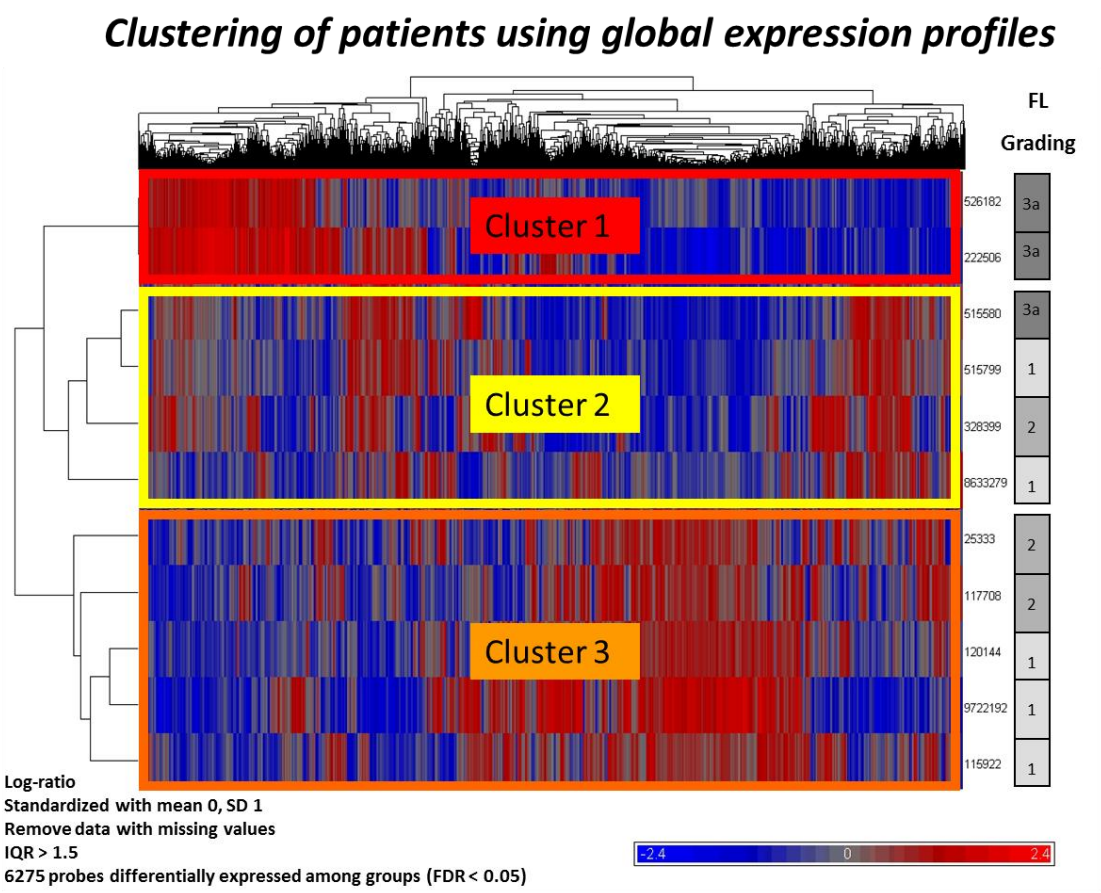


Figure 2.15 - Clustering the patients using global expression profiles of log-ratio (DLBCL vs. FL) showed three distinct clusters with two, four and five cases each. Cluster #1 included two high-grade FL3A, and both cluster #2 and #3 had cases with low-grade FLs (grades 1 and 2). The third FL3A was included in cluster #2. Neither grade nor LCP correlated significantly with GEP clustering, except for #1 including two FL grade 3A lymphomas.

GEP	FL grade	LCP (%)	
		FL	DLBCL
Cluster 1	FL3A (2x)	80	95
Cluster 2	FL1 (2x) FL2 FL3A	76.25	92.5
Cluster 3	FL1 (3x) FL2 (2x)	70	93

Figure 2.16 - Correlation of clusters with FL grade & Lymphoma-Cell-Purity (LCP). Neither grade nor LCP correlated significantly with GEP clustering, except for cluster #1 including two FL grade 3A lymphomas.

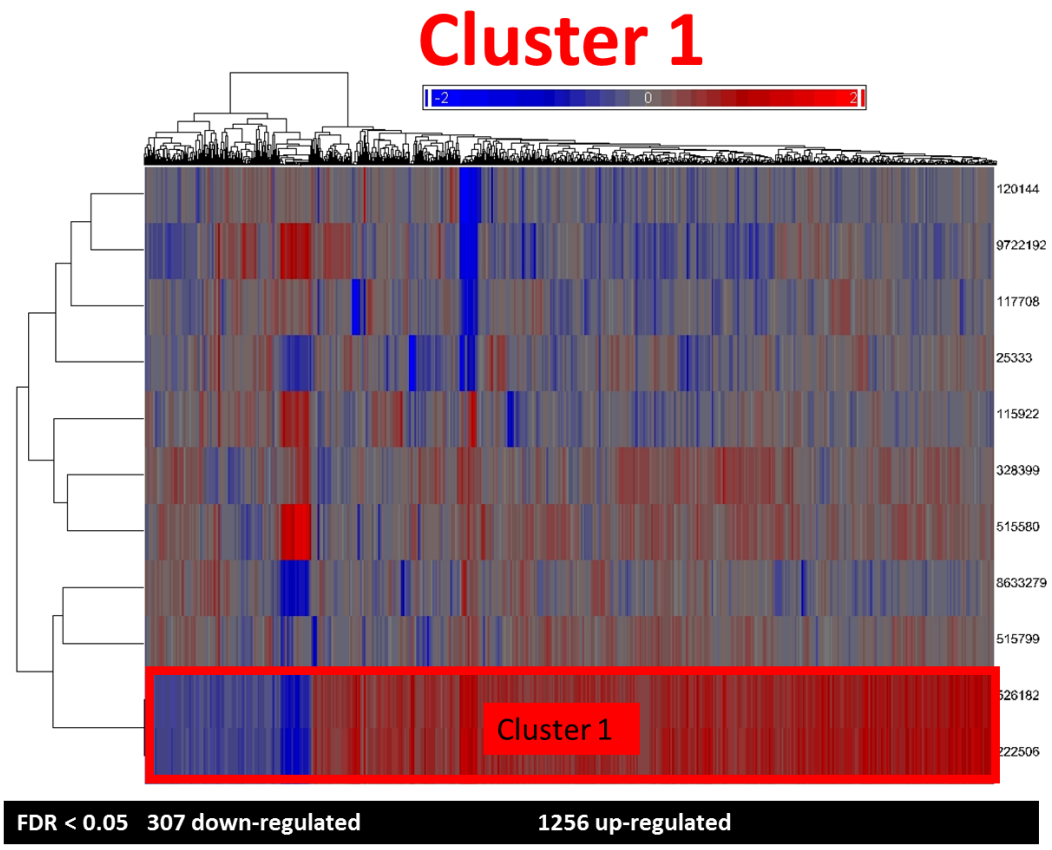
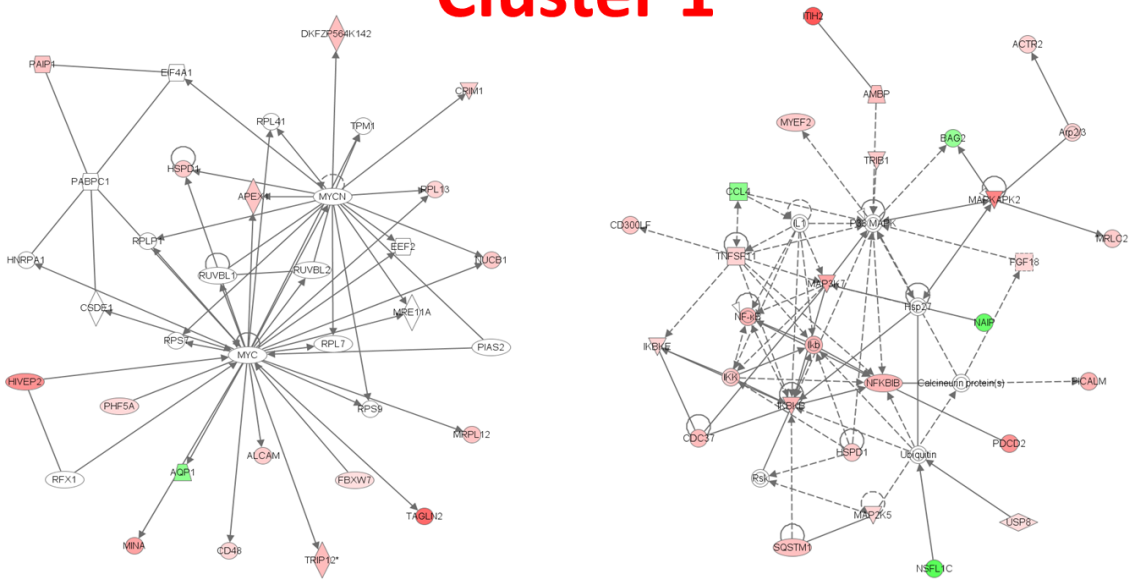


Figure 2.17 - GEP of Cluster #1.

Cluster 1



Genes from cluster 1 mapped to network containing MYC and p38MAPK

Figure 2.18 - Cluster #1 profiling network analysis was done using Ingenuity Knowledge Database software. It shows relatively more consistent expression profile with a predominance of gene over-expression with up-regulation enriched with genes involved in cell cycle, cell growth and proliferation, including several oncogenes and target genes mapped to MYC and MAPK pathway networks.

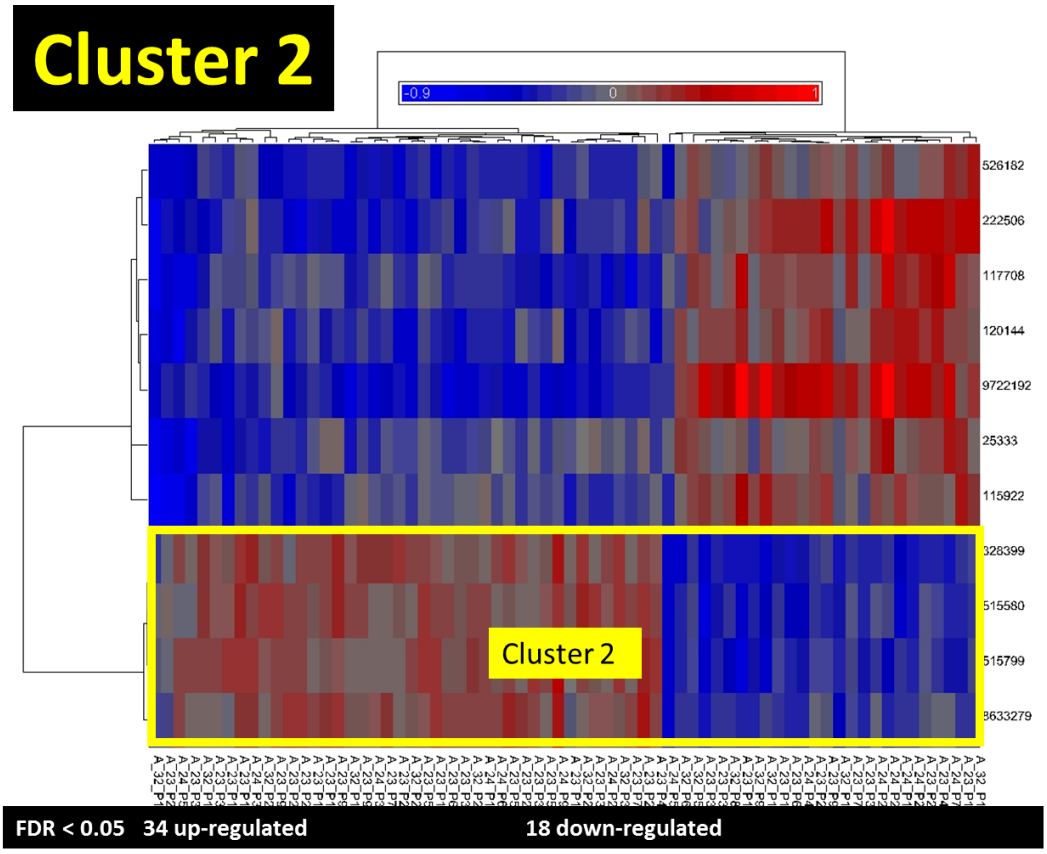


Figure 2.19 - GEP of Cluster #2.

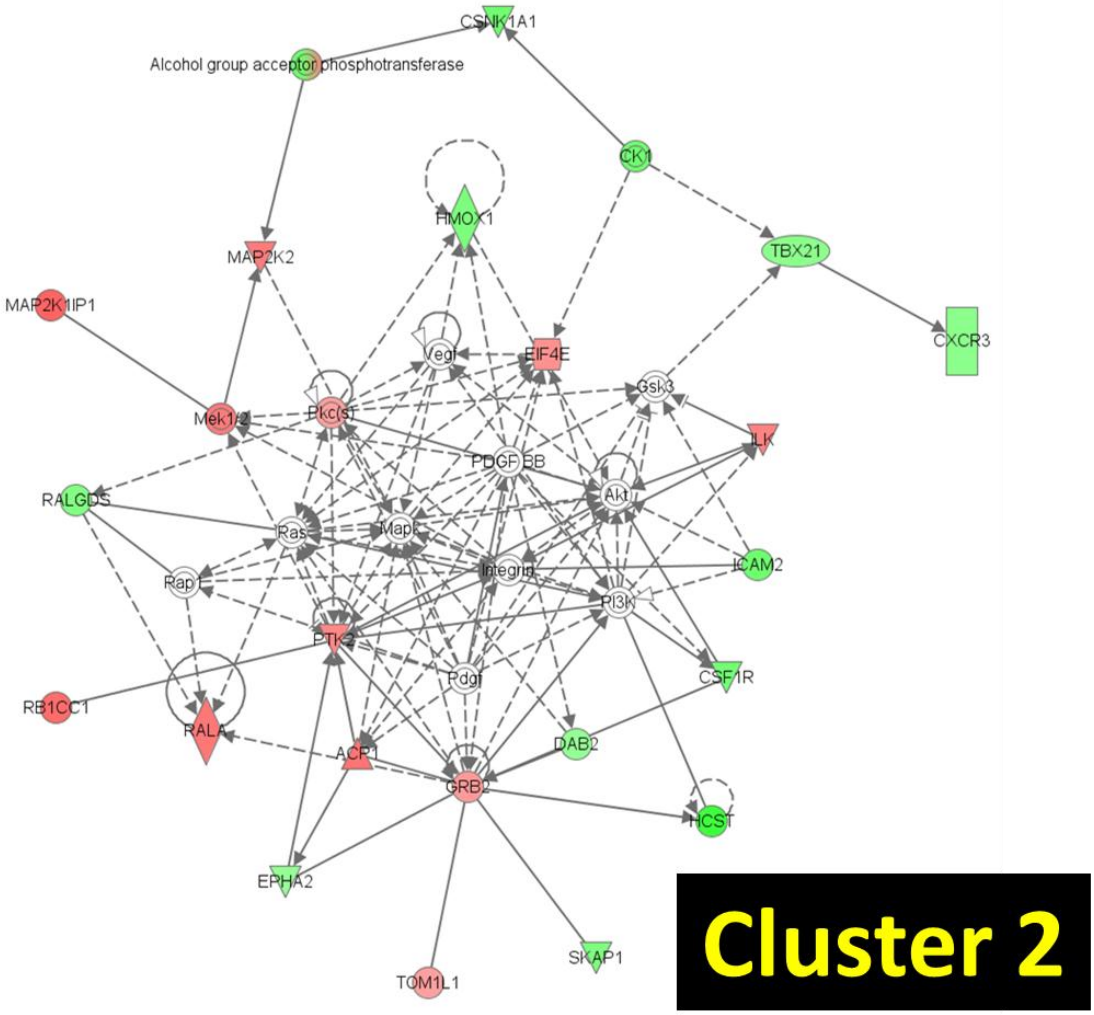


Figure 2.20 - Cluster #2 profiling network analysis was done using Ingenuity Knowledge Database software. It is characterized by a lower number of significantly differently expressed genes with predominance of poorly annotated ones with diverse cell functions. No significant network was highlighted. Yet, several interesting genes are referenced, such as *GRB2*, *PTK2* and *RALA*.

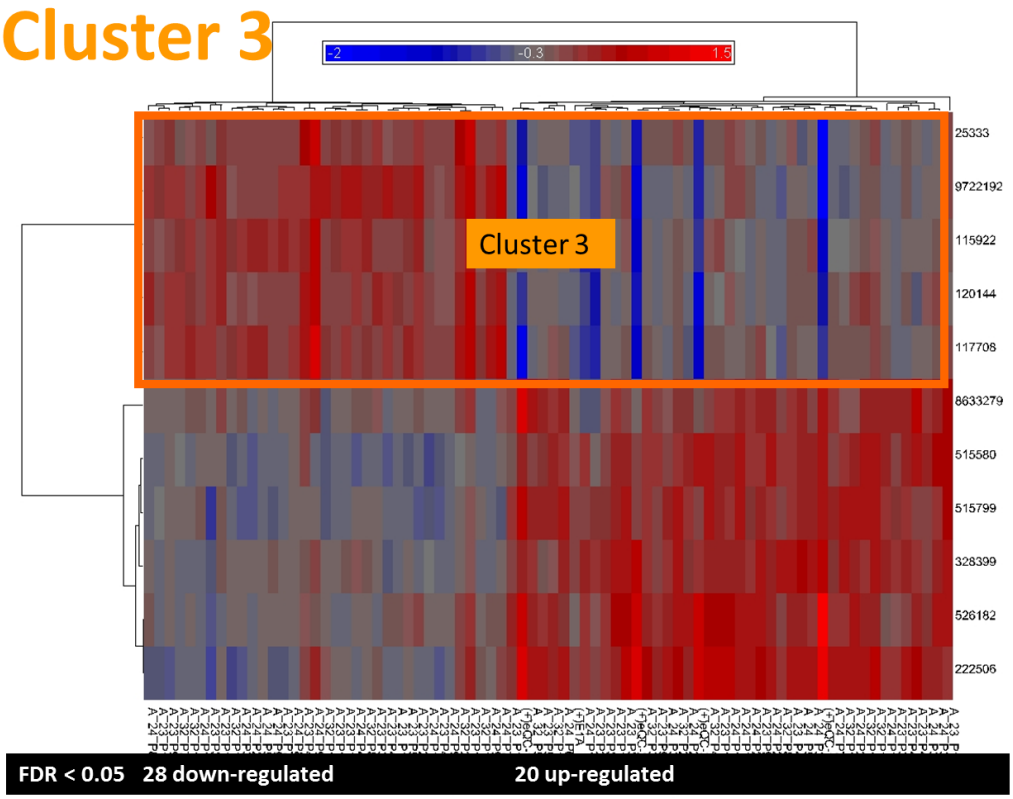


Figure 2.21 - GEP of Cluster #3.

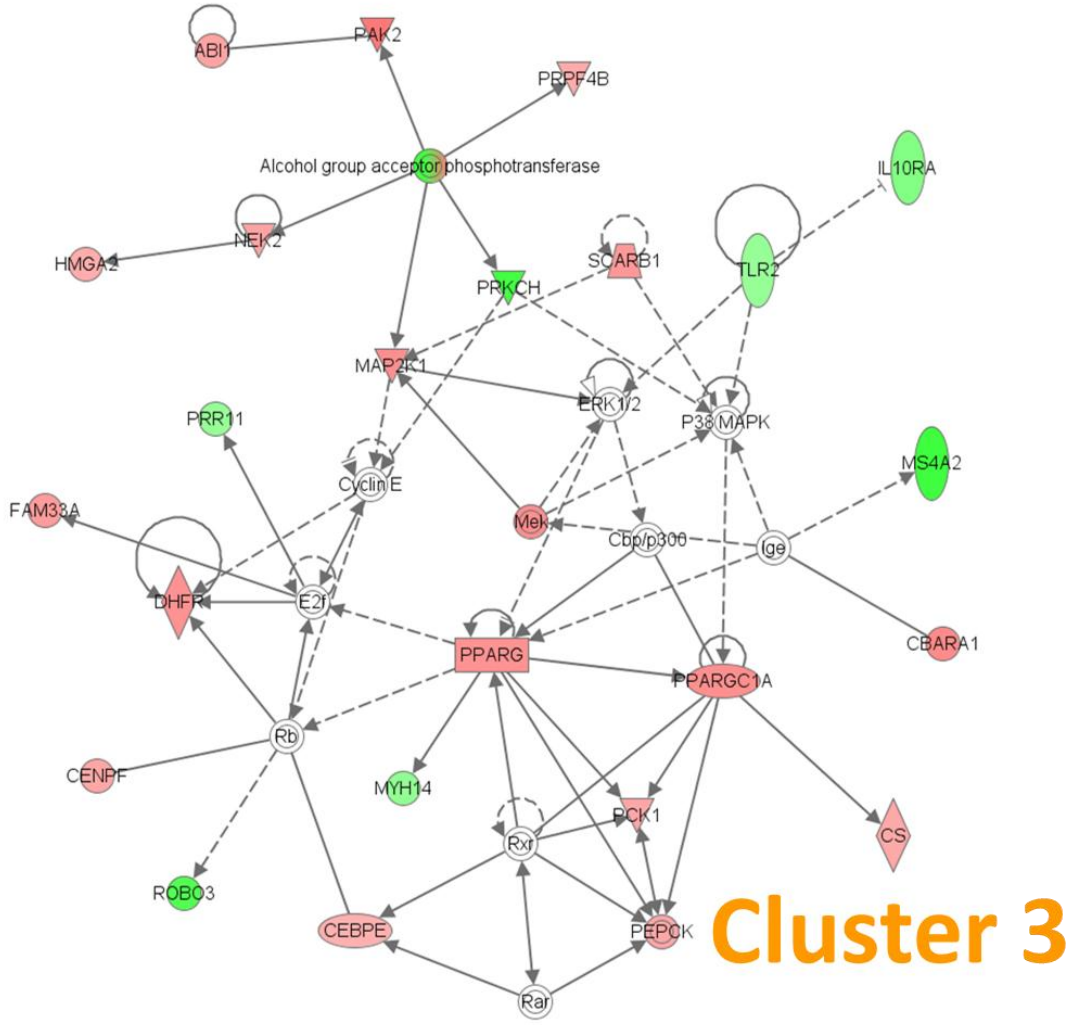


Figure 2.22 - Cluster #3 profiling network analysis was done using Ingenuity Knowledge Database software. It is characterized by a lower number of significantly differently expressed genes with predominance of poorly annotated ones with diverse cell functions. No significant network was highlighted. Yet, several interesting genes are referenced, such as *PPARG* and *PPARGC1A*.

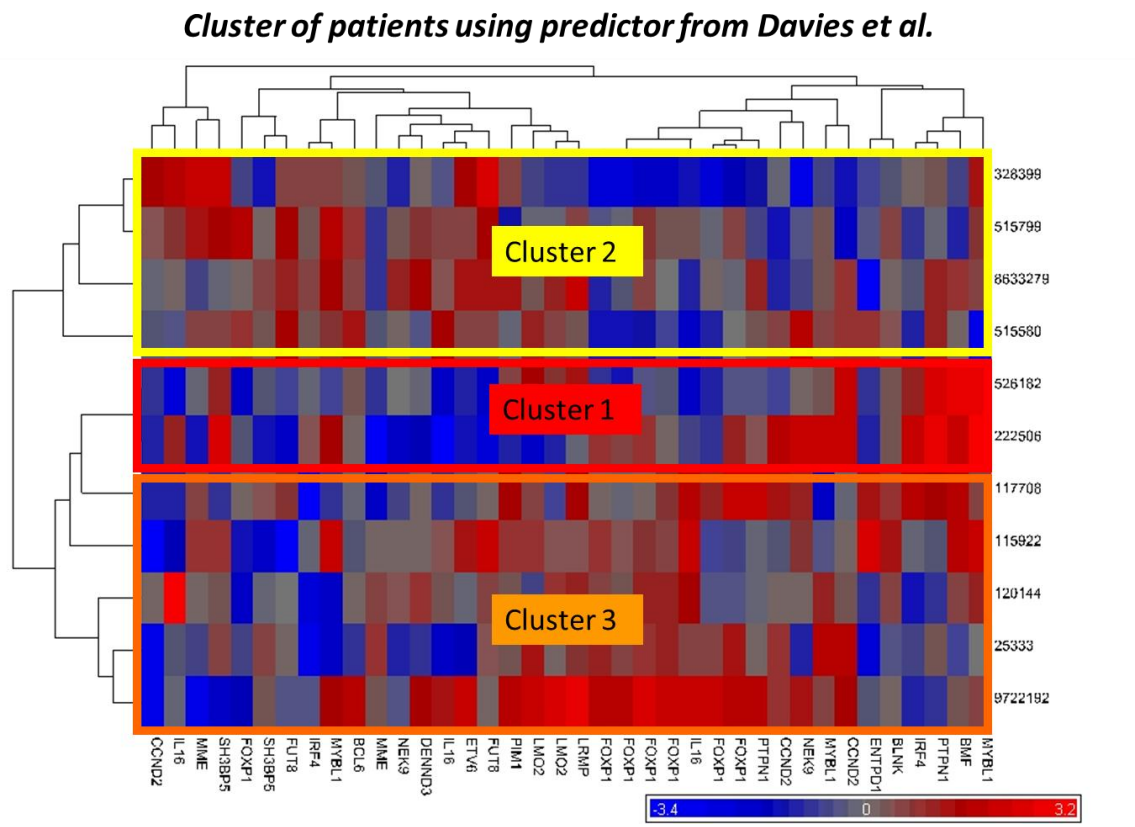


Figure 2.23 - Using the signature gene profiles from Davies et al, a similar three cluster structure is observed.

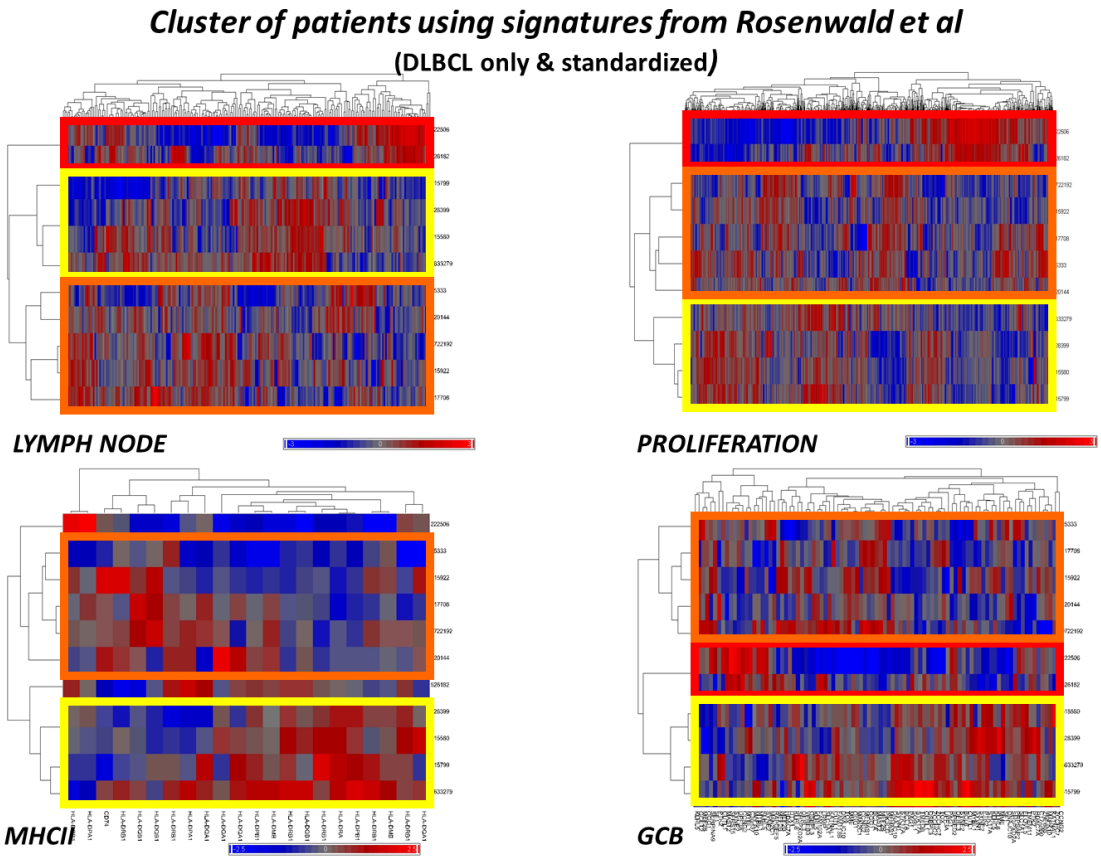


Figure 2.24 - Using the four gene signatures profiles from Rosenwald et al, a similar three cluster structure is observed, except for the MHCII one.

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Section 3: New Biological Predictors of Survival and Transformation in Follicular Lymphoma: The Microenvironment

3.1 INTRODUCTION

FL retains many of the morphological features of the normal follicle suggesting functional crosstalk between malignant B cells and non-malignant cells, including T cells, follicular dendritic cells (FDCs), macrophages, mast cells and endothelial cells. T cell ligands and derived cytokines, (such as CD40 ligand and IL4) play a major role in the growth of both non-neoplastic B cells and follicular lymphoma B cells, indicating that responsiveness to paracrine factors is not a preserved characteristic of the malignant phenotype (1). Important trophic signals from FDCs/macrophages and T cells are essential for the neoplastic B cells which are highly dependent on these for survival and proliferation, as evidenced by the difficulty of establishing FL cell-lines without cytokine support or feeder layers (2).

In solid tumours, it has been shown that tumour-associated macrophages (TAMs) can promote not only neoplastic growth but also angiogenesis (3). Angiogenesis plays a vital role in growth and progression of tumours. The "angiogenic switch" results from interactions of vessels with cancer cells but also with non-neoplastic cells in the microenvironment, including macrophages (4). The abundance of macrophages, microvessel density (MVD) and recently of a subset of T cells, regulatory T cells (Tregs) associated with tumours has been reported to correlate with poor prognosis in solid tumours (5). These cells can be recruited and “educated” by tumours using a range of growth factors and chemokines, so that they modulate tumour microenvironment characterized by “trophic” protumoural signalling, immunosuppression, matrix breakdown, tumour-cell motility and angiogenesis.

In FL, the role of cellular components of the microenvironment in tumour progression and transformation is not as well studied. Recent GEP studies have shown that the immune response plays a role in predicting the outcome and clinical aggressiveness of FL (6, 7). The LLMPP consortium study revealed that gene expression derived from non-neoplastic immune cells were the primary

contributors to the outcome predictor. One signature appeared to be derived from reactive T cells in the lymph node biopsies and conferred a favourable outcome (so-called IR-1, immune response-1). The other signature revealed a gene expression pattern most reminiscent of macrophages and/or FDCs and was associated with inferior survival (IR-2, immune response-2) (6). However, the determination of the cellular components within the tumoural microenvironment responsible for the gene signatures and the linkage to prognosis was less clear. Moreover, the two studies referenced produced conflicting results that may be dependent on methodology as well as the heterogeneity of therapeutic regimens given to the patients in both study populations (8). Importantly, these conflicting data raise the important question of whether specific cell populations of non-neoplastic cells can directly impact both survival and transformation risk in FL and might be targeted by novel, future therapies.

3.2 AIM

We planned to survey both malignant and non-malignant cells of FL microenvironment as relevant and promising biomarkers that might have translation into clinical practice using a uniformly treated cohort of patients.

3.3 MATERIAL & METHODS

3.3.1. Patients & Samples Selection

The BCCA is the primary referral center for patients diagnosed with lymphoid malignancies in the province of British Columbia, Canada. The BCCA coordinates cancer care for the entire province of BC with most patients receiving treatment directly under the guidance of a province-wide medical oncology network (http://www.bccancer.bc.ca/HPI/ChemotherapyProtocols/Lymphoma/). In addition, most of the diagnostic materials both FF and FFPET are centrally reviewed and processed at the Vancouver Cancer Centre of the BCCA. In this study, we used BCCA´s clinical and pathological electronic database. FL transformation was defined as histologically proven biopsy demonstrating

diffuse large B cell lymphoma (DLBCL), or alternatively those patients with clinical evidence of transformation as defined by one or more of the following criteria: 1) a sudden rise in serum lactate dehydrogenase (LDH) to more than twice the normal level, 2) a rapid, discordant localized nodal enlargement, and 3) a new or unusual extranodal involvement of organs such as brain, lung and bone (9). All samples were reviewed by two hematopathologists (PF and RDG) based on the criteria defined by the 2008 World Health Organization classification of tumours of hematopoietic and lymphoid tissue (10). This study was performed in accordance with the Declaration of Helsinki after receiving ethical approval from the University of British Columbia and the BCCA Research Ethics Board.

In this study, consecutive patients seen at the BCCA between July 1987 and May 1993 were offered enrolment in a phase II trial consisting of multi-agent chemotherapy (bleomycin, cisplatin, etoposide, doxorubicin, cyclophosphamide, vincristine and prednisone) followed by involved field irradiation to sites of original nodal involvement (hereafter referred to as BP-VACOP). Patients were eligible for enrolment if they were aged 16 to 61 years, with newly diagnosed, treatment-naïve, HIV-negative and had advanced-stage indolent non-Hodgkin's lymphoma. Advanced-stage disease was defined as Ann Arbor stage III or IV, or stage II with B symptoms, non-radioencompassable disease, or bulk$\geq$10 cm in maximum diameter at any individual tumour site. The source of pathology specimens for diagnosis included only lymph node biopsies. One-hundred and twenty six patients with FL were included in the study. Clinical files were reviewed and the clinical data collected included the following information: age, sex, stage (Ann Arbor), performance status (Eastern Cooperative Oncology Group – ECOG), LDH levels, presence of bulky disease and B symptoms (fever, night sweats, weight loss). Date of death or last clinical follow-up visit as well as date of progression of FL or transformation to aggressive disease was annotated. The International Prognosis Index (IPI) factors were determined. Due to the era of the study, the FLIPI index was unavailable.

3.3.2. Tissue Microarray & Immunohistochemistry

Only those FL cases with adequate biopsy material and available blocks were included. Of the126 cases of FL, paraffin blocks were available for 110 patients. Of these, 105 had adequate material remaining in the block to be used for the TMA including 75 cases in FFPET and 78 in with B5-fixed material. Duplicate 1.0mm cores were used to construct the TMA. Slides from the TMA block were cut at 4 microns. Hematoxylin & Eosin (H&E) sections and representative regions were selected and duplicate cores were done and used to build one FFPET TMA and one B5-fixed TMA (Beecher Instruments, Inc. Sun Prairie, WI, USA). 43 patients were represented in both fixatives which resulted in 40% of the cohort having quadruplicate cores of disease (Figure 3.1). Slides from the TMA block were cut at 4 microns and an H&E stain of the TMA was prepared using routine methods. All cores contained neoplastic follicles (average of 3 follicles per core) and no major discrepant results were seen between duplicate cores. A battery of immunohistochemical stains were performed including CD20 (clone L26), CD3 (clone Cell Marque/Novo Mix), CD4 (clone 4B12), CD7 (clone CD7-272), CD8 (clone C8/144B), CD10 (clone 56C6), CD21 (clone 1F8), CD25 (clone IL2-R), CD34 (clone QBEnd-10), CD57 (clone NK-1), CD68 (clones Kp1 and PGM1), CD163 (clone 10D6), S100A8 (clone CF-145), KI67 (clone MIB-1), TIA1 (clone 2G9A10F5), FOXP3 (collaboration Roncador, G et al, CNIO), PD1 (clone NAT150), BCL2 (clone 124), BCL6 (clone GI191E/A8), BCLxL (clone 2H12) and NOXA (114C307) (Table 3.1) using a Dako® autostainer and the EnVision polymer detection system. Pressure cooking antigen retrieval was used for all antibodies. A variety of buffers were used depending on the specific antibody to allow optimal detection of the antigens. The chromogen in all cases was diaminobenzidine. The immunostained TMA slides were first screened with CD20 to ensure tumour cell content.

The different immunostained TMA slides were scored both for architectural pattern and the number of positively stained cells. Prior to counting the morphological patterns and cell content were first evaluated for their consistency between duplicate cores and between TMAs (B5 vs. formalin for the 43 overlap cases). Several biomarkers of different cell types were analyzed in this study.

3.3.2.1. Malignant B Cells

Malignant cells were studied using antibodies against CD10, CD20, BCL6, BCL2 and two BCL2-family proteins, one anti-apoptotic with reported impact in FL prognosis, BCLxL (11) and a BCL2 homology 3-only member that promotes apoptosis, NOXA. This latter gene/protein has been reported in DLBCL and FL transformation GEP studies (12, 13). We included NOXA in the study after querying the publicly available GEP database of the Lymphoma/Leukemia Molecular Profiling Project (LLMPP) consortium on FL (http://llmpp.nih.gov) which showed *NOXA* to correlate with better OS (P=0.049) (Figure 3.2). All these immunohistochemical markers were scored qualitatively, as duplicate cores were either completely positive or negative. In addition to the qualitative CD10 scoring, the presence or absence of interfollicular CD10+ neoplastic cells was noted. NOXA protein expression has not been previously reported in FL, thus we sored it using a histoscore, NOXA Protein Expression (NPE) by combining the intensity (1-3) and percentage of positive cells (400Xfield) with scores ranging between 0 and 300. Cases were dichotomized in high vs. low NPE (Figure 3.3). The proliferation rate of the neoplastic cells was scored based on the percentage of MIB1-positive cells within neoplastic follicles (MIB1 <10% = 1, MIB1 >10 and <50% = 2, MIB1 > 50% = 3).

3.3.2.2. Follicular Dendritic Cell (FDC)

FDC meshworks (CD21+) were morphologically classified either as follicular pattern (tight concentric FDC cytoplasmic processes) or expanded when there was dissolution of the normal pattern and coalescence of FDC processes in a loose arrangement between follicles (Figure 3.4).

3.3.2.3. Macrophages

Three macrophages markers were used: CD68 and two markers referenced in some reports as M2-like macrophages, CD163 and a less studied marker S100A8. The initial analysis using CD68 revealed that macrophage content could be divided into two groups, cases with either none or few cells positive vs. many positive cells. To make the determination of macrophage numbers more objective, we counted CD68+ cells using high power magnification (Olympus BX40; 1,000X oil lense). This high magnification was used so as to avoid counting neutrophils, mast cells and intercellular debris occasionally stained

with this antibody. Five representative fields per case were counted. We chose to count in areas where the staining was the strongest and most uniform. In virtually all of the cases scoring was made easier by the fact that cases were either clearly positive or negative (Figure 3.5). FDCs did not stain with any macrophage marker nor did the neoplastic B cells in any case.

S100A8+, also known as MRP8 or Calgranulin A, is a member of the calcium-binding-S100 protein family and is expressed by monocytes and macrophages. Recently, it has been shown to be characteristic of late-stage inflammatory macrophages, with anti-inflammatory homeostatic functions and is up-regulated by glucocorticoids and IL10 (14). These are features suggestive of a M2 phenotype. In a MALT lymphoma mouse model, *S100A8* up-regulation was the single gene associated with the lymphoma stage when compared with gastric chronic inflammation (15). Interestingly, we queried the reported data by Dave *et al* and *S100A8* was predictive of survival in both univariate and multivariate analysis in the 191 FLs (6) [IPI: RR =4.6, 95% CI =1.8-11.5, P=0.001; S100A8: RR =1.7, 95% CI =1.1-2.7, P=0.012]. S100A8 positive cells stain cells mostly in the interfollicular areas (Figure 3.6).

3.3.2.4. T Cells

T cells markers (CD3, CD4, CD7, CD8, CD25, CD57, TIA1, FOXP3 and PD1) were evaluated both for their content and distribution in relation to the neoplastic follicles (intrafollicular or perifollicular/interfollicular compartments).

The total number of positive cells (FOXP3, CD57, PD1 and CD25) was counted per core (X100 lenses). Immunoarchitectural patterns of distribution of the different T cell markers were determined in relation to the neoplastic follicles (Figure 7, 8 & 9). A FDC meshwork stain (CD21) was also used to better classify the several T cell patterns. Whenever the majority of positive cells were within the follicles in each core, cases were labelled as having a follicular pattern. From the remaining cases with predominance of positive cells outside the follicles, those with a predominant perifollicular rim of positive cells were labelled perifollicular. For purposes of analysis both groups, those with a follicular distribution of positive cells and those with a perifollicular distribution, were grouped together as having a “follicular” pattern (Figure 3.10).

Assignment of the CD4 pattern and the combined CD4 vs. CD8 were scored semi-quantitatively and in the CD4 *vs.* CD8 pattern determination, both stains were evaluated using sequential sections by overlaying both slides on the microscope. Cases with a follicular predominance of CD4 *vs*. CD8 cells were identified as having a "follicular" pattern, whereas cases with a similar distribution of CD4 *vs*. CD8 cells were defined as "diffuse" (Figure 3.9).
Cytotoxic granule TIA1+ cells were scored qualitatively, with cases called positive in the presence of more than 10% of positive T cells.

3.3.2.5. Microvessel Density & Follicle Size

For microvessel density (MVD), 84 cases had formalin-fixed paraffin blocks with adequate material remaining in the block to be used for whole sections. Immunohistochemistry was performed routinely for CD20 and CD34 using a Dako® autostainer and the EnVision polymer detection system.
Images of whole sections were stained for CD34 and captured using a cooled charge-coupled device (CCD) camera, a motorized stage and customized NIH software (16). Thresholding was applied to identify CD34+ objects. The motorized stage allowed for tiling of adjacent microscopic fields, thereby allowing reassembly of the entire tumour section at high resolution (Figure 3.11). High-resolution images of tumour vasculature over the entire whole sections of each case, defined "distance maps". The distance from each tumour cell in the tissue to the nearest CD34+ pixel was measured automatically and the mean of distances of all cells to the nearest vessel in the whole section was used to calculate the tumour to vessel distance that encompassed 90% of the tumour (TVD_{90}).
Average follicle size per case was visually ranked according to the following groups: 0 - loss of follicular pattern; 1 - small follicles (dense staining), and 2 - large follicles (sparse staining) – (Figure 3.12).

3.3.3. Data analysis Statistics and Survival Analysis

Progression-free survival (PFS) was defined as the interval between diagnosis and progression of lymphoma or death due to lymphoma or toxicity of treatment. Overall survival (OS) was defined as the interval from date of diagnosis until death from any cause and Risk of Transformation (RT) was

defined as the interval from diagnosis until clinical or biopsy-confirmed transformation into aggressive disease. The following variables: IPI score, histologic grade, age, gender and each of the biomarkers (see Table 3.2) were evaluated for prognostic significance. Survival estimates were calculated using the Kaplan-Meier method (17). Significance levels, estimates of hazard ratios (HR), and their 95 percent confidence intervals (CI), were calculated using the proportional-hazards regression model (18). Data were analyzed using the Statistical Software Package for the Social Sciences (SPSS version 10.1 for Windows; SPSS Inc®, Chicago, IL).

3.4 RESULTS

3.4.1. Clinical Characteristics & Outcome (Table 3.1)

105 patients were included in this study, representing the FL patients from the original clinical cohort (n = 126) with available blocks, sufficient tissue remaining in the blocks and successful TMA construction and interpretation.

The median age of the evaluable patients was 45 years (range 19-61 years). There were 51 women and 54 men. The distribution of IPI scores included 65 patients with IPI scores of 0/1 (group 1), 41 with IPI score of 2/3 (group 2) and only a single patient with an IPI score of 4/5 (group 3). For analysis purposes, the single patient in IPI group 3 was included with IPI group 2 in order to eliminate a clinical group with only one patient. The median follow-up of living patients (n = 51) was 17.1 years and 20 patients underwent transformation. The median OS was 15.5 years. The IPI was highly significant as a predictor of OS (HR=2.4, 95% CI=1.4-4.2, P=0.001) and PFS (HR=2.0, 95% CI=1.2-3.3, P=0.006), but not RT. Estimated 10-year OS and RT for all patients was 65% and 20%, respectively. Importantly, this cohort was treated uniformly with aggressive therapy and thus the “treatment variable” was held constant.

3.4.2. Pathology Variables

A small subset of cores was lost during the preparation of the sections or was uninterpretable due to poor fixation and/or inadequate staining (varies with biomarker). The percentage of informative individual cores was 96%, which varied with the different biomarkers (1-18%) (see Table 3.2).

Histologic grading of FL subtypes according to the WHO - 2008 classification (10) included 80 with grade 1, 17 grade 2 and 7 cases with grade 3A. A total of 15 patients were determined to have marginal zone differentiation, as defined by a zone of pale cells at least three cells in thickness, expressing CD20 and surrounding neoplastic follicles. Neither histologic grade nor the presence of marginal zone differentiation had an effect on OS, PFS or RT (data not shown).

3.4.2.1. Malignant B Cells Markers

The percentage of the positive cases for BCL2, BCL6 and CD10 were in line with previous reports. In total, 16% of the CD10+ FL had no or minimal interfollicular involvement by CD10+ neoplastic cells. Only a small number of cases (14%) had more than 10% BCLxL+ cells. None of these markers had impact on survival. Of the 100 evaluable FLs with NPE, 50 had a high NPE ≥ 20 and median OS of 16.3 years while 50 had low NPE<20 and median OS of 10.7 years (P=0.057) – (Figure 3.13). Increased NPE correlated with both higher grade (X^2, P=0.04) and increased proliferation index (X^2, P=0.02) It did not correlated with any clinical features.

There was a correlation between the proliferative rate (MIB1) and the histologic grade, at least for the small number of grade 3a cases. The majority (83%) of grade 3a FL cases had a proliferative rate > 50%. Grade 2 FL cases showed more heterogeneity, with 62% having a proliferative rate between 10 and 50%, while the bulk of the remaining cases had >50% MIB1+ cells. Only 50% of the grade 1 FL cases revealed < 10% MIB1+ cells, with the majority of the remaining cases demonstrating proliferative rates between 10 and 50%. These data indicate the imperfect relationship between histological grade and proliferative rate. Importantly, neither grade nor MIB1 score were predictive of outcome in the setting of uniform therapy.

3.4.2.2. Follicular Dendritic Cell marker

The follicular FDC pattern (CD21) was disrupted with expansion of the meshwork and fusion of the follicles in 27% of the cases. This pattern had no impact on prognosis.

3.4.2.3. Macrophages Markers

The numbers of CD68+ macrophages or lymphoma-associated-macrophages (LAM) were initially determined using low power magnification. None of the cases showed staining of neoplastic B cells with anti-CD68 antibody. It was clear that most cases had few or no macrophages, while a small number had many (Figure 3.5). The distribution of these CD68+ cells was uneven throughout cores with both intrafollicular and perifollicular macrophages observed. We then used high power magnification (1,000X oil lense) to count the CD68+ macrophages, which ranged between 1 and 25 cells per high power field (hpf). Because many of the cases had no or few macrophages, we established a cut-off of 15 cells/hpf for analysis purposes.

Importantly, the majority of the negative cases had less than 10 cells (82%) and positive cases more than 20 cells (58%) per hpf, respectively. Table 3 shows the distribution of clinical and pathologic variables for the FL cases with high LAM

(n=12) versus those with low LAM content (n= 87).

Cases with less and more than 15 CD68+ macrophages/hpf had median OS of 16.3 and 5.0 years, respectively (P=0.0003)(Figure 3.14). Cases with less and more than 15 CD68+ macrophages/hpf had a median PFS of 7.05 and 1.69 years, respectively (P=0.001). The inferior PFS and OS of the patients with a high LAM score cannot be explained by a lower response rate to treatment (Table 3.3), nor an increased risk of histologic transformation nor lack of appropriate treatment for relapse (all patients with relapse were treated with systemic chemotherapy, three with high dose chemotherapy and hematopoietic stem cell transplant). All but one of the ten deaths among the patients with high LAM scores resulted from progressive lymphoma.

As expected, cases with increased S100A8+ cells (14 cases) correlated significantly with LAM cases ((n=12) - X2, P<0.001)). S100A8+ stained cells located in the interfollicular areas (Figure 3.6). A subset of 14 cases showed high density of S100A8+ cells defined by > 5 per high power field (100x lense). Like LAM, cases with increased S100A8+ cells had inferior OS (P=0.04), but S100A8+ cells did not predict RT (P=0.4) (Figure 3.15).

3.4.2.4. T Cell Markers

The reactive T cells showed a predominantly perifollicular distribution (CD3) in the majority of the cases (70%) as well as a CD4 phenotype (76%). In 71% of the cases there was a clear follicular pattern with CD4 cells inside the follicles surrounded by CD8+ cells outside the follicles, whereas in other cases the distribution was diffuse for both subsets. CD57+ cells represented more than 10% of the T cells in the majority of the cases (64%) and they had a clear follicular pattern in 68%, with most of the positive cells localized inside the follicles. In 82% of these cases the CD57 follicular pattern correlated with the CD4/CD8 follicular pattern and both CD57+ and CD4+ cells had an equal distribution within the follicles. Cases with more than 10% cytotoxic TIA1-granule+ cells were found in 45 % of the cases.

Follicular vs. diffuse pattern of distribution of CD4 vs. CD8+ cells was associated with PFS only (HR=0.54, 95% CI=0.29-1.0, P=0.05) (Figure 3.9), CD25 pattern was significant for both PFS (HR=2.4, 95% CI=1.4-3.9, P=0.001), and OS (HR=1.8, 95% CI=1.1-3.1, P=0.024) but not risk of transformation. Only the FOXP3 score had a significant impact in PFS, OS and risk of transformation (Table 3.2). Cases with a follicular vs. diffuse FOXP3+ pattern of distribution had a median OS of 7.13 years and not reached, respectively (HR=3.1, 95% CI=1.8-5.4, P<0.0001) (Figure 3.16) and median PFS of 2.2 and 8.8 years, respectively (HR=2.7, 95% CI=1.6-4.6, P=0.0003). Finally, cases with follicular vs. diffuse patterns had median risks of transformation of 13.3 years and not reached, respectively (HR=3.7, 95% CI=1.5-8.9, P=0.004) (Figure 3.16). Histological grade, IPI, and bone marrow involvement were evenly distributed between these two groups (Table 4). Interestingly, all but one case with a high content of LAM had a FOXP3+ follicular pattern and a follicular pattern of CD25+ cells. The pattern of CD25+ and FOXP3+ cells were also significantly correlated (Table 3.4).

Cases revealed zero to 731 FOXP3+ cells per core (median of 242 cells per core) and zero to 654 PD1+ cells per core (median of 102 cells per core). There was a predominantly intrafollicular or perifollicular localization of positive cells (“ follicular pattern”) in 38 and 43 patients for FOXP3 and PD1, respectively. There was no significant correlation between cell density (χ^2=0.1) or pattern (χ^2=0.8) between markers. Separately, cell density of both FOXP3 (P=0.7) and PD1 (P=0.16) did not impact prognosis. Yet, when quartiles were combined and

dichotomized, cases with high cell content of FOXP3+/PD1+ cells correlated with inferior OS & increased RT in univariate (P=0.002 & P=0.01) (Figures 3.8 & 3.17) and multivariate Cox models (RR=2.18, 95% CI=1.2-3.9, P=0.01 & RR=3.4, 95% CI=1.3-8.8, P=0.015).

In univariate analysis, a PD1 follicular pattern correlated with inferior OS (P=0.027) but did not impact on RT. When combined with follicular FOXP3 cases, a subset of 15 "double follicular" FOXP3+&PD1+ cases correlated with inferior OS in univariate (P=0.0016) and multivariate Cox models (RR=2.1, 95% CI=1-4.3, P=0.04). They also showed a trend for increased RT (P=0.09) (Figure 3.9).

3.4.2.5. Microvessel Density

There were 84 suitable cases for analysis. TVD_{90} ranged from 50.3 to 144 microns (median 78.4) and these data were divided into quartiles. In univariate analysis the quartile (21 cases) with low TVD_{90} (i.e. high MVD) showed inferior OS (P=0.0001) and an increased RT (P=0.01) (Figure 3.18). Clinical characteristics are summarized in Table 3.5. As most cases showed a distinct perifollicular pattern, we correlated TVD_{90} with follicle size in each case. There was a significant association between low TVD_{90} and small follicle size (x^2, P=0.01). Low TVD_{90} also correlated with younger age (x^2, P=0.034), increased IPI (x^2, P=0.01) and increased $CD68^+$ macrophages (x^2, P=0.025). In univariate analysis, TVD_{90} and IPI are significant for both OS and RT, but not age or follicle size. In this cohort, CD68+ macrophages predicted OS (P=0.0004), but not RT (P=0.38).

3.4.3. Multivariate Analysis

A multivariate proportional hazards model (Cox model) was constructed for all of the variables significant by univariate analysis including the NPE, FOXP3+ immunoarchitectural pattern, LAM content, TVD_{90} and IPI and all were shown to be independent predictors of OS; NPE (RR=1.89, 95%CI=1.1-3.3, P=0.03), FOXP3 (HR=2.9, 95% CI=1.7-5.1, P=0.001), LAM content (HR= 2.4, 95%CI=1.3-4.5, P=0.004); TVD_{90} (RR=2.5, 95% CI=1.1-5.0, p=0.01) and IPI (HR=2.1, 95% CI=1.1-3.7, P=0.008). Interestingly, in the multivariate Cox model

for Risk of Transformation only FOXP3 positivity showed significance (HR = 3.9, 95% CI =1.5-9.9, P=0.004) and TVD_{90} showed borderline significance (P=0.08).

3.5 DISCUSSION

FL represents a lymphoma subtype characterized by a significant degree of clinical heterogeneity (19). New clinical prognostic models, namely FLIPI 1 & 2, may help to define those patients at risk of failing current therapies, but the degree to which these clinical variables will be helpful in planning initial therapy for newly diagnosed patients remains undefined (20, 21). Importantly, both models include clinical surrogates for the underlying molecular genetic alterations and thus, will not further our understanding of the biology of FL and clearly do not help to identify novel targets for therapy. An improved understanding of the molecular abnormalities that underlie the survival differences among patients with FL may lead to improvements in risk-stratification, but more importantly would offer the possibility of identifying new targets for rationale design of future treatments. To a large extent, the future of tailored or personalized therapies in cancer will depend on this very hypothesis. Most published data for FL suggest that the molecular alterations that characterize this lymphoma result from a combination of abnormalities present at the time of diagnosis and ongoing stochastic events that occur in association with clonal evolution & selection (6, 12, 22-25). Because FL is a germinal center derived neoplasm and frequently reveals intra-clonal heterogeneity at the level of the immunoglobulin heavy chain, there is a belief that additional molecular alterations are occurring over time and contribute to disease progression. However, a number of other possibilities exist to explain disease progression in FL, including the outgrowth of subclones that may have been present as minor populations at diagnosis, as resting lymphoma-originating-cells (22, 26). Whatever the relative contribution of these two processes, there is little doubt that the molecular signature present in initial diagnostic biopsies plays a prominent role in predicting outcome in FL. GEP studies of the LLMPP have clearly shown that the signatures from co-ordinately expressed genes play a dominant role in affecting survival, as evidenced by widely divergent survivals (6). The most important gene expression signatures in the recently developed

molecular predictor were not derived from neoplastic B cells but instead were expressed by non-neoplastic immune response cells. One of these signatures, IR-2, included a number of genes that appeared to correspond to the repertoire expressed by macrophages and was associated with inferior survival. Thus, our finding of high LAM associated with inferior survival is consistent with the GEP data identifying increased IR-2 as a poor prognostic finding. Data supporting such a role for reactive macrophages as biomarkers in cancer have been described in a number of different solid tumours (3). A suspected role in lymphoma was untested until the aforementioned recent studies by the LLMPP consortium (6).

3.5.1. Neoplastic FL B Cells

Of the biomarkers studied and expressed by the neoplastic cells only NOXA protein expression significantly correlated with a favourable OS in FL. In this uniformly & aggressively treated cohort from the BCCA, NPE was an independent predictor of outcome. This is an interesting result as in the literature (see Section 1) only anti-apoptotic genes & proteins members of the BCL2-family have been reported as relevant and with clinical impact in FL outcome, such as MCL1 and BCLxL (11, 27). None of the pro-apoptotic members of the family, specially the apoptosis regulators or "sensitizers/derepressors" class BH3–only, such as NOXA, have been correlated with FL prognosis. *NOXA* is a *TP53* target gene, which is physiologically up-regulated in cell stress and damage (28, 29). Not surprisingly, it is over-expressed by hypoxia (HIF1α) (30). In accordance with our study NPE correlated with increasing grade and proliferation index, features that did not impact survival. The significant impact in survival, independent of both clinical and microenvironment, is quite interesting as it has been shown that intracellular levels of NOXA in human tumours like melanoma, myeloma or mantle cell lymphoma can be increased by therapeutic agents such as proteasome inhibitors into levels that trigger NOXA-mediated apoptosis (31, 32) (Figure 3.19).

3.5.2. Macrophages

The current study clearly shows that LAM content is an independent predictor of survival in uniformly treated FL. The content of LAM was predictive of survival, including OS (P=0.003) and PFS (P=0.001) and remained an independent variable distinct from the IPI in a Cox multivariate model (P=0.004). These data agree with similar observations made in epithelial cancers that increased numbers of macrophages portend a worse outcome (3, 33). Moreover, these results also validate the recent GEP studies and provide indirect validation at the protein level for the role of IR-2. The precise biological function of tissue-based macrophages in this setting remains largely undefined, but suggests that these cells may be providing important growth signals to the FL cells in the form of cytokine stimulation or specific chemokine production. Macrophage heterogeneity is well studied in the mouse but less is known about human macrophages (34-36). The plasticity of macrophage function is evident in the diverse range of functions of these cells, including inflammatory responses, immune reactions, tissue remodelling and morphogenesis. Depending on the microenvironment, macrophages appear to be either “classic” M1 (inflammatory responses induced by IL12 and TNFα) or “alternative” M2 (tissue remodelling and morphogenesis induced by IL4, IL13 and IL10) subtypes, referred to as polarized macrophages. These two types differ in their receptor expression and cytokine/chemokine profiles (37). In advance of this study we studied macrophage content and distribution in a series of reactive lymph nodes (n=10, data not shown). Virtually all cases showed large numbers of tissue-based macrophages both within and between reactive lymphoid follicles. Thus, we were surprised to find that most cases of FL in this study had no or very few macrophages. We hypothesize that in the minority of cases of FL with large numbers of macrophages the neoplastic B cells display signals that attract and/or retain predominantly M2-type macrophages. Alternatively, an inflammatory microenvironment might be created by the anti-tumour responsive T cells that attract or retain the macrophages. Once in the lymph node, these cells might provide the neoplastic B cells with trophic/survival signals, facilitate tumoural invasion by disrupting basement membranes, promote angiogenesis by secreting angiogenic factors, alter the phenotype and/or function of antigen-presenting dendritic cells or amplify Tregs leading to an immunosuppressive intra-tumoural environment (38).

3.5.3. Regulatory T Cells

It has been almost a century since immunosurveillance was proposed as a mechanism responsible for the repression of cancer growth (39). Since then, our understanding of tumour immunobiology has increased indicating that immunosurveillance represents one dimension of the complex relationship between the immune system and cancer (38). It is now a widely held belief that the growth of tumours is influenced by non-neoplastic cells of the immune system. These cells are thought to exert selective pressure on the malignant cells. Ultimately, the malignant cells escape immune recognition and destruction and much like tolerance to self-antigens, tolerance to tumour-associated antigens may arise and permit or even promote disease progression (38).

Tregs were initially defined as a subset of suppressor T cells that mediate immune tolerance by inhibiting autoreactive T cells (40). Naturally occurring Tregs play a vital role in maintenance of tolerance to self-antigens and immunologic homeostasis through the antigen specific suppression of effector CD4+ and CD8+ T cells (41). Yet, mechanisms whereby Tregs, including peripherally induced cells, bring about the inhibition of other T cell subtypes are still not well understood (42). They include direct suppression by cell contact and/or cytokine production, as well as inhibition of antigen-presenting cell function. The T cell receptor repertoire of Treg cells is as broad and diverse as that of other CD4+ T cells, but it is skewed towards recognizing complexes of self-peptides and MHC.

Many of the tumour-associated antigens recognized by autologous T cells in cancer are antigenically normal self-constituents. This suggests that Tregs normally engaged in the maintenance of self-tolerance may also suppress immunosurveillance against autologous tumour cells. Depletion of these Treg cells before a tumour challenge encourages effective immune responses to tumours in mouse models (43). In mice with advanced tumours, including lymphoma, the administration of anti-CD25 or intratumoural agonistic anti-GITR antibodies reduces tumour-infiltrating Foxp3+CD25+CD4+ Tregs and results in enhanced anti-tumour immunity with a significant decrease of the tumour burden without causing autoimmune disease (44, 45). In humans, T cells reactive to tumour-associated-antigens can expand and become detectable in

the peripheral blood when Tregs are first depleted (46, 47). Tregs accumulate within different types of tumours draining lymph nodes and the peripheral blood (5, 48-51). FOXP3+ T cells present in the tumour microenvironment contribute to the growth of the tumour and their presence is associated with unfavorable prognosis in patients with ovarian carcinoma (5). This study by Curiel *et al.* highlights the importance of the chemokine network in the recruitment and function of these cells. Decreased CCL22 produced by the tumour cells and/or TAM reduced the recruitment of the Tregs that characteristically express the CCL22 cognate receptor CCR4.

In reactive lymphoid tissues, FOXP3+ Tregs are found mostly at the borders between T and B cells and within germinal centers where they suppress T cells as well as B cell responses through both inhibition of immunoglobulin class switch recombination (52) or generation of "Treg-epitopes" identified in the Fc region of the immunoglobulin molecule itself (53).

Intratumoural $CD4^+$ T cells ($CD25^+$ or $CD25^-$) expressing FOXP3 are increased in FL compared to reactive lymph nodes (54). These $FOXP3^+$ cells are either attracted (CCL22) or induced locally by lymphoma cells through cell contact without stimulation of TCR but by expressing CD70 (54-56). These $FOXP3^+$ cells suppress intra-tumoral CD4+ or CD8+ T-cells either through TCR activation, cell contact or by producing TGFβ (54, 56, 57). In a small series of FL cases purified using fluorescence activated cell sorting; we found that CCL22 was one of the chemokine genes most highly expressed by the malignant B cells (unpublished observation 2008, RDG). Moreover, Tregs have also been shown to induce alternatively-activated macrophages, the so-called M2-type (58), which are also associated with pro-tumoural immunity. Finally, in combination with CpG vaccination, antibody-mediated T cell modulation, mostly by depletion of Tregs through anti-folate receptor 4 (FR4) antibodies or functionally blocking them using anti-GITR, anti-CTLA4 or anti-PDL1 antibodies, correlates with a significant reduction of tumour burden both in mouse models and a phase II clinical trial in patients with recurrent FL (59, 60).

In concordance with most published data, including mouse models and a number of different primary human tumours as well as *in vitro* and functional data in FL, where Tregs are associated with a pro-tumoral immunity, we found a follicular pattern of FOXP3+ Tregs to be associated with inferior survival in FL.

Importantly, this follicular pattern also predicted an increased risk of transformation. Superficially, this finding appears to contradict previous retrospective studies of the survival impact of Tregs in FL using TMAs and immunohistochemistry (61, 62). However, in contrast to those studies, our analysis found the distribution of Tregs to be more important than their numbers. In addition, our patients received uniform therapy, thus holding this important variable constant. Recent data convincingly demonstrate the dramatically variable impacts that different therapies have on the non-neoplastic immune cells in the microenvironment (8, 63-65). Indeed, studies of the microenvironment in FL become nearly impossible to interpret when patients receive very heterogeneous treatments, because the specific chemotherapeutic agents, radiation and biological agents differentially impact benign immune cells. As was shown in several recent series studying the impact of LAM in FL, the specific therapeutic regimens used decisively influenced the prognostic impact of non-neoplastic cells in the microenvironment (8, 63-66).

In FL, where the content and distribution of various immune-related cells is markedly heterogeneous, we reasoned that the topographic distribution of Tregs relative to the neoplastic follicle might reflect their function and thus their clinical impact. Qualitative (architectural patterns) or semi-quantitative biomarkers (cell counts) such as those measured in this study may be difficult to reproduce (67). However, the use of TMAs and immunohistochemistry at least partially overcomes this difficulty and has been shown to provide a reliable strategy to assess large numbers of clinical samples and validate novel biomarkers discovered by GEP (25, 63).

In this study, we analyzed Tregs within FL biopsies using three markers known to be characteristically expressed by Tregs: CD4, CD25 and FOXP3. As previously described, Tregs belong to the CD4+CD25+ T cell subset. However, Tregs can be CD25- and CD8+CD25+ T cells can exhibit regulatory activity (68). FOXP3 is the single best marker of activation of Tregs (69). Simple enumeration of cells identified using these markers showed no impact on survival or risk of transformation. However, we found a follicular distribution of these $CD25^+$ or/and $FOXP3^+$ cells strongly correlates with outcome (Table 3). Interestingly, the survival associations in our cohort followed a pattern linked to specificity for the regulatory phenotype: CD4/8 pattern had an impact just on

PFS, CD25 showed significance for PFS and OS and FOXP3 had an impact on PFS, OS and, importantly, RT.

Programmed death-1 (PD1) belongs to the immunoglobulin CD28 receptor family and is characteristically expressed by germinal center-associated T cells. These cells have immunoregulatory activity and are FOXP3 negative (70). In the current study, we showed that combined cell density and distribution within FL of differently expressed immunoregulatory cell markers are important predictors of OS & RT in advanced-stage FL patients treated uniformly with an aggressive treatment regimen.

This impact of either follicular or perifollicular FOXP3+ cells suggests a functional suppression of immunosurveillance within the tumour microenvironment that is present at the time of diagnosis. The malignant cells may have the capacity to “recruit” or “skew” Treg activity towards the suppression of anti-tumoral T-cells, thus allowing the malignant cells to “escape” from the regulatory activity (38). Interestingly, eleven of 12 cases with a high content of LAMs in this series had a follicular FOXP3 pattern. Both Tregs and macrophages may cooperate, in a subset of cases, providing the neoplastic B cells with trophic and survival signals that promote tumour progression, the accumulation of further genetic damage and transformation.

3.5.4. Vessels

Angiogenesis plays a crucial role in the growth and progression of human solid tumours (4). In most tumour types, increased MVD correlates with increased disease progression and decreased survival. Similar results have been reported in hematopoietic tumours, including multiple myeloma and lymphoma (71-74). In murine lymphoma models and in lymphoma patients, circulating endothelial cells and serum vascular endothelial growth factor (VEGF) levels appear to correlate with lymphoma volume and increased angiogenesis (75, 76). In contrast, previous studies of MVD in FL have resulted in conflicting results. Koster et al showed increased MVD to be associated with a more favourable OS in a series of 36 uniformly treated patients given CVP and interferon (IFN) α2b followed by IFN maintenance (77). In a later report, Jorgensen *et al*. analyzed 107 FL cases with heterogeneous treatments and found increased interfollicular MVD predicted inferior OS and increased transformation to

DLBCL(78). In both studies, MVD was calculated using quantification of focal "vessel hot spots" within the tumour. In FL however, the vessels show a heterogeneous distribution. Therefore, in order to avoid a scoring bias, we studied whole sections of FL biopsies and quantified automatically the average distance from CD34 stained vessel that included 90% of tumour cells – TVD_{90}. We believe this is a more accurate measurement of vessel density within the lymph node.

Because of vessel predominance within the interfollicular and perifollicular areas, TVD_{90} was correlated with the average size of the follicles in each case. Interestingly, cases with low TVD_{90} correlated with small sized follicles although follicle size itself did not affect survival or risk of transformation.

In the current study, all 84 patients were treated uniformly with multi-agent chemotherapy and radiation, while in the series by Koster et al, all 36 patients were treated with CVP chemotherapy and IFN followed by IFN maintenance. In addition to the difference in cohort size, different therapeutic regimens may explain the contradictory results. IFN has both immunomodulatory and anti-angiogenic effects and thus may have been more effective in tumours with increased MVD (79). Radiation has been shown to induce tumour cells to secrete cytokines capable of inhibiting apoptosis in endothelial cells, thereby diminishing treatment response (80, 81). It remains possible that this treatment modality influenced survival and transformation risk in our study.

It is well known that angiogenesis in cancer is critically influenced by the local tumour microenvironment (82). In this study using the same uniformly treated cohort, increased numbers of LAM is associated with adverse outcome (63). Similar to solid tumour-associated macrophages, these LAM, possibly originating from bone marrow derived myeloid cells, may be attracted by hypoxia and tumour-derived chemotactic factors and show a distinct phenotype that promotes angiogenesis (83-85). Consistent with this hypothesis, we show a significant association between TVD_{90} and LAM. In previous published NHL studies that included FL cases, increased angiogenesis and macrophage density simultaneously correlated with pathological progression in lymphoma (86, 87). Given the inferior outcome associated with increased LAM in FL, macrophages are a good candidate to be supportive of angiogenesis in FL. S100A8+ is characteristic of late-stage inflammatory macrophages with anti-

inflammatory homeostatic functions and is up-regulated by glucocorticoids and IL10 (14). These are features suggestive of a M2 phenotype. We show in the current study a significant association between S100A8 and TVD90, consistent with this hypothesis (Figure 3.20 & 3.21).

3.6 CONCLUSION

This study provides compelling data that support a role for benign macrophages, vessels and Tregs in predicting outcome in aggressively treated FL patients and further substantiate the gene expression profiling data highlighting the importance of non-neoplastic cells in determining prognosis in FL (Figure 3.22). These biomarkers may become useful in initial therapeutic decisions of patients with FL, and may even provide a rationale for trials of new therapies, such as anti-angiogenic therapy in FL patients with increased MVD. This cohort has the important and rare advantage of its patients being treated uniformly. Heterogeneous treatment modalities within and between different series of FL has been considered responsible for the controversy in the published literature (Figure 3.23). Yet, at present, these significant biomarkers related to the non-malignant microenvironment remain interesting clinic-pathological findings, but they represent functionally untested hypotheses. An improved understanding of the interactions between non-neoplastic macrophages, T cells and vessels with neoplastic B cells in FL should provide important insights into the biology of this common tumour. Assessment of these markers in prospective clinical trials will expedite their validation.

Ultimately, it may be possible to manipulate the microenvironment of the lymph node in favour of the host and turn off tumoural immunosuppression or trophic survival signals derived from non-neoplastic tissue cells (Figure 3.24).

3.7 TABLES

Antibody	Clone	Source	Dilution
Bcl-2	124	DAKO	1:20
Bcl-X_L	2H12	ZYMED	1:50
Bcl-6	PG-B6p	DAKO	1:10
NOXA	114C307	Abcam	1:50
CD3	-	Cell Marque/Novocastra	1:100
CD4	4B12	Novocastra	1:50
CD7	CD7-272	Novocastra	1:30
CD8	C8/144B	DAKO	1:50
CD10	56C6	Novocastra	1:50
CD20	L26	DAKO	1:500
Ki67	MIB1	DAKO	1:100
CD21	1F8	DAKO	1:30
CD57	NK-1	Novocastra	1:30
TIA1	2G9A10F5	Beckman	1:250
CD25	4C9	Novocastra	1:100
FOXP3	236A/E7	Abcam	1:2
PD1	NAT105	Abcam	1:50
CD34	QBEnd-10	Cell Marque	1:100
CD68	KP1	DAKO	1:2000
CD163	10D6	Novocastra	1:50
S100A8	CF-145	Acris Abs	1:50

Table 3.1 - Antibodies used in the study.

Biomarker	# Failed	# Positive (%)	Morphological Pattern	Comments	OS PFS	RT
Ki67 (MIB1)	5	62 (62)	-	>50% nuclei	NS	NS
CD4 pattern	2	78(76)	Follicular	Follicular predominance	NS	NS
CD8 pattern	1	91(88)	Perifollicular	Perifollicular predominance	NS	NS
CD4/CD8 pattern	**5**	**71 (71)**	**Follicular**	**Follicular CD4 / perifollicular CD8**	**PFS**	NS
CD7 pattern	1	73 (70)	Inter-follicular	CD7 predominance	NS	NS
CD57 content	1	67 (64)	-	>10% of T-cells	NS	NS
CD57 pattern	13	68(63)	Follicular	Intra-follicular CD57	NS	NS
Tbet content	33	10(14)	-	>1%	NS	NS
TIA1 content	1	47(45)	-	>10% T-cells	NS	NS
CD25 content	6	51(52)	-	Number of cells	NS	NS
CD25 pattern	**5**	**40(40)**	**Follicular**	**Follicular & perifollicular**	**OS/PFS**	**S**
FOXP3 content	3	34(33)	-	Number of cells	NS	NS
FOXP3 pattern	**3**	**38(36)**	**Follicular**	**Follicular & perifollicular**	**OS/PFS**	**S**
PD-1 pattern	4	43 (41)	**Follicular**	**Follicular & perifollicular**	**OS/PFS**	NS
PD1 & FOXP3	6	-	-	Quartiles	**OS/PFS**	**S**
CD21 pattern	19	27 (31)	Expanded follicles	Coalescence of FDC	NS	NS
CD34 (vessels)	6	-	Follicular	Vessel density	NS	NS
CD68 content	**6**	**12(12)**	-	**>15 cells / hpf**	**OS/PFS**	NS
S100A8 content	**7**	**16(15)**	**interfollicular**	**>5 cells / hpf**	**OS/PFS**	NS

Table 3.2 - Biomarkers related to FL microenvironment.

Features	# Patients with high LAM (%)	# Patients with low LAM (%)	Total (%)	p-value
Number	12 (12)	87 (88)	99 (100)	-
Clinical Features				
IPI Group 1 (0/1)	4 (33)	54 (62)	58 (59)	
Group 2 (2/3)	8 (66)	32 (37)	40 (40)	**0.058***
Group 3 (4/5)	0 (0)	1 (1)	1 (1)	
BM Involvement	6 (50)	44 (51)	50 (51)	
Treatment Response				
CR	6 (50)	48 (55)	54 (55)	
PR	6 (50)	36 (41)	42 (42)	**NS****
NR	0 (0)	3 (3)	3 (3)	
Pathology Features				
FL Grade				
Grade 1	6 (50)	67 (77)	73 (74)	
Grade 2	4 (33)	16 (18)	20 (20)	**0.046*****
Grade 3	2 (17)	4 (5)	6 (61)	
MZ Differentiation	3 (25)	22 (25)	25 (25)	**NS**
Transformation	**0 (0)**	**8 (9)**	**8 (8)**	**NS**

Table 3.3 - Distribution of clinical and pathology variables between high and low Lymphoma-Associated-Macrophages (LAM); IPI = International Prognostic Index score, BM = bone marrow, CR = complete remission, PR = partial remission, NR = no response; *IPI group 1 vs. 2/3; **CR vs. PR/NR; ***Grade 1 vs. 2/3A.

Feature	# Patients with Follicular FOXP3+ cells (%)	# Patients with Diffuse FOXP3+ cells (%)	Total (%)	p-value
Number	38 (37)	64 (63)	102 (100)	-
Clinical Features				
Median age (y)	45	45	-	**0.9**
Female (%)	15 (40)	37 (58)	-	**0.6**
Median follow-up (y)	17	16	-	**.011**
IPI Group 1 (0/1)	19 (50)	43 (67)	62 (61)	
Group 2 (2/3)	19 (50)	20 (32)	39 (38)	**0.14**
Group 3 (4/5)	0 (0)	1 (1)	1 (1)	
BM Involvement	22 (60)	29 (45)	52 (51)	**0.3**
Treatment Response				
CR	18 (47)	40 (63)	58 (57)	
PR	19 (50)	22 (34)	41 (40)	**0.3**
NR	1 (3)	2 (3)	3 (3)	
Pathology Features				
FL Grade				
Grade 1	29 (76)	51 (80)	80 (78)	
Grade 2	6 (16)	10 (16)	16 (16)	**0.8**
Grade 3	3 (8)	3 (4)	6 (6)	
FOXP3 cells (MD; SD)	312 (145)	200 (125)	-	**<0.001**
LAM >15cells HPF	11	1	12(12)	**<0.001**
CD4/8 pattern (F)	29	40	69(68)	**0.2**
CD25pattern (F)	31	7	38 (37)	**<0.001**
Transformation	12 (32)	9 (14)	20(20)	**0.04**

Table 3.4 - Distribution of clinical and pathology variables between Follicular and Diffuse pattern of FOXP3$^+$ cells; IPI = International Prognostic Index score, BM = bone marrow, CR = complete remission, PR = partial remission, NR = no response; *IPI group 1 vs. 2/3; **CR vs. PR/NR; ***Grade 1 vs. 2/3A.

Feature	# Patients with Low TVD 90 (%)	# Patients with High TVD 90 (%)	Total (%)	p-value (χ2)
Number	21 (25)	63 (75)	84 (100)	-
Clinical Features				
Median age (y)	39	46	-	**0.034**
Female (%)	52	48	-	**0.63**
Median follow-up (y)	13.9	14.3	-	**.083**
IPI Group 1 (0/1)	8 (38)	45 (71)	62 (61)	**0.01***
Group 2 (2/3)	13 (62)	17 (27)	39 (38)	
Group 3 (4/5)	0 (0)	1 (2)	1 (1)	
BM Involvement (%)	14 (66)	28 (44)	42 (50)	**0.13**
Treatment Response				
CR	8 (38)	39 (62)	47 (56)	**0.08****
PR	13 (62)	23 (36)	36 (43)	
NR	0 (0)	1 (2)	1 (1)	
Pathology Features				
FL Grade				
Grade 1	17 (80)	49 (78)	66 (78)	**0.76*****
Grade 2	4 (20)	10 (16)	14 (17)	
Grade 3	0 (0)	4 (6)	4 (5)	
Large follicles (%)	1 (5)	20 (32)	21 (25)	**0.01**
↑ S100A8+ cells # (%)	10 (48)	4 (7)	14	**<0.001**
Transformation (%)	8 (38)	12 (19)	20(24)	**0.086**

Table 3.5 - Distribution of clinical and pathology variables between high TVD90 and low TVD90 cases. IPI = International Prognostic Index score, BM = bone marrow, CR = complete remission, PR = partial remission, NR = no response; *IPI group 1 vs. 2/3; **CR vs. PR/NR; ***Grade 1 vs. 2/3A.

3.8 FIGURES

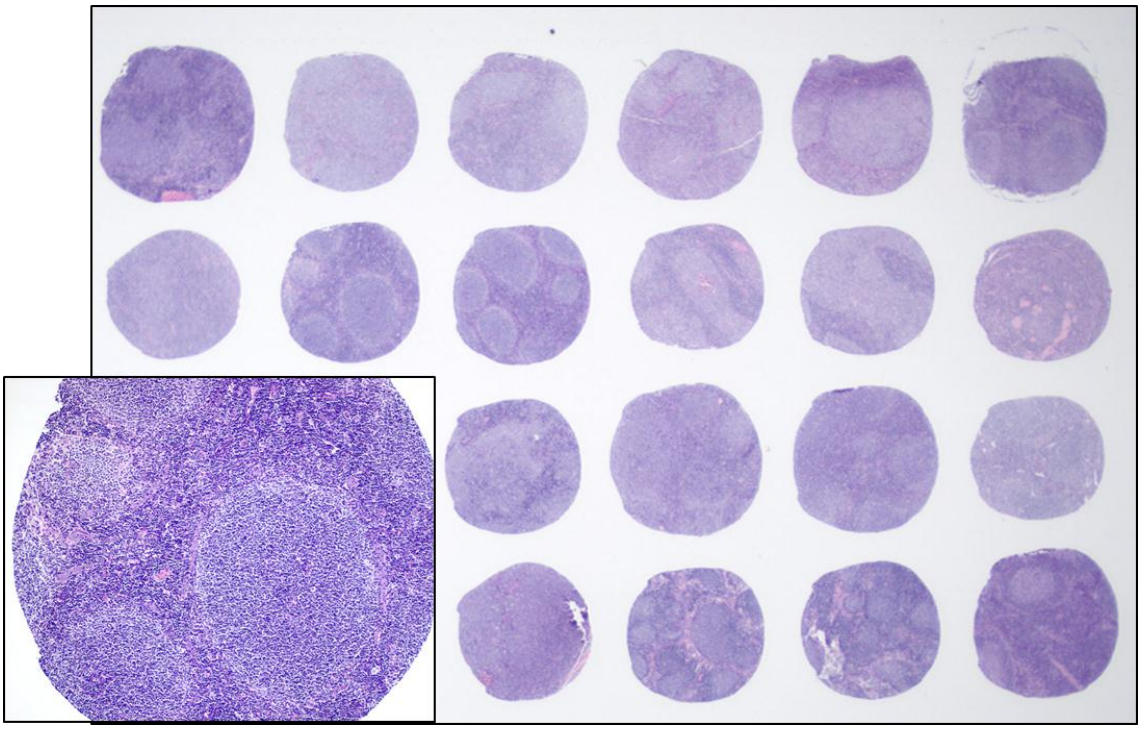

Figure 3.1 - Representative tissue microarray cores of follicular lymphoma patients (H&E) (Microscope NIKON Eclipse E600; Digital Camera Dxm1200).

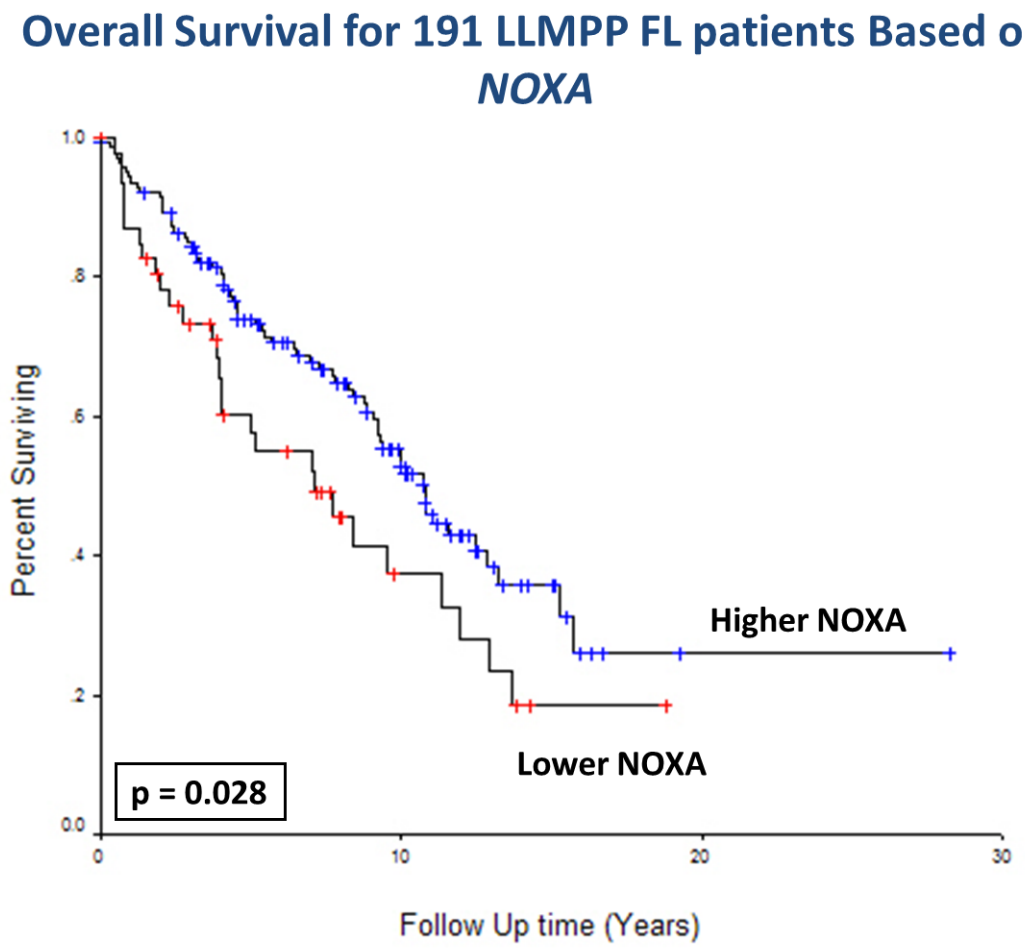

Figure 3.2 - Overall survival curve based on *NOXA* gene expression from diagnostic biopsies on 191 cases of FL from the freezers of LLMPP institutions detected by gene expression profiling using Affymetrix® U133A/B arrays. The upper curve represents cases with increased *NOXA* and the lower curve those cases with decreased *NOXA*.

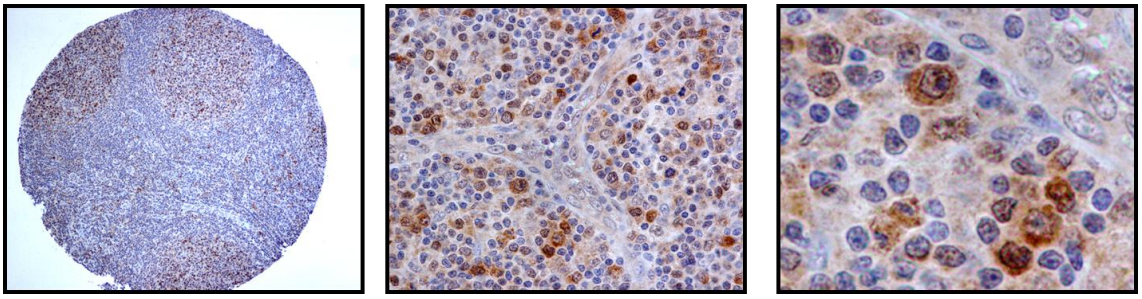

Figure 3.3 - Representative tissue microarray cores of follicular lymphoma patients showing NOXA Protein Expression by both malignant and histiocytic-like cells (Microscope NIKON Eclipse E600; Digital Camera Dxm1200).

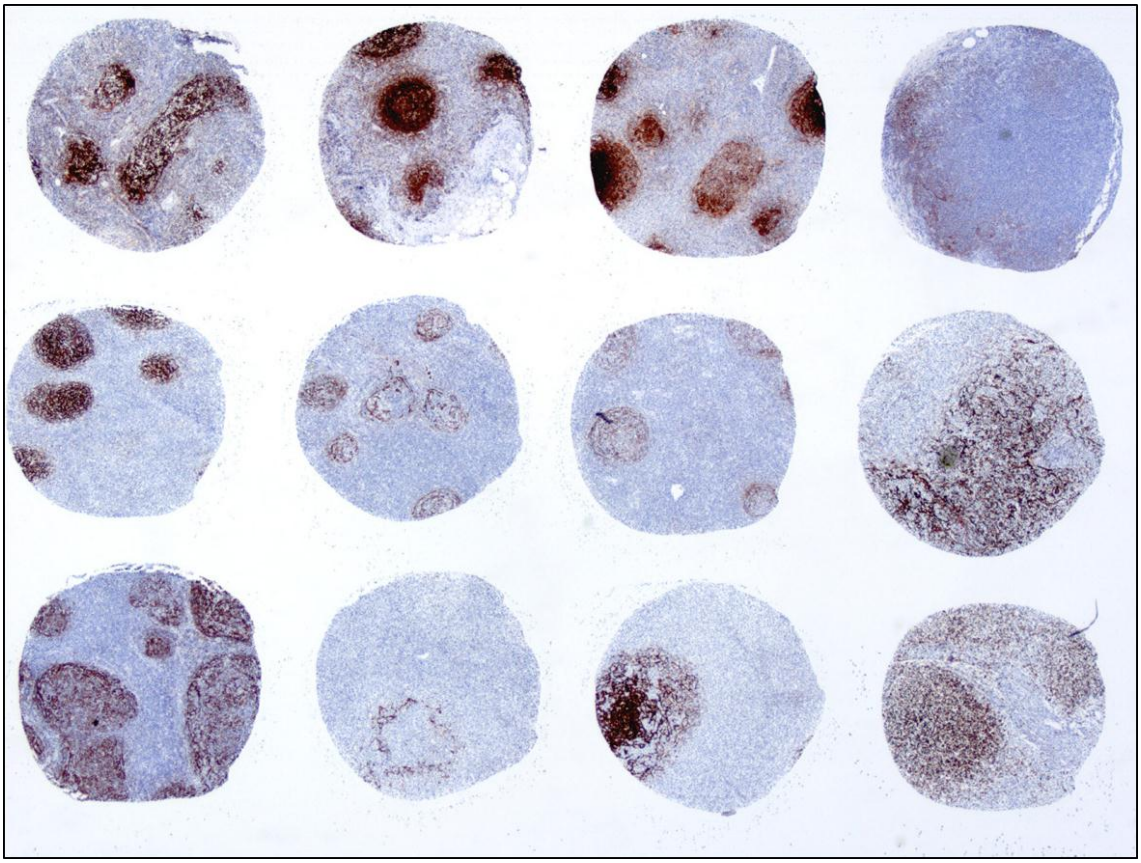

Figure 3.4 - Representative tissue microarray cores of follicular lymphoma patients showing different staining intensity & architectural patterns of CD21+ Follicular Dendritic Cells meshwork (Microscope NIKON Eclipse E600; Digital Camera Dxm1200).

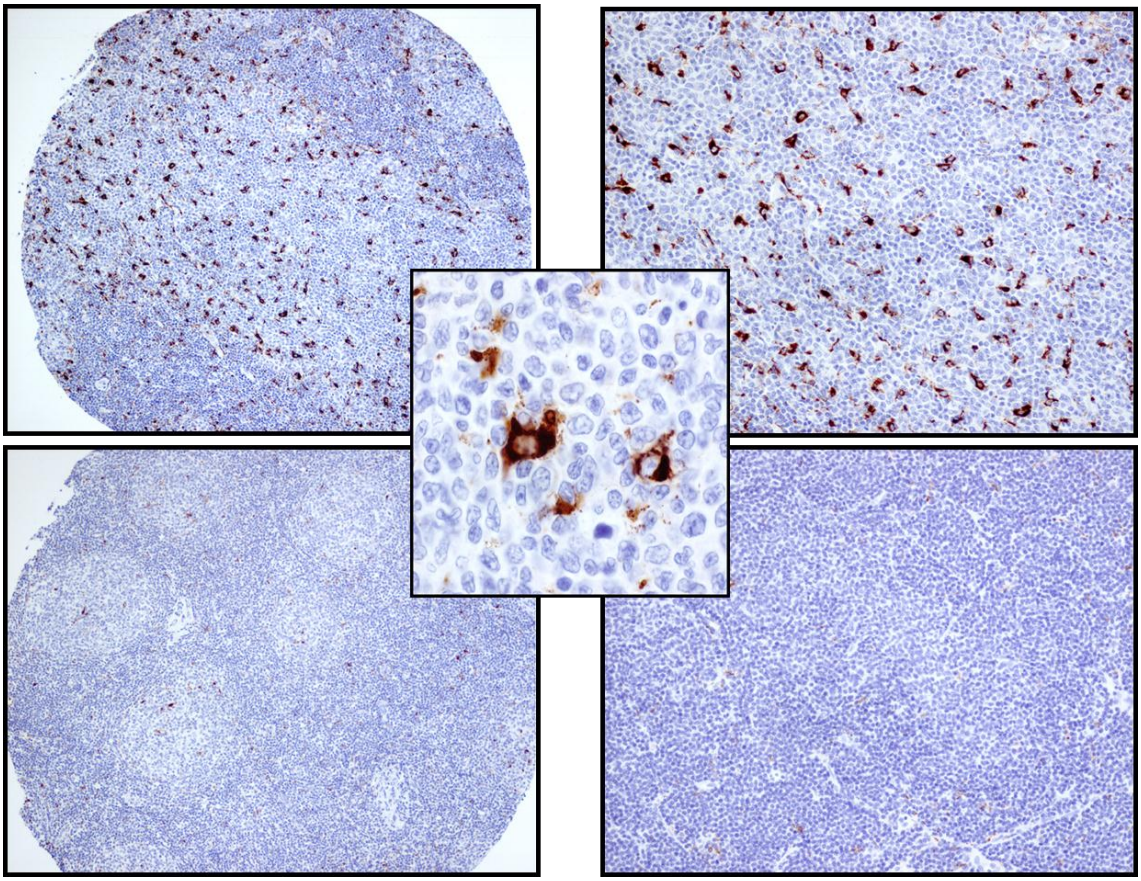

Figure 3.5 - Representative tissue microarray cores of follicular lymphoma patients. The image on the left shows the uncommon finding of large numbers of macrophages both within and surrounding neoplastic follicles (n=12). The image on the right shows the more common finding of very macrophages (n=87). The insert shows the typical strong cytoplasmic staining of reactive macrophages with anti-CD68 antibodies (Microscope NIKON Eclipse E600; Digital Camera Dxm1200).

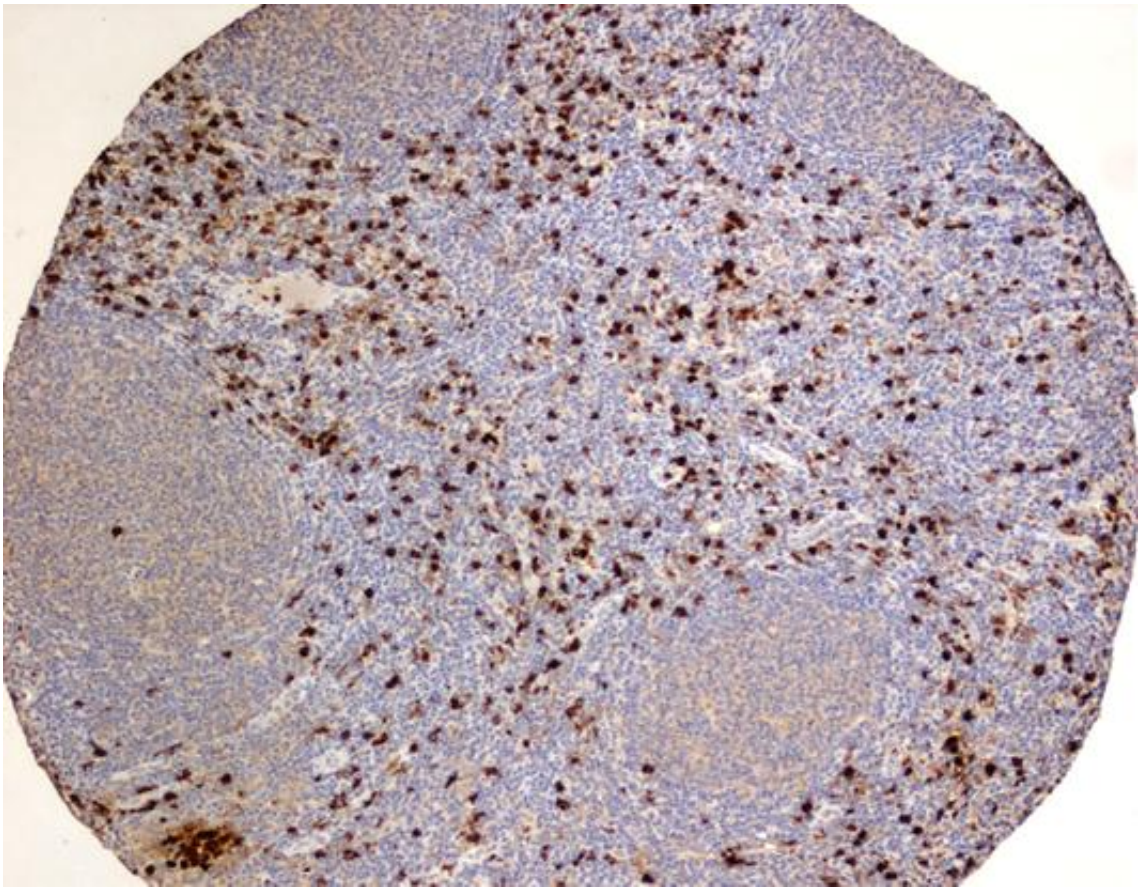

Figure 3.6 - Representative tissue microarray core of a follicular lymphoma patient. The image shows a case with increased S100A8+ / Calgranulin A+ cells. They have a preferential interfollicular distribution (Microscope NIKON Eclipse E600; Digital Camera Dxm1200).

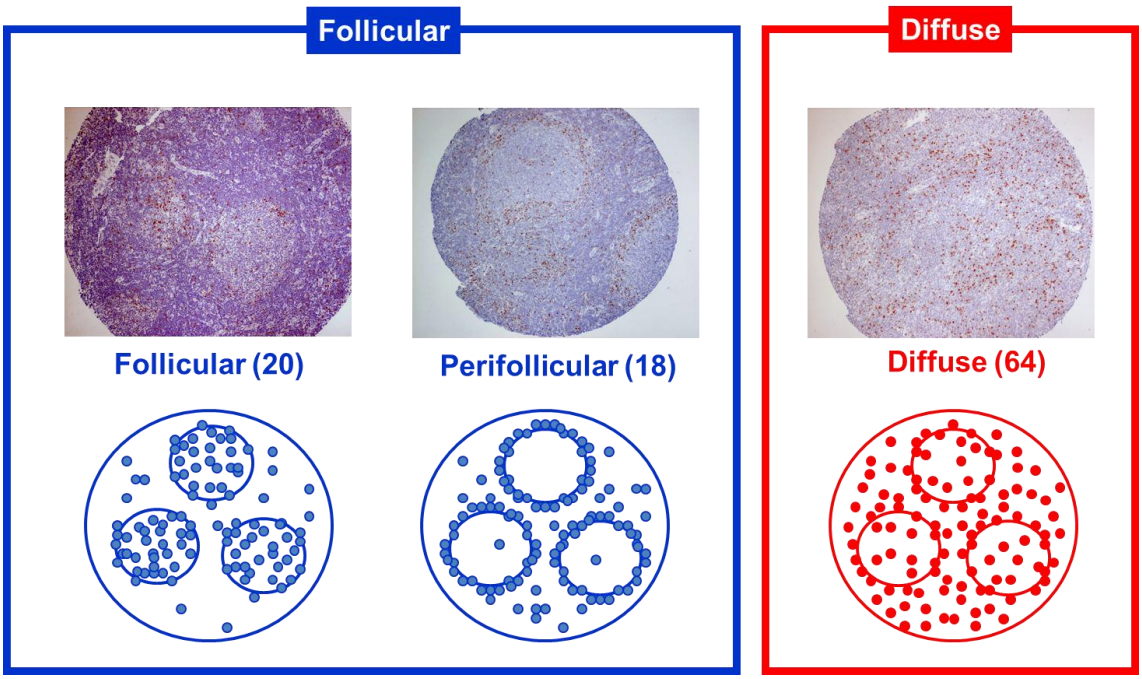

Figure 3.7 - Representative tissue microarray cores of follicular lymphoma patients showing three distinct FOXP3+ T cells architectural patterns (Microscope NIKON Eclipse E600; Digital Camera Dxm1200).

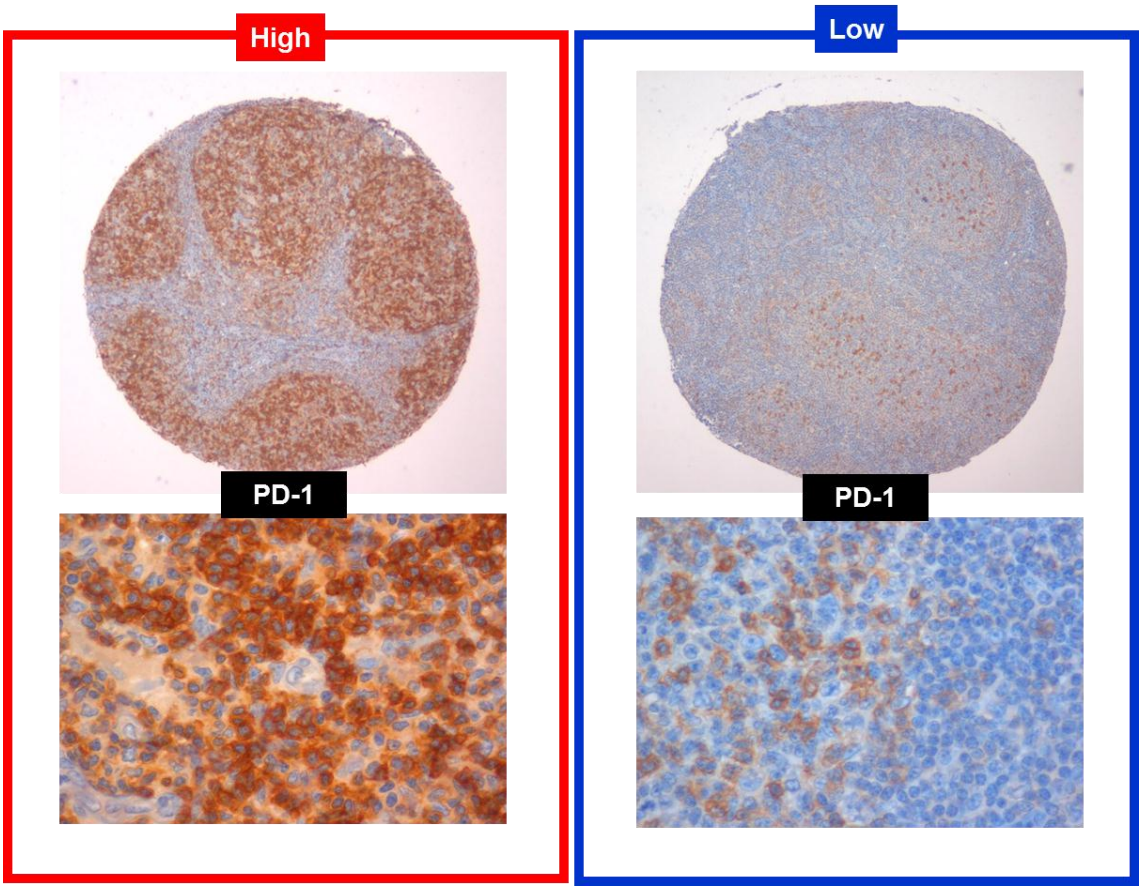

Figure 3.8 - Representative tissue microarray cores of follicular lymphoma patients showing one case with high and low PD1 cell content (Microscope NIKON Eclipse E600; Digital Camera Dxm1200).

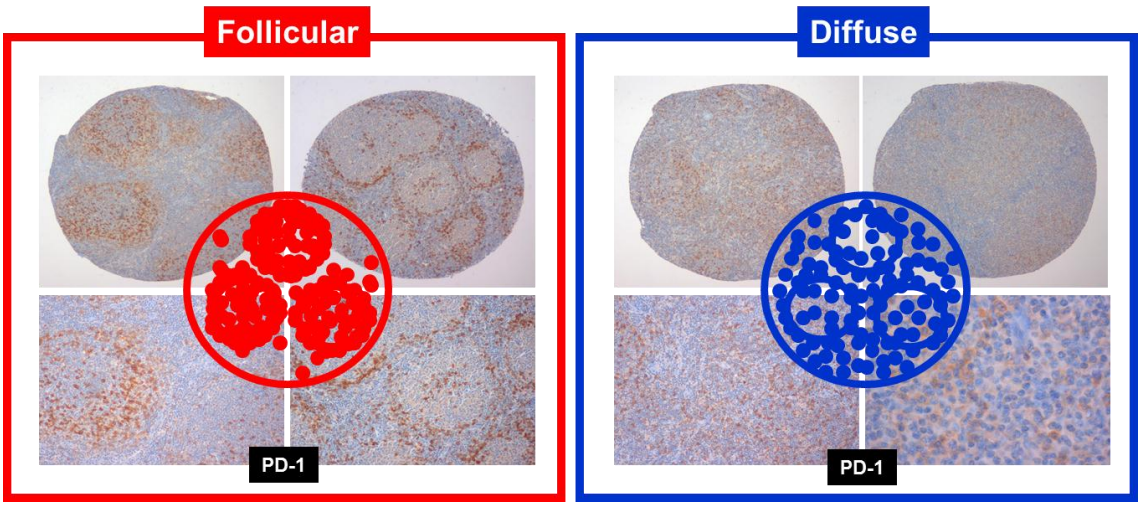

Figure 3.9 - Representative tissue microarray cores of follicular lymphoma patients showing two distinct PD1+ T cells architectural patterns, follicular and diffuse (Microscope NIKON Eclipse E600; Digital Camera Dxm1200).

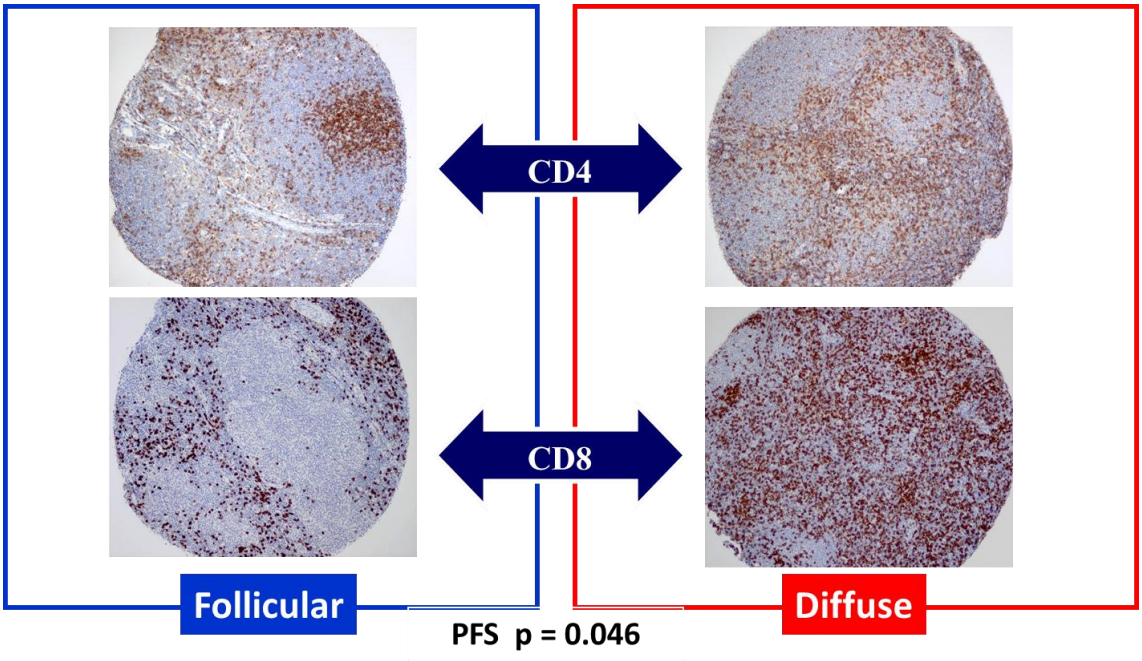

Figure 3.10 - Representative tissue microarray cores of follicular lymphoma patients showing two major patterns of CD4 & CD8 T cells (Microscope NIKON Eclipse E600; Digital Camera Dxm1200).

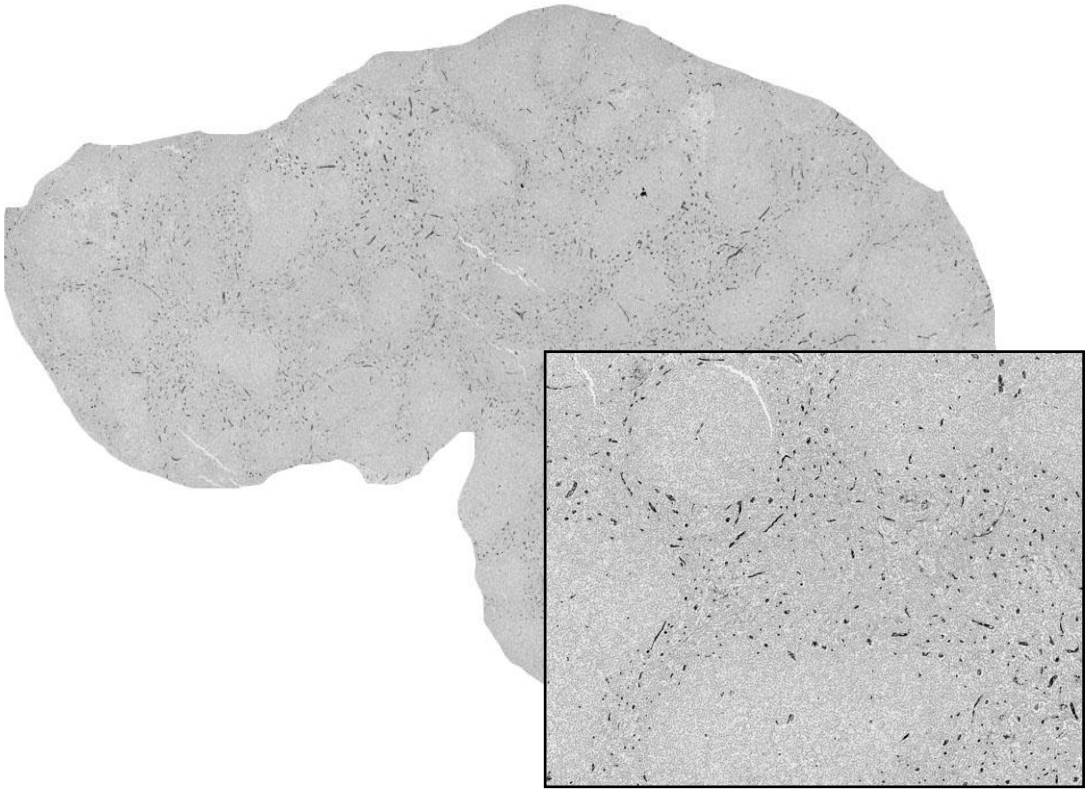

Figure 3.11 - TVD90. Whole sections were used and edges and staining artifacts were cropped prior to analysis (Microscope NIKON Eclipse E600; Digital Camera Dxm1200).

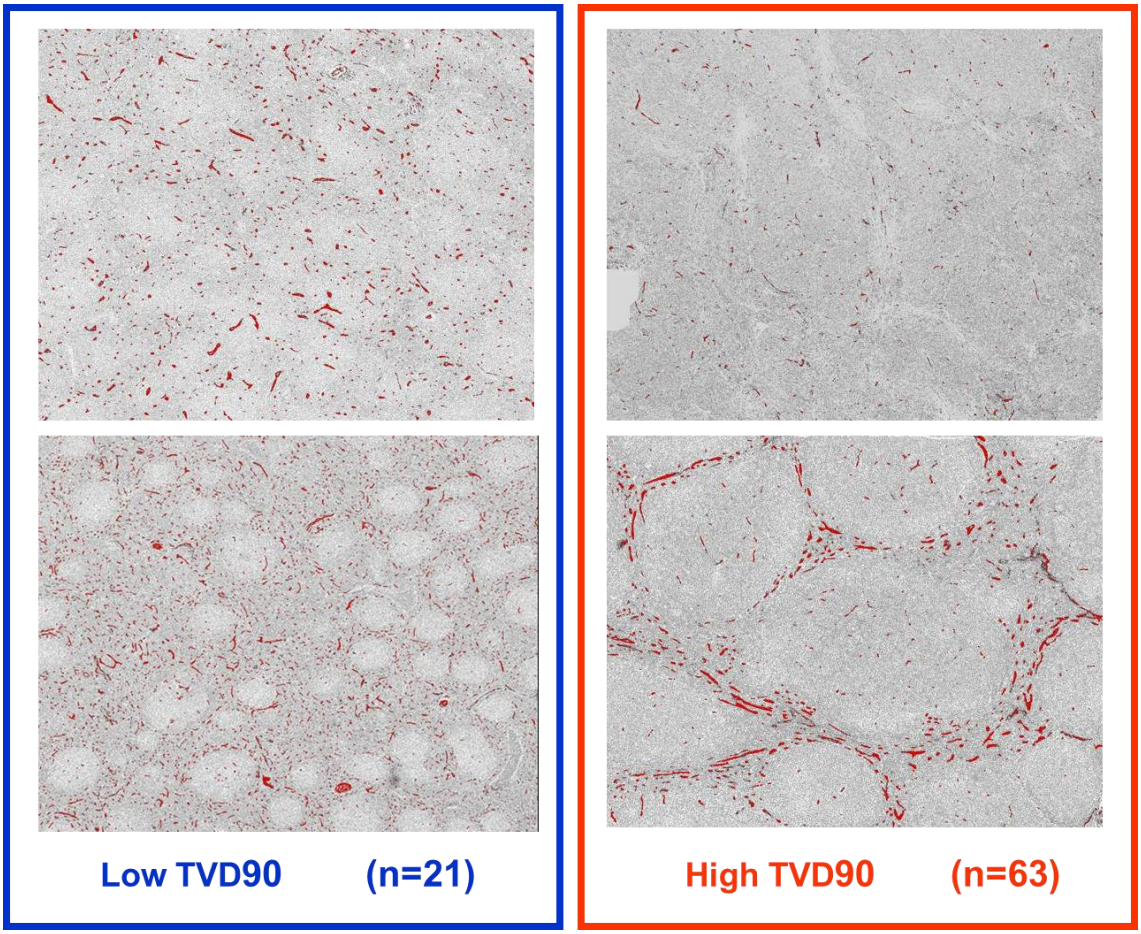

Figure 3.12 - Representative sections of follicular lymphoma cases with low and high TVD90 (Microscope NIKON Eclipse E600; Digital Camera Dxm1200).

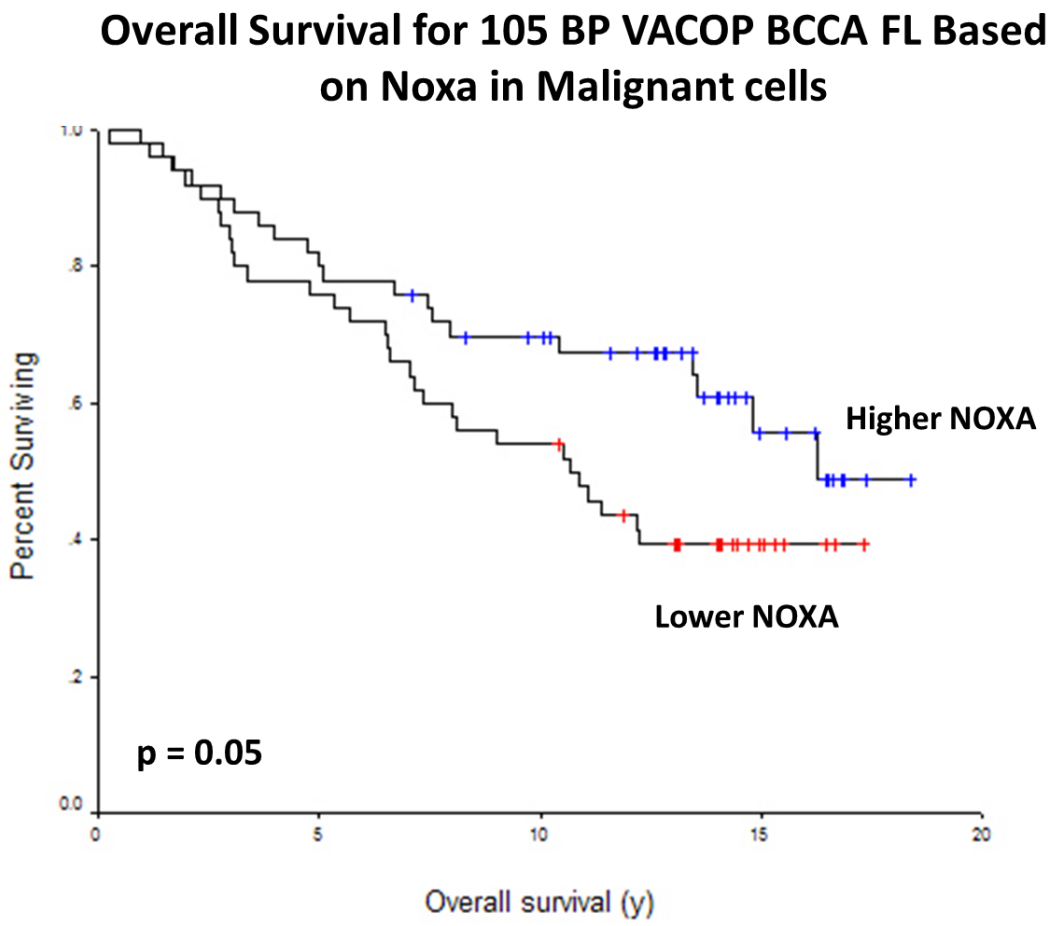

Figure 3.13 - Overall survival curve based on NOXA Protein Expression (NPE). The upper curve represents cases with increased NPE and the lower curve those cases with low NPE.

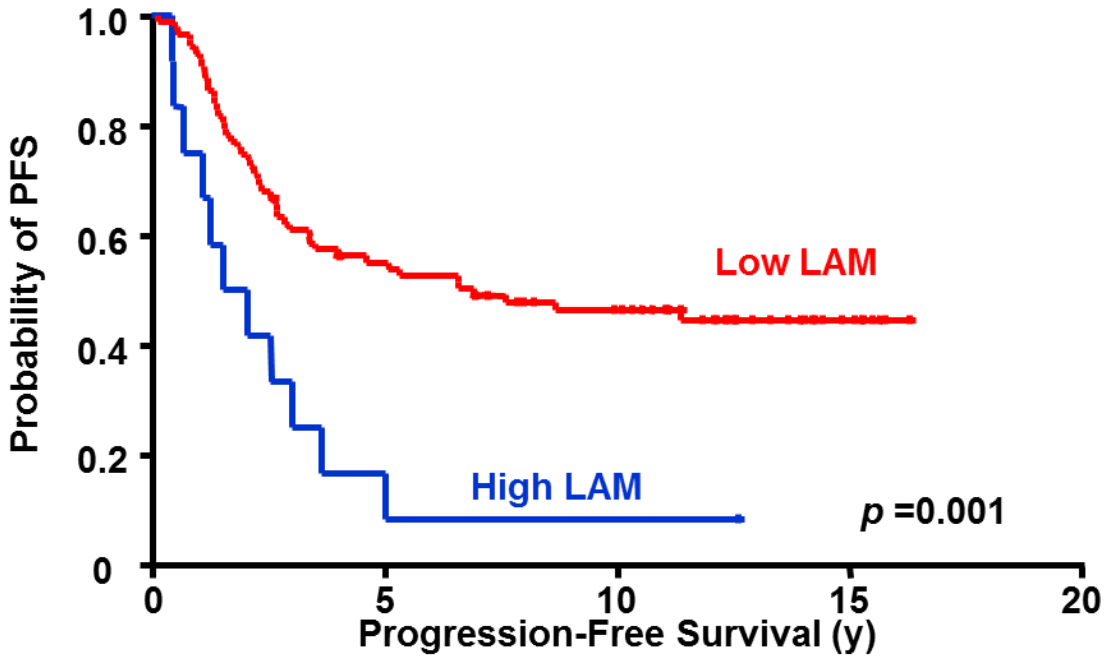

Figure 3.14 - Progression-free survival curve based on lymphoma-associated-macrophage (LAM) content. The upper curve represents cases with <15 CD68+ macrophages/hpf and the lower curve those cases with >15 CD68+ macrophages/hpf. The median PFS were 7.05 and 1.69 years, respectively (P=0.001).

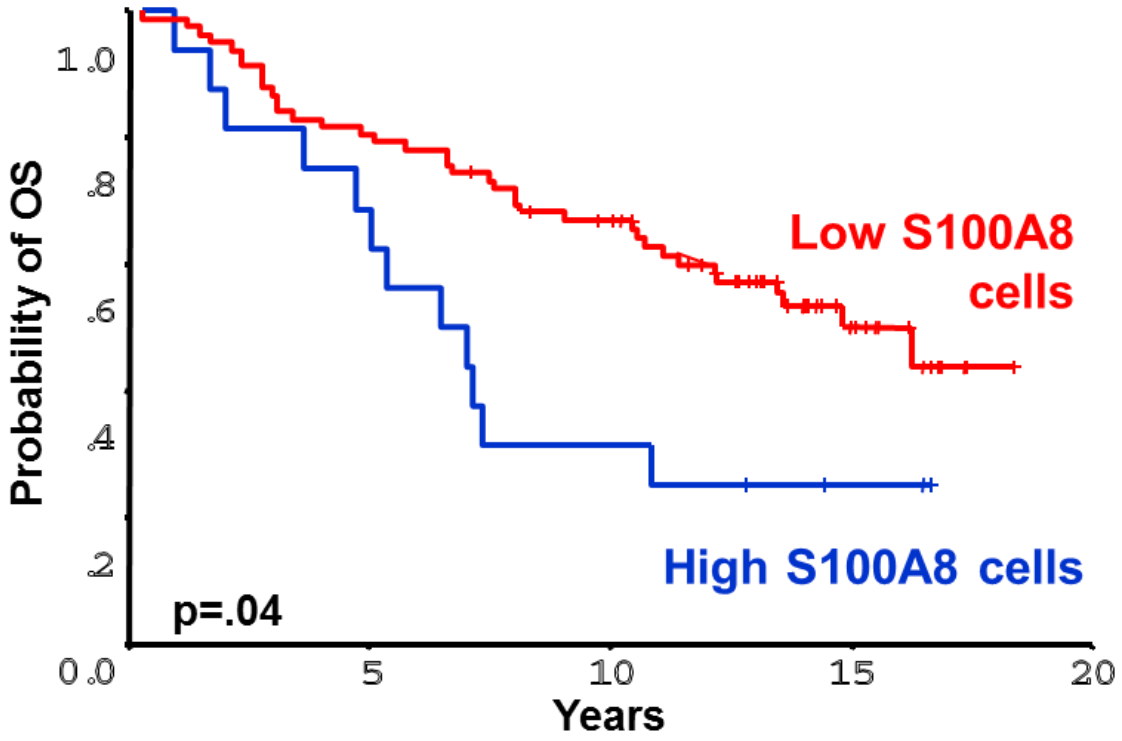

Figure 3.5 - Overall survival curve based on S100A8+ or Calgranulin A+ cells content. The upper curve represents cases with low number of cells and the lower curve those cases with increased cell content (n=14).

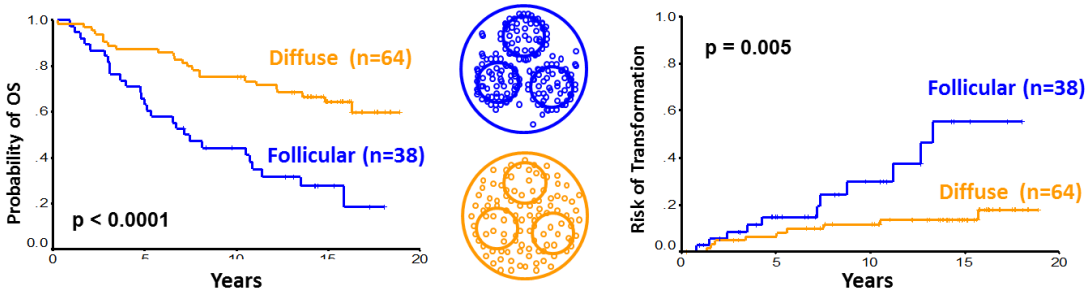

Figure 3.16 - Overall survival & Risk of Transformation curves based on architectural patterns of FOXP3+ T cells.

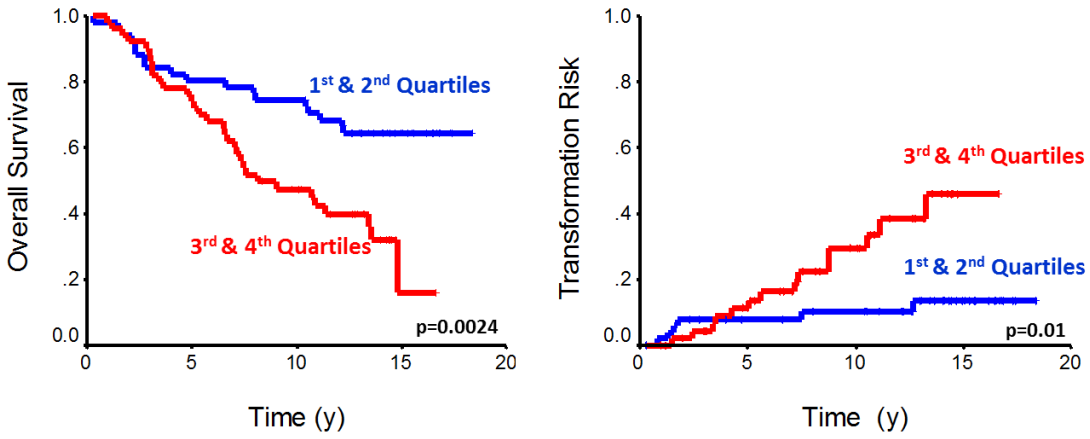

Figure 3.17 - Overall survival & Risk of Transformation curves based on combined cell content of FOXP3+ & PD1+ cells.

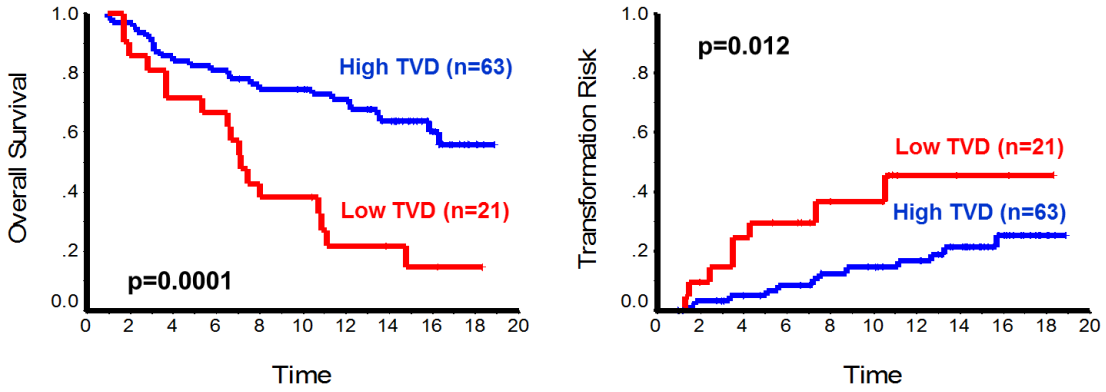

Figure 3.18 - Overall survival & Risk of Transformation curves based on Microvessel density using TVD90 – Tumor to Vessel density including 90% of the malignant cells.

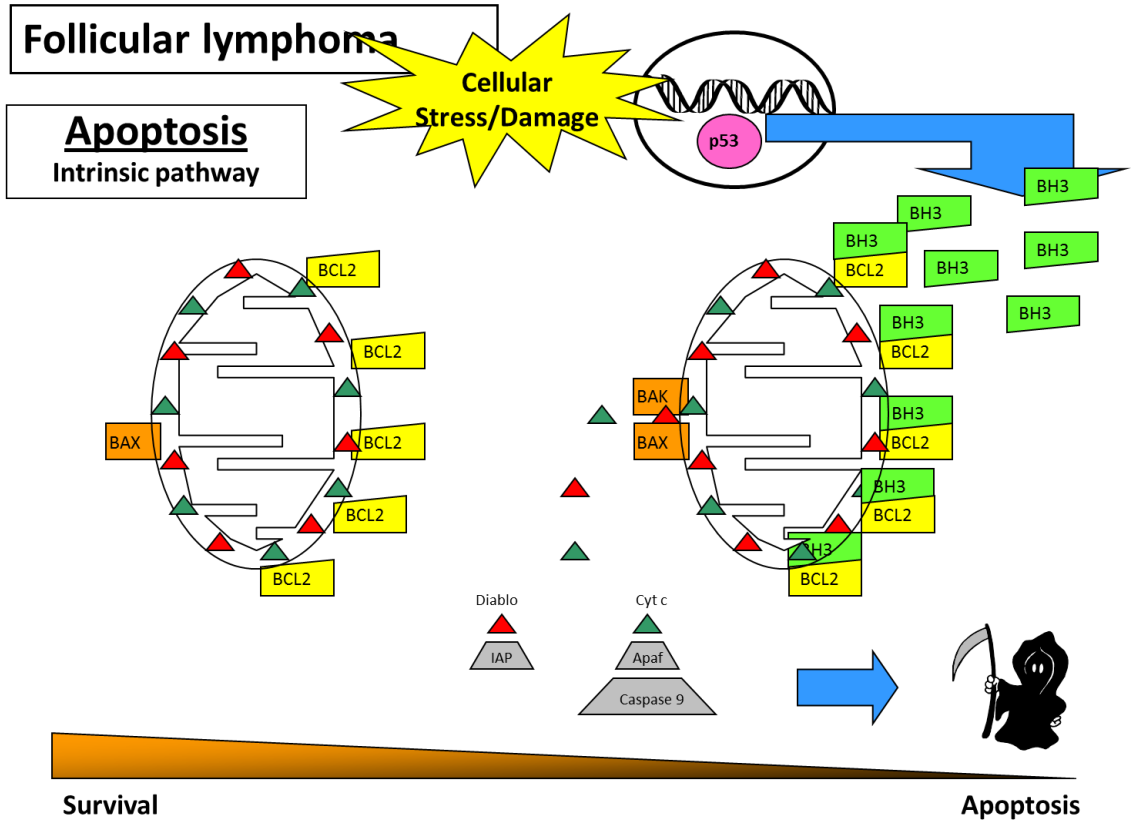

Figure 3.19 - NOXA is a pro-apoptotic member of the BCL2 protein family, belonging to the BH3-only sub-family. BCL2 family members can form hetero- or homodimers, and they act as anti-or pro-apoptotic regulators that are involved in a wide variety of cellular activities. The expression of NOXA is regulated by the tumor suppressor TP53, is involved in TP53-mediated apoptosis and is one of the major “sensitizers / derepressors” favoring apoptosis.

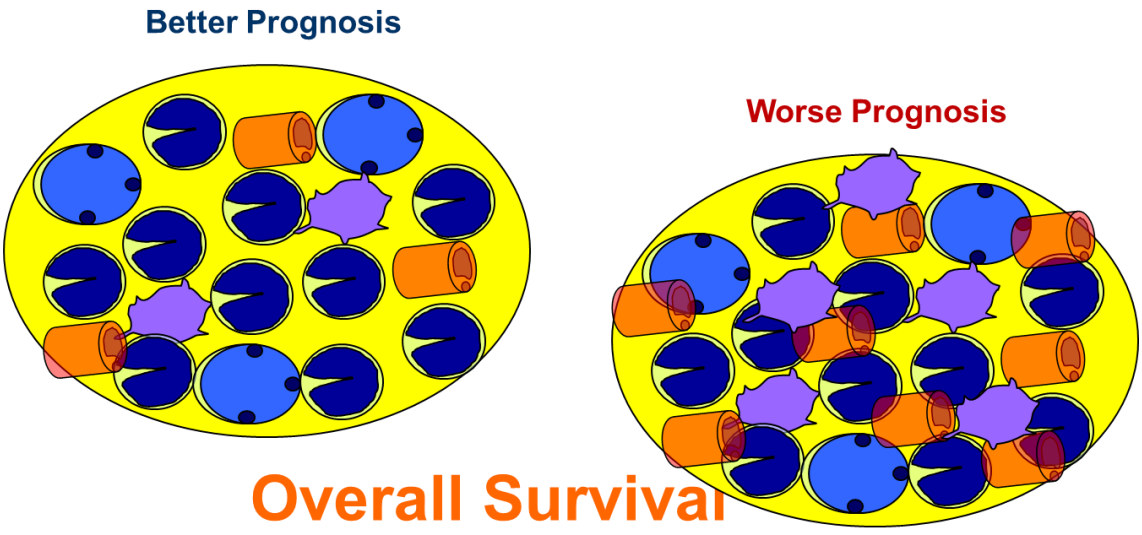

Figure 3.20 - Increased microvessel density correlated with increased LAM and S100A8 positive cells promoting a local pro-tumoral immunity within the microenvironment…

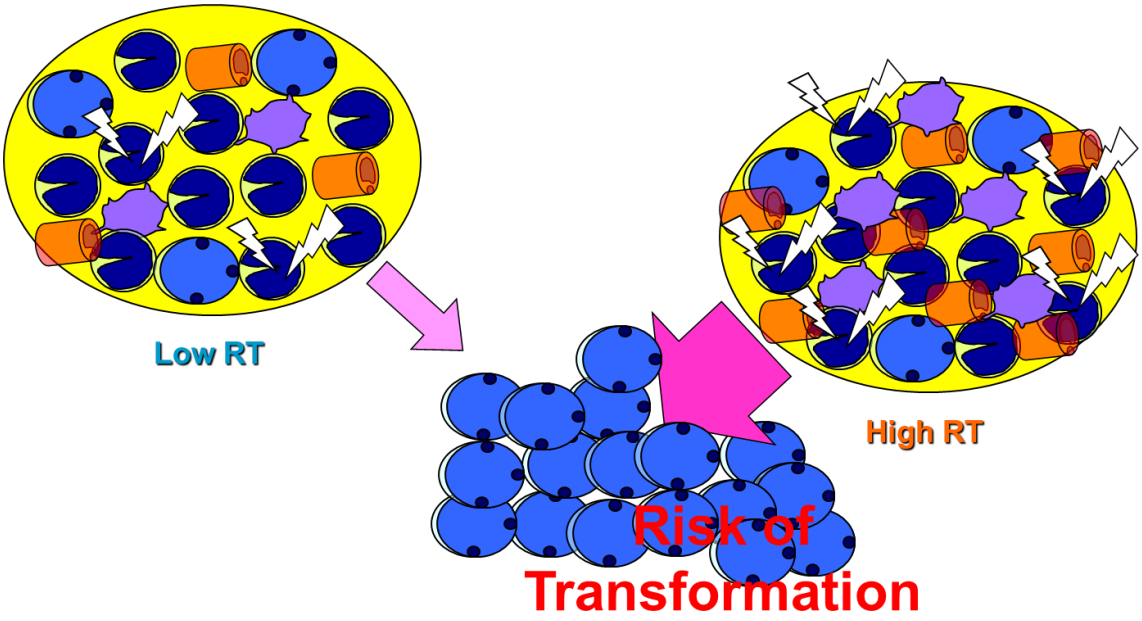

Figure 3. 21

… this is significantly correlated with increased risk of transformation.

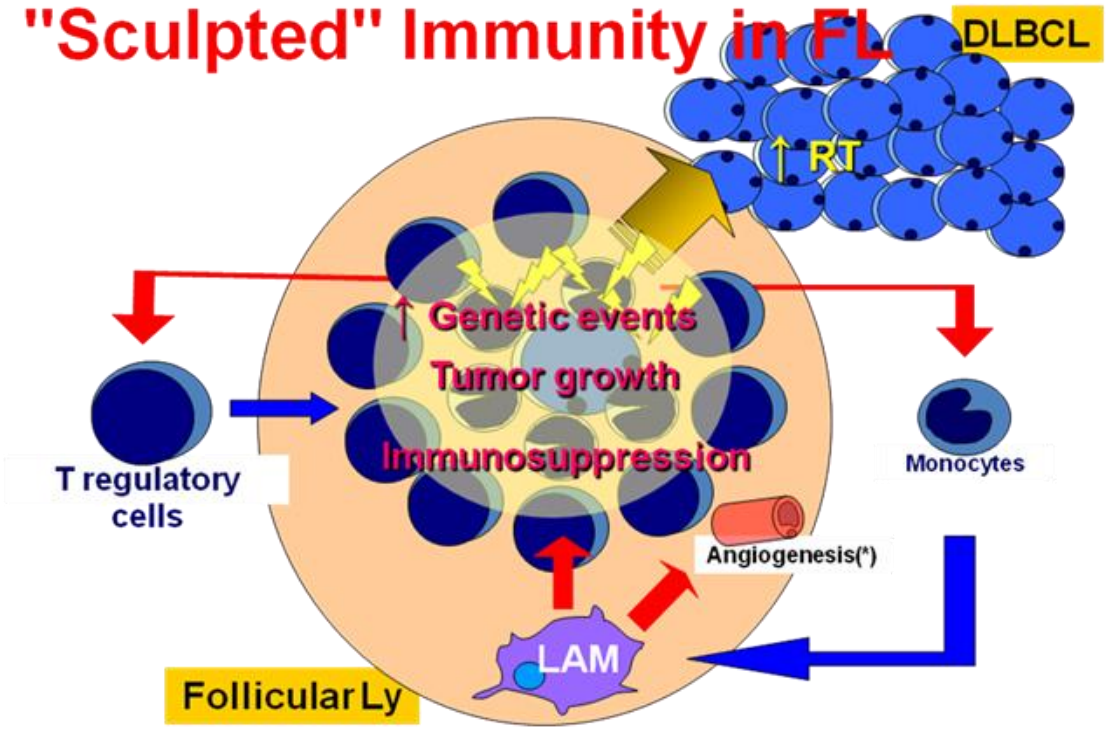

Figure 3.22 - A model of protumoral immunity within follicular lymphoma. The malignant cells have the capacity to “recruit” or “skew” both monocytes and Treg activity towards the suppression of anti-tumoral T cells, thus allowing the malignant cells to “escape” from the regulatory activity within the tumor microenvironment that is present at the time of diagnosis. Both Tregs and macrophages cooperate, in a subset of cases, providing the neoplastic B cells not only with trophic and survival signals but also promoting tumor progression through the accumulation of further genetic damage that will eventual lead to transformation into high grade disease, most commonly a Diffuse Large B cell lymphoma.

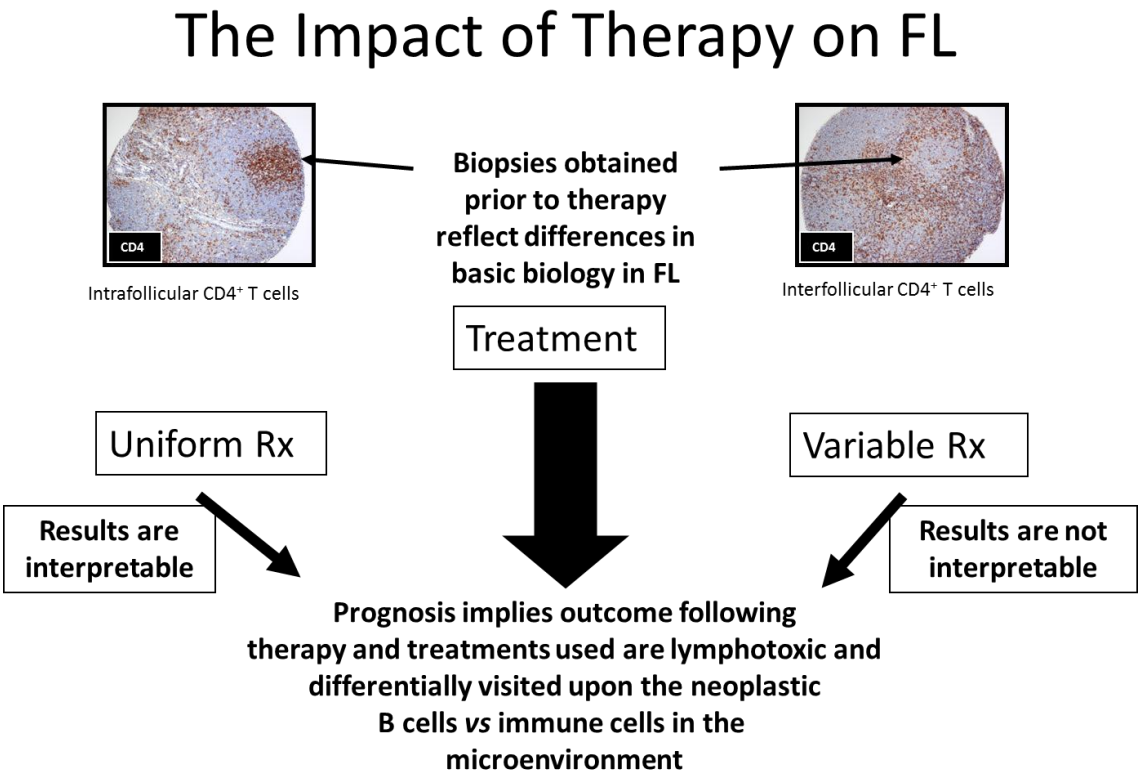

Figure 3.23 - Impact of microenvironment in FL prognosis: distinct differences in most non-malignant cell content and patterns of distribution within FL are present in diagnostic biopsies. Some have been reported to impact survival often with contradictory results among studies. The unique difference characterizing these series is different therapeutic regimens used within the same cohort and among different series. Limited knowledge on how chemotherapy impact on microenvironment limits any interpretation of series treated with different protocols. Uniformity of treatment is mandatory. Rx-therapeutic regimens

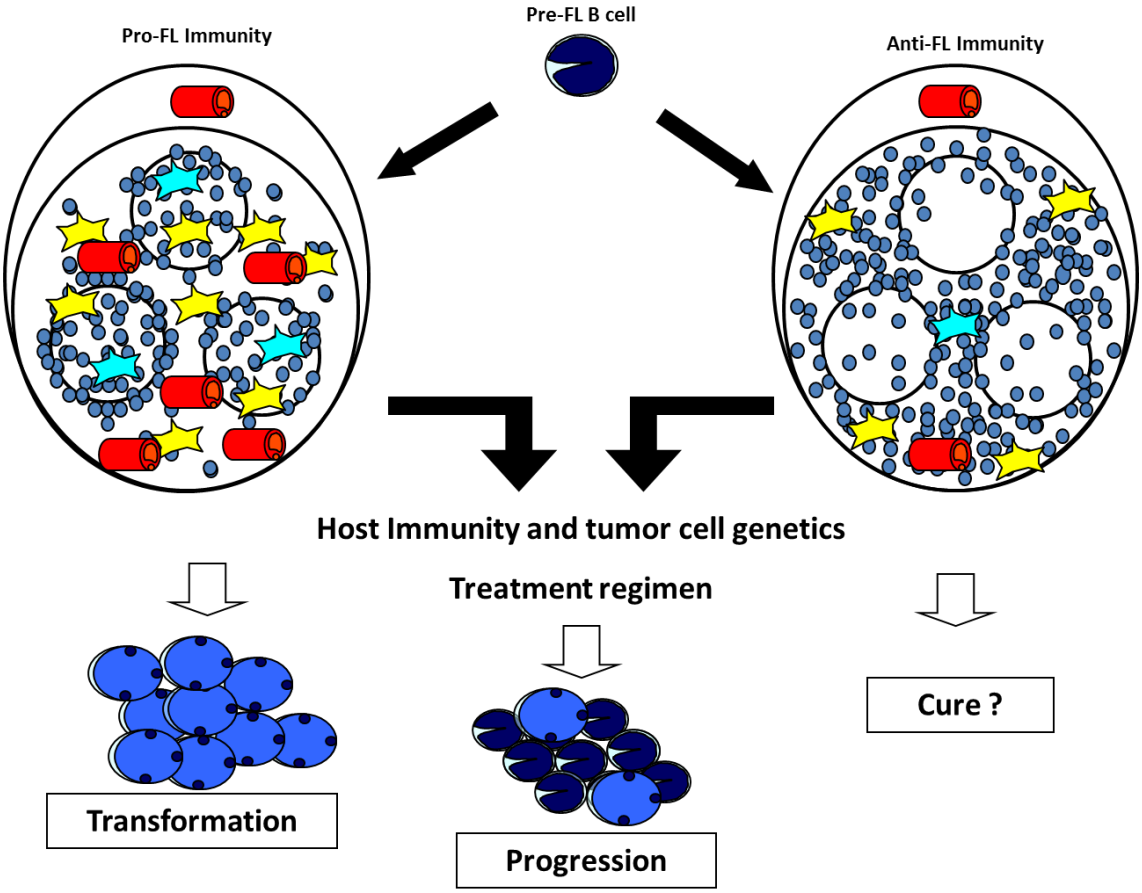

Figure 3.24 - Dual model for the role of tumor immunity in FL and its impact on survival and/or transformation. A follicular polarization of CD4+ cells, especially FOXP3 Tregs (○), increased numbers of vessels (▭), mast cells (✶) or macrophages (✶) represent cases where malignant cells edited local immunity to support tumor growth. Alternatively, lack of follicular localization of those T cells, low numbers of mast cells, macrophages and vessels may be characteristic of an anti-lymphoma immunity. The survival outcome or transformation risk is heavily dependent on the type of therapy used and baseline immune-response characteristics. These latter factors are poorly studied and indeed are major challenges for the future of FL treatment.

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Section 4: Microenvironment in Follicular Lymphoma: Monocyte & Macrophage Interactions in Patients with Follicular Lymphoma Harboring a t(14;18): Is There a Clonal Relationship?

4.1 INTRODUCTION

FL is somewhat unique amongst B cell neoplasms as it retains many of the morphological features of the normal follicle. Cellular interactions between tumoural B cells and their surrounding T cells, follicular dendritic cells (FDC) and macrophages are maintained in FL. Survival signals from these non-neoplastic cells appear to be important for the neoplastic B cells (1). In the NOD/SCID mice transplanted with human FL samples, contrary to other lymphoma types, these cells fail to maintain their histology for more than one graft generation. The malignant cells either die or transform into higher grade disease through an apparent EBV-driven mechanism (unpublished observation, PF).

As discussed in the previous chapter, the Leukemia/Lymphoma Molecular Profiling Project (LLMPP) using gene expression profiling (GEP) revealed that non-neoplastic immune cells were predominantly responsible for the gene expression signatures contributing most to an outcome predictor (2). Immune response-1 (IR-1) appeared to be derived from reactive T cells in the lymph node biopsies and conferred a favorable outcome; while immune response-2 (IR-2) revealed a gene expression pattern most reminiscent of macrophages and conferred an inferior survival on FL patients. Hypotheses raised by these observations led us to demonstrate that Lymphoma-Associated Macrophage (LAM) content is an independent adverse prognostic factor in advanced-stage FL patients treated uniformly (see Section 3), further validating the impact of IR-2 on FL survival (3).

These cells may be recruited and “educated” by tumours using a range of growth factors and chemokines, so that they adopt a “trophic” role that facilitates matrix breakdown, tumour-cell motility and angiogenesis (4).

However, the precise biological function of tissue-based macrophages in this setting remains largely undefined. Macrophage heterogeneity is better studied in the mouse but less is known about human macrophages despite the increasingly number of reports on these cells (5-7). The plasticity of macrophage function is evident in the diverse range of functions of these cells, including inflammatory responses, immune reactions, tissue remodeling and morphogenesis. One of the many markers differentially expressed in the different types of macrophages is CD163, a receptor that scavenges hemoglobin by mediating endocytosis of haptoglobin-hemoglobin complexes. It is member of the scavenger receptor cysteine-rich family and exclusively expressed on cells of the monocyte lineage. CD163 expression is suppressed by pro-inflammatory mediators like lipopolysaccharide (LPS), interferon-gamma (IFNγ), and tumour necrosis factor alpha (TNFα), whereas IL6 and the anti-inflammatory cytokine IL10 strongly up-regulate CD163 mRNA in monocytes and macrophages. This expression pattern implies a functional role of CD163 in the anti-inflammatory response of monocytes, characteristic of M2 cells reported to be pro-tumoural (8) – see Sections 1 & 3.

FL is characterized by the t(14;18)(q32;q21), which juxta-poses the *BCL2* gene on chromosome 18 with the immunoglobulin heavy chain locus on chromosome 14, resulting in the constitutive expression of BCL2 protein, which is not significantly expressed by normal follicular center B cells. Although 15% of FLs are t(14;18)-negative, the translocation has been used as a genetic marker for FL. It has been postulated that it occurs as an error during VDJ rearrangement at the pre-B cell stage of differentiation in the bone marrow and is insufficient but necessary for the malignant transformation (1). Although t(14;18) translocation and ectopic BCL2 expression are critical early events in the natural history of lymphoma pathogenesis, further B cell differentiation and additional oncogenic events are required for full malignant transformation. The t(14;18) has been found frequently in the blood of healthy blood donors as well as in germinal centers of patients in the absence of manifest clinical lymphoma, and who only develop FL several years later (9, 10). The favored hypothesis regarding the pathogenesis of FL suggests that B cells harboring a t(14;18) arise at the pre-B stage in the marrow. These cells eventually enter the

circulation, home to the lymph node and upon antigenic challenge enter the germinal center where they expand and are submitted to the processes of somatic hypermutation (SHM), class-switch recombination(CSR) mediated by activation-induced cytidine deaminase (AID). Thus, the bulk of circulating t(14;18)+ cells detected in healthy individuals and in FLIS lesions is not formed by naïve B cells but with already GC-experienced and expanded t(14;18) B cell clones. They can seed the follicles and reside, ”inoffensively”, in a resting state with a low risk of proliferation, genomic instability or malignant progression. Only upon accumulation of additional genetic hits in cells “blocked” as GC like B cells, a process rich in double-strand DNA breaks. In such cells the “ectopic” expression of BCL2 will allow the hazardous rescue from physiological apoptosis, resulting in their accumulation, sustained proliferation, in and off target SHM and CSR, favouring second and subsequent additional genetic hits, some of which lead to the development of FL (11, 12).

In vitro studies have shown that precursor cells of the B cell lineage can differentiate into other cell types including tissue-based macrophages (13-15). Interestingly, it has been shown that in FL, a subpopulation of endothelial cells show the characteristic t(14;18) translocation of FL (16).

These findings may indicate, among other hypotheses, plasticity of a neoplastic precursor cell in the marrow that maintains the ability to differentiate into different mature cell types, including macrophages. In the lymph node, these cells would provide the neoplastic B cells with a trophic environment in cooperation with Tregs cells leading to a sculpted immunosuppressive intra-tumoural milieu. A subset of these monocytes/macrophages might be genetically related to lymphoma-originating cells and be recruited to lymphoma sites.

4.2 AIM

Our aim is to study intratumoural monocytes & macrophages in FL patients and verify the clonal relationship of these “non-neoplastic” cells with the malignant B cell clone.

4.3 MATERIALS & METHODS

4.3.1. Patients & Sample Selection

In this study we used BCCA´s Centre for Lymphoma Cancer clinical and pathological electronic database. All samples were reviewed by two hematopathologists (PF and RDG) based on the criteria defined by the 2008 World Health Organization classification of tumours of hematopoietic and lymphoid tissue (17). The study was performed in accordance with the Declaration of Helsinki.

The study started with a pilot study using 7 FFPET samples including cases from a cohort of patients uniformly treated with a BP-VACOP regimen described in the previous section (see Section 3). A FICTION technique was optimized using these samples. Then the study was expanded using fresh FL samples. These were sorted from consecutive fresh lymph biopsies submitted to BCCA´s Immunology laboratory during an 8-month period between October 2005 and May 2006. After all routine diagnostic proceedings were preformed following BCCA´s Lab Med protocols (http://www.bccancer.bc.ca/HPI/labservices/), touch imprints of the lymph nodes were taken. The slides were stained with Giemsa and those cases suggestive of reactive lymph nodes or low-grade follicular lymphoma were archived and correlated with the final diagnosis often involving flow cytometry, histology and cytogenetics or molecular biological ancillary studies. A proportion of the samples were submitted for FICTION (see Section 1).

4.3.2. FICTION Technique

As previously described this technique preserves cell morphology and combines immunofluorescence to detect cellular antigen and fluorescence *in-situ* hybridization (FISH) to detect chromosomal abnormalities. This technique has already been successfully applied on FFPET but with a certain level of inconsistency due to technical problems, such as poor target signal intensities and nonspecific background. These problems have been attributed to the intra- and extracellular protein cross-linkages caused by the fixation process, which result in a reduction in the accessibility of the target DNA to the specific FISH probes. Recently, a pressure-cooking method in combination with Tris-EDTA or EDTA buffer as antigen retrieval has been applied in some FISH studies on

paraffin tissue resulting in good signal intensity (18). On the other hand, heat retrieval and calcium-chelating agents such as EDTA have been shown to improve the quality of immunostaining when used on paraffin sections (19). Previous studies have identified optimal conditions of antigen retrieval in order to obtain consistently good results with the FICTION method on routinely processed tissue samples, obtaining a critical duration of antigen retrieval essential for a good equilibrium between FISH and immunofluorescence. Long periods of pressure cooking could increase FISH signal, but at the expense of also damaging the cell membrane, resulting in a decrease of the fluorescence signal (20). We used this approach as a template to optimize the FICTION protocol first in FFPET samples of the pilot study and later for touch imprints samples. The final protocol used in the study is summarized in Figure 4.1.

4.3.2.1. Paraffin Samples

Biopsy samples from FL and reactive lymph nodes were obtained from the BCCA Pathology Department archive after having been fixed in buffered formalin and paraffin embedded by conventional techniques. Six samples were FL included in the BP-VACOP study (described in Section 3) and were selected based on: 1) the presence of *BCL2* rearrangement in four of 6 cases and; 2) LAM cell content including three cases LAM-rich and three LAM-poor (3). One reactive lymph node with follicular hyperplasia was added.

Tissue sections (3 to 4um) were cut and placed on salinized slides (Dako, Glostrup, Denmark) and incubated at 56ºC overnight. The slides were then deparaffinised twice using xylene (10 min, room temperature in a hood). The slides were hydrated in a series of ethanol solutions (100%, 95% and 75% for 5min each) and then washed with PBS.

4.3.2.2. Touch Imprint Samples

In an eight month period, 388 fresh lymph node samples were screened. Eighty one FLs and 38 reactive nodes (RN) confirmed by both histology and phenotypic and/or genotypic studies were selected. Based on the quality of the touch preps and tumour cell content fifty four samples (42FLs & 12 RNs) were selected for FICTION. Touch preparations obtained from the imprints of fresh nodes were air dried for 30 to 60 minutes at room temperature. They were

archived in a freezer at -20ºC. Just before FICTION the slides were fixed in either buffered formalin or acetone for more than one hour at room temperature.

4.3.2.3. Paraffin & Touches Antigen Retrieval

Slides were first washed slowly in tap water and placed in a jar with the retrieval solution, 50mM Tris/EDTA (pH9) - Dako Target Retrieval Solution (pH 9)®., in a stainless-steel 5l pressure cooker with an operating pressure of 120 PSI at 120ºC for 2 minutes followed by 30 minutes of cooling time. Thereafter, slides were washed slowly in tap water and placed in PBS.

4.3.2.4. Immunofluorescence Staining

After checking the tissues/cells using the microscope the first antibody, CD163 (clone 10D6), monoclonal mouse antibody Novocastra (NCL-CD163), dilution 1:50, was applied (50µl/slide) with plastic lids for 30min to one hour. Slides were then washed in PBS 0,1%Tween 20 three times for 5 minutes each and the second antibody applied (50 µl /slide) for one hour in the dark, Alexa Fluor 594 (excitation/emission maxima ~590/617 nm) Goat anti-Mouse in 1:200 dilution - Invitrogen®. Subsequently, they were washed in PBS 0,1%Tween 20 three times for 5 minutes each.

Before FISH was done, slides were covered and DAPI II and coverslip and checked for positive fluorescence. Up to 50 CD163 positive cells were mapped per case using MetaSystems® platform and image analysis software.

4.3.2.5. Fluorescence In-Situ Hybridization (FISH)

Slides were placed in 1xPBS till the coverslip felt (5 minutes on average) and then dehydrated through a series of ethanol washes (70%, 80% and 100% for 2 min each). A *BCL2* probe from Vysis®, Vysis LSI BCL2 Dual Color, Break Apart Rearrangement Probe was denatured separately at 72ºC for 3 minutes and then applied to the slide and co-denatured at 65ºC for 90 seconds. It was left overnight in a humid chamber at 37ºC in the dark. A rapid first wash was done with 2xSSC/0.3% NP40 at 70ºC for 2 minutes and repeated at room temperature for 5 minutes and finally with 1xPBS for 5 minutes. The slides were dried in the dark for at least 30 minutes and covered with DAPI II (5 to10 µl) and cover-slipped and stored at 4ºC.

The breakapart probe is easier to score in tissues and smears but only documents a break and/or rearrangement within the *BCL2* locus with no information on the translocation partner. A *BCL2* fusion probe (Vysis® LSI BCL2 Dual Color, Break Apart Rearrangement Probe) was used in selected cases to confirm the *BCL2* rearrangement and translocation to the *IGH* locus.

4.3.3. Image Capturing and Analysis

Tissues and smears images were captured using the MetaSystems® platform. The whole slides were screened for both CD163 positive and negative cells and nuclei counted for FISH probe signals. Up to two hundred CD163 negative cells and 50 CD163+ cells were counted. Data were analyzed using the Statistical Software Package for the Social Sciences (SPSS version 10.1 for Windows; SPSS Inc®, Chicago, IL).

4.4 RESULTS

4.4.1. Paraffin Samples – Pilot Study

In six out of seven samples, FICTION was successful with good fluorescence and FISH signals. Bearing in mind that these are samples from early 90s this was a very promising result. Four of the six FL showed a *BCL2* translocation. In all cases with confirmed translocation we were able to confirm the rearrangement in the CD163-negative cells (Table 4.1). CD163+ cells were captured in all cases varying between 8 and 80 cells with a median of 44 cells (representing the reactive case with the highest number of cells). Most cells had normal FISH signals (two red & green signals with no split signals), with roughly 6 to 75 cells counted (median 36). Abnormal FISH signals including split probe signals or extra signals were also present in the CD163 positive cells (Figures 4.2 & 4.3) but this was not found in all CD163 expressing cells. In order to confirm that these few cells with abnormal signals were not just a technical artifact of the technique, we used in two cases a fusion probe from Vysis®, which confirmed the translocation in some cells. The abnormal FISH signals were present in very low numbers in all samples including BCL2-negative FL and in the reactive case (Figure 4.4), with 2 to 8 cells identified (median 7). The percentage of CD163+ cells with abnormal FISH signals ranged from 6% to 33% (median 18%). Despite the low number of samples and cells there was no

significant difference in the percentage of the CD163+ cells with abnormal FISH signals between FL with (median 15%, ranging 10% to 33%) or without *BCL2* rearrangement (23%) or the reactive sample (6%). Similarly, FL rich in LAM (median of 11% with abnormal FISH) were not significantly different than cases with low numbers of LAMs (median of 18% with abnormal FISH).

In conclusion, in the pilot study we have found CD163+ cells with abnormal signals (Figures 4.2 to 4.4) but the overall results showed no clear evidence that macrophages could harbour the same translocation characteristic of the malignant cells.

There are several reasons that might explain these results. First, there were limited or only very rare cells that could be clearly evaluated by FICTION, as many of the cells showed partial or completely overlapping nuclei making it impossible to evaluate the FISH signals. Secondly, a loss in intensity of the fluorescence signal which also reduced the number of evaluable cells and lastly overlap of nuclei in most of the tissue sections. The number of cells with abnormal signals was very limited in all cases (although considerable in relation to the total number of cells assessed) and not significant in terms of the FISH analysis in FFPET. In most studies using commercially available probes like the ones used in this study of whole tissue sections, the threshold value to consider a positive result above background is about 20% for breakapart probe signals.

Thus, we expanded our study changing the type of samples, trying to avoid the problem with overlapping nuclei present in FFPET sections.

4.4.2. Touch Imprints Samples

FICTION was performed using 54 samples including 42 FLs and 12 RNs. After optimization of the technique in both reactive and FL samples, we achieved successful results in 29 samples and the technique failed in 25. The vast majority of the latter were due to poor fixation of the cells resulting in deficient antigen retrieval and very limited sample to be analyzed with rare CD163+ cells to be evaluated.

Interestingly, some of the features were related to the sample type as only 2 (17%) out of 12 RN failed while 23 (55%) out of 42 FL failed. Of the 19 FLs with results 14 had a *BCL2* translocation and 5 were negative for this rearrangement. In all cases with confirmed *BCL2* translocation we were able to

confirm the rearrangement in the CD163 negative cells (Table 4.2). CD163+ cells were captured in all cases varying between 5 to 148 cells with a median of 21 cells. Most cells had normal FISH signals (two red & green signals with no split signals), varying between 4 to 100 cells (median 21). Abnormal FISH signals including split probe signals or extra signals also present in the positive CD163 cells but not found in all CD163+ cells. CD163+ cells with abnormal signals were present in very low numbers in all FL samples with or without *BCL2* rearrangement, varying from 1 to 48 cells (median 4). No abnormal signals, suggestive of split signals were observed in the RNs. The percentage of CD163+ cells with abnormal FISH signals ranged from 2% to 33% (median 21%).

In the 10 RNs the total cells assessed was between 5 to 50 cells with no cells showing documented abnormal signal constellations (Figures 4.5 & 4.6).

In the five FL cases negative for *BCL2* rearrangement total, CD163+ cells ranged from 5 to 51 cells and from these only 1 to 3 cells had abnormal FISH signals. The percentage of these cells ranged from 2 to 20% (Figure 4.7).

In the fourteen FL with *BCL2* rearrangement, total CD163+ cells ranged from 6 to 148 cells and from these between 2 to 48 cells had abnormal FISH signals. The percentage of these cells ranged from 16 to 33% (Figures 4.8 to 4.15).

The differences in the cell content of CD163+ cells with abnormal FISH between the three groups, reactive LNs, FL with or without *BCL2* rearrangement were significant for the number of cells with FISH abnormal signals (χ^2, $P<0.001$) as well as the percentage of these cells (χ^2, $P=0.001$) but not for the total number of cells assessed (χ^2, $P=0.74$). *BCL2*+ FL had significant more cells and higher percentage of CD163+ with abnormal FISH has both *BCL2*-negative lymphomas and reactive lymph nodes.

4.5 DISCUSSION

In this study we describe convincing preliminary evidence suggesting that a minority of LAMs appear to harbour the *BCL2* translocation characteristic of the FL B cells. This is not a new finding as Streubel *et al.* previously reported that microvascular endothelial cells within FL samples shared the *BCL2* rearrangement (16). Using various markers, they found that cells lining the

microvasculature and expressing an endothelial phenotype, in most cases using CD31 but also CD34, von Willebrand factor or *Ulex europaeus* lectin, also shared the genetic hallmark of FL B cells. Interestingly, CD31 is also expressed by a subset of circulating monocytes with angiogenic functions that are recruited into hypoxic tissues including tumours (21).

Four mechanisms can be considered to explain our findings:

First, the reported findings of macrophages with abnormal FISH signals indicating *BCL2* translocation could represent a technical artifact resulting from nuclei of lymphoma cells phagocytized by macrophages, thus explaining why only a minority of cells show the abnormal signals. Furthermore, the pattern of signals observed in CD163+ and CD163 – are identical for each case (Figures 4.9 & 4.10). This is a very strong argument favouring FL nuclei within LAM cytoplasm, which makes mandatory the validation of these results using other techniques such as cell sorting followed by cell culture and repeat FISH studies. Yet, the significant differences found in the abnormal signals between cases positive and negative for *BCL2* rearrangement, independent of the number of cells assessed, favours that these abnormal signals reside within macrophages.

Second, cell fusion could explain the results (22, 23). If true then it should result in tetraploid karyotype, an event easily detected using a technique such as FICTION. However, some reports have shown that tetraploid hybrids undergo reduction divisions resulting in diploid daughter cells (23).

Third, eventual gene transfer could result from uptake of apoptotic bodies from lymphoma cells (24).

Finally, both FL cells and macrophages derive from a common precursor cell in the bone marrow. Cells of the hematopoietic system are derived from common precursors that differentiate into lineages with distinct morphologic, immunophenotypic, and functional characteristics (25). Most theories of hematopoietic cell differentiation have proposed that as cells differentiate fully, they become “lineage committed.” Recent data suggests there is more plasticity between the B cell and myeloid lineages than previously thought (26). This plasticity is associated with changes in transcription factor expression and/or activity. Within the lymphoid system, C/EBPα was shown not only to divert uncommitted progenitors to the myeloid lineages but also to induce the transdifferentiation of committed B lymphocytes into functional macrophages

that still retain immunoglobulin gene rearrangements as evidence of their B cell origin (15). This transdifferentiation of mature B cells into macrophages is associated with inhibition of the B cell commitment transcription factor PAX5 (27). Conditional deletion of *PAX5* causes mature B cells to dedifferentiate into uncommitted precursors in the bone marrow (28). PAX5-deficient progenitors carrying rearranged *IG* genes could differentiate into myeloid cells and T cells, but not into B cells. Moreover, bipotential B-macrophage progenitors have been demonstrated in adult bone marrow from mice (29). Hou *et al.* reported the identification in healthy human bone marrow specimens of a small fraction of primitive $CD34^{+}/CD19^{+}/CXCL4^{-}$ cells termed B cell/myeloid progenitors (BMPs) (30). When placed in B-lymphoid culture conditions, BMPs acquired characteristics of mature B cells, including surface light chain expression. In myeloid culture conditions, however, the BMPs formed myeloid colonies. This plasticity of hematopoietic cells observed in experimental models in which modulation of specific transcription factors can reprogram cells to enter different differentiation pathways have been shown to recapitulate in the clinical setting. It has been reported previously that two different hematopoietic populations in the same patient may share identical genetic changes or abnormalities, raising the possibility that tumours expressing the phenotype of one hematopoietic lineage might “transdifferentiate” into a genetically similar but phenotypically distinct tumour of a different lineage. Recently, the occurrence of histiocytic and dendritic cell (H/DC) sarcomas has been described in patients with FL in which the sarcomas shared both the *IGH* and *BCL2* rearrangements with the FL. This may represent a de-differentiation event of the B cell due to loss of PAX5 activity (31). All evaluable H/DC tumours were negative for PAX5, despite genotypic evidence of a B cell derivation. In contrast, CEBPβ and PU.1 were highly expressed in the same tumours and both of these transcription factors play a major role in mediating macrophage and myeloid differentiation (27).

Interestingly, sporadic H/DC tumours as well as Langerhans cell histiocytosis (LCH) have also shown *IGH* gene rearrangements at a higher frequency than expected: 30% (14/46) of LCHs cases had clonal *IGH*@ (4 cases), *IGK*@ (5 cases) or *TRG*@ (9 cases) gene rearrangements, respectively, but failed to express T cell or B cell lineage-specific or associated markers (32). 39% (9/23) of cases of H/DC sarcomas showed clonal *IGH* (+/-IGK) gene rearrangements,

whereas 2 (9%) cases showed only clonal *IGK* gene rearrangements, which were further validated and confirmed by direct DNA sequencing. One histiocytic sarcoma also showed t(14;18). Notably, all H/DC sarcomas were negative for PAX5 (33). The results provide genotypic evidence supporting the notion of lineage plasticity of hematopoietic cells and their associated neoplasms. H/DC tumours contained both clonal *IGH* gene rearrangements and t(14;18), both which are believed to occur as recombinase-mediated events in the bone marrow in the pre-B stage of differentiation by normal or illegitimate *VDJ* rearrangement, respectively (34). Thus, a postulated common precursor would be differentiated at least to the point of recombination activating gene (*RAG*) complex activity. It is well established that clonal expansion of B cells carrying the t(14;18) translocation are commonly detected in healthy persons, termed FL-like B cells (9, 35) . These circulating clonal cells which are much more common than FL, appear to lack additional oncogenic events to develop into clinical FL. Yet, they represent the pool of cells expressing high levels of BCL2 and having already passed through the germinal center from which secondary hits in germinal centers reactions can drive these cells toward FL as well as eventually modifying transcription factors may induce reprograming into macrophage phenotype with pro-lymphoma features. Cobaleda *et al.* showed that Bcl2-mediated survival cooperated with pax5 loss in a murine model, leading to pax5-deficient progenitors carrying rearranged IG genes that could differentiate into myeloid cells (28).

Thus, macrophages with *BCL2* translocation may have resulted from a cell already committed to the lymphoid B cell lineage. These cells have been shown to have enormous plasticity when PAX5 is deleted or down regulated and can give rise to other cell lineages including macrophages (13). We may even speculate that this transdifferentiation could eventually happen in the FL microenvironment as most CD163+ cells with an abnormal FISH signal showed an identical pattern present in the malignant cells, favouring a close clonal relation.

4.6 CONCLUSION

Despite the limitations of the study our findings are still provocative and may indicate lymphoma precursor cell plasticity and ability to differentiate into mature-like monocytes & macrophages providing the neoplastic B cells with a trophic / pro-survival microenvironment. Further validation is mandatory to convincingly demonstrate these findings.

4.7 TABLES

CASE	GRADE	LAM content	BCL2 Rear	CD163 NEG cells			CD163 POS cells		
				FISH NEG	FISH POS	% FISH POS	FISH NEG	FISH POS	% FISH POS
FL	FL1	High	POS	20	80	80	68	8	10
FL	FL2	High	POS	65	43	40	47	7	12
FL	FL1	Low	POS	25	75	75	6	8	25
FL	FL1	Low	POS	43	36	46	14	7	33
FL	FL1	Low	NEG	89	2	2	26	8	23
RFH	RFH	RFH	NEG	100	1	1	75	5	6

Table 4.1 – FICTION results of formalin fixed paraffin embedded samples.

CASE	GRADE	BCL2 Rearrangement	CD163 NEG cells			CD163 POS cells		
			FISH NEG	FISH POS	% FISH POS	FISH NEG	FISH POS	% FISH POS
FL	FL1	NEG	34	2	5	48	2	4
FL	FL2	NEG	65	3	4	48	3	5
FL	FL2	NEG	23	1	4	4	1	20
FL	FL3a	NEG	15	0	0	49	1	2
FL	FL1	NEG	10	0	0	18	1	5
FL	FL1	POS	21	65	75	18	6	25
FL	FL2	POS	45	54	54	4	2	33
FL	FL1	POS	65	45	40	15	6	28
FL	FL2	POS	87	35	28	6	2	25
FL	FL1	POS	56	90	61	42	9	17
FL	FL2	POS	45	76	62	20	5	20
FL	FL1	POS	78	60	43	16	4	20
FL	FL2	POS	23	45	66	94	32	25
FL	FL1	POS	98	78	44	5	2	28
FL	FL1	POS	67	56	45	100	48	32
FL	FL1	POS	87	32	26	25	8	24
FL	FL2	POS	78	34	30	13	5	27
FL	FL2	POS	98	56	36	10	2	16
FL	FL1	POS	30	111	78	48	24	33
RFH	RFH	NEG	98	2	2	21	0	0
RFH	RFH	NEG	155	3	1	5	0	0
RFH	RFH	NEG	123	0	0	19	0	0
RFH	RFH	NEG	98	1	1	8	0	0
RFH	RFH	NEG	148	0	0	30	0	0
RFH	RFH	NEG	121	0	0	32	0	0
RFH	RFH	NEG	89	0	0	50	0	0
RFH	RFH	NEG	165	3	1	15	0	0
RFH	RFH	NEG	198	2	1	20	0	0
RFH	RFH	NEG	134	1	0	15	0	0

Table 4.2 - FICTION results of touch imprints from fresh samples

4.8 FIGURES.

MonoMacs FICTION Protocol

Paraffin samples

1.	56ºC overnight	
2.	Deparaffinize – Xylene (2x)	10 min Room Temp
3.	ETOH – 100% / 95% / 75%	5 min each

Smears samples

1.	Air dried	>30 minutes
2.	Freezer (-20ºC)	
3.	Formalin or Acetone fixative	>1 Hour

Paraffin & Smears samples

1.	Wash (tap water, slowly)	
2.	Jar with Buffer TRS High pH (DAKO)	
3.	Pressure Cooker	
4.	Wash (tap water, slowly)	
5.	Check tissues/cells in the microscope	
6.	First Antibody (50ul/slide) with plastic lids on top	30 min/1hour
7.	Wash 3x PBS 0.1%Tween 20	5 min
8.	Second Antibody (50ul/slide) with plastic lids on top	30 min/1hour
9.	Wash 3x PBS 0.1%Tween 20	
10.	Check fluorescence – DAPI II & coverslip	
11.	Map cells	
12.	1x PBS (let coverslip fall)	5 min
13.	ETOH – 70% / 80% / 100%	2 min each
14.	Denature probe separately 72ºC	3 min
15.	Apply probe on slide and co-denature	90 secs
16.	Overnight 37ºC	
17.	Wash 2xSSC/0.3%% NP40 - 70ºC	2 min
18.	Wash 2xSSC/0.3%% NP40 – Room temp	5 min
19.	1x PBS – Room temp	5 min
20.	Dry in the dark	30 min
21.	DAPI II (5-10µl)	
22.	Store at 4ºC	

Figure 4.1 – Optimized FICTION protocol for detection of CD163 positive cells in FFPET and touch imprints.

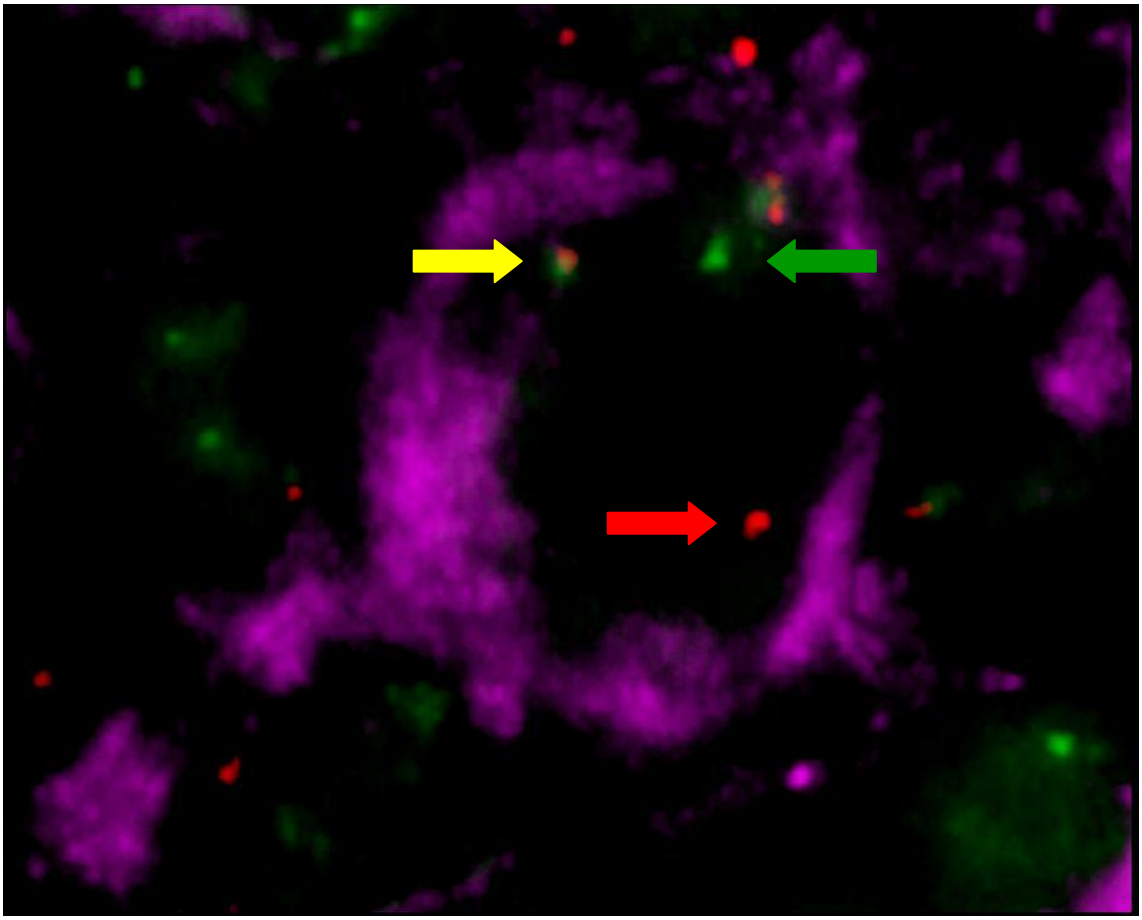

Figure 4.2 - FICTION analysis of one BCL2+ FFPE FL. A CD163 positive cell (purple) with one fused signal and one split apart signal (Vysis® LSI BCL2 Dual Color, Break Apart Rearrangement Probe).

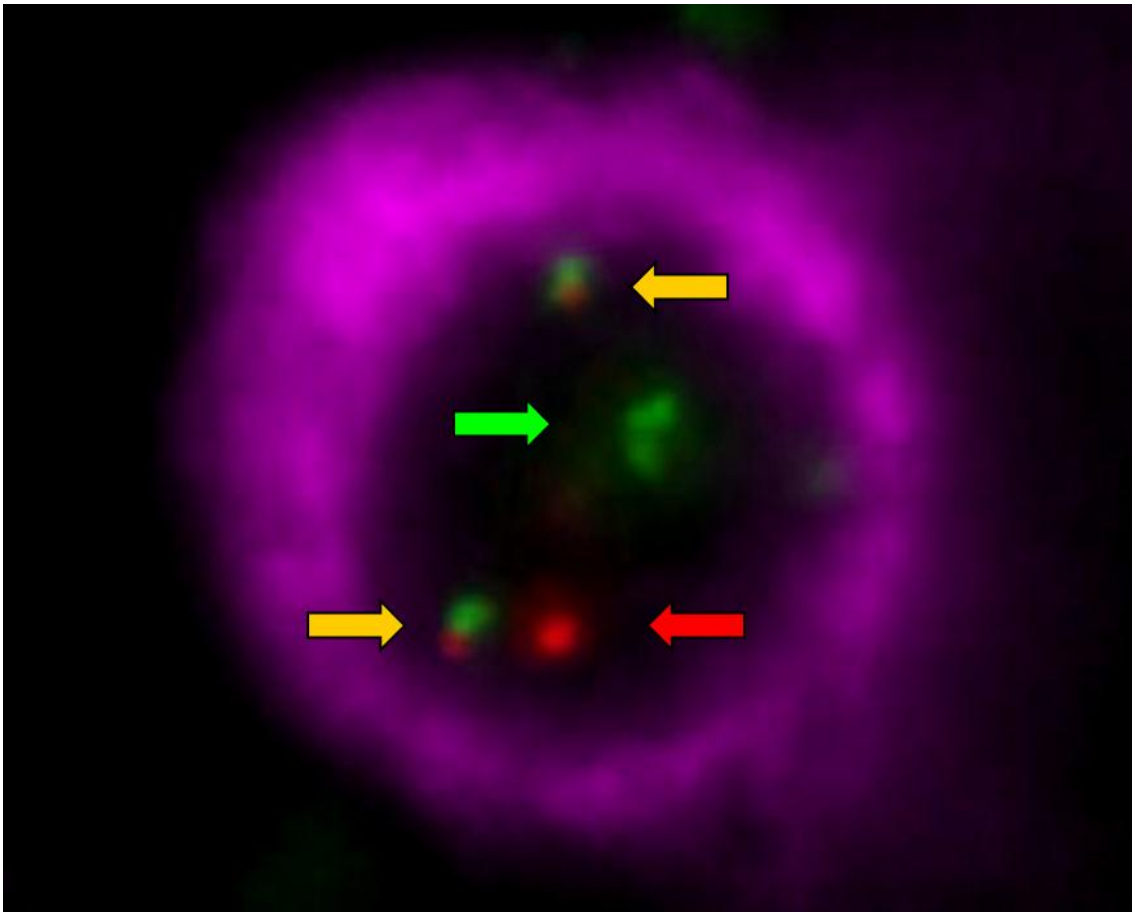

Figure 4.3 - FICTION analysis of one BCL2+ FFPE FL. A CD163 positive cell (purple) with two fusion signals using a Vysis® LSI IGH/BCL2 Dual Color, Dual Fusion Translocation Probe. This probe was used in selected cases to confirm the presence of fused signals IGH & BCL2.

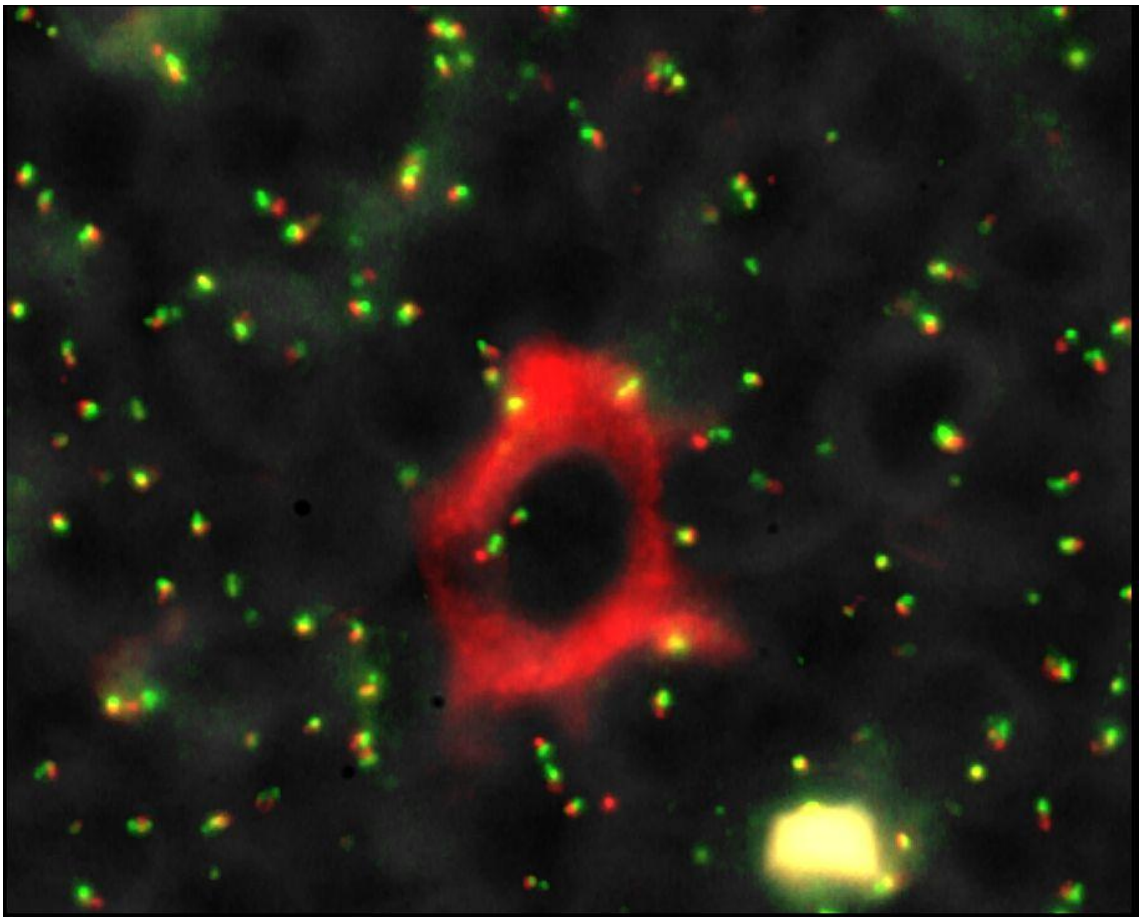

Figure 4.4 - FICTION analysis of one reactive FFPE LN. A CD163 positive cell (red) with two fused signals (Vysis® LSI BCL2 Dual Color, Break Apart Rearrangement Probe).

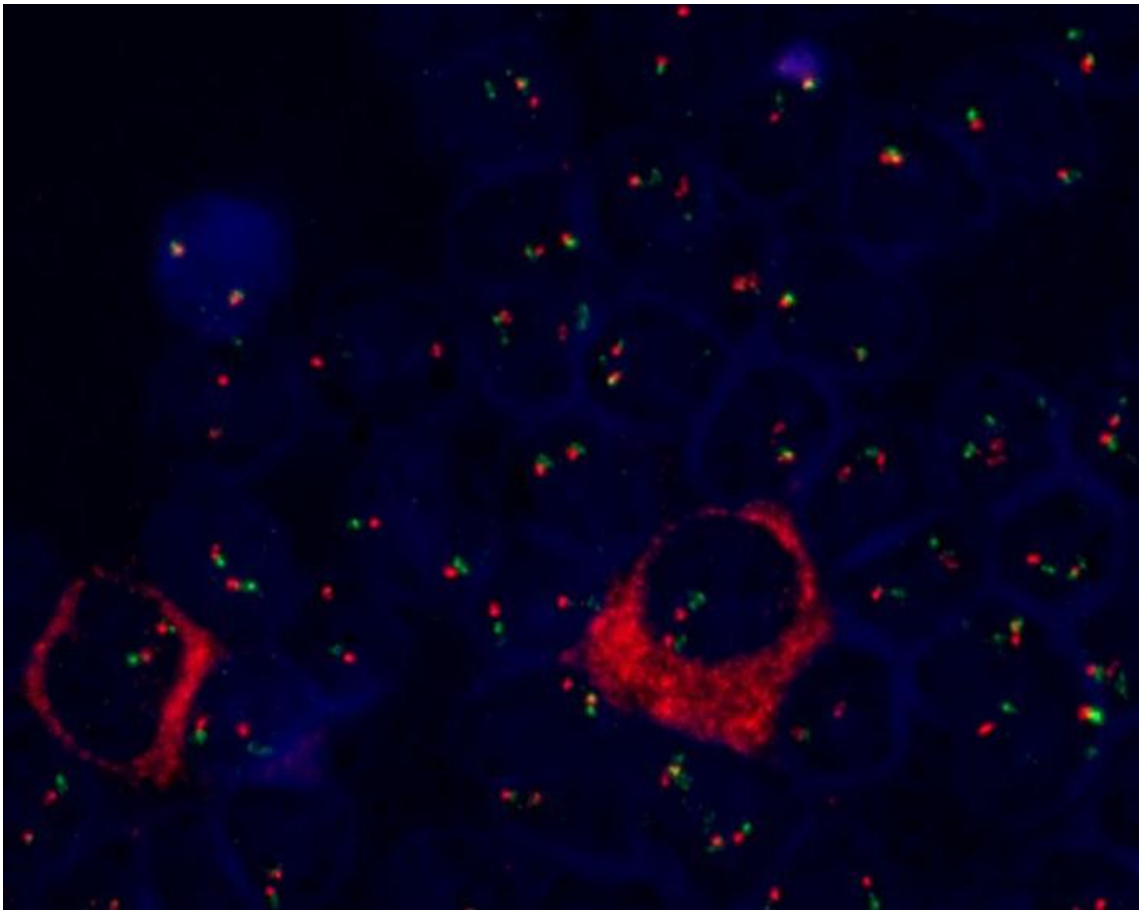

Figure 4.5 - FICTION analysis of one Reactive Fresh LN. Two CD163 positive cells (red) with two fused signals (Vysis® LSI BCL2 Dual Color, Break Apart Rearrangement Probe).

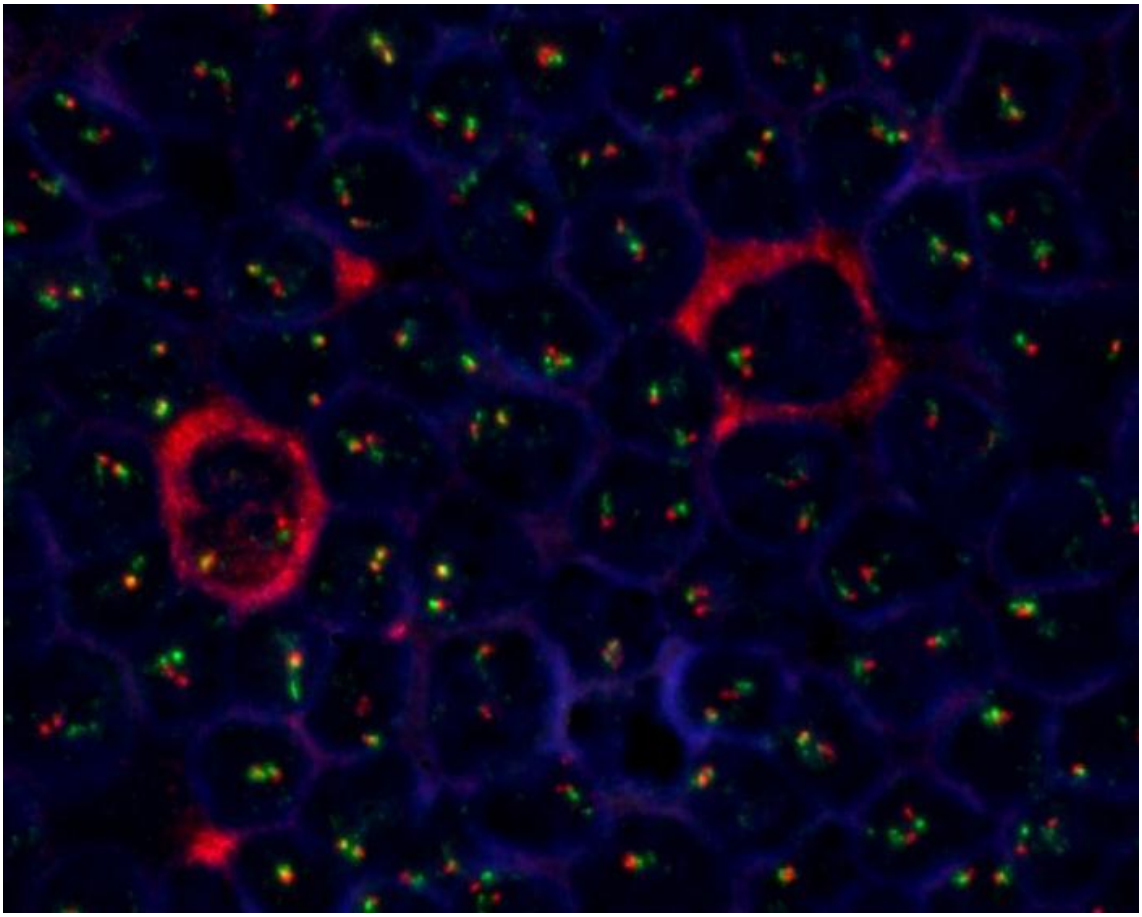

Figure 4.6 - FICTION analysis of one Reactive Fresh LN. Two CD163 positive cells (red) with two fused signals (Vysis® LSI BCL2 Dual Color, Break Apart Rearrangement Probe).

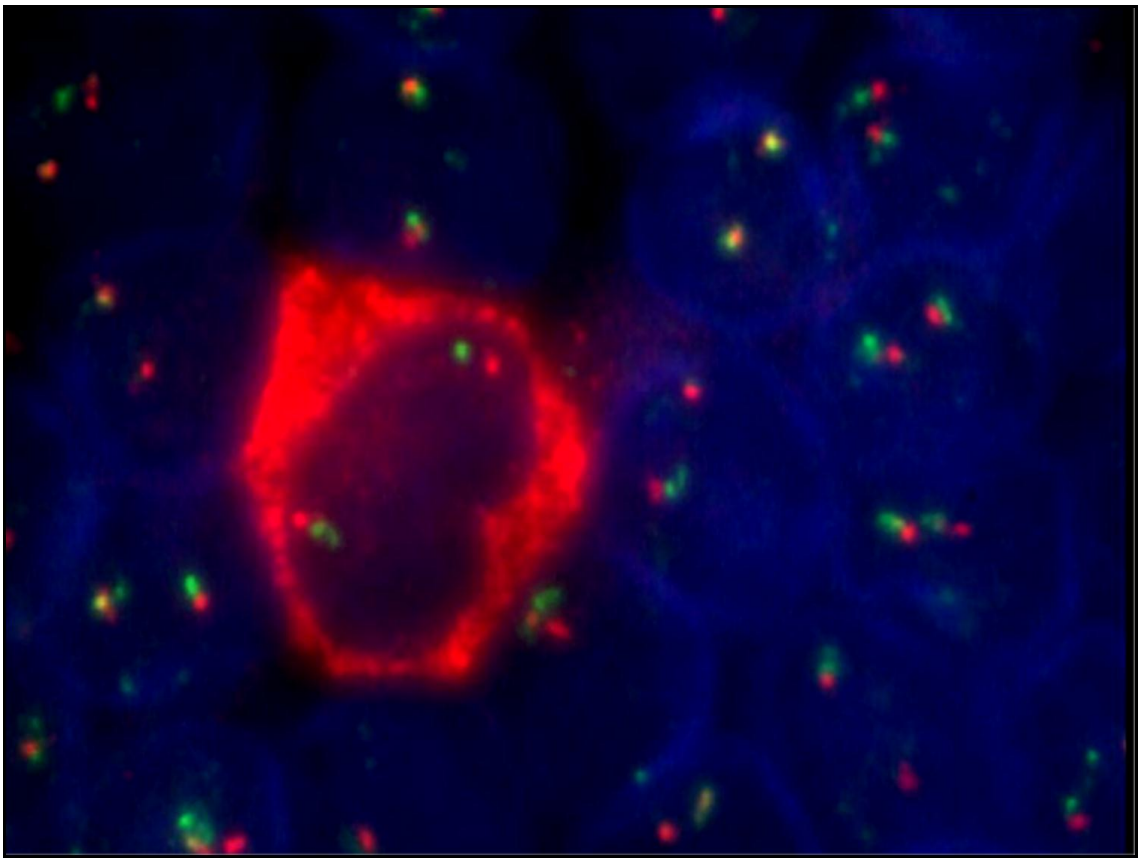

Figure 4.7 - FICTION analysis of one BCL2 negative Fresh FL. One CD163 positive cell (red) with two fused signals (Vysis® LSI BCL2 Dual Color, Break Apart Rearrangement Probe).

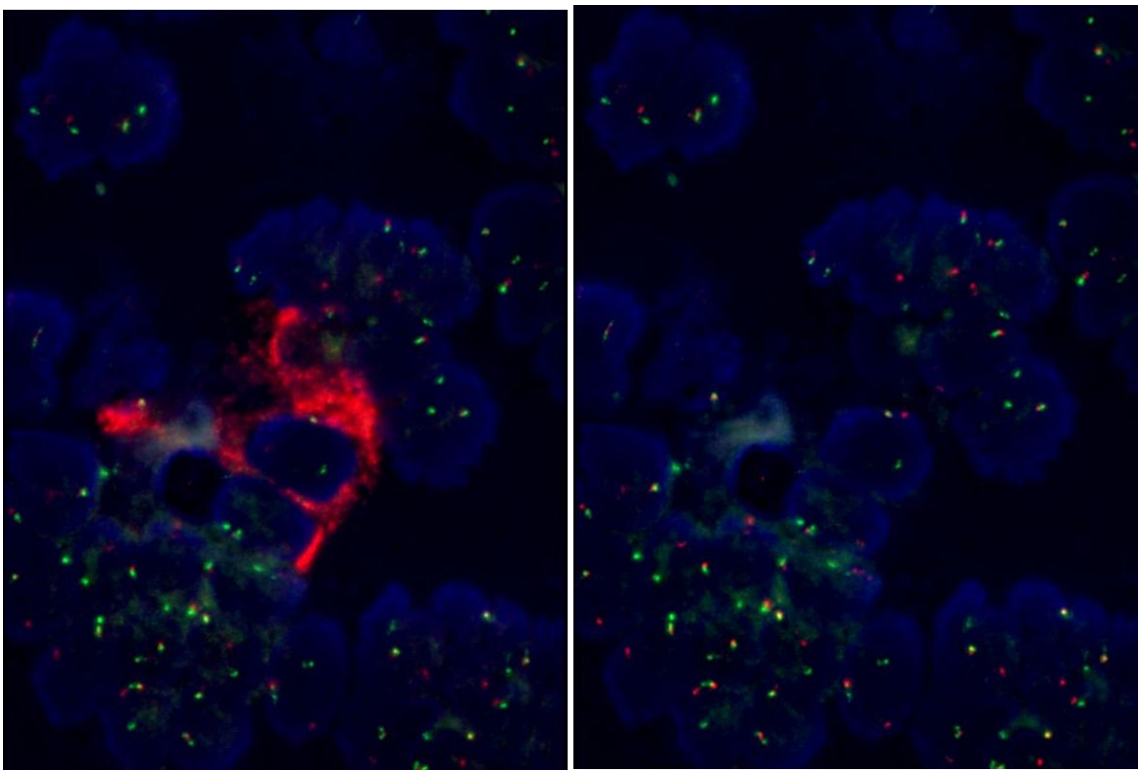

Figure 4.8 - FICTION analysis of one BCL2+ Fresh FL. One CD163 positive cell (red) with one fused and one split signals (Vysis® LSI BCL2 Dual Color, Break Apart Rearrangement Probe).

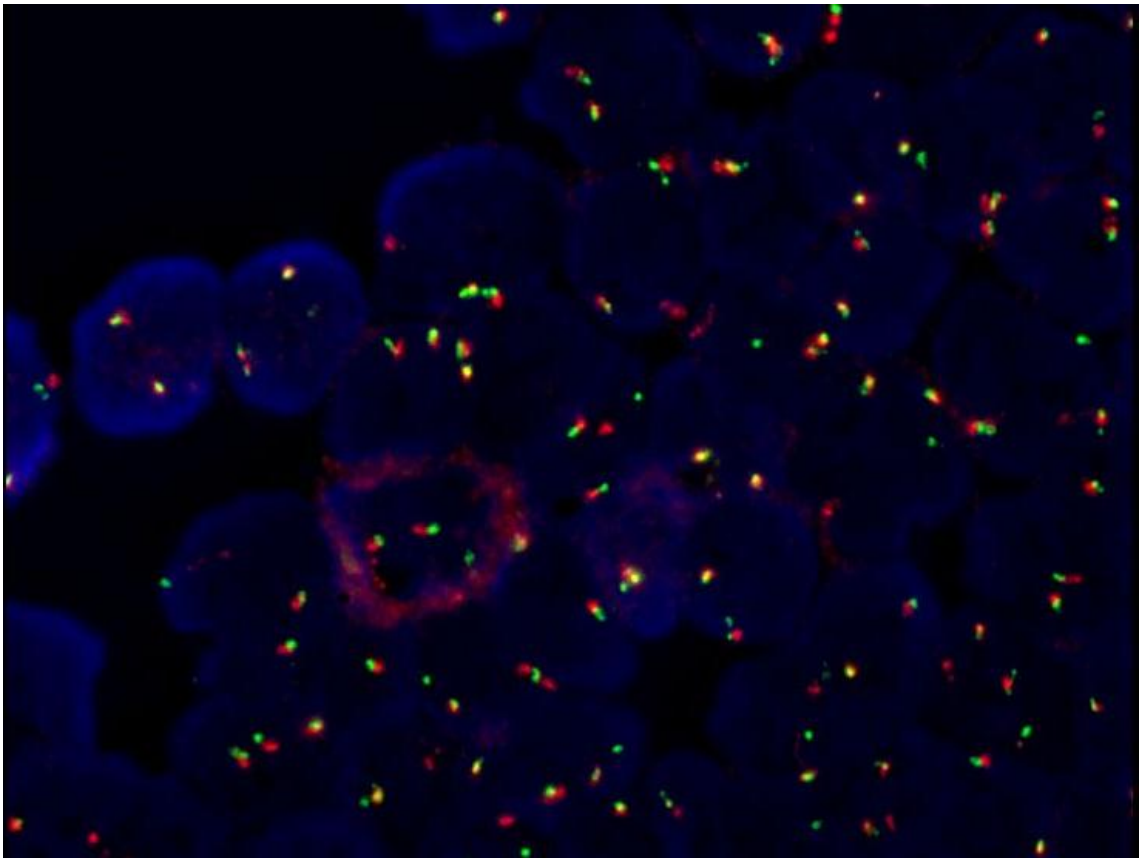

Figure 4.9 - FICTION analysis of one BCL2+ Fresh FL. One CD163 positive cell (red) with two fused and one extra green signal (Vysis® LSI BCL2 Dual Color, Break Apart Rearrangement Probe).

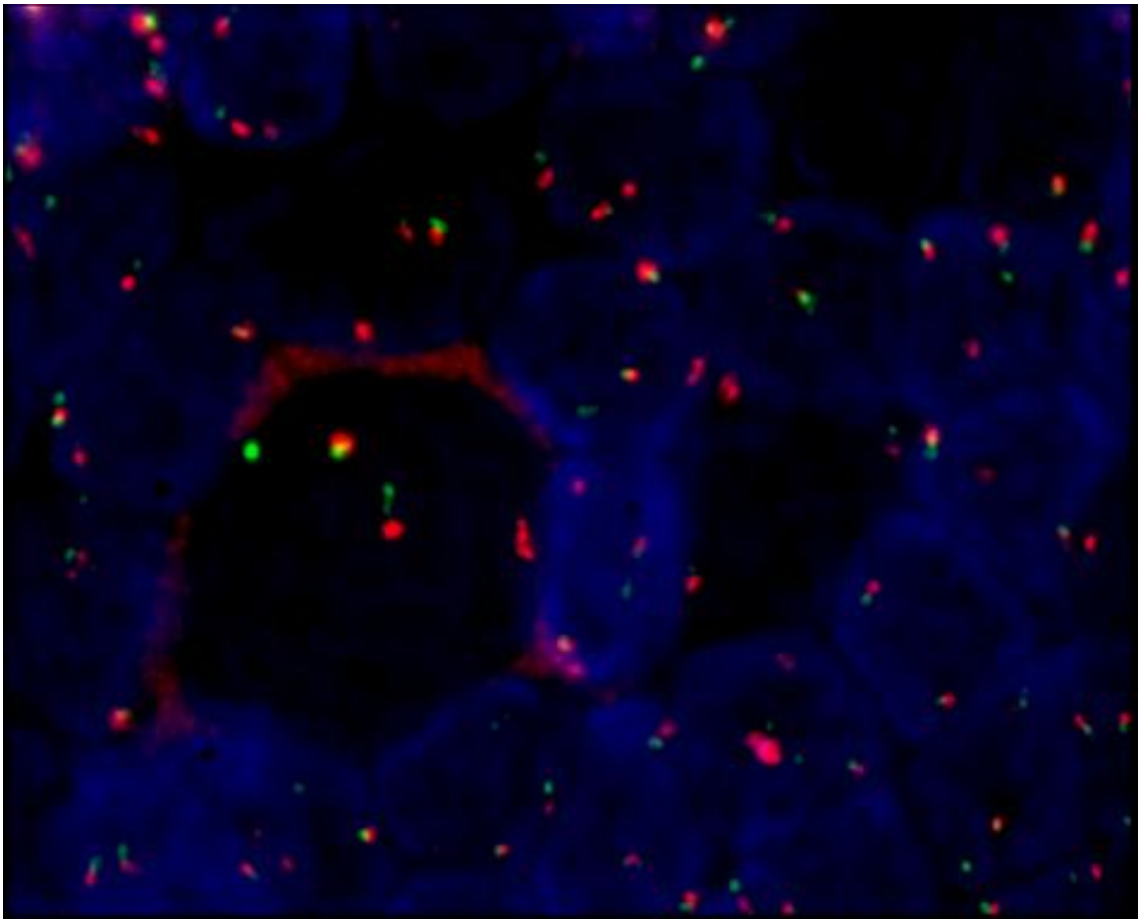

Figure 4.10 - FICTION analysis of one BCL2+ Fresh FL. One CD163 positive cell (red) with two fused and one extra green signal (Vysis® LSI BCL2 Dual Color, Break Apart Rearrangement Probe).

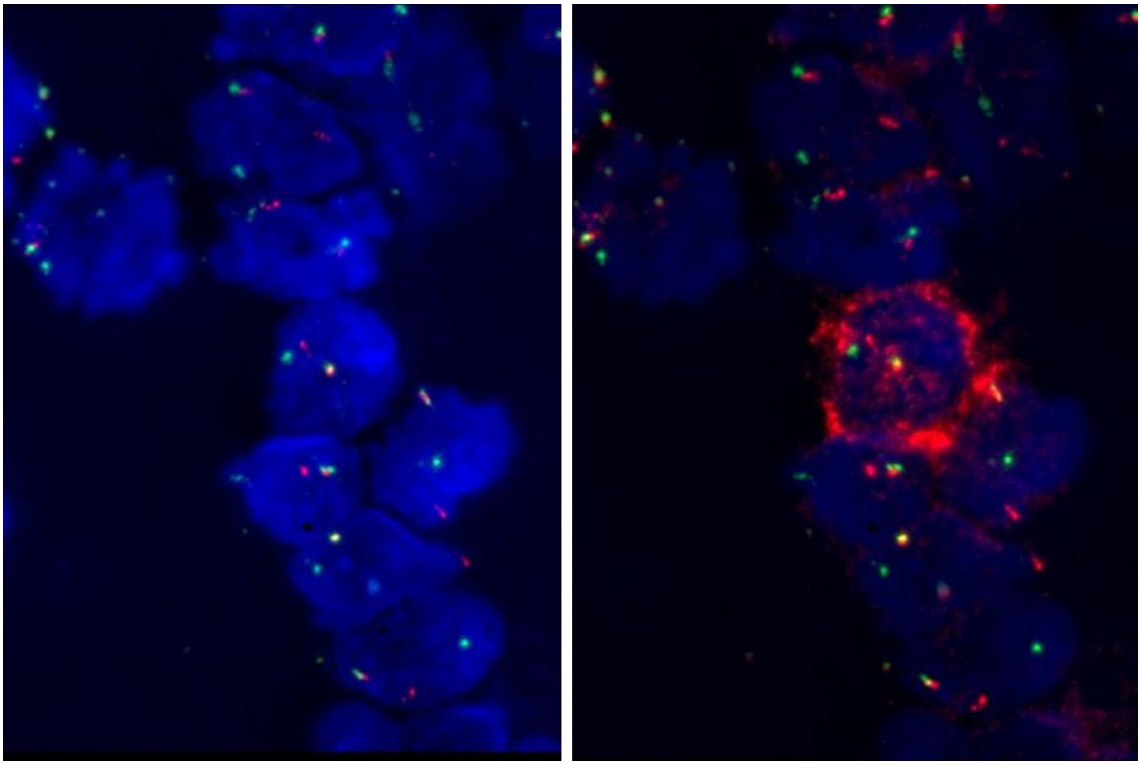

Figure 4.11 - FICTION analysis of one BCL2+ Fresh FL. One CD163 positive cell (red) with one fused and one split signals (Vysis® LSI BCL2 Dual Color, Break Apart Rearrangement Probe).

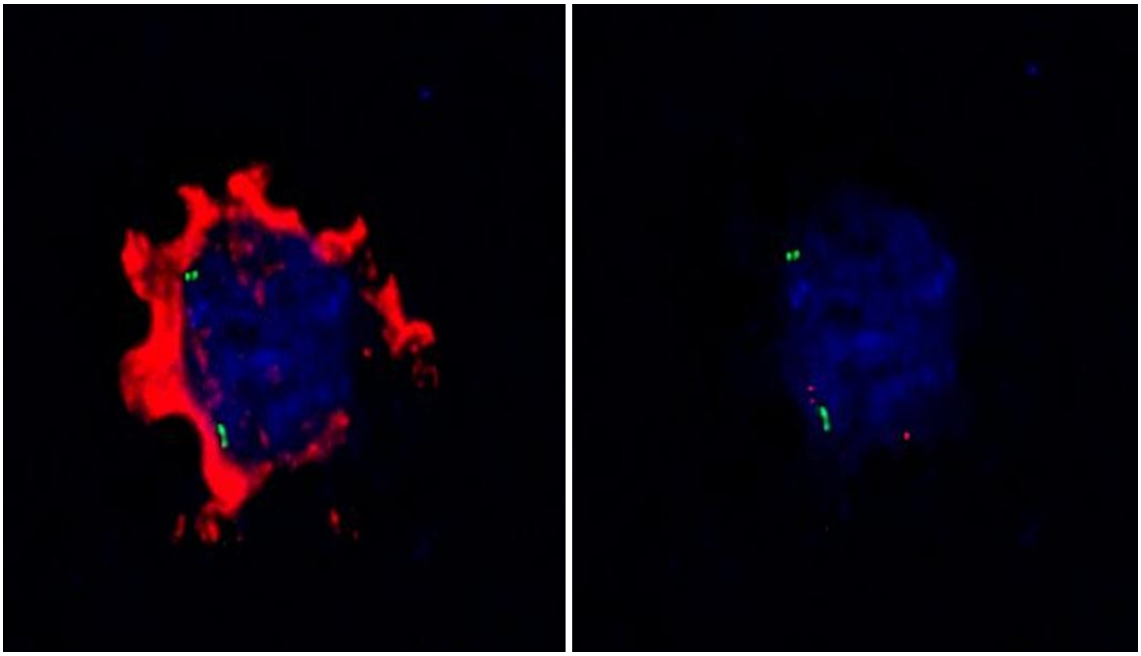

Figure 4.12 - FICTION analysis of one BCL2+ Fresh FL. One CD163 positive cell (red) with one fused and one split signals (Vysis® LSI BCL2 Dual Color, Break Apart Rearrangement Probe).

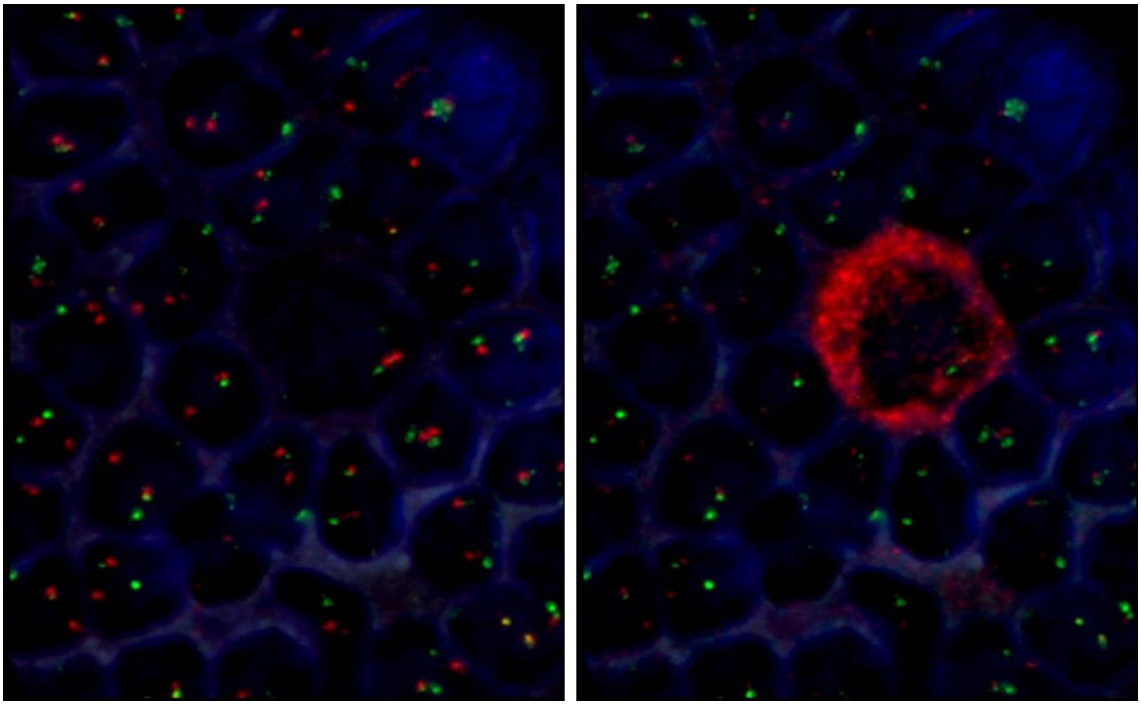

Figure 4.13 - FICTION analysis of one BCL2+ Fresh FL. One CD163 positive cell (red) with one fused and one split signals (Vysis® LSI BCL2 Dual Color, Break Apart Rearrangement Probe).

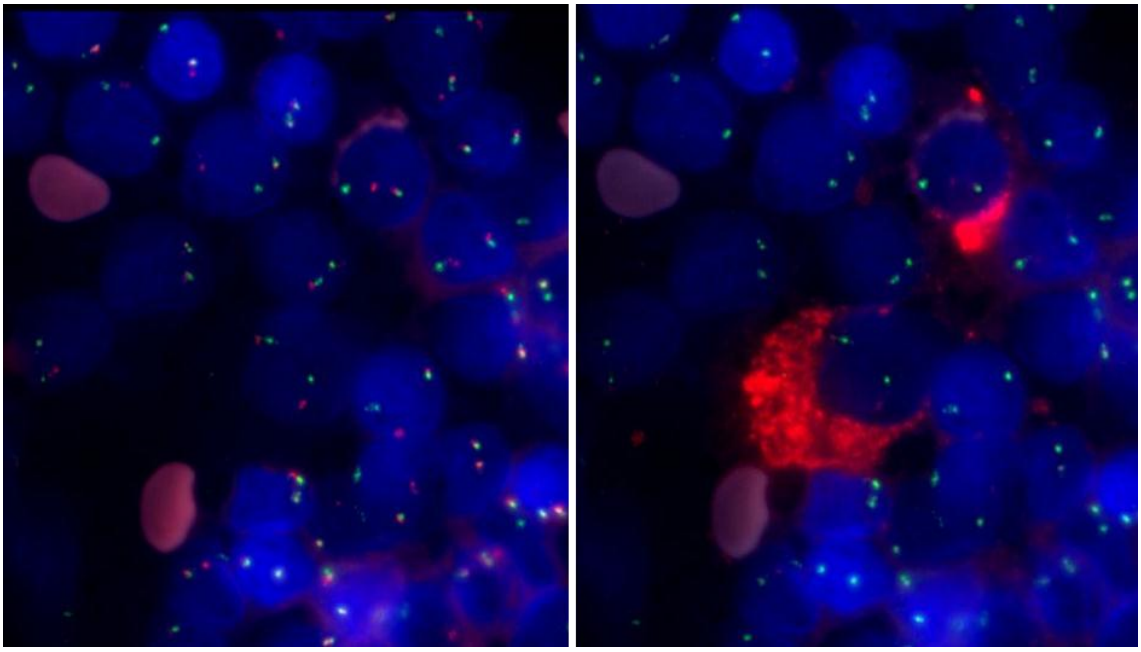

Figure 4.14 - FICTION analysis of one BCL2+ Fresh FL. Two CD163 positive cells (red) with one fused and one split signal (Vysis® LSI BCL2 Dual Color, Break Apart Rearrangement Probe).

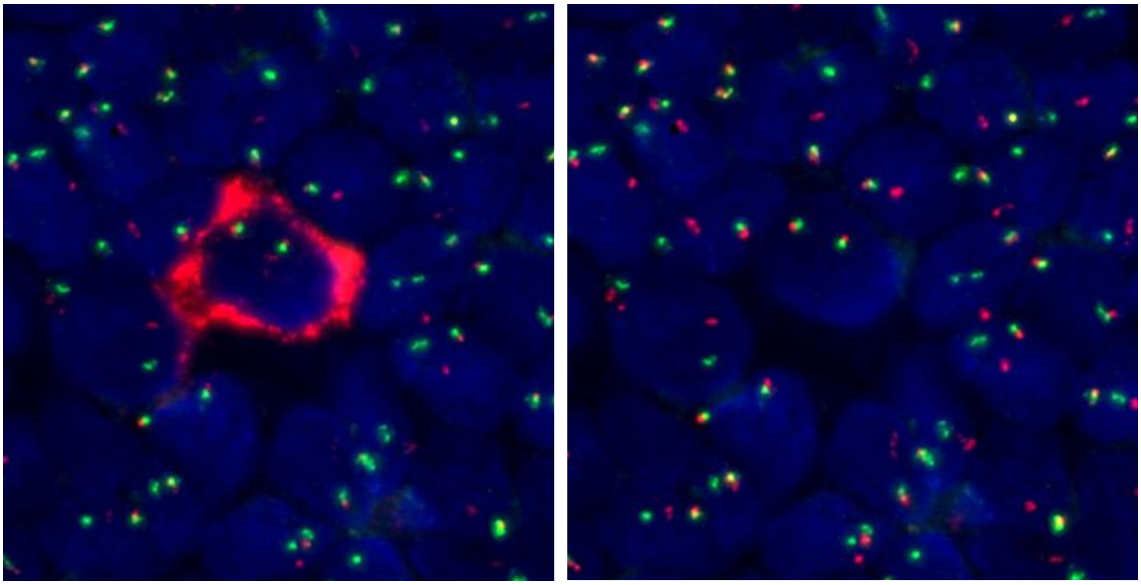

Figure 4.15 - FICTION analysis of one BCL2+ Fresh FL. One CD163 positive cell (red) with two fused signals in a background of CD163 negative cells with one fused and one split signal. (Vysis® LSI BCL2 Dual Color, Break Apart Rearrangement Probe).

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Section 5: New Biological Predictors of Survival in Diffuse Large B Cell Lymphoma

5.1 INTRODUCTION

Diffuse large B cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin lymphoma, accounting for 30-40% of newly-diagnosed cases (1). Although curable in the majority of cases with anthracycline-based combination chemotherapy and the monoclonal antibody rituximab (R), approximately 30-40% of patients with DLBCL will relapse after standard first-line therapy (2, 3). This variability in clinical outcome likely relates to genetic heterogeneity within DLBCL, reflected in a wide array of cytogenetic and molecular abnormalities. Recently, microarray gene expression profiling studies have identified multiple genes of potential prognostic significance in DLBCL, and have led to the subdivision of DLBCL into two major biological categories based on presumed cell of origin: germinal centre B cell (GCB), and activated B cell (ABC) (4, 5). The cell-of-origin (COO) distinction provides a prognostic and biologically relevant sub-classification of DLBCL and was subsequently validated at the protein level as germinal center B cell-like (GCB) and non-GCB-like DLBCL subtypes (6). However, the prognostic value of biological markers in DLBCL remains controversial, and these have not yet been incorporated into routine clinical practice (7).

Previous to the introduction of R as standard of care (R-CHOP), several biologic factors have been studied as prognostic factors in DLBCL, including *TP53* mutations or TP53 overexpression, *BCL2* rearrangement or BCL2 expression, BCL6 expression and *BCL6* rearrangement, CD10 and COO in an effort to better understand lymphomagenesis and to potentially identify patients who may benefit from specific treatment approaches (6, 8-13).

Since the introduction of R-CHOP as the current standard of care for the treatment of DLBCL, two established biomarkers, BCL2 and BCL6 expression, appear to have lost their prognostic significance (14, 15). The specific mechanism(s) responsible for this effect is/are largely unknown. Rituximab may preferentially prevent chemotherapy failure in DLBCLs that express BCL2

protein or fail to express BCL6. It is known that BCL2 over-expression and absence of BCL6 protein are more common in the non-GCB subtype. Thus, R may benefit mostly non-GCB cases, which were associated with an inferior outcome in the pre-R era.

As therapy changes, all previously identified biomarkers need to be re-evaluated. Moreover, new candidate prognostic markers need to be identified in order to make improvements in risk-stratification and better define the biology of treatment failure.

In this chapter I will focus on previously identified and new candidate biomarkers in DLBCL treated with R-CHOP:

TP53 mutation and expression (Section 5.1)

Cell-Of-Origin distinctions (Section 5.2)

BCL6 rearrangements (Section 5.3)

MYC rearrangements (Section 5.4)

Section 5.1: Strong TP53 expression is an independent predictor of outcome in *de novo* diffuse large B cell lymphoma treated with either CHOP or R-CHOP

S5.1.1 INTRODUCTION

TP53 is the most extensively studied tumor suppressor gene and a key regulator of cellular homeostasis. It can be activated by different stimuli associated with tumorigenesis, resulting in the inhibition of the tumour cell growth. This inhibition can involve a number of different cellular pathways, including cell-cycle arrest, senescence, differentiation and apoptosis (16) (http://p53.free.fr/). And not surprisingly, it is lost or mutated in a sizable proportion of cases of almost all cancer types, including lymphoma.

TP53 has 11 exons, 10 of them coding. It is a transcription factor with an important central DNA binding domain that is the target of approximately 95% of *TP53* mutations found in human cancers (Figure 5.1.1). These are mostly point mutations, of which 80% are missense. In cancer, TP53 function is lost due to mutation of the gene in about half of all human tumours and there are many

reports correlating the presence of mutant *TP53* with more advanced cancer and worse prognosis (16, 17). 95% of the mutations target in the central DNA binding domain of the protein (amino acids 100–300) (16). In most of the cases, these mutations are single amino acid substitutions in the form of point missense mutations, leading to the synthesis of a stable but functionally inactive protein that tetramerizes with wild-type protein, inducing its accumulation in the nucleus via a so-called "dominant negative effect" (18, 19). These mutated TP53 (missense) proteins have a prolonged half-life compared with wild-type (wt) protein as a result of protein stabilization. Thus, in many studies TP53 protein over-expression has been used as a surrogate marker of mutated *TP53.* Almost every residue of the DNA binding domain has been found mutated, but the end result of different mutations in the function of the protein is variable. Six well-studied hot spots for mutation comprise 30% of the mutations (http://p53.free.fr/). Thus, this selection bias for certain mutations in cancer suggests inactivation of the protein and that the dominant-negative effect does not represent the whole story. Reports have documented mutant proteins that regulate gene expression and induce cell growth, cell death or apoptosis, independently of wt *TP53* (20, 21). Studies using mice generated to express well-known mutated TP53 displayed a considerable different spectrum of tumour types and frequencies than simple double *TP53* knockout mice, suggesting the presence of a mutated protein influences tumorigenesis in a manner distinct and more proactive than simply deleting wt *TP53* (20, 22). Importantly, in common to the vast majority of these engineered TP53 in mice, there are very high levels of mutant protein expressed in the tumour cells. Different mechanisms have been proposed for this oncogenic gain-of-function effect of TP53, such as specific activation of certain promoters by the mutant proteins, or the ability of some of these mutants to block TP53 homologues, such as TP63 and TP73 (21, 23). Thus, the presence of TP53 overexpression in cancer cells may represent both classic dominant-negative loss-of-function and oncogenic gain-of-function, suggesting that assessing protein overexpression maybe the best single method to study TP53 and its overall impact on tumours such as lymphoma. Several translational studies have shown correlation of TP53 protein expression detected by

immunohistochemistry with the prognosis of human tumours, including lymphoma (24-27).

Despite this level of understanding, the relationship between *TP53* mutations and their impact on the protein function is not always straightforward and is likely dependent of several parameters, including the mutation site, the resulting amino acid substitution and even functional alterations associated with some single nucleotide polymorphisms (21). The correlation between TP53 accumulation and *TP53* mutation is about 80% as frameshift and nonsense mutations do not usually lead to protein accumulation. Moreover, in some extreme benign conditions, such as major hypoxia, there is transient accumulation of the protein. To distinguish between mutated or wt accumulations it is useful to correlate TP53 expression with TP53-regulator proteins, like HMD2 or TP21. Most *TP53* mutations cannot induce these molecules resulting in overexpression of TP53 in the absence HMD2 and TP21.

Despite the use of different detection methods, including both mutational analysis and immunohistochemistry, in the CHOP era *TP53* mutations in DLBCL were reported in18 to 30% of the cases and were associated in most but not all studies with an adverse prognosis (24-30). Correlation of TP53 expression with COO DLBCL subtypes have also been reported (31).

S5.1.2 AIM

Assess the prognostic value of TP53 overexpression and its correlation with *TP53* mutational status in routine clinical diagnostic formalin-fixed paraffin-embedded tissue (FFPET) biopsies using a population-based cohort of DLBCL patients treated with either CHOP or CHOP-R. We combined TP53 protein expression detected by immunohistochemistry (IHC) with *TP53* mutational status using an array platform (AmpliChip p53 Test®) of DLBCL also distinguished by COO subtypes.

S5.1.3 MATERIAL & METHODS

S5.1.3.1. Patients & Samples Selection

The patients included in this study were a subset of the 292 patients and published previously by Sehn et al (32). This is a population-based retrospective analysis examining outcomes for all patients with advanced-stage DLBCL in a single Canadian province during a 3-year interval, September 1, 1999, through August 31, 2002, including the 18 months before and the 18 months after the introduction of rituximab plus CHOP into clinical practice in British Columbia. All patients were older than 15 years of age and had biopsy proven, newly diagnosed DLBCL within the study interval. In addition, all patients had advanced stage disease: stage III or IV; stage I or II with “B” symptoms or bulky disease (10cm), or a contraindication to radiation; or testicular DLBCL of any stage. All patients received a CHOP-like chemotherapy regimen with curative intent, whereas R-CHOP was used after March 01, 2001. Patients were excluded if they were HIV-positive, had CNS involvement at presentation, or had evidence of transformation from an antecedent indolent lymphoma.

Approval was obtained from the University of British Columbia institutional review board. Informed consent was provided according to the Declaration of Helsinki.

S5.1.3.2. Tumour Specimens - Formalin-Fixed Paraffin-Embedded Tissue Samples

A total of 155 patients were analyzed based on available formalin-fixed paraffin-embedded tissue (FFPET) blocks with sufficient tissue of the appropriate fixative and that had interpretable immunohistochemistry for all antigens. All samples were diagnostic biopsies of *de novo* DLBCL expressing CD20 (clone L26). Tissue microarrays (TMA) were built using duplicate 0.6mm cores (Beecher® WI, USA) (Figure S5.1.2) and stained with commercially available antibodies against CD3, CD10, BCL6, MUM1, BCL2, TP53 (clone DO7) and TP21. DLBCL cases were assigned to GCB or non-GCB subgroups based on the method of Hans et al (6). Nuclear expression of the TP53 antibody was assessed using a TP53 histoscore combining TP53 intensity (ranging 1 - 3) multiplied by % of positive malignant cells per TMA core. Strong expression was defined as high intensity (3/3) expression in 50% of the malignant cells (Figure S5.1.3). TP21 expression was considered positive when expressed in >10% of

the malignant cells. The TP53 gene mutational analysis was performed on this subset of cases with strong TP53 nuclear expression with DNA extracted from FFPET samples using Amplichip p53® (Roche Molecular Systems®). In these samples tumour cell purity was estimated in %, combining CD20 staining malignant cells and a CD3 stain for non-malignant T cells.

S5.1.3.3. DNA Extraction

DNA was extracted from FFPET sections using the AmpliChip p53 Lysis Reagent®. Lysis reagent was added to each 1.5mL sterile tube containing a tissue section. Samples mixed with 200 uL of lysis reagent were incubated at 98°C for 30 minutes. Samples were then cooled for 5 minutes prior to the addition of Proteinase K where samples were then incubated for one hour at 65°C. Proteinase K (2 mg/mL) was inactivated after lysis by heating at 98°C for 15 minutes. Centrifugation of samples was done to remove debris from the microcentrifuge tube. The lysate was carefully removed after centrifugation to another sterile tube.

S5.1.3.4. Roche's AmpliChip p53 Assay(Figure S5.1.4 & S5.1.5)

The AmpliChip p53 Test is a product which is currently under development at Roche Molecular Systems, Inc. The AmpliChip p53 Test reagents were used to amplify products encompassing the coding regions of the *TP53* gene in two reactions (A and B) for all samples including a reference wt DNA. Exons 2, 5, 8, 10, exon 4 upstream sequences, and internal control were in the Primer Mix A. Primer Mix B was designed and contained primers for exons 3, 6, 7, 9, 11, exon 4 downstream sequences, and an internal control. After thermalcycling, the products from Primer Mixes A and B were combined. The products generated from the A and B reactions were cleaved by a mix containing DNase I. Fragmentation is performed by recombinant DNase I to generate small DNA fragments of an average size of 50-100 nucleotides. The Alkaline Phosphatase in the Working Fragmentation Mix destroys the residual dNTPs from the amplification reactions. The fragmented DNA amplicons are subsequently labeled with biotin at their 3' termini by the action of Terminal Transferase using AmpliChip TdT Labeling Reagent as substrate. The biotin-labeled *TP53* target DNA fragments are added to the hybridization buffer containing the AmpliChip

B1 Oligonucleotide Solution which functions as a hybridization control. The mixture is hybridized to the oligonucleotides located on the AmpliChip p53 Microarray using the Affymetrix GeneChip Fluidics Station 450Dx and an AmpliChip p53 specific protocol. The hybridized AmpliChip p53 Microarray is washed and stained with a streptavidin-conjugated fluorescent dye (phycoerythrin).

S5.1.3.5. Chip Design

The AmpliChip p53 microarray is designed by RMS® and manufactured by Affymetrix® using technology that combines photolithographic methods and combinatorial chemistry. The microarray consists of a square grid of 228,484 spotted probes, with each spot being 11 microns in diameter. Each probe type is located in a specific area called a probe cell, which contains approximately 10^6 copies of a specific oligonucleotide sequence. Probe microarrays are manufactured by light-directed combinatorial chemistry in a series of cycles. The glass substrates are coated with linkers containing photolabile protecting groups. A mask is then applied that exposes selected portions of the probe microarray. Illumination removes the photolabile protecting groups enabling selective nucleoside phosphoramidite addition only at the previously exposed sites. Next, a different mask is applied and the cycle of illumination and chemical coupling is repeated. By repeating this cycle, a specific set of oligonucleotide probes is synthesized, with each probe type in a known location. A single probe set for an interrogating base position includes five probes, one probe to hybridize to the wt, three probes to detect three possible single base pair mutations, and one probe to detect single deletions (Figure S5.1.6). There are at least 24 probe sets for each nucleotide position, including both sense and antisense probe sequences. A total of 1300 nucleotide positions of coding regions of exons 2 – 11 are tiled on AmpliChip p53. After staining, the AmpliChip p53 Microarray is scanned by an Affymetrix GeneChip Scanner 3000Dx using a laser that excites the fluorescent label bound to the hybridized p53 target DNA fragments. The amount of emitted light is proportional to bound target DNA at each location on the probe microarray.

S5.1.3.6. Data Analysis of Microarray Signals

The *TP53* mutation status was determined by a *TP53* Re-sequencing Algorithm developed by RMS®, which is designed to detect single base pair substitutions and single base pair deletions of a sample in a background of wt p53 DNA probe intensities.

The Re-sequencing Algorithm first reads the probe intensities generated by the GeneChip Operating Software (GCOS) Ver 1.1 provided by Affymetrix. Based on these raw data, the algorithm performs an initial exon quality test to detect the presence of distinct problems in each PCR product. If an exon fails the initial quality test, the exon failure is reported and no further analysis is made. If an exon passes the test, the probe intensities are normalized using quartile normalization in order to correct array-to-array variability. The quality of each probe set is then examined to eliminate unreliable probe set data for further computation. Using only probe sets that have passed the quality tests, the algorithm makes a tentative call for each base position. Possible base calls for each nucleotide position are wt, single base substitution, single base deletion, or no call (unable to make a call). In the case of a single base substitution, the algorithm identifies the mutated base (e.g., G to A). After the tentative calls are made, the reliability of each base call is re-examined by the algorithm to fine tune the calls using various parameters calculated from the neighbouring base positions. Each exon quality is also re-examined based on the final base calls. If there are too many no calls and/or mutation calls in one exon, these data are considered as 'noisy' and the exon fails the quality test. If an exon fails, the exon failure is reported and no calls are reported for that exon.

S5.1.3.7. Statistical Analysis

Prognostic variables were compared between the groups, using the independent samples *t* test for continuous variables and the X^2 test for categorical variables (Table S5.1.1). Overall Survival (OS) was calculated as the time from date of diagnosis until death as a result of any cause or date of last follow-up. OS was assessed using the Kaplan-Meier method and compared between groups using the log-rank test (33). A multivariate analysis was performed using a Cox proportional hazards model to assess the independent effect of TP53 protein expression in the two treatment era OS, after controlling

for relevant clinical prognostic factors (34). Data were analyzed using the Statistical Software Package for the Social Sciences (SPSS version 10.1 for Windows; SPSS Inc®, Chicago, IL).

S5.1.4 RESULTS

Clinical data was available in all 155 patients, including 77 patients treated with CHOP and 78 treated with R-CHOP. Their clinical characteristics, including the IPI factors, were evenly matched between CHOP and R-CHOP groups (Table S5.1.1). The two treatment regimens represent two consecutive eras of therapy and thus the median follow-up of living patients was 5.1 and 4.0 years for CHOP *vs.* R-CHOP, respectively. The 5-year survival estimate for all patients was 61% and survival outcomes were significantly better, as expected, for the patients treated with R-CHOP. For all 155 patients, the effect of adding rituximab to CHOP therapy had a significant impact on OS (P=0.0035).

Of the 155 cases, 75 had a GCB phenotype and 80 were non-GCB. There were 19 lymphomas showing strong TP53 nuclear expression (19/155 or 12%). All these cases had striking differences of TP53 histoscore between positive cases and those called negative, as seen by the values of the median and standard deviation of the histoscore (Figure S5.1.7). Of the 19 TP53-positive cases, 10 had a GCB phenotype and 80 were non-GCB (Figure S5.1.8). All the 19 strong TP53 cases were negative for TP21 expression, this combination typically associated with *TP53* mutations.

Seventeen samples were successfully arrayed in the AmpliChip p53 microarray. In all these cases the tumour cell content was higher than 65% (ranging from 65% to 90%). Eighteen mutations and four polymorphic mutations were found in the 17 samples (Table S5.1.2). Table S5.1.2 summarizes all the detected mutations, as well as the common SNP involving codon 72, whose effect in carcinogenesis is unclear and controversial. There was only one case with no mutation but harbouring a polymorphic SNP. All of the mutations were missense mutations (including the SNP) and with the exception of one, all have been previously described in the lymphoma literature. The distribution of these mutations in the protein follows the reported literature in lymphoma, as shown in the frequency plot of the reports of *TP53* mutations in lymphoma along with our

results (Figure S5.1.9). The vast majority are, as expected, within the DNA binding domain. The only outlier belongs to a case with two mutations.

In univariate analysis, both IPI and strong nuclear TP53 expression had significant prognostic impact (P<0.0001) (Figure S5.1.10). A Cox model revealed both variables to be independent variables of OS (P=0.005 & P<0.0001, respectively).

As previously reported, the addition of rituximab to the treatment regimen resulted in a dramatic improvement in survival (Figure S5.1.11). Importantly, when analyzed by treatment era, the IPI was significant in the pre-R era (P<0.0001) but not for the post-R era (P=0.2). While strong TP53 was significant in both groups (P=0.015 for CHOP and P=0.012 for R-CHOP) (Figure S5.1.12).

When we separate the patients by COO subtype, where strong TP53 expression resulted in inferior survival only in the GCB group (P<0.0001) vs. (P=0.34) for the non-GCB group (Figure S5.1.13).

S5.1.5 DISCUSSION

In this study, we describe for the first time a previously identified prognostic marker in DLBCL that did not lose its impact in the new R-CHOP therapy era. Using an easily assessable biomarker, we identify cases that over-expressed the protein due to missense mutations with resistance to both CHOP and R-CHOP therapy. Despite the small number of cases with adverse prognosis (12%) these patients could be reasonably seen as candidate for new alternative therapies.

Experimental data on the different functional effects of individual *TP53* mutations is increasing and is a crucial goal for the design of future anti-cancer targeted therapies (21, 30). However, many factors such as epigenetic changes, RNA editing, post-transcriptional protein modifications underlie the complex relationships between genotypic and phenotypic abnormalities. As mentioned previously, in human cancer there is a bias for certain “malignant” mutations but there are also many “benign” mutations with minimal effects on the TP53 pathway. Different and divergent functional results of different *TP53* mutations studied either *in vitro* or *in vivo* are well known (21). Thus, isolated

mutational analysis *per se* might not be the best approach to determine the clinical impact of *TP53* gene abnormalities including mutations. The assessment of a common final end-result such as TP53 protein expression abnormalities seems to be the better strategy to recognize abnormalities with functional impact that might influence clinical outcome. In this study we show that increased expression of the TP53 protein is associated in all except one case to missense, previously reported, “pathologic” mutations. Thus, immunohistochemical assessment of routine diagnostic FFPET is an easy and efficient technique to evaluate *TP53* status.

Semi-quantitative determination of protein expression of biomarkers using FFPET samples has been controversial in many different settings due to variability related to fixation time and tissue quality, antigen retrieval techniques, antibody clone and dilution, and finally scoring variability that is heavily dependent on observer subjectivity (35). Like many other genes with impact in human cancer, the correlation between protein and gene mutations is not linear (16). But, in contrast to other tumour suppressor genes where large delections or frameshift mutations resulting in loss of the protein are common, single amino acid substitution missense mutations in *TP53* result in strong expression of the protein. Only rarely, absence of TP53 protein expression may be associated with nonsense or frameshift mutations. On the other hand, cells exposed to a stressful microenvironment, such as occurs with hypoxia may physiologically increase TP53 overexpression. However, the normal feedback loop of the TP53 pathway keeps the level of expression under tight control. This mechanism is lost in cases with missense mutations of the core binding domain of TP53 as was seen in this study, resulting in striking protein accumulation.

Importantly, in the human cancer literature there is a significant correlation between mutations, increased protein expression and adverse prognosis (16). In experimental studies, normal cells have less than 500 TP53 molecules per cell. Physiological induction such as occurs with hypoxia can lead to ~ 5,000 molecules per cell, while in cases of DNA binding domain missense mutations the total number rises to 50,000. Thus, there is a log2 difference in TP53 protein quantities between reactive and neoplastic mutated *TP53* settings (36-39). This log difference is in the range of differential assessment by immunohistochemistry, using an optimal protocol. We established our protocol

by defining as a positive control – the homogeneous nuclear expression of TP53 by the lower basal epithelial cells in tonsil squamous epithelium. Next, we chose a TP53 antibody with its epitope located outside the DNA binding domain and thus infrequently mutated. The DO7 antibody recognizes the epitope defined by amino acids 21 to 25 of the amino terminus of the protein. Finally, we defined a cut-off value for positivity in maximum protein expression (3/3) in the majority of the neoplastic cells (>50%).

In order to confirm the mutated form of TP53 protein, we evaluated the expression of TP21. This is a downstream effector of TP53 activation resulting in cell cycle arrest and is physiologically increased in cases of increased TP53 expression. The accumulation of mutated TP53 is unable to induce TP21 expression. Consequently, a strong TP53/negative TP21 phenotype is highly correlated with mutated *TP53* (27, 40). None of the 19 cases with strong nuclear TP53 showed expression of TP21. To confirm this hypothesis we arrayed the 19 strong-positive TP53 cases and all but one case showed missense mutations. Five SNPs were also found. All cases had mutations localized to the DNA binding domain and this location overlaps with the known mutational data in human cancer and lymphoma in particular (Figure S5.1.9 & Table S5.1.2). Curiously, the only case not showing a "pathological" mutation had a well-described polymorphic alteration whose role in cancerigenesis is controversial, though reported to have a pro-tumoral effect through the TP53 related transcriptional factor TP73 (23).

The 19 cases with strong TP53 expression distributed equally between COO subtypes (Figure S5.1.8). As recently described by Visco *et al*, strong TP53 expression significantly correlated with an adverse outcome in the GCB-like lymphomas (Figure S5.1.13) (31). The mechanism remains elusive, but we suggest aberrant signalling whereby a mutated TP53, in contrast to normal TP53, is not down-regulated by BCL6 protein that characterizes GCB-like DLBCLs (41).

S5.1.6 CONCLUSIONS

Strong TP53 protein expression is an easy biomarker to assess in routine FFPET diagnostic biopsies of DLBCL patients and correlates almost universally

with *TP53* missense mutations. Importantly, strong TP53 protein expression is an independent prognostic factor for patients with DLBCL even when treated with R-CHOP. *TP53* mutations were found in both GCB and non-GCB subtypes, but of prognostic importance only in the GCB subgroup. Finally, the prognostic impact of TP53 is not diminished in the era of CHOP-R, identifying a subgroup of patients with inferior survival deserving alternative therapies.

S5.1.7 TABLES

	CHOP (77)	CHOP-R (78)	p (χ^2)
High IPI (%)	73	70	0.8
Female (%)	40	42	0.8
Median Age	66 (22-85)	58 (20-84)	0.1
Median follow-up time living patients	5.1 years	4 years	nd
Strong p53 (%)	12	13	0.8
p53 mutations	9/9	9/10	0.9
p53 histoscore	38 (0-300)	45 (0-300)	0.8
GCB (%)	49	47	0.7

Table S5.1.1 - Clinical & pathological characteristics, including the IPI factors, were evenly matched between CHOP and R-CHOP groups.

Exon	Codon	Codon change	Amino Acid change	Effect	NHL Ref June 2006
2	7	A to T	Asp to Val	Missense	-
4b	107	T to G	Tyr to Asp	Missense	1
5	133	T to C	Met to Thr	Missense	7 (2)
5	134	T to A	Phe to Ile	Missense	4
5	151_1	C to T	Pro to Ser	Missense	78 (3)
5	151_2	C to A	Pro to His	Missense	26 (2)
5	173	G to A	Val to Met	Missense	66 (1)
6	195	T to C	Ile to Thr	Missense	72
6	197	T to A	Val to Glu	Missense	8 (1)
6	216	T to A	Val to Glu	Missense	5 (1)
7	234	A to G	Tyr to Cys	Missense	102 (8)
7	241	C to T	Ser to Phe	Missense	85 (1)
7	245_1	G to A	Gly to Ser	Missense	366 (6)
7	245_2	G to A	Gly to Asp	Missense	138 (3)
7	248_1	C to T	Arg to Trp	Missense	625 (14)
7	248_2	G to A	Arg to Gln	Missense	727 (36)
7	251	T to G	Ile to Ser	Missense	22 (1)
8	273	G to A	Arg to His	Missense	656 (24)
8	285	G to A	Glu to Lys	Missense	140 (1)
4b	72_2	G to C	Arg to Pro	Missense	SNP (?)

Table S5.1.2 - TP53 mutations found in the cohort. All but one missense mutation have been previously reported in lymphoma.

S5.1.8 FIGURES

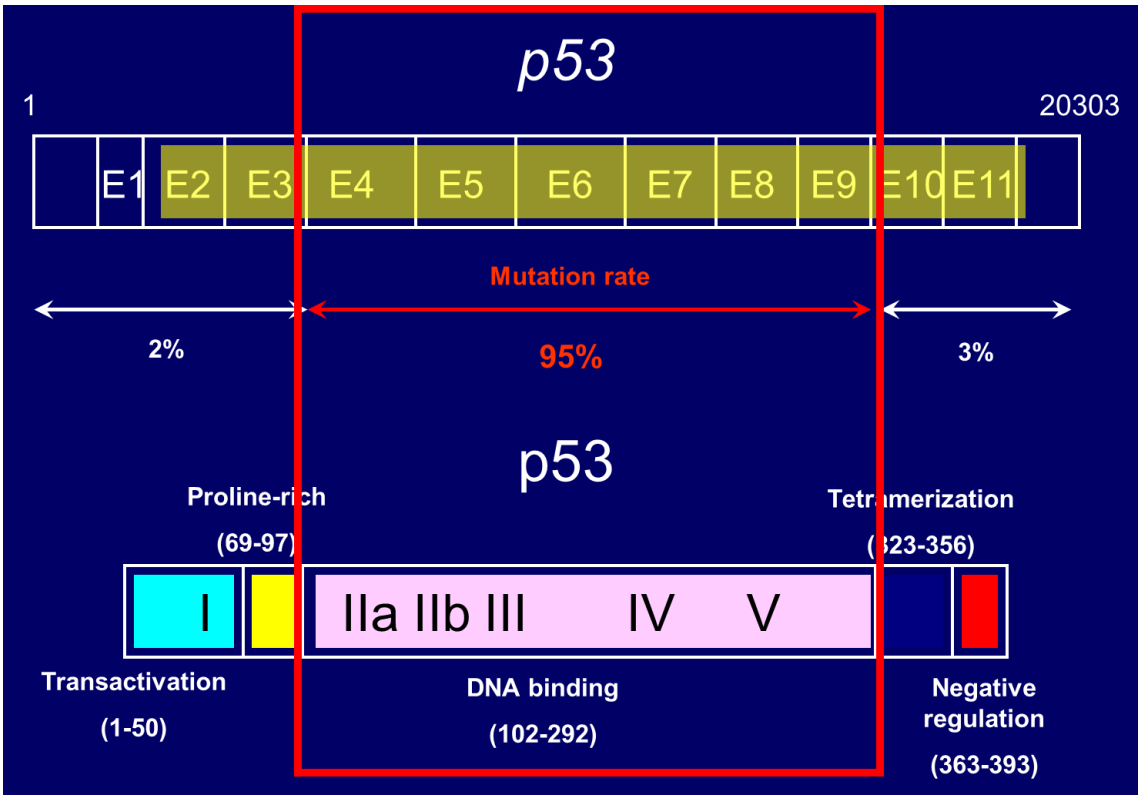

Figure S5.1.1 - *TP53* gene structure with 11 exons, 10 of them coding (yellow box). It is a transcription factor with an important central DNA binding domain that is the target of approximately 95% of TP53 mutations found in human cancers.

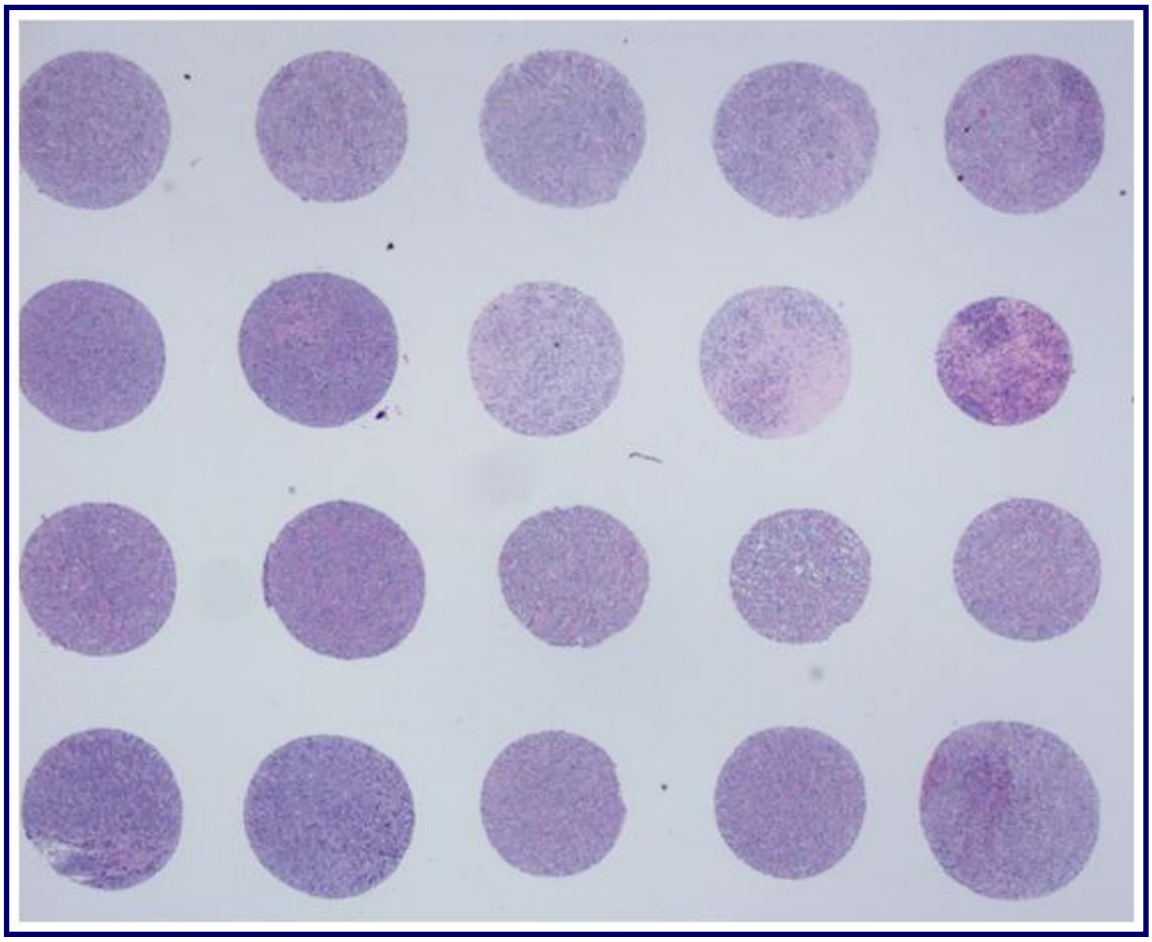

Figure S5.1.2 -Tissue microarrays (TMA) were built using duplicate 0.6mm cores.

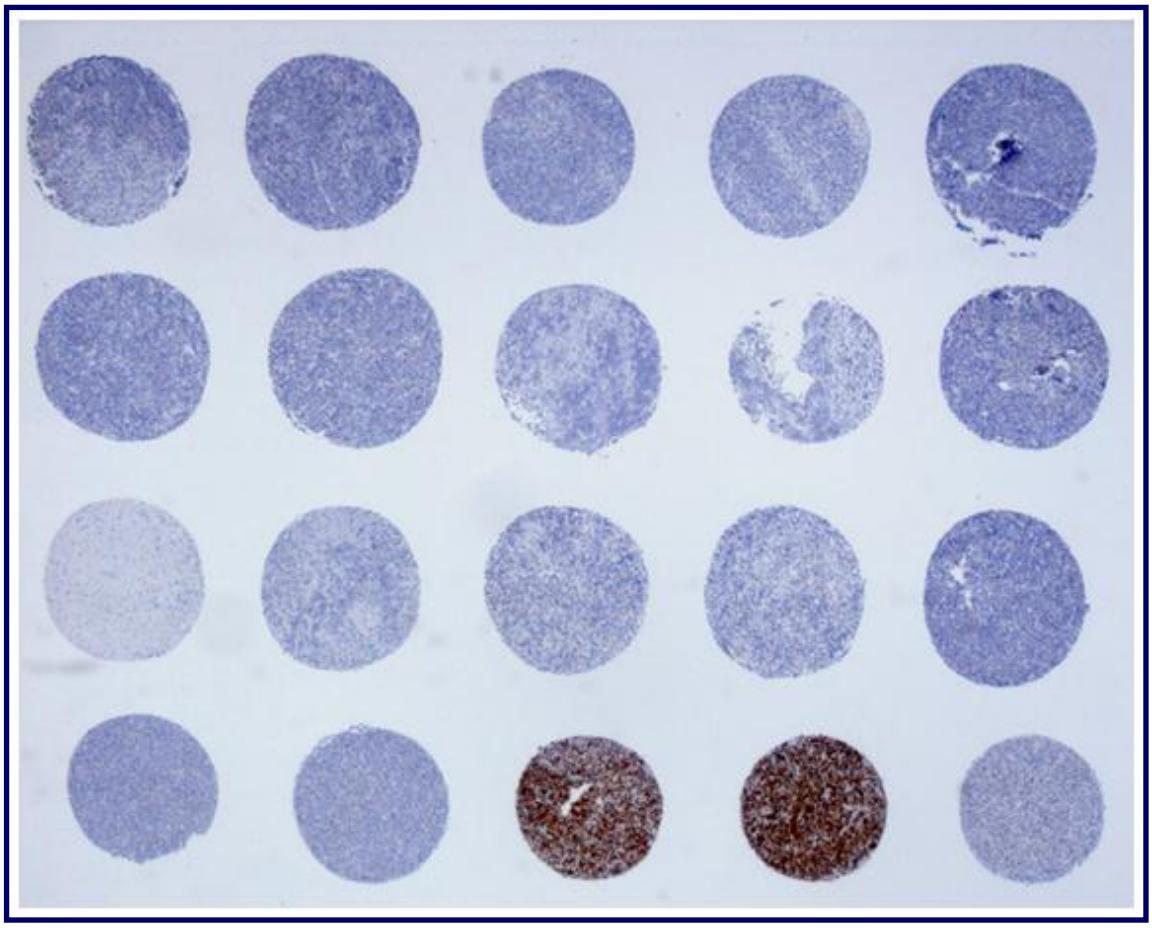

Figure S5.1.3 - TP53 protein expression. Nuclear expression of the TP53 antibody was assessed using a TP53 Histoscore combining TP53 intensity (ranging 1 to 3) multiplied by % malignant cells per TMA core. Strong expression was defined as high intensity (3/3) expression in >50% of the malignant cells (one pair shown in the lower row).

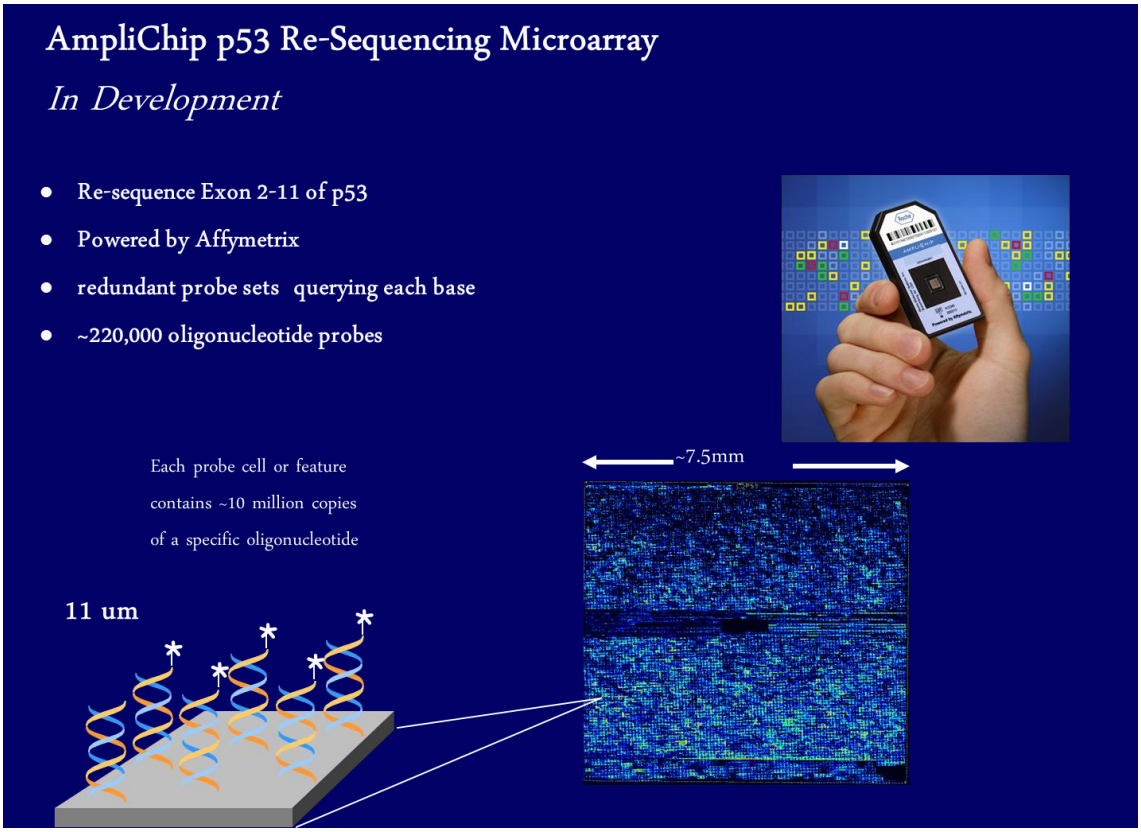

Figure S5.1.4 - Roche's AmpliChip p53 Assay

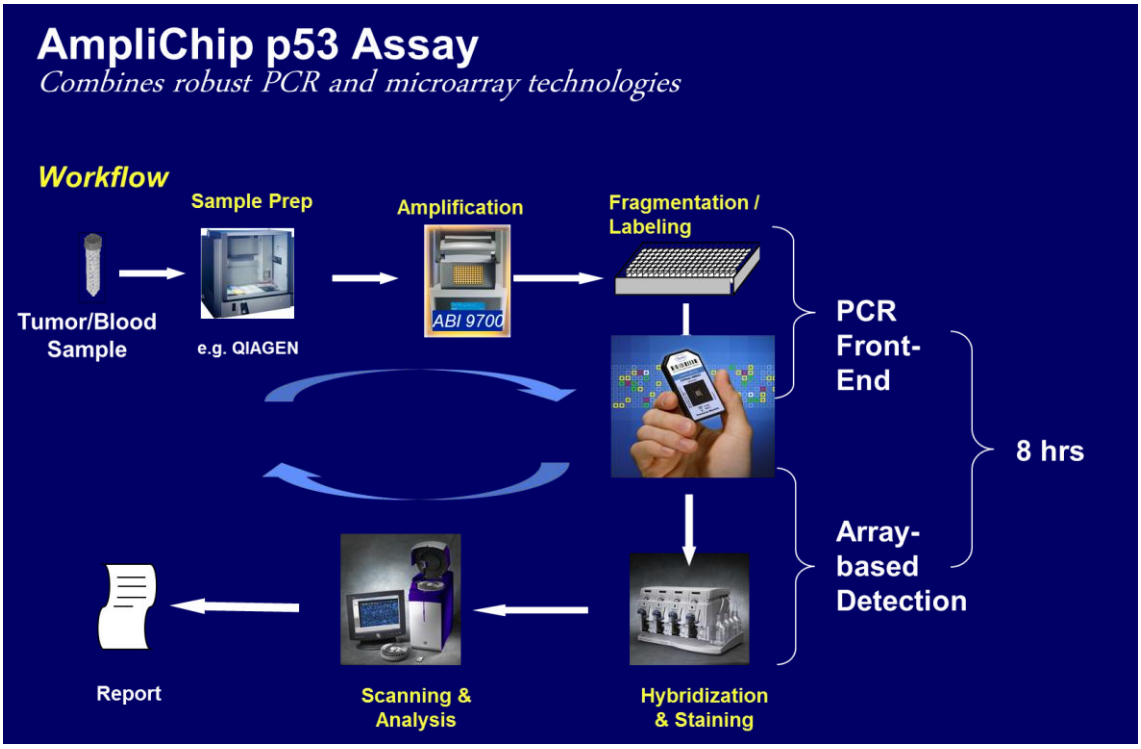

Figure S5.1.5 - AmpliChip p53 Microarrays are manufactured using technology that combines photolithographic methods and combinatorial chemistry. Over 220,000 different oligonucleotide probes are synthesized on a glass surface to analyze both sense and antisense strands of an amplified target DNA specimen. Each probe type contains approximately 10^6 copies of a given probe. After staining, the AmpliChip p53 Microarray is scanned using a laser that excites the fluorescent label bound to the hybridized p53 target DNA fragments. The amount of emitted light is proportional to bound target DNA at each location on the probe microarray.

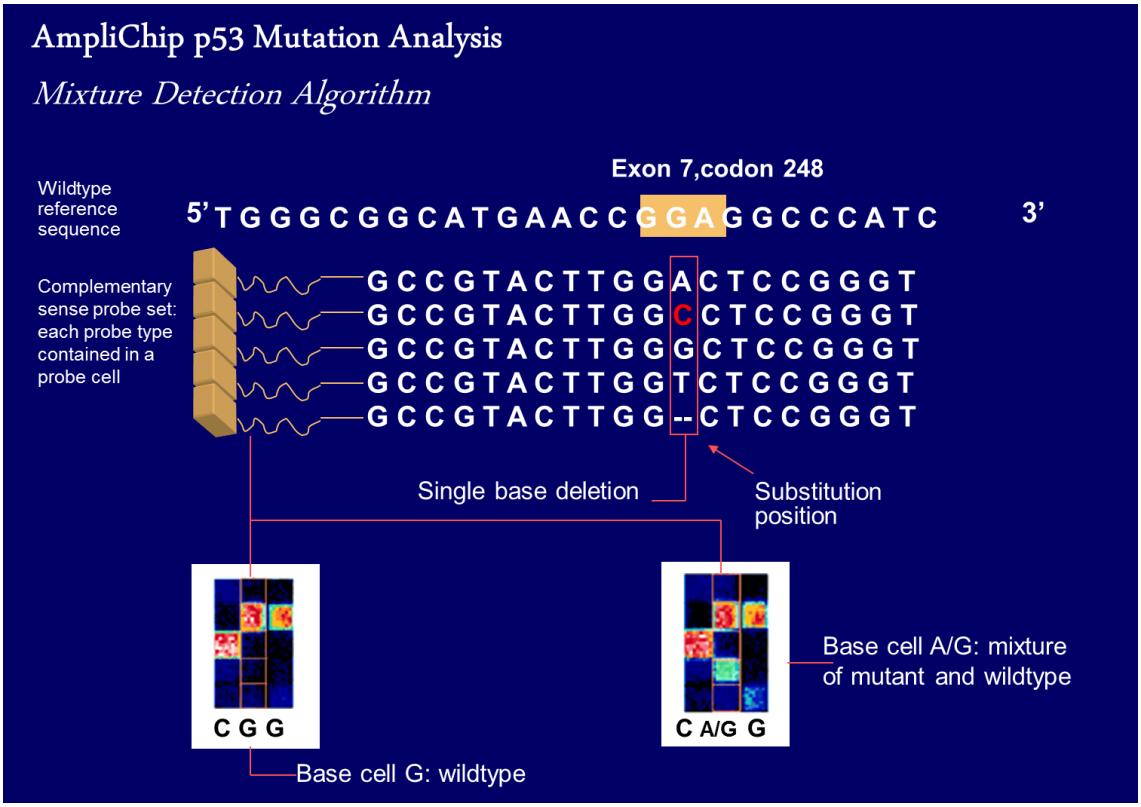

Figure S5.1.6 - Each sequence from a known reference is present in different probes, differing only by one base. Thus, this allows all different point mutations to be detected, like in this example with one substitution of Guanine to Adenine.

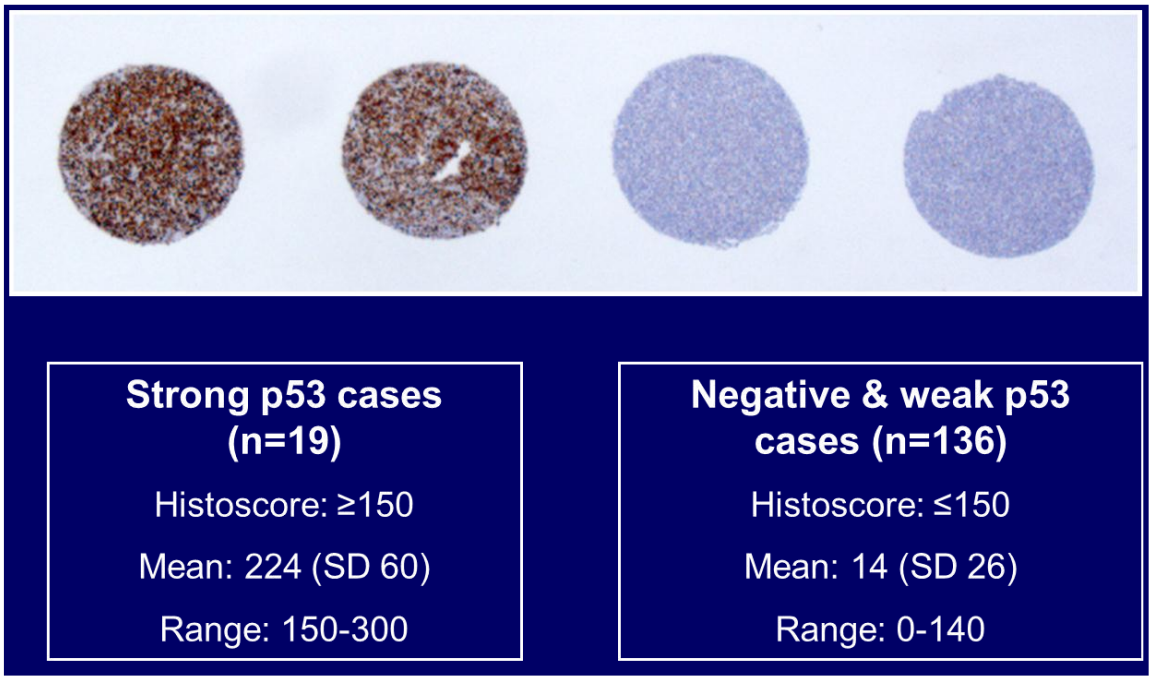

Figure S5.1.7 - TP53 Histoscore. The differences in staining were striking between strong nuclear staining and negative cases.

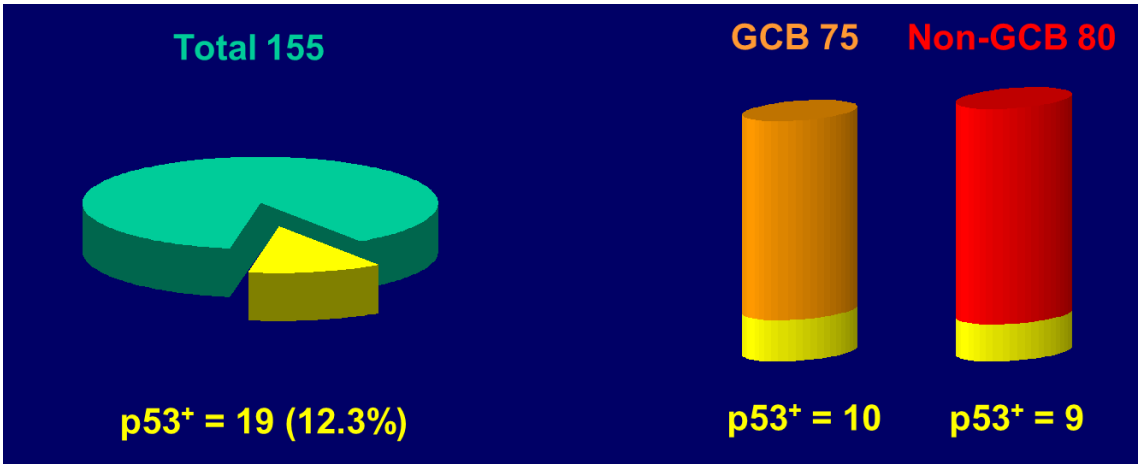

Figure S5.1.8 - Distribution of the cases with strong nuclear TP53 expression.

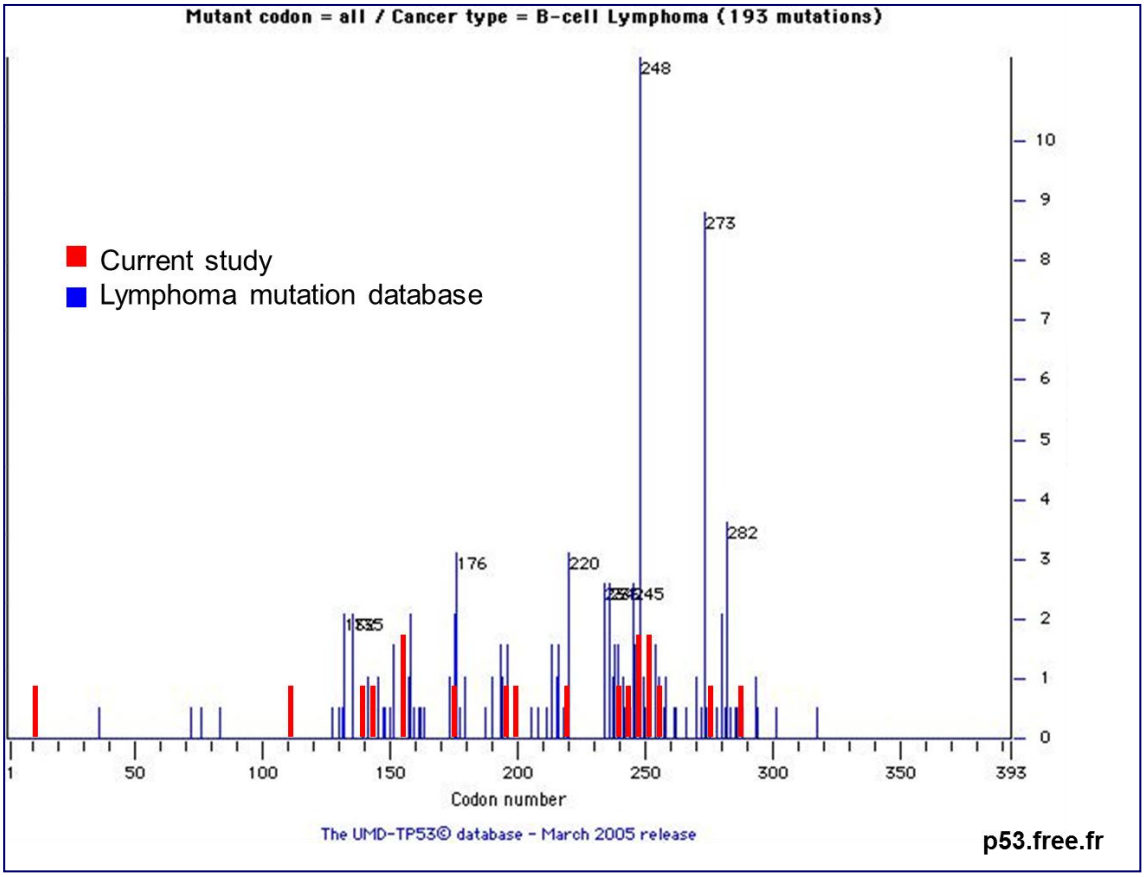

Figure S5.1.9 - The distribution of *TP53* mutations found in the current study (red) follows the reported literature in lymphoma, as shown in the frequency plot of the reports of *TP53* mutations in lymphoma (blue).

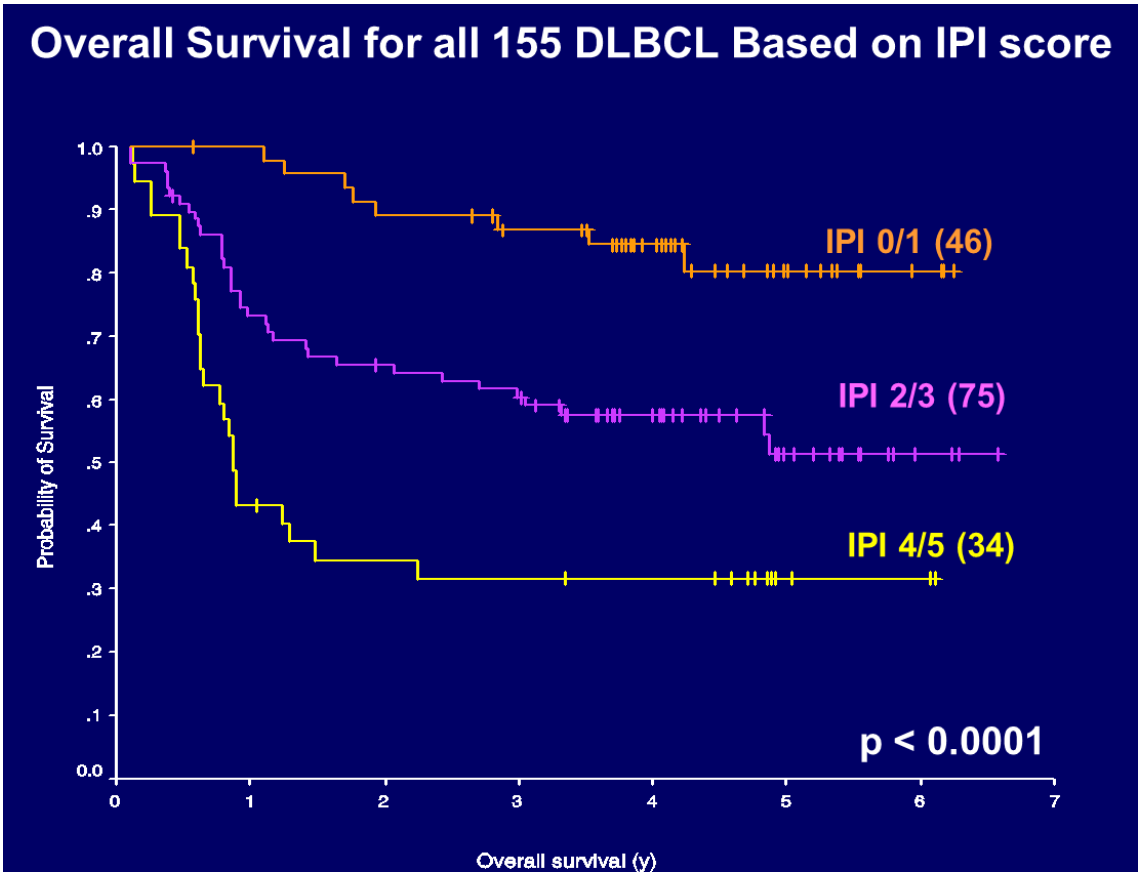

Figure S5.1.10 - In univariate analysis the IPI had prognostic impact on OS (P<0.0001).

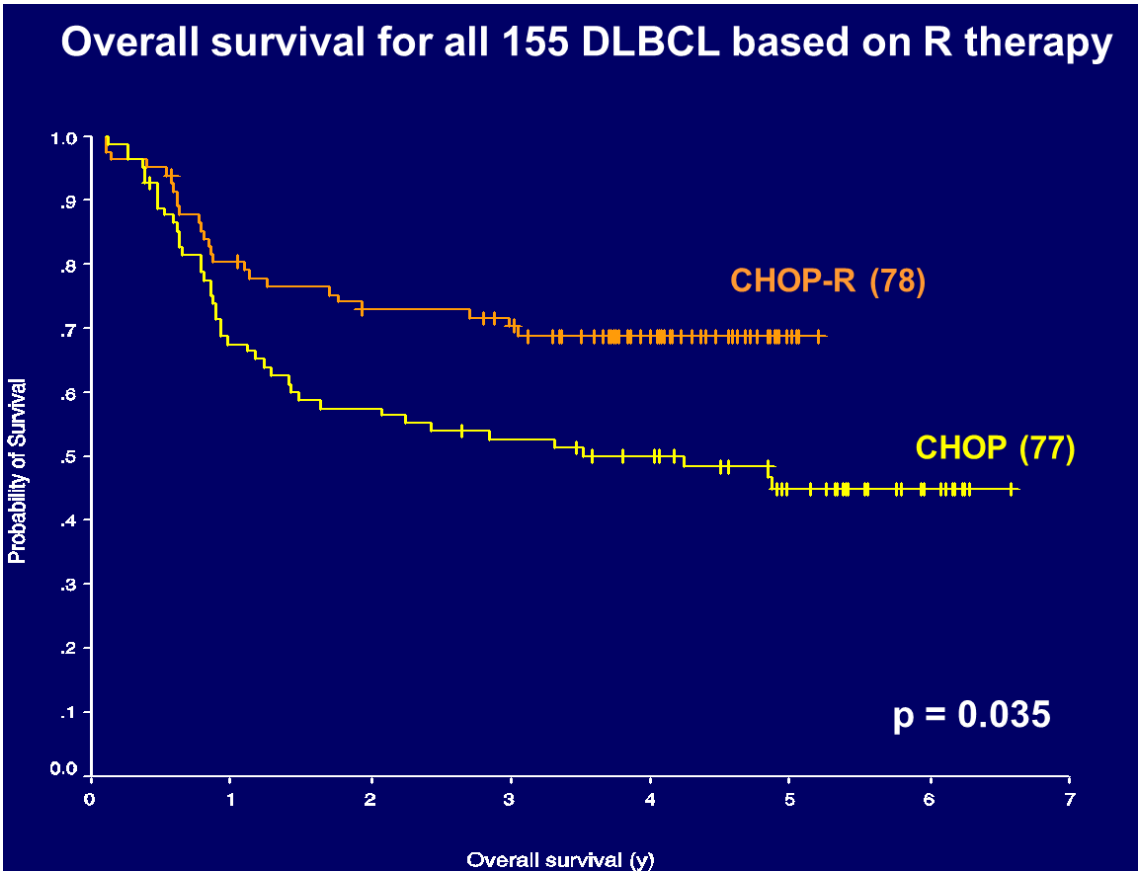

Figure S5.1.11 - Impact of R therapy addition in DLBCL overall survival. As previously reported, the addition of R in the whole group resulted in a dramatic improvement in survival.

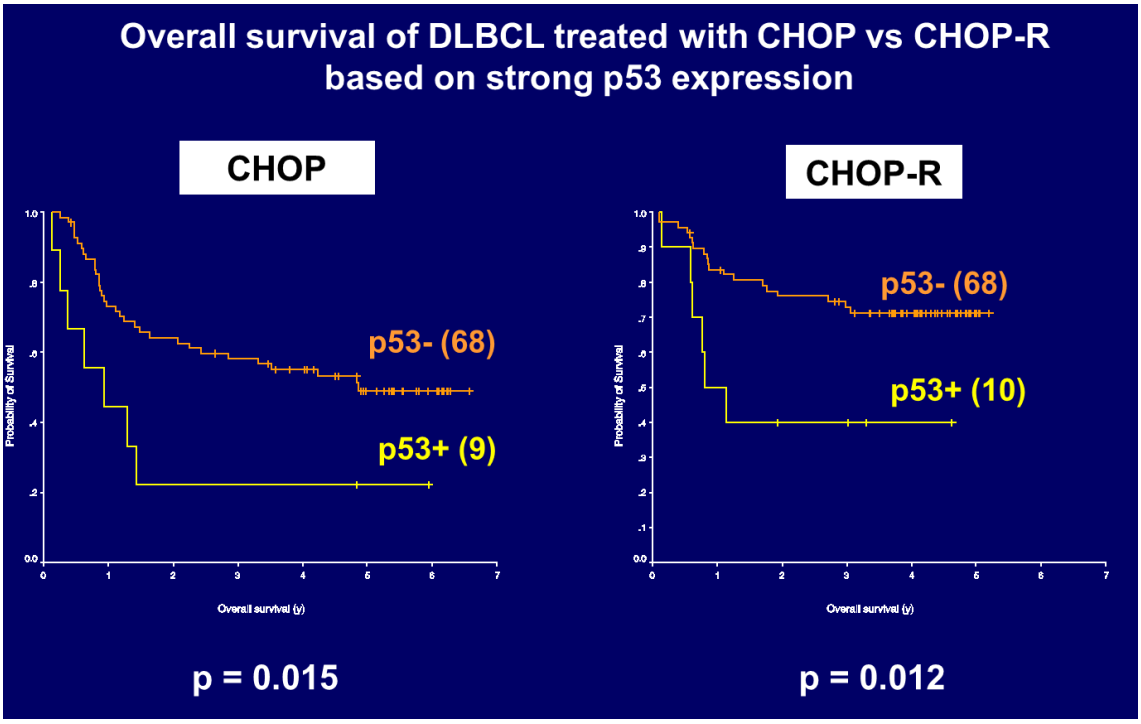

Figure S5.1.12 - Strong TP53 was significant in both treatment era groups.

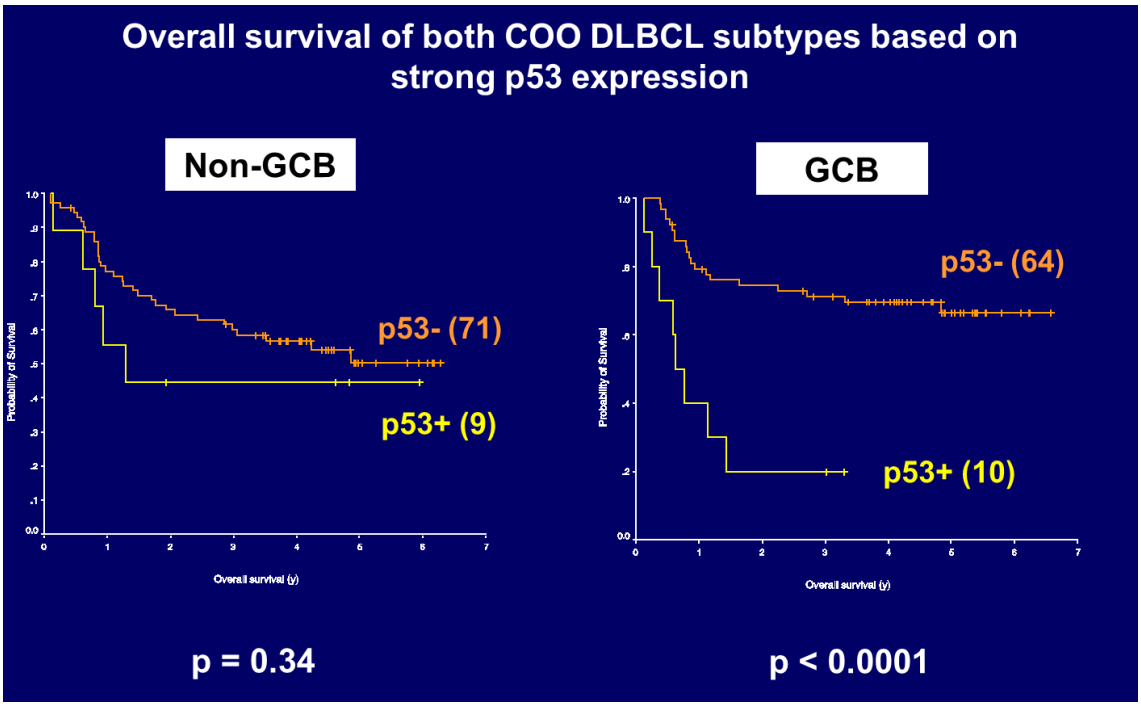

Figure S5.1.13 - Separated by COO subtype, strong TP53 expression resulted in inferior survival only in the GCB group (P<0.0001) vs. (P=0.34) for the non-GCB group.

S5.1.9 REFERENCES

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Section 5.2: Addition of Rituximab (R) to CHOP Improves survival in non-GCB subtype of DLBCL

S5.2.1 INTRODUCTION

The Cell-of-Origin (COO) distinction in DLBCL is well established, since the initial studies using gene expression profiling identifying two distinct subgroups of DLBCL: germinal center B cell–like (GCB) activated B cell–like (ABC) (1). The GCB subgroup is characterized by expression of a germinal center B cell molecular signature, can be associated with the *BCL2* translocation, t(14;18)(q32;q21) (2, 3) and carries a better prognosis than the ABC subgroup (4). The molecular signature of the ABC subgroup is distinctly different and is characterized by overexpression of a group of genes that are up-regulated in peripheral-blood B cells activated by mitogenic stimulation *in vitro*, many of the genes included in the nuclear factor-kappa B (NFκB) pathway. (1, 4) (Figure S5.2.1). Tissue microarrays (TMA) and immunohistochemical staining can be used to provide biomarker data that correlates closely with the results of gene expression profiling in predicting outcome (5) (Figure S5.2.2).

BCL2 is an anti-apoptotic factor that is important in normal B cell development and differentiation (6, 7). The t(14;18)(q32;q21) brings the *BCL2* gene under the control of immunoglobulin heavy-chain gene (*IGH*) enhancer and leads to overexpression of BCL2 protein. However, BCL2 expression can also be up-regulated by alternate mechanisms, including copy number increases in the gene and downstream of NFκB signalling, as is often observed in the ABC subgroup of DLBCL, which lacks this translocation. BCL2 overexpression provides a survival advantage for malignant B cells and is thought to play a critical role in resistance to chemotherapy. As a result of these biologic functions, BCL2 overexpression should be of prognostic importance in DLBCL. However, correlation of BCL2 expression with survival in patients with DLBCL is controversial, with studies showing no difference in overall survival (OS) (8, 9) or decreased OS (10-12). BCL2 expression is highly correlated with the t(14;18)(q32;q21) in the GCB subgroup, but the translocation had no impact on survival (3). However, the influence of BCL2 expression on survival within the ABC subgroup of DLBCL is not clear. The existing controversy regarding BCL2

expression and survival may be related to studying DLBCL as a single entity. In contrast, analyzing BCL2 expression in the context of DLBCL subgroups might indeed clarify its clinical relevance.

At BCCA, we previously studied a group consisting of 94 patients with *de novo* DLBCL, all with a clonal karyotype at diagnosis and treated with curative intent in the pre-R era. A TMA was constructed with duplicate 0.6mm cores and stained for BCL2, CD10, BCL6, MUM1 and FOXP1. Cases were called positive if more than 30% of the tumor cells expressed a given protein. Cases were defined as GCB if they were CD10+. Non-GCB was defined as CD10-, MUM1+ and/or FOXP1+. Cytogenetic studies were performed routinely and locus-specific FISH was performed using commercially available Vysis probes (dual-color LSI *IGH/BCL2*) to detect the t(14;18). Unbalanced increases in *BCL2* gene copy number were determined by comparison of *BCL2* and *IGH* signals. The IPI was highly predictive of OS (P<0.00001). The t(14;18) was detected by both routine cytogenetics and FISH in 24 (25%) cases, but did not predict survival (P=0.78). None of the non-GCB cases harbored a t(14;18) and importantly, the presence of t(14;18) and isolated *BCL2* copy number gain were mutually exclusive (Figure S5.2.3). Expression of BCL2 protein and non-GCB-type immunostaining profile both predicted a poor OS (P=0.008 and P=0.03, respectively). Expression of BCL2 was imperfectly correlated with either t(14;18) or a non-GCB immunostaining profile, but was highly correlated with cases harboring an increased gene copy number for *BCL2* (see Figure S5.2.4, χ^2 P=0.005). Increased *BCL2* gene copy number did not predict OS (P=0.43), a not unexpected finding as it accounts for only a proportion of BCL2 protein-positive cases. Cases lacking both the t(14;18) and increased *BCL2* copy number were deemed cytogenetically “normal”, accounting for 47 cases. These cases were distributed between the GCB (42%) and non-GCB subtypes (62%). Of the cytogenetically “normal” cases, 33% of the GCB and 75% of the non-GCB expressed BCL2 protein. In this study, we concluded that multiple mechanisms are responsible for BCL2 expression in DLBCL. DLBCL cases with a t(14;18) are always GCB and never show BCL2 gene copy number gains in addition to the *BCL2* translocation, suggesting that these two events are mutually exclusive. Non-GCB cases do not harbour the t(14;18), more

commonly have isolated *BCL2* gene copy number gain and have a higher percentage of cytogenetically “normal” BCL2 protein-positive cases. The latter finding suggests a prominent role for transcriptional up-regulation resulting from constitutive activation of NFκB. Other mechanisms, such as epigenetic changes or promoter hypomethylation that might be deregulating BCL2 expression requires further study. Importantly, in this small cohort treated with CHOP & CHOP-like protocols, BCL2 protein expression and COO (GCB vs. non-GCB) were predictive biomarkers in DLBCL (13).

The addition of R to CHOP chemotherapy has shown a significant improvement in the outcome of patients with DLBCL (14, 15). The underlying mechanism(s) responsible for this effect *in vivo* is still largely unknown. When classical DLBCL biomarkers were studied in the new R-CHOP era, R was show to preferentially prevent chemotherapy failures in DLBCLs that express BCL2 protein and in those patients whose biopsies failed to show expression of BCL6 protein (16, 17) (Figure S5.2.5). These two proteins are well known to be differentially expressed between the two COO DLBCL subtypes, as the non-GCB phenotype is usually more often associated with both over-expression of BCL2 and down regulation of BCL6. Thus, it is expected that the effect of the introduction of R is greater in this group, the non-GCB lymphomas.

S5.2.2 AIM

The aim of our study was to examine the clinical impact of R added to in DLBCL distinguished by COO subtype.

S5.2.3 MATERIAL & METHODS

S5.2.3.1. Patient & Sample Selection

The patients included in this study were a subset of the 292 studied published previously by Sehn *et al* (15). This is a population-based retrospective analysis cohort examining outcomes for all patients with DLBCL in a single Canadian province during a 3-year interval, September 1, 1999, through August 31, 2002. Rituximab was added to CHOP as the standard of care for DLBCL on March 01,

2001. All patients were older than 15 years of age and had biopsy proven, newly diagnosed DLBCL within the study interval. In addition, all patients had advanced-stage disease: stage III or IV; stage I or II with “B” symptoms or bulky disease (10cm), or a contraindication to radiation; or testicular DLBCL of any stage. All patients received a CHOP-like chemotherapy regimen with curative intent. Patients were excluded if they were HIV-positive, had CNS involvement at presentation, or had evidence of transformation from an antecedent indolent lymphoma.

Approval was obtained from the University of British Columbia institutional review board. Informed consent was provided according to the Declaration of Helsinki.

S5.2.3.2. Tumor Specimens Formalin-Fixed Paraffin-Embedded Tissue (FFPET) Samples

We identified 163 patients with DLBCL treated with either CHOP or R-CHOP with available paraffin blocks and interpretable immunostaining. A total of 155 patients were analyzed based on available FFPET blocks with sufficient tissue of the appropriate fixative that had interpretable immunohistochemistry for all antigens. All were diagnostic biopsies of *de novo* DLBCL expressing CD20 (clone L26). TMA were built using duplicate 0.6mm cores (Beecher® WI, USA) and stained with antibodies against CD3, CD10, BCL6, MUM1 and BCL2 (Figure S5.2.6 & Table S5.2.1). DLBCL cases were assigned to GCB or non-GCB subgroups based on the method of Hans et al (5). BCL2 positivity was defined as ≥ 30% malignant cells positive.

S5.2.3.3. Statistical Analysis

Prognostic variables were compared between the groups, using the independent samples *t* test for continuous variables and the X^2 test for categorical variables (Table S5.2.1). OS was calculated as the time from date of diagnosis until death as a result of any cause or date of last follow-up. OS was assessed using the Kaplan-Meier method and compared between groups using the log-rank test (18). A multivariate analysis was performed using a Cox proportional hazards model to assess the independent effect of BCL2 and COO protein expression in the two treatment eras after controlling for relevant clinical

prognostic factors (19). Data were analyzed using the Statistical Software Package for the Social Sciences (SPSS version 10.1 for Windows; SPSS Inc®, Chicago, IL).

S5.2.4 RESULTS

Clinical data were available in all 163 patients including 81 patients treated with CHOP and 82 treated with R-CHOP. Their clinical characteristics, including the IPI, were evenly distributed between the two treatment cohorts (Table S5.2.2). The median follow-up of living patients was 4.6 years. The IPI was predictive of OS (P<0.0001) for the entire study population (Figure S5.2.7). Six cases had uninterpretable immunostains resulting in 74 cases with a GCB phenotype and 83 with a non-GCB phenotype (n=157) (Figure S5.2.8). Overall, 71% and 75% of the cases over-expressed BCL2 and BCL6, respectively (Figure S5.2.9). When comparing GCB vs. non-GCB groups there was no statistical difference in the number of cases expressing BCL2; as BCL2 protein was expressed in 70% of the GCB cases and 73% of the non-GCB (P= 0.72), while, as expected, BCL6-positive cases were over-represented in the GCB group as BCL6 was expressed in 96% GCB cases and 63% non-GCB cases (P<0.0001) (Figure S5.2.10). The COO phenotype and both BCL2 and BCL6 expression profiles were evenly distributed between the two treatment groups (Table S5.2.2). As previously reported, the addition of R in the whole group resulted in a dramatic improvement in survival (Figure S5.2.11). In univariate analysis, the addition of R was associated with a better prognosis in the non-GCB cases (P=0.02), but not in the GCB cases (P=0.3) (Figure S5.2.12). The addition of R to CHOP chemotherapy and IPI were independent predictors of OS in non-GCB DLBCL (P=0.02; P=0.016, respectively).

Next, we evaluated the effect of R on survival in relation to both BCL2 and BCL6 expression profiles. As described, lymphomas over-expressing BCL2 benefit significantly with immunochemotherapy. The addition of R was also of prognostic importance in the lymphomas over-expressing BCL2 (P=0.0081).The effect on BCL6-negative cases was of borderline significance. However, the number of cases was small, precluding any definitive conclusions (Table S5.2.3). Bearing in mind the significant effect of R in the BCL2-positive cases,

and that BCL2 expression was evenly distributed between both COO subtypes, we analysed the survival of BCL2-positive and negative cases within each COO subtype. This analysis revealed that only the non-GCB cases expressing BCL2, but not the GCB cases, benefitted from the addition of R to the treatment regimen (Table S5.2.4). Thus, this survival difference was not solely explained by either BCL2 or BCL6 expression, suggesting an important role of the biological mechanism responsible for the expression of these proteins within COO subtypes.

S5.2.5 DISCUSSION

The addition of R to standard chemotherapy, as we show in the current study and in agreement with several previous studies, overcomes BCL2 over-expression associated resistance to chemotherapy in patients with DLBCL (16). BCL2 is a member of the family of proteins that regulate programmed cell death. Its overexpression in B cell NHL is thought to result in chemotherapy resistance of the lymphoma cells both *in vitro* and *in vivo* (20, 21). As a consequence, BCL2 protein overexpression is generally associated with poor survival in patients with DLBCL who are treated with standard chemotherapy (10, 11, 20, 21). R has eliminated the negative effect of BCL2 protein overexpression in patients with DLBCL, and more specifically, we showed R to overcome this adverse influence on survival mostly due to its impact on non-GCB DLBCL.

Despite the widespread use of R in the treatment of B cell NHL, the mechanisms by which R exerts its anti-lymphoma effect are still not fully understood. Evidence from *in vitro* studies that have used lymphoma cell lines or fresh tumor cells, and from *in vivo* animal studies, suggest that R acts through three different mechanisms including, 1) complement-dependent cytotoxicity (22-24) , 2) antibody-dependent cell-mediated cytotoxicity (25); and 3) induction of apoptosis (26, 27). Among these, induction of apoptosis may be particularly important for a chemotherapy-sensitization effect of R. It appears to induce apoptosis through multiple signalling pathways that inhibit at least two anti-apoptotic proteins, namely BCL2 and BCLxL (28, 29). Studies of two Epstein-Barr virus–positive Burkitt lymphoma cell lines (Ramos and Daudi) have

shown that the binding of R to CD20 inhibits both the NFκB and the ERK1/2 pathways via an increase in Raf-1 kinase inhibitor, which results in decreased expression of BCLxL and BCL2 (30-32).

When the COO subgroups were analyzed separately, BCL2 expression level was associated with inferior survival only in the non-GCB subgroup, suggesting that the addition of rituximab to chemotherapy for DLBCL may be of benefit mainly in the subgroup with BCL2 expression. Thus, BCL2 expression could be a biomarker of more aggressive disease in the non-GCB but not the GCB type. Alternate mechanisms promoting BCL2 expression unrelated to the t(14;18) must be occurring in ABC DLBCL. As previously reported NFκB is constitutively expressed in ABC DLBCL and has a critical role in its pathogenesis (33). *BCL2* is a target gene for NFκB (34) and in many ABC DLBCL patients, BCL2 up-regulation may be mediated through the NFκB pathway. Another possible mechanism is the amplification of the chromosomal locus 18q21 where the *BCL2* gene resides. Studies using comparative genomic hybridization have shown that approximately 20% of DLBCL patients have 18q gains (35). As mentioned, we previously investigated the incidence of 18q21 using the FISH technique, finding gain or amplification of 18q21 more frequently in the non-GCB DLBCL subgroup compared with the GCB DLBCL subgroup (13). As BCL2 is up-regulated by different mechanisms in the GCB vs. non-GCB DLBCLs, BCL2 overexpression is a biomarker for events that are responsible not only for poor prognosis (e.g., NFκB activation or 18q21 amplification) but also for being a good target for R therapy. *In vitro*, R negatively regulates NF-κB and down regulates *BCL2*. Thus, R lowers the apoptotic threshold of the lymphoma cells, facilitating cell death induced by the accompanying cytotoxic agents. In the GCB subgroup, BCL2 expression is mainly a result of the t(14;18), thus representing a completely different mechanism of expression, appearing to be less affected by R.

In summary, our study has demonstrated immunochemotherapy using R-CHOP is associated with better OS for patients with DLBCL due largely to its effect on the non-GCB subgroup. As previously reported, BCL2-positive lymphomas benefited significantly from the addition of R to CHOP chemotherapy. Although the frequency of BCL2 expression was not statistically different between COO

subtypes, only non-GCB BCL2-positive lymphomas showed an improved outcome.

The suggestive inhibition of the NFκB pathway by R is a possible candidate mechanism to explain the differences in OS between GCB-type and non-GCB type DLBCL (Figure S5.2.13). Finally, assessing the prognostic impact of the expression of BCL2 and eventually other biomarkers on survival without taking subgroup distinction into consideration may result in erroneous conclusions.

S5.2.6 CONCLUSIONS

Immunochemotherapy using R-CHOP is associated with better OS in DLBCL, due largely to its effect on the non-GCB subgroup. Although BCL2 expression does not contribute to the determination of COO distinctions, the OS of BCL2-positive DLBCL patients is significantly improved by the addition of R. These results provide insight into the possible mechanisms by which R exerts its beneficial therapeutic effect.

S5.2.7 TABLES

Antibody	Clone	Dilution
BCL2	124	1:40
BCL6	PG-B6p	1:10
CD10	56C6	1:50
MUM1	MUM1p	1:50

Table S5.2.1 – Antibodies used in the study.

Therapy (# patients)	CHOP (81)	CHOP-R (82)	χ^2 p-value
High IPI (%)	60	56	0.42
Female (%)	32 (40%)	35 (43%)	0.68
Median Age (y)	66	58	0.07
Median follow-up time (y)	5.1	4.0	nd
GCB subtype	38	36	0.69
Bcl-2 expression	56	55	0.67
Bcl-6 expression	57	66	0.15

Table S5.2.2 - Clinical and pathological characteristics of the entire cohort.

BCL2 & BCL6 (n# cases)		CHOP (2 year OS)	CHOP-R (2 year OS)	Kaplan Meyer OS (p)
BCL2	BCL2 + 111	53%	75%	0.008
	BCL2 - 45	64%	66%	0.93
BCL6	BCL6 + 123	61%	70%	0.125
	BCL6 - 33	43%	68%	0.092

Table S5.2.3 - Effect of R on DLBCL survival in relation to both BCL2 and BCL6 expression profiles. DLBCLs over-expressing BCL2 benefit significantly with immunochemotherapy. The effect on BCL6 negative cases was of borderline significance, precluding any conclusions.

COO & BCL2 (no cases)		CHOP (2 year OS)	CHOP-R (2 year OS)	Kaplan Meyer OS (p)
GCB	BCL2 + 51	61%	69%	0.32
	BCL2 - 22	66%	67%	0.83
Non-GCB	BCL2 + 60	45%	75%	0.017
	BCL2 - 23	59%	64%	0.70

Table S5.2.4 - Effect of R on DLBCL survival in relation to COO phenotype and both BCL2 and BCL6 expression profiles. Only the non-GCB cases expressing BCL2, benefited from the addition of R to the treatment regimen.

S5.2.8 FIGURES

COO	Activated B Cell (ABC)	Germinal Center B cell (GCB)
Clinical outcome (CHOP)	Inferior	Superior
RNA	Constitutive NF-κB activation	NF-κB pathway not increased
DNA	*BCL2* gene amplification or transcriptional up-regulation	t(14;18)(q32;q21)

Figure S5.2.1 - Cell-of-Origin distinction in DLBCL.

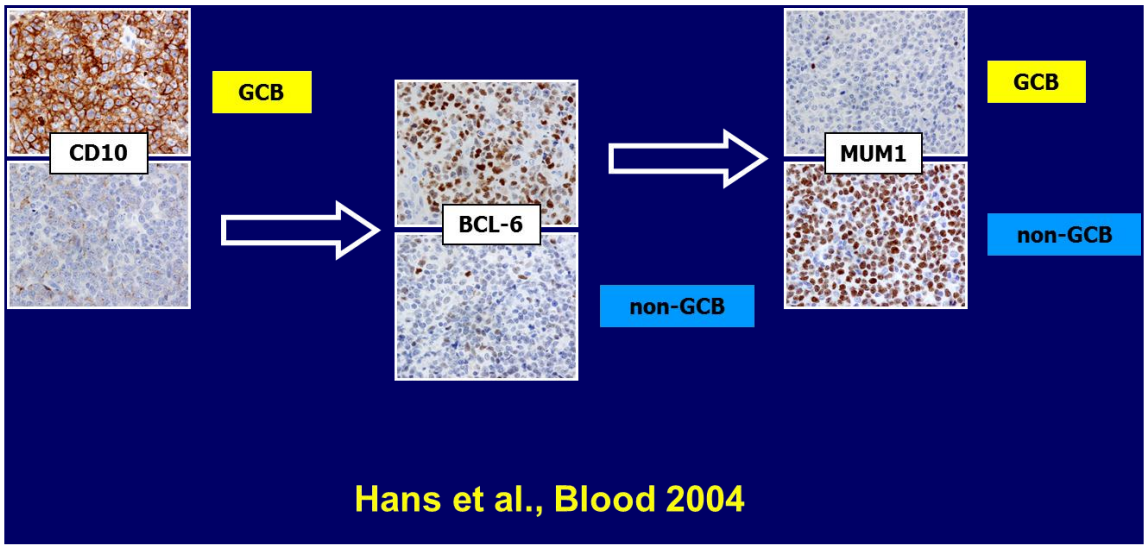

Figure S5.2.2 - Cell-of-Origin distinction using tissue microarrays (TMA) and immunohistochemical staining algorithm.

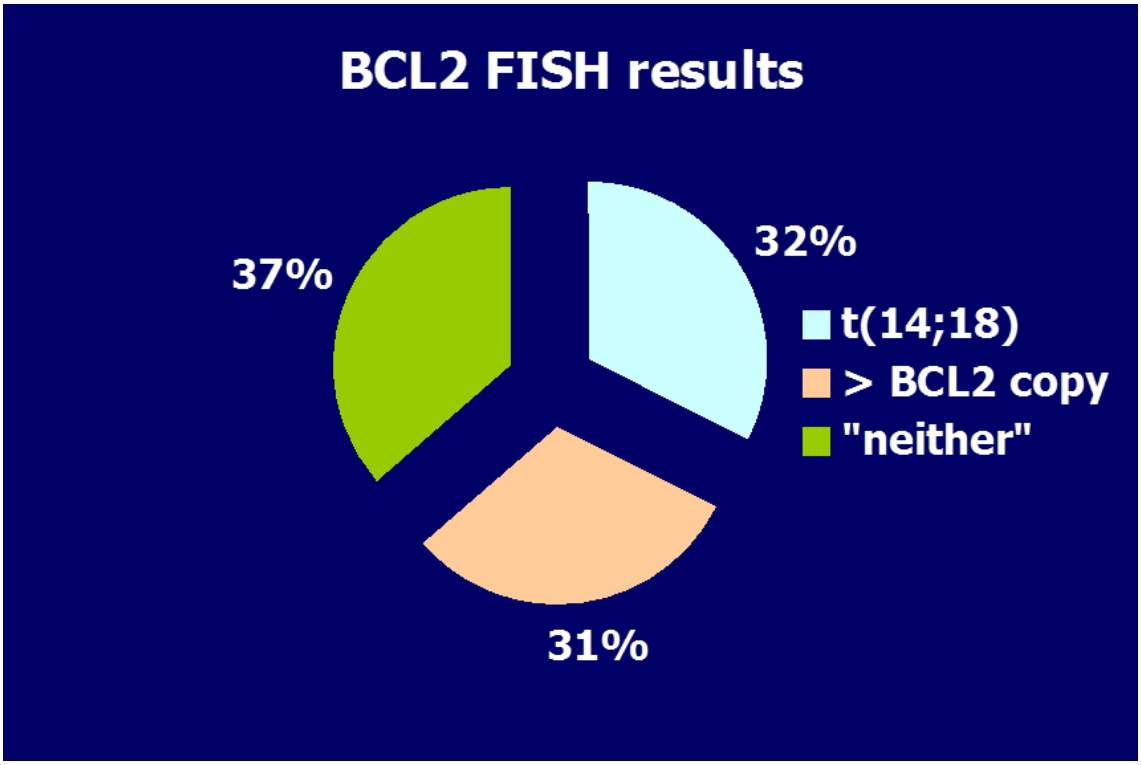

Figure S5.2.3 - FISH *BCL2* results in 100 DLBCL patients diagnosed at BCCA. Increased gene copy number and t(14;18) were mutually exclusive.

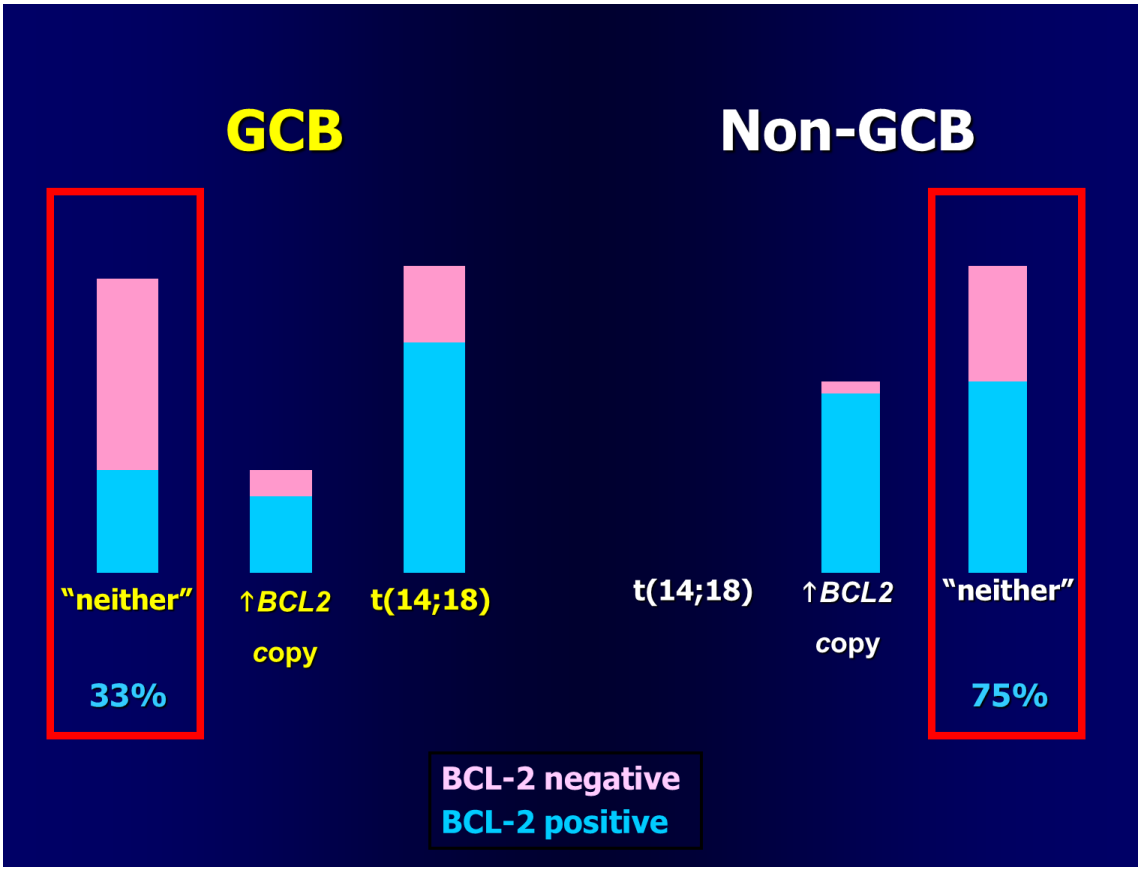

Figure S5.4 - Correlation between *BCL2* FISH results, BCL2 expression and COO phenotype. Cases lacking both the t(14;18) and increased *BCL2* copy number (47 cases) were distributed between the GCB (42%) and non-GCB cases (62%). Of these, only 33% of the GCB compared with 75% of the non-GCB expressed BCL2 protein.

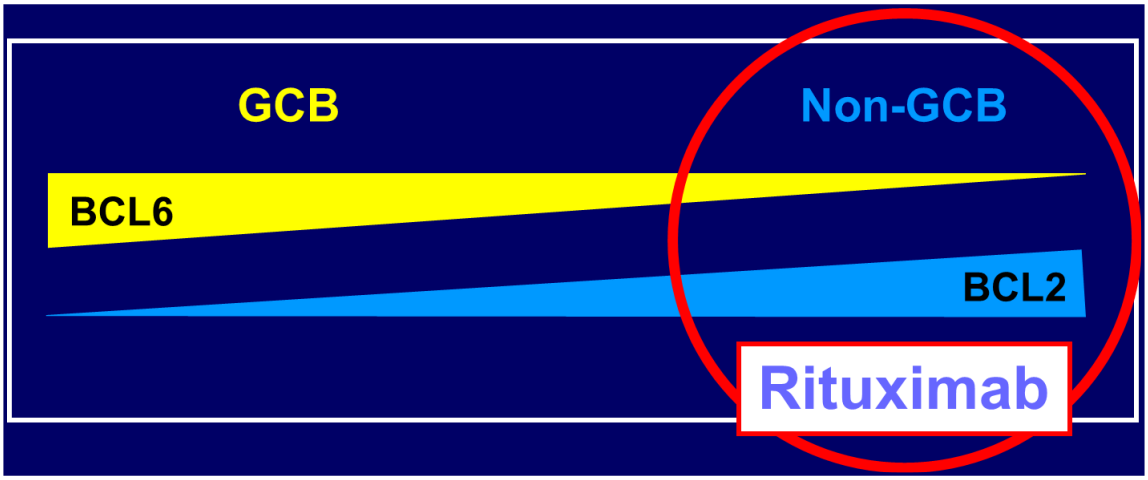

Figure S5.2.5 - In the new R-CHOP era, rituximab shows to preferentially prevent chemotherapy failures in DLBCLs that express BCL2 protein and failed to express BCL6 protein.

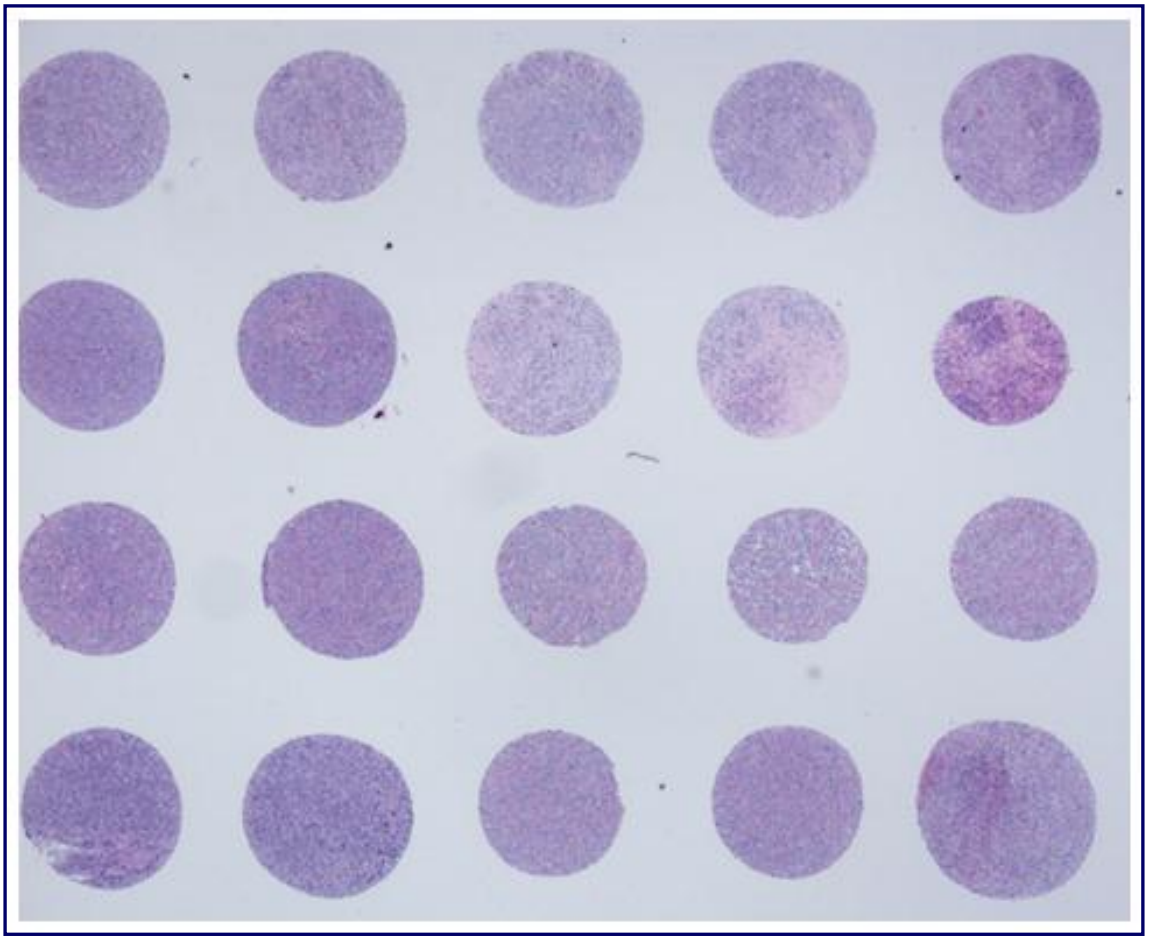

Figure S5.2.6 - Tissue microarray (TMA) built using duplicate 0.6mm cores from diagnostic DLBCL FFPET samples.

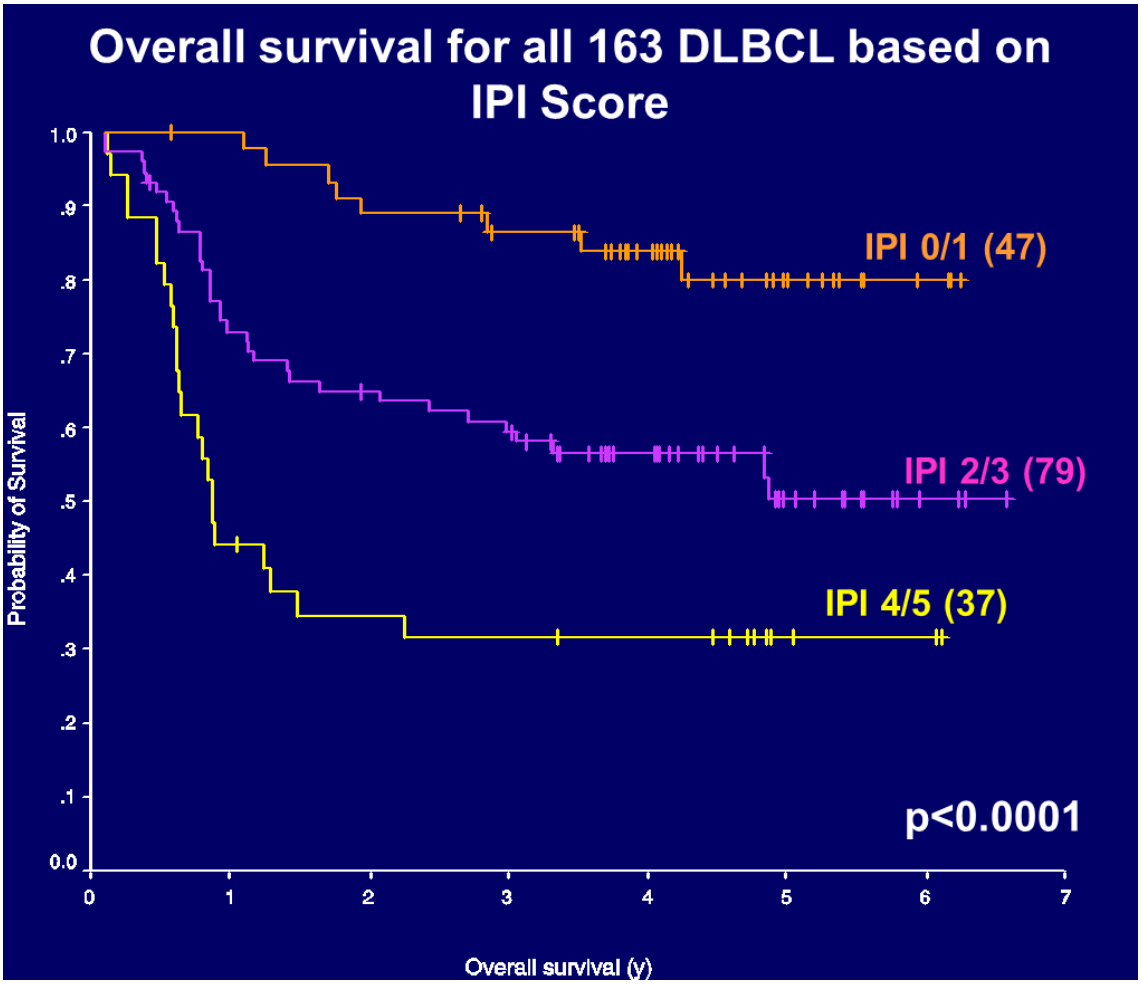

Figure S5.2.7 - The IPI was predictive of overall survival (OS) (P<0.0001) for the entire study population.

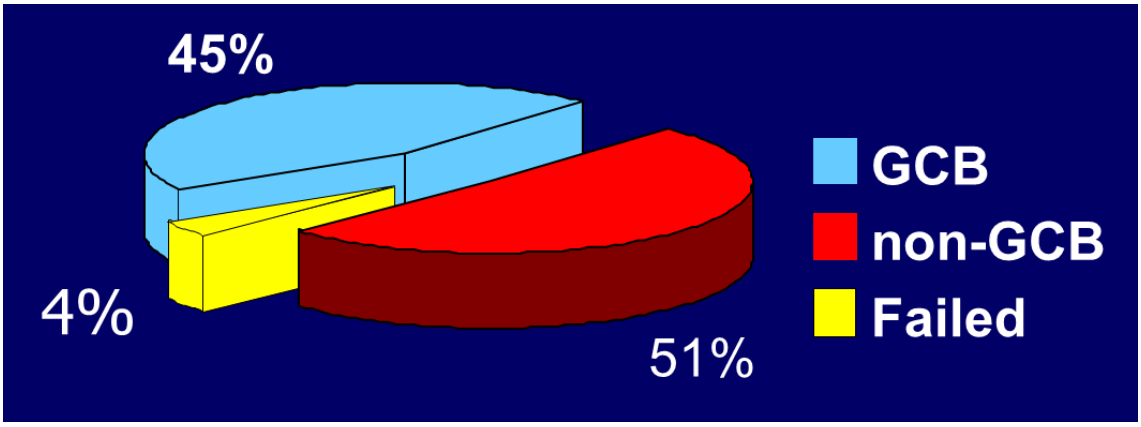

Figure S5.2.8 - Cell-of-Origin phenotype sub-types.

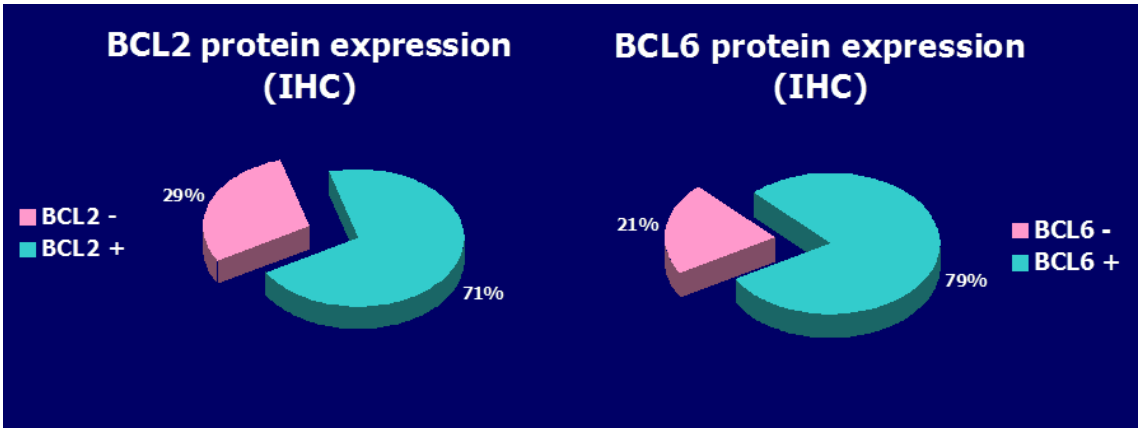

Figure S5.2.9 - BCL2 & BCL6 expression: Overall, 71% and 79% of the cases over-expressed BCL2 and BCL6, respectively.

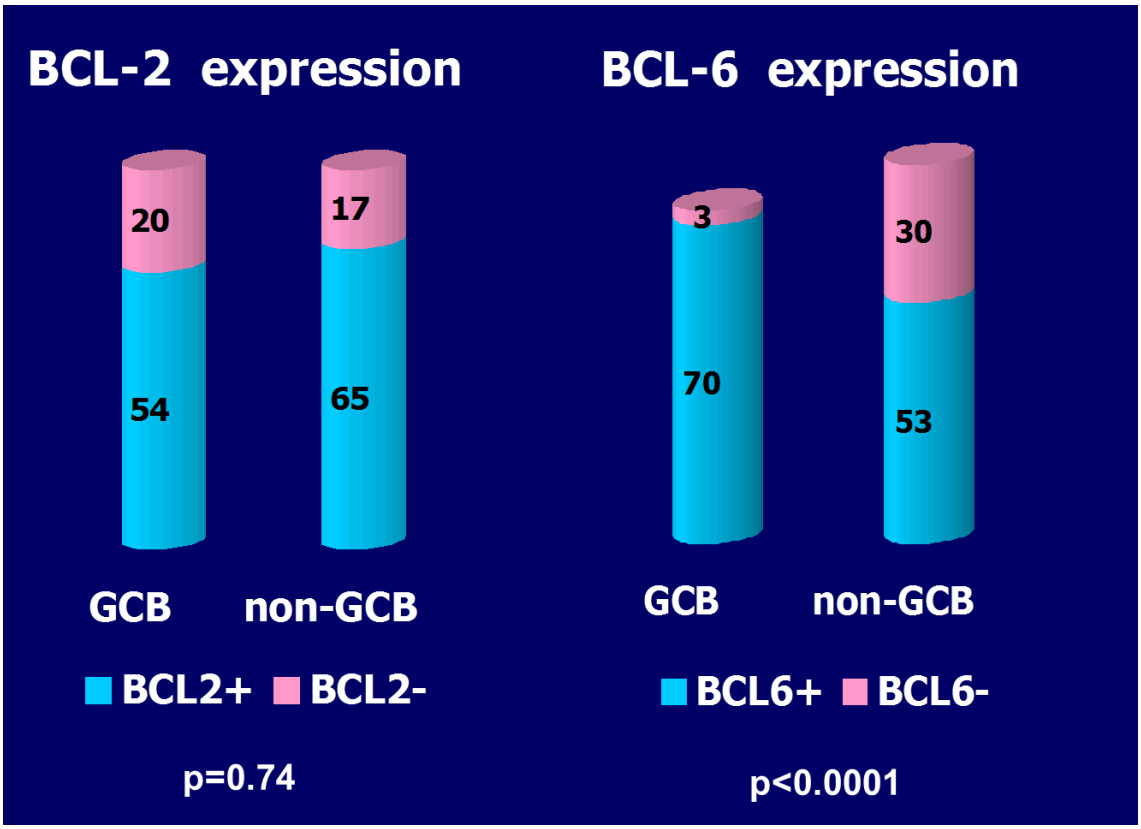

Figure S5.2.10 - Correlation of BCL2 & BCL6 with COO phenotype. When comparing GCB vs. non-GCB groups there was no statistical difference in the number of cases expressing BCL2, while, as expected, BCL6-positive cases were over-represented in the GCB group.

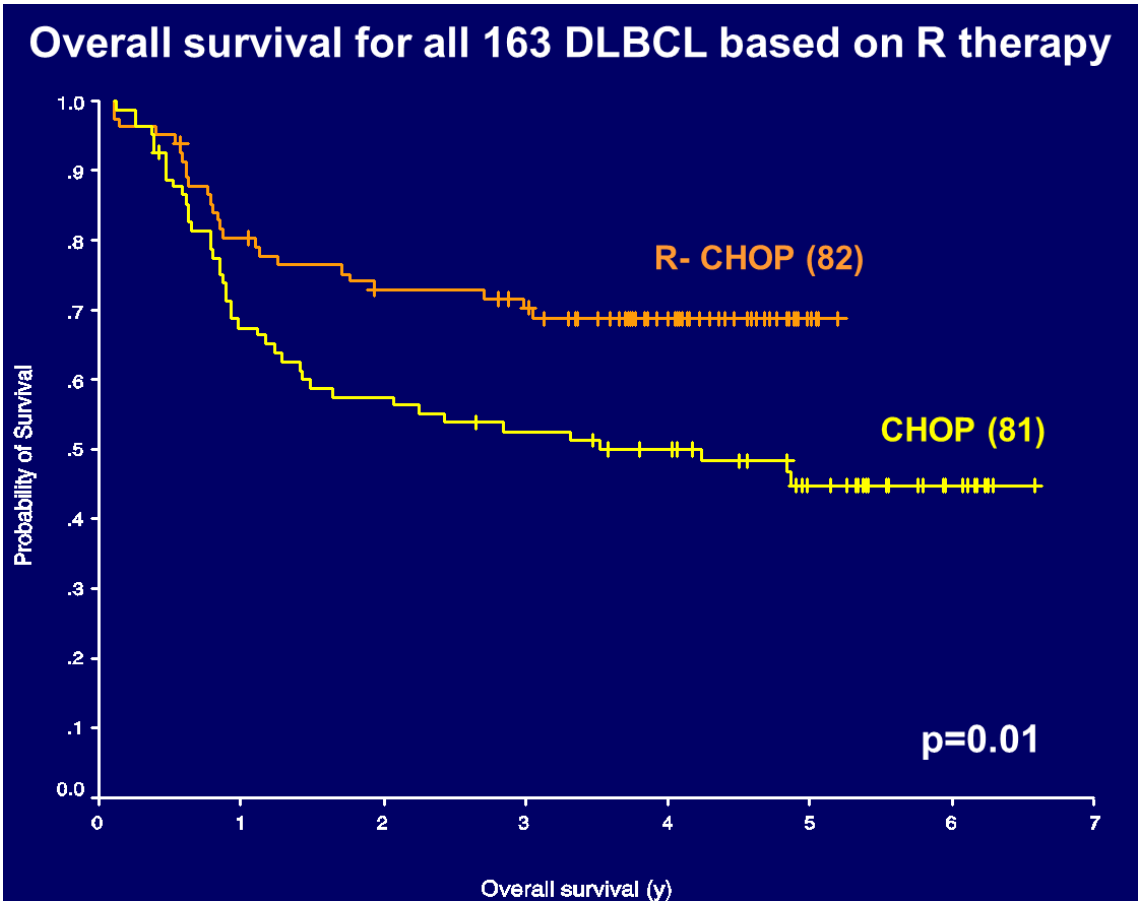

Figure S5.2.11 - Impact of R therapy addition in DLBCL overall survival. As previously reported, the addition of R in the whole group resulted in a dramatic improvement in survival.

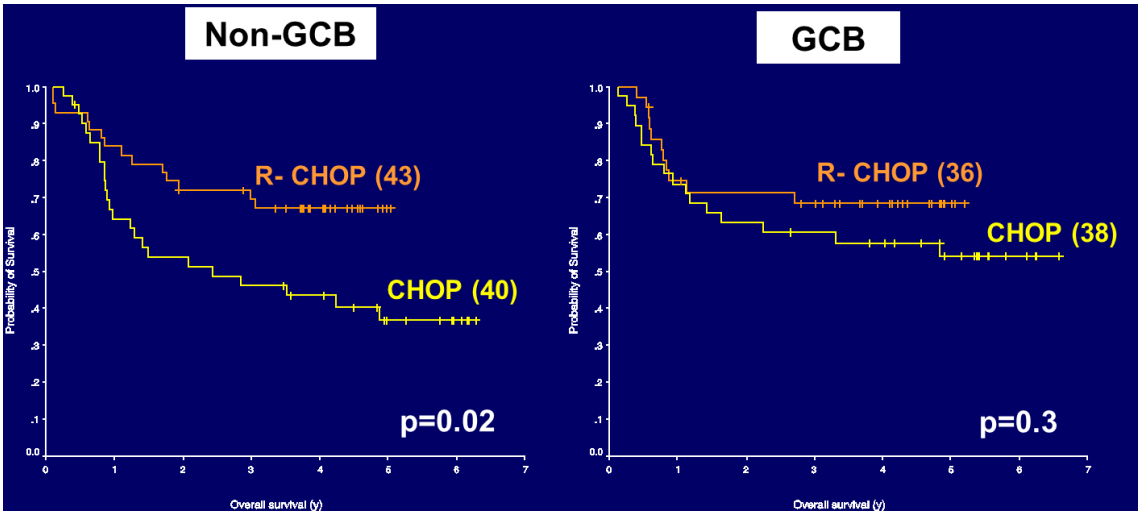

Figure S5.2.12 - Impact of R therapy addition in DLBCL COO subgroups overall survival. The addition of R was associated with a better prognosis in the non-GCB cases, but not in the GCB cases. The significant result in the non-GCB was independent of IPI group in a Cox multivariate analysis.

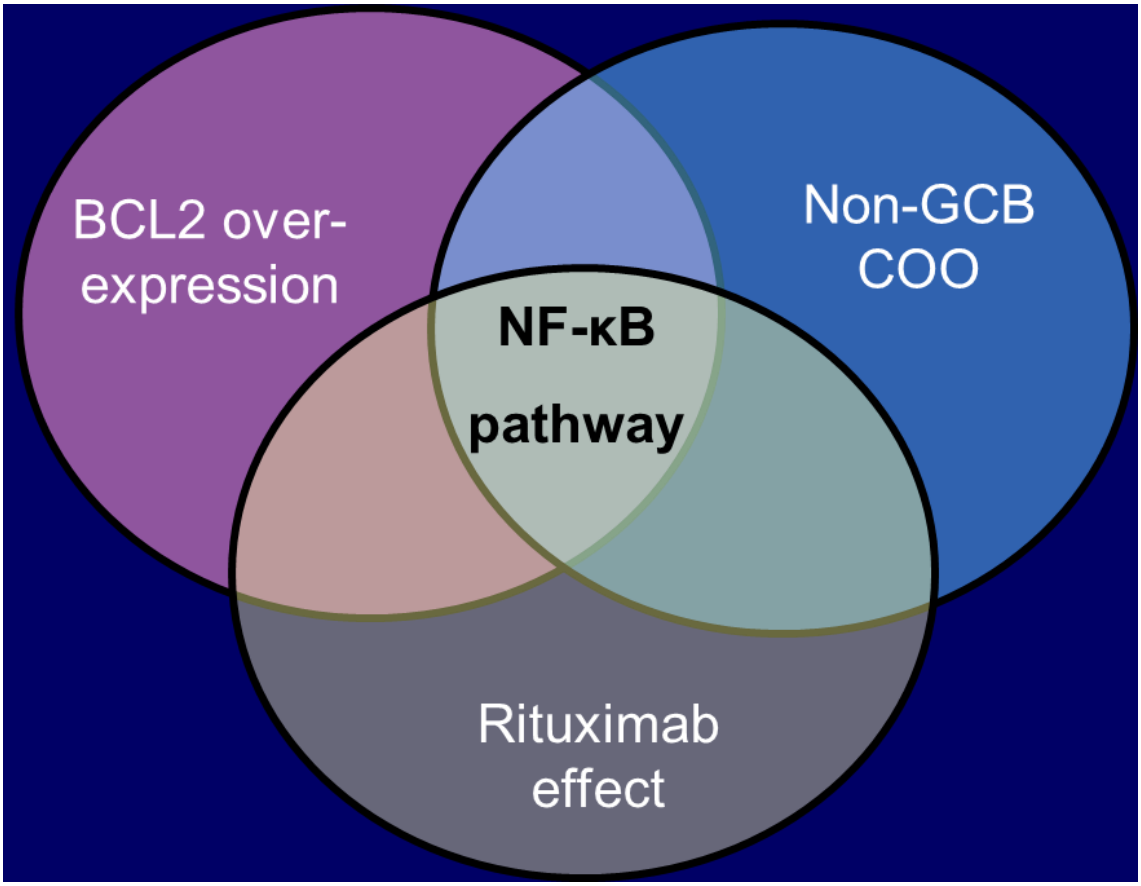

Figure S5.2.13 - NFκB pathway. The candidate mechanism responsible for differences in BCL2 expression, response to Rituximab therapy and OS between GCB-type and non-GCB type DLBCL.

S5.2.9 REFERENCES

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Section 5.3: Prognostic Significance of *BCL6* Rearrangements Detected by Fluorescence in situ Hybridization (FISH) in Diffuse Large B Cell Lymphoma

S5.3.1 INTRODUCTION

As a master regulator of germinal center (GC) formation, the BCL6 protein plays a critical role in promoting rapid proliferation, survival, attenuating sensing and response to DNA damage, and blockade of terminal differentiation. Given the unusual tolerance of germinal centre B cells to simultaneous proliferation and somatic hypermutation, it is perhaps not surprising that a majority of B cell lymphomas arise from GC B cells, among which the DLBCL is the most common subtype. Constitutive expression of BCL6 is often observed in DLBCL, frequently in association with promoter translocations or point mutations (1). Indeed, rearrangement of the *BCL6* proto-oncogene located at chromosome band 3q27 is one of the most frequent cytogenetic abnormalities in DLBCL, occurring in up to 35% of cases (2-4). The *BCL6* gene, a zinc-finger transcription factor, may be translocated with diverse partners in DLBCL, including both immunoglobulin heavy-chain (IGH) and non-IGH loci (5, 6). Clinical studies investigating the prognostic impact of *BCL6* rearrangement in DLBCL have yielded contradictory results, variably demonstrating favourable (2), intermediate (3, 4, 7), or adverse outcome (8, 9). However, an association has recently been reported between *BCL6* rearrangement and ABC phenotype (10). This Cell-Of-Origin (COO) profile was initially demonstrated as an adverse biological marker in DLBCL patients treated with CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone), and has been shown to retain its predictive impact in patients treated with CHOP plus rituximab (R-CHOP) (11).

The introduction of R to standard first-line therapy has significantly improved clinical outcome in DLBCL, and may alter the prognostic impact of both clinical and biological factors in this disease (12, 13). The prognostic significance of *BCL6* rearrangement has not yet been reevaluated since the introduction of R to DLBCL therapy. We have used a tissue microarray (TMA)-based fluorescence *in-situ* hybridization (FISH) strategy to analyze *BCL6* rearrangement status in a retrospective cohort of patients with DLBCL.

S5.3.2 AIM

The objectives of this study were to compare the effect of *BCL6* rearrangement on survival in DLBCL patients treated with CHOP and R-CHOP, and to evaluate the relationship between *BCL6* rearrangement and other clinical and biological prognostic variables in this disease, including COO phenotype.

S5.3.3 MATERIAL & METHODS

S5.3.3.1. Patients & Sample Selection

We included all patients identified through the Centre for Lymphoid Cancer database of the BCCA who met the following criteria: 1) confirmed diagnosis of DLBCL (excluding primary mediastinal B cell lymphoma) by pathology review, 2) treatment with either cyclophosphamide, doxorubicin, vincristine and prednisolone (CHOP) alone or in combination with rituximab immunotherapy (R-CHOP), 3) available diagnostic paraffin material on a TMA, 4) negative HIV status 5) treated at the BCCA between the years 1999-2007. Ethical approval for this study was obtained from the University of British Columbia – British Columbia Cancer Agency Research Ethics Board (UBC BCCA REB).

S5.3.3.2. Fluorescence *in-situ* Hybridization and Immunohistochemistry Using Tissue Microarrays

For TMA construction archival, formalin-fixed, paraffin-embedded tissue (FFPET) diagnostic biopsy specimens were selected and 0.6mm duplicate cores were obtained from representative areas containing large B cells with typical morphology. FISH was performed according to a standard protocol for paraffin material as described elsewhere (10, 14) using commercially available Vysis LSI *BCL6* Dual Color, Break Apart Rearrangement Probes (Abbott Molecular, IL, USA). Cases were recorded as rearranged for the *BCL6* locus (*BCL6*+) if break apart occurred at least in one signal of more than 5% of nuclei. All other signal constellations were regarded as negative (*BCL6*-). Immunohistochemical analysis was performed using monoclonal antibodies for

BCL2 (Dako, clone 124, Denmark), BCL6 (Ventana Medical Systems, Tucson, Arizona), CD10 (Ventana Medical Systems, Tucson, Arizona), and MUM1 (Dako, Denmark), following routine protocols for automated immunohistochemistry on the Ventana Benchmark XT (Ventana Medical Systems, Tucson, Arizona). Cases were then categorized as germinal centre B cell-like (GCB) or non-GCB using a standard algorithm (15). Accordingly, for each case, the core with the highest percentage of tumor cells stained was used for analysis. Cases were considered positive if 30% or more of the tumor cells were stained with an antibody.

S5.3.3.3. Statistical Analysis

Group comparisons were performed by means of X^2 and student t tests. For time to event analyses we used SPSS Software Version 11.0.0 applying Kaplan Meier survival estimates (16), univariate and multivariate Cox proportional hazard models with the endpoint of overall survival (OS) (17), defined as the time from initial diagnosis to death from any cause. P-values < 0.05 were reported as significant.

S5.3.4 RESULTS

A total of 174 patients with DLBCL who met all inclusion criteria were identified. FISH was successfully performed in 164 out of 174 cases (94.3%), of whom 65 received CHOP and 99 received R-CHOP. *BCL6* rearrangement was detected in 32 out of 164 cases (19.5%), including 17 CHOP-treated patients and 15 R-CHOP-treated patients. Representative *BCL6*+ FISH images of the TMA sections are shown (Figure S5.3.1).

Baseline clinical characteristics according to *BCL6* rearrangement status are listed in Table S5.3.1. There were no significant differences in initial treatment regimen between the two subgroups, with similar proportions of *BCL6*+ (15/32) and *BCL6*- (84/132) patients receiving R as part of initial therapy. A higher proportion of patients in the *BCL6*+ subgroup had a high-intermediate or high-risk International Prognostic Index (IPI) score (IPI 3-5) (62.5% *vs.* 40.2%, P=0.02). Analyzing the individual IPI parameters, the proportion of patients with elevated serum lactate dehydrogenase (LDH) was higher in the *BCL6*+ group

(P=0.007). No significant difference in age, performance status, Ann Arbor stage or number of extranodal sites was found between subgroups.

Immunohistochemical characteristics according to *BCL6* rearrangement status are listed in Table S5.3.2. BCL6 staining and COO classification (15) was successfully performed in 162 of 164 patients, respectively. Representative BCL6 staining results are shown in Figure 1B. BCL2 staining was available in 115 of 164 (70%) patients. The presence of a *BCL6* rearrangement was significantly correlated with COO; 68% of *BCL6*+ patients were of non-GCB subtype, as compared with 48% of *BCL6*- patients (P=0.006). No association was found between *BCL6* rearrangement and either BCL6 (P=0.78) or BCL2 (P=0.95) protein expression.

The median follow-up duration was 2.9 years (range, 0.33-8.68). Kaplan-Meier analysis of the entire cohort revealed an OS benefit for patients treated with R-CHOP compared to CHOP alone (5-year OS, 66.6% *vs.* 44.1%, P=0.007), as previously reported (18), and a trend toward decreased OS in patients with a *BCL6* rearrangement (5-year OS, 59.4% *vs.* 43.4%,P=0.07). Within treatment groups, *BCL6* rearrangement was associated with a trend toward inferior OS (5-year OS, 45.3% *vs.* 70.1%; P=0.08) among patients treated with R-CHOP, but not among patients treated with CHOP (5-year OS, 41.2% *vs.* 45%; P=0.64) (Figure S5.2.2). Conversely, R use was significantly associated with an OS benefit in *BCL6*- patients (5-year OS, 70.1% *vs.* 45.0%, P=0.009), but not in *BCL6*+ patients (5-year OS, 45.3% *vs.* 41.2%, P=0.70). In agreement with previously published data, BCL6 protein expression was associated with a trend toward prolonged OS, but only in CHOP-treated patients (5-year OS, BCL6+ *vs.* BCL6-, 52.2% *vs.* 31.8%, P=0.11)(19).

In R-CHOP treated patients, high IPI score (IPI 3-5) predicted significantly worse OS (5-year. OS, 46.1% *vs.* 77.8%, P=0.002), while non-GCB immunohistochemical profile was associated with a trend toward inferior outcome (50.8% *vs.* 76.8%, P= 0.07). A Cox regression analysis was performed for the R-CHOP-treated cohort using the following four variables: IPI score, COO immunophenotype, BCL2 protein expression, and *BCL6* gene rearrangement status. In multivariate analysis, only high IPI score was found to be an independent adverse prognostic factor for overall survival (P=0.002).

S5.3.5 DISCUSSION

The *BCL6* proto-oncogene encodes a transcriptional repressor essential for normal germinal center formation, whose deregulated expression has been implicated as an important pathway in lymphomagenesis (20). Recently, in collaboration with others, we have shown that BCL6 binds to the promoters of approximately 3000 genes, representing more than 10% of the genes in the whole genome microarrays used for these assays. This number of target genes is consistent or even slightly less in number than those identified for other transcription factors, such as MYC, HES, NOTCH1, and several of the E2F isoforms (21). This indicated that BCL6 regulates a biologically coherent set of pathways such as DNA repair, cell cycle, chromatin formation, and regulation, protein stability and transcriptional regulation that showed represented in both normal and malignant B cells (21).

This study analyzed the clinical impact of *BCL6* gene rearrangement in a retrospective cohort of DLBCL patients, treated in both the pre- and post-R eras. Our data suggest an association between *BCL6* rearrangement and inferior outcome in patients treated with R-CHOP, but not in patients treated with CHOP. However, *BCL6* rearrangement also correlated with the presence of high-risk clinical features and did not demonstrate prognostic significance independent of the IPI.

The effect of *BCL6* rearrangement on outcome in DLBCL has remained uncertain despite previously published work. In a seminal study by Offit *et al.*, *BCL6* rearrangement was found to be a favorable prognostic marker in DLBCL, possibly due to an association with less extranodal disease (2). Most subsequent investigators failed to demonstrate any influence of *BCL6* rearrangement on prognosis in DLBCL, and similarly our analysis showed this marker to have no predictive value in patients treated with CHOP alone (3, 4, 7, 10). A more recent analysis, restricted to DLBCL patients with primary nodal disease, reported inferior survival in patients with *BCL6* rearrangement, as well as a correlation between this marker and non-GCB immunophenotype (8). In agreement with our results, several prior investigators have reported a correlation between *BCL6* rearrangement and adverse clinical features (6), but this has not been a uniform finding. These discrepancies may be explained in

part by heterogeneity between study populations due to selection biases and to differences in molecular diagnostic techniques and their interpretation.

The addition of R to first-line combination therapy has led to a significant survival improvement in patients with DLBCL, requiring a re-evaluation of established prognostic markers (13). Biological prognostic markers in particular may be influenced by changes in standard therapy, due to the differential effects of new agents within previously defined biological subgroups. It has recently been reported that BCL2 protein expression, an adverse prognostic marker in CHOP-treated patients, loses its prognostic effect in the R-CHOP era, due to a disproportionate benefit from R in BCL2+ cases (22). In our analysis, the addition of R appeared primarily to benefit patients without *BCL6* rearrangement, and was associated with significantly prolonged OS in this subgroup. In contrast, little improvement in outcome was observed with the addition of R among *BCL6*+ patients, in whom long-term survival remained less than 50% even with R-CHOP. *BCL6* rearrangement was associated with a trend toward poorer outcome in R-CHOP-treated patients, suggesting that it may have value as a predictive biomarker in DLBCL in the R era.

The IPI remains the most successful clinical prognostic instrument in DLBCL (5, 23). However, the IPI appears to lose discriminatory power in R-CHOP-treated patients, and in a recent analysis was unable to identify a subgroup with less than a 50% chance of long-term survival (13). Biologic markers may help to refine risk-stratification in DLBCL, and to identify high-risk subgroups which might benefit from intensified or novel therapies. However, to be clinically useful these markers must provide prognostic information complementary to the IPI. In our study, the presence of *BCL6* gene rearrangement correlated with high IPI score, and in multivariate analysis this biomarker did not show independent prognostic significance. *BCL6* rearrangement also showed an association with non-GCB immunophenotype, previously demonstrated to be an unfavorable prognostic marker in CHOP-treated patients with DLBCL (15). However, in our cohort of R-CHOP-treated patients, COO phenotype did not emerge as an independent prognostic variable by multivariate analysis. BCL6 protein expression has been identified as a hallmark of GCB derivation in DLBCL, and has been associated with favorable clinical outcome (15, 24). However, in keeping with prior studies, we did not find a correlation between *BCL6*

rearrangement and BCL6 protein expression, but demonstrated a relationship between *BCL6* rearrangement and non-GCB phenotype (8, 10). This seemingly paradoxical finding may relate to several factors. First, mechanisms other than gene rearrangement have been shown to deregulate *BCL6* expression in DLBCL, including targeted mutations that may interrupt negative gene auto-regulation (20). Second, the functional effect of *BCL6* rearrangement may vary depending on other biological factors, including the chromosomal translocation partner and cellular context. One group of investigators has demonstrated significantly lower *BCL6* expression with non-*IGH*/*BCL6* than with *IGH*/*BCL6* rearrangements, and has also reported inferior survival in DLBCL patients with non-*IGH*/*BCL6* translocations (9, 25). A more recent study by the Leukemia/Lymphoma Molecular Profiling Project (LLMPP) examined the effect of *BCL6* rearrangement on gene expression, in patients with both GCB- and ABC-subtype DLBCL (10). In this analysis, *BCL6* rearrangement was associated with increased *BCL6* mRNA levels in patients with ABC-subtype DLBCL, but not in patients with GCB-subtype DLBCL. The clinical significance of these biological subdivisions remains to be clarified.

S5.3.6 CONCLUSION

In summary, the introduction of R may have altered the prognostic impact of *BCL6* rearrangement in patients with DLBCL. However, to be incorporated into clinical practice as a useful prognostic biomarker, *BCL6* rearrangement must be shown to provide prognostic information additional to the IPI. *BCL6* rearrangement also correlates strongly with non-GCB immunophenotype, and the interdependence of these two prognostic variables requires further study. Correlative biomarker analysis within prospective randomized clinical trials will be needed to determine the prognostic value of this marker in the R era.

S5.3.7 TABLES

	BCL6+ ‡ (n=32)	BCL6- (n=132)	P value
Age (years), median (range, sd)	65.5 (35-85, 14.1)	63 (16-92, 14.8)	*P*=NS
Gender (%)			
Male	63	61	*P*=NS
Female	37	39	
Mass size (cm), median	10	6	*P*=NS
(range, sd)	(3-20, 5.4)	(1-25, 5.0)	
Lactate dehydrogenase (%)			
Normal	25	51	*P*=0.007
Elevated	75	49	
Extranodal sites (%)			
0-1	68	75	*P*=NS
>1	32	25	
Site of presentation(%)			
Primary nodal	87	75	*P*=NS
Primary extranodal	13	25	
Stage, Ann Arbor (%)			
I-II	31	43	*P*=NS
III-IV	69	57	
IPI score (%)			
0-2	38	60	*P*=0.022
3-5	62	40	

‡*BCL6+: BCL6 gene rearrangement present; BCL6-: BCL6 gene rearrangement absent; sd: standard deviation.*

Table S5.3.1. Clinical characteristics of 164 DLBCL patients subject to successful FISH for the *BCL6* locus

	BCL6+ (n=32)	BCL6- (n=132)	P value
BCL2 protein expression (%)			
Positive	78	71	*P*=NS
Negative	22	29	
BCL6 protein expression (%)			
Positive	69	71	*P*=NS
Negative	31	29	
Cell-of-origin* (%)			
GCB	32	59	*P*=0.006
Non-GCB	68	41	

*According to Hans et al.[17]

Table S5.3.2 - Immunophenotypic characteristics of 164 DLBCL patients subject to successful FISH for the *BCL6* locus.

S5.3.8 FIGURES

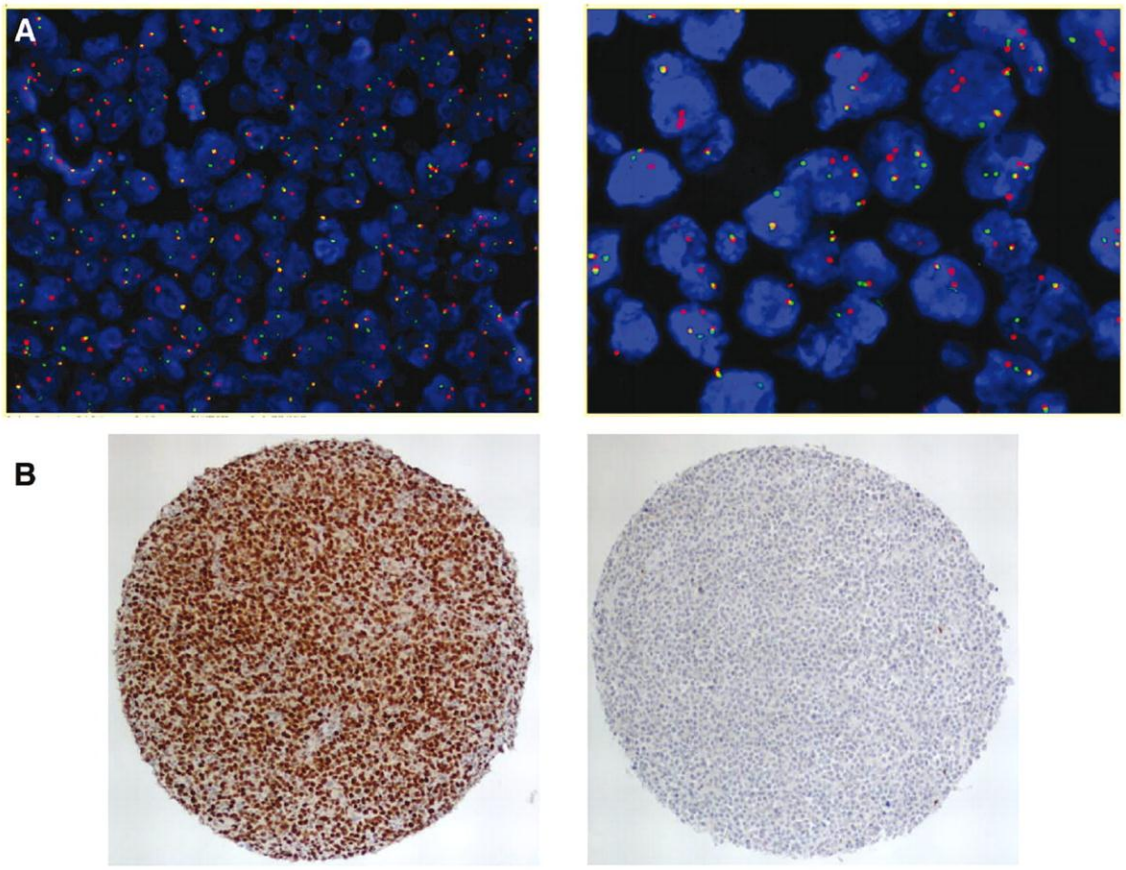

Figure S5.3.1 - **(A)** FISH on FFPET sections on a TMA. Left panel: Interphase nuclei with *BCL6* break apart (*BCL6*+). The most common signal pattern is 1 fusion signal (yellow) and 2 split signals (1 red, 1 green) visualized at 200x magnification. Right panel: *BCL6*+ interphase nuclei with break apart (split signals) and polyploidy (multiple fused signals) of the *BCL6* locus (high power field). **(B)** Immunohistochemistry for BCL6 on TMA cores. Left panel: Positive staining in virtually all large B cells Right panel: Negative staining.

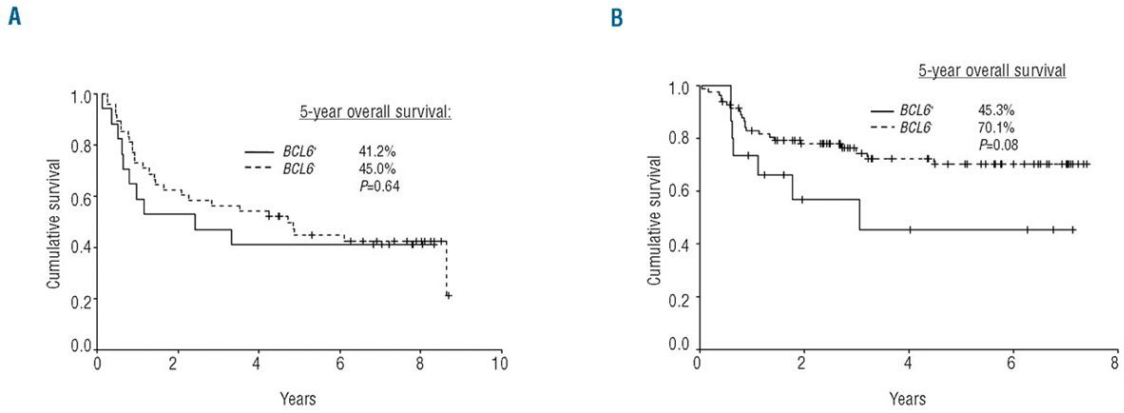

Figure S5.3.2 - Overall survival in *BCL6*+ vs. *BCL6*- disease. A. OS, CHOP-treated patients (n=65). B. OS, R-CHOP-treated patients (n=99).

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Section 5.4: *MYC* Aberrations are Associated with a Poor Prognosis in DLBCL Patients Treated with R-CHOP Immunochemotherapy

S5.4.1 INTRODUCTION

Diffuse large B cell lymphoma (DLBCL) is recognized to be a heterogeneous group of diseases with clinical, morphological, immunohistochemical and molecular subtypes defined in the new World Health Organization (WHO) classification (1). Further, a new category has been created defined as 'borderline cases' which are considered B cell lymphomas, unclassifiable, with features intermediate between DLBCL and Burkitt lymphoma (2). Morphologically, these tumors typically have a mixture of medium to large sized cells, a high proliferation rate and 35-50% of cases have an 8q24/*MYC* translocation (2). However, approximately 5-10% of DLBCLs with typical morphology also harbour a *MYC* rearrangement and these cases are considered in the category of DLBCL, not otherwise specified (NOS) in the updated WHO classification (3). Although approximately 20% of these cases may also harbor a concurrent *IGH-BCL2* translocation i.e. t(14;18)(q32;q21), the so-called 'double-hit' or dual translocation lymphomas (4), which is known to be associated with a poor outcome, there is very little information of the prognostic importance of *MYC* aberrations in isolation in DLBCL.

Gene expression profiling studies suggest that some *MYC*+ DLBCL have a molecular signature that is distinct from Burkitt lymphoma (5, 6). *MYC*+ cases of aggressive B cell lymphoma lacking a molecular Burkitt's lymphoma signature had an inferior prognosis, however, this analysis was not confined to DLBCL and almost half had a concurrent t(14;18) (6). A recent study suggests that *MYC* aberrations identified by fluorescence *in situ* hybridization (FISH) in pathologically defined DLBCL patients treated with CHOP-like chemotherapy is associated with an inferior prognosis (7). However, the presence of concurrent *IGH-BCL2* translocation was not assessed and none of the patients in this analysis had received rituximab (R) containing treatment regimens. With the

established superiority of R-CHOP compared to CHOP (8-10), the importance of *MYC* rearrangements in this population must be re-established.

S5.4.2 AIM

The purpose of this study was to screen an unselected series of patients with DLBCL for *MYC* aberrations to determine the frequency of this occurrence and whether if there were any pathologic or clinical defining features as well as to assess the prognostic impact of *MYC* aberrations in DLBCL patients treated with R-CHOP immunochemotherapy.

S5.4.3 MATERIAL & METHODS

S5.4.3.1. Patients & Samples Selection

The BCCA Centre for Lymphoid Cancer Database was screened to identify adult patients (> 15 years of age) with newly diagnosed DLBCL treated with CHOP in combination with R (R-CHOP) with curative intent. Patients that were HIV-positive were excluded. Those cases with paraffin blocks available were utilized to construct a tissue microarray (TMA). For this analysis, only those cases that are considered to be DLBCL not otherwise specified (DLBCL-NOS) by the updated WHO classification of lymphomas recently published were included (3). Clinical information including for the calculation of the International Prognostic Index (IPI) was determined (11). This study was approved by the BCCA Research Ethics Board.

S5.4.3.2. Tissue Microarray & Immunohistochemistry

The TMA was constructed using duplicate 0.6mm cores from formalin-fixed paraffin-embedded tissue (FFPET) derived from newly diagnosed cases of DLBCL. Immunohistochemistry (IHC) was performed on archived FFPET using CD20 (L26, Dako, CA), CD3 (Dako, CA), CD10 (Clone 56C6, Vector, CA), Ki-67 (Dako, CA) and BCL2 (clone 124, Dako, CA). The TMAs were stained using automated immunohistochemistry on a Ventana Benchmark using standard protocols. Cases were considered positive for CD10 or BCL2 if $\geq$ 30% of the B cells were positive. The Ki-67 antibody MIB1 was used to determine the

proliferation rate and ≥80% was defined as 'high proliferation' as previously described (12).

S5.4.3.3. Cytogenetic Analysis

All cases were screened for a *MYC* rearrangement using a Vysis dual color FISH break-apart probe (Abbott Molecular, Abbott Park, IL). Cases harbouring a *MYC* rearrangement were also screened for the presence of a t(14;18) rearrangement using the LSI *IGH/BCL2* dual color, dual fusion translocation probe (Abbott Molecular Abbott Park, IL). Partner chromosomes were determined using additional BAC probes.

S5.4.3.4. Determination of DLBCL Cell of Origin Subtypes

For 62 patients, frozen tissue was available for gene expression profiling analysis. RNA was extracted using the ALL PREP kit (Qiagen) and following reverse transcription it was hybridized to the U133-2 Plus arrays (Affymetrix) according to the manufacturer's protocol. CEL files were normalized using robust multi-chip analysis (RMA). The cell of origin (COO) phenotype was determined using model scores for the activated B-cell type (ABC) and germinal centre type (GCB) using the Bayesian formula previously described (13). For the remaining patients, the COO was determined to be GCB or non-GCB using immunohistochemistry according to the Hans criteria (14).

S5.4.3.5. Statistical Analysis

Progression-free survival (PFS) was determined from the date of the pathological diagnosis to the date of relapse, progression or death due to lymphoma or treatment toxicity. Overall survival (OS) was determined from the date of diagnosis to the date of death of any cause. The time to central nervous system (CNS) relapse was defined from the date of diagnosis to the date of documented relapse in the CNS. The X^2 test was used to compare baseline characteristics between *MYC*+ and *MYC*- – cases. Survival curves were plotted using the Kaplan Meier method and compared using the log-rank test (15). The Cox Proportional Hazards model was used to determine the impact of multiple factors on PFS, OS and time to CNS relapse (16). All statistical analyses were

performed using SPSS software (SPSS version 11.5 for Windows; SPSS Inc®, Chicago, IL).

S5.4.4 RESULTS

A total of 137 newly diagnosed cases of DLBCL treated with R-CHOP chemotherapy and with paraffin blocks available for the construction of the TMA were included in this analysis. FISH was successful in 135 cases at defining the presence or absence of a *MYC* rearrangement and 2 cases were considered technical failures. In total, 12/135 (8.8%) cases of DLBCL were positive for *MYC* in this series. Patients that were DLBCL *MYC*+ were predominantly male (75%) with a median age of 69 (22-85) with no particular high risk defining clinical features at disease presentation compared to *MYC*- cases (Table S5.4.1). Half were early stage and were in a good risk group by the IPI (Table S5.4.1). Only 33% had >1 extranodal site of involvement and testicular involvement was more common than in *MYC* negative patients.

S5.4.4.1. Cytogenetics, Immunohistochemistry and Cell of Origin

The partner chromosome information for the *MYC* rearrangement was available in 6/12 cases and 3 involved the *IG* loci t(8;14) and 3 involved a variant partner chromosome (non-*IG*). Three of the *MYC* positive cases (25%) had a concurrent *BCL2* t(14;18) so-called dual translocations or 'double-hit'. In total 8/12 cases were BCL2+ (67%) using the Dako antibody with no difference in frequency observed compared to the MYC^- cases (Table S5.4.1). Of interest, 2/3 of the *MYC*+ cases that were also t(14;18)+ were BCL2- by IHC using the DAKO antibody. *MYC*+ cases were more likely to have a high proliferation rate (Table S5.4.1).

In the updated WHO classification of lymphomas, the COO phenotype distinguishes two molecular subtypes of DLBCL-NOS, germinal centre-like (GCB) and activated B cell-like (ABC) (3, 13). This was further validated at the immunohistochemical level into GCB and non-GCB subgroups based on the presence or absence of CD10, BCL6 or MUM1 (14). The COO phenotype was available for 131 cases (97%) (4 missing in the *MYC*- group); 62 were assigned by gene expression profiling (GEP) (13) and 69 by the Hans algorithm (14). For

the purpose of the COO assignment two groups were considered: GCB and non-GCB (the latter including those assigned ABC by GEP). There was no difference in frequency of GCB vs. non-GCB between the *MYC*+ and *MYC*- cases (Table S5.4.1). Not surprisingly, the 3 cases with dual translocations had a GCB phenotype.

S5.4.4.2. Impact on Survival of *MYC* Rearrangements in R-CHOP Treated Patients

The 5-year PFS (65% *vs.* 31%, P=0.006) and OS (72% *vs.* 33%, P=0.016) was inferior in cases of DLBCL treated with R-CHOP that harboured a *MYC* rearrangement (Figure S5.4.1a and 1b). Within the favourable GCB COO (n=68), cases that harboured a *MYC* translocation had an inferior PFS (P=0.049) and OS (P=0.014). However within the non-GCB subgroup (n=63), there was an inferior PFS (P=0.033) but not OS (P=0.303). In univariate analysis, in addition to the presence of a positive *MYC* break-apart assay, the IPI, a non-GCB phenotype, extranodal involvement and bone marrow involvement with DLBCL were all associated with an inferior PFS (Table S5.4.2). Similar results were observed for OS, although only a trend for an inferior outcome was observed with a non-GCB phenotype. Of note, BCL2 expression and a high proliferation rate, including variable cutoffs for Ki-67 ($\geq$ 80%, $\geq$90% or $\geq$95%) were not prognostic for PFS or OS (Table S5.4.2). Multivariate analysis using a Cox proportional hazard model was performed on the R-CHOP cohort including factors that had a P-value of < 0.1 in univariate analysis. The presence of a *MYC* rearrangement remained a significant factor for both PFS (HR 3.28 (1.49, 7.21), P=0.003) and OS (HR 2.98 (1.28, 6.95), P=0.011) (Table S5.4.3). Of note, if the *MYC*+ cases with a dual translocation (n=3) are removed and the analysis is repeated, *MYC* retains prognostic significance for both PFS (P=0.009) and OS (P=0.05).

S5.4.4.3. Risk of CNS Relapse in R-CHOP Treated Patients with a *MYC* Rearrangement

Given that CNS relapse is a known consequence in Burkitt lymphoma, we evaluated whether there was an increased risk of CNS relapse in cases of DLBCL that harbour a *MYC* translocation. Only one patient with testicular

involvement received intrathecal prophylaxis at the time of primary therapy, otherwise CNS prophylaxis was not utilized as part of the primary therapy. Using the time to CNS relapse as the endpoint, the presence of a *MYC* break-apart (P=0.018) was predictive of a CNS relapse in R-CHOP treated patients (Figure S5.4.2). In multivariate analysis, the presence of a *MYC* break-apart (HR 8.0 (1.33, 48.03) P=0.023) and kidney involvement (HR 25.28 (2.60, 245.86) P=0.005) remained significant in the Cox proportional hazards model after adjusting for the IPI, extranodal sites>1 or other high-risk extranodal sites (bone marrow, testicular involvement and sinus involvement). Of interest, a high proliferation rate was not predictive of an increased risk of CNS relapse.

S5.4.5 DISCUSSION

The recently published updated WHO defines three subgroups of aggressive lymphomas that may harbor a *MYC* gene rearrangement: Burkitt lymphoma; a new category defined as B cell lymphoma, unclassifiable with features intermediate between DLBCL and Burkitt lymphoma (BCLU) as well as DLBCL-NOS (1). Overall, the frequency of *MYC* aberrations in DLBCL ranges from approximately 5-15%, however, in many studies it is often unclear how the patients are selected for cytogenetic analysis and some older studies may have included so-called 'Burkitt-like' cases.

A limited number of studies have evaluated the prognostic importance of *MYC* in patients treated with CHOP-like regimens (7). The German High-Grade Non-Hodgkin's Lymphoma Study Group (DSHNHL) recently evaluated 177 patients who were treated in the BH1 and BH2 clinical trials which compared CHOP-like regimens (7). Patients were included if they had tumor tissue available for the construction of a TMA to assess for the presence of a *MYC* gene rearrangement. In this comprehensive analysis, the presence of a *MYC* aberration was associated with an inferior OS (P=0.047) and there was a trend to a reduction in EFS (P=0.062). It is unclear from this analysis what proportion of cases had a concurrent t(14;18) translocation. In a Japanese study, 11% of cases of DLBCL were found to harbor a *MYC* translocation however, the cases evaluated were those that had abnormal karyotypes that may have been selected based on high risk features, which is also reflected by the high

proportion of cases with a concurrent *BCL2* translocation (17). Nevertheless, the presence of a *MYC* translocation was associated with an inferior prognosis, particularly if in concert with a t(14;18). In contrast, one study found that the presence of a *MYC* rearrangement did not predict for a worse outcome, however, the treatment received is not detailed and it is possible patients may have received more dose-intensive regimens (18).

The present study is the first published to evaluate whether the presence of *MYC* gene rearrangements is still of clinical relevance in patients treated in the R-CHOP, which is now the accepted standard of care in the treatment of DLBCL given confirmed superiority in a number of randomized clinical trials. In this 'unselected' population-based registry cohort of DLBCL, the frequency of *MYC* aberrations was 8.8%, which is comparable to the German study (7.9%). Similar to the German study, we did not identify any high-risk clinical features at presentation in the *MYC* positive group. Although there was a tendency for the tumours in our study to have a high Ki-67 score, a wide range was seen and this feature cannot be relied upon to identify patients at higher risk to harbor a *MYC* rearrangement. Further, our results contrast the DSHNHL study in which no correlation between a high proliferation rate and the presence of a *MYC* rearrangement was found which may reflect the high variability and poor reproducibility of this immunohistochemical marker (19). Regardless, a high proliferation rate was not associated with outcome in this analysis. In contrast, the presence of a *MYC* rearrangement retained prognostic significance in R-CHOP treated patients in multivariate analysis for both PFS and OS and thus it is independent of clinical risk factors and COO. Further, we were able to define a poor risk group of patients with DLBCL with *MYC* rearrangements within the favorable GCB subgroup.

In our series, 72% of cases were BCL2+ using the commercially available Dako antibody. In the R-CHOP treated subgroup 4/12 cases were BCL2- (including 2 $t(14;18)^{+}$) and interestingly, 3 of 4 are long-term survivors compared to 2 of 8 who were BCL2+. Although it is possible that false-negative results may occur based on the sensitivity of the Dako BCL2 antibody, it would appear that the combination of *MYC* driving proliferation and the anti-apoptotic effect of BCL2 are critical in determining outcome. However, given the small number of patients in this study, this requires further validation. In a recently described

large series of dual *MYC/BCL2* translocations (double-hit cases) from the BCCA, it was shown that overall survival of patients with these concurrent translocations was extremely poor (20). Cases with of DLBCL with dual translocations had an improved but still unsatisfactory outcome compared to those that were aggressive B cell lymphoma unclassifiable and were more likely to be BCL2- (20). Thus, cytogenetic studies and BCL2 status should be tested in all DLBCL *MYC*+ cases to determine the critical factors impacting prognosis in this population.

Central nervous system relapse is a known risk in patients with Burkitt lymphoma and as a result, chemoprophylaxis is incorporated into treatment regimens for this disease. Similarly, we found an increased risk of CNS relapse in cases of DLBCL treated with R-CHOP that harbored a *MYC* aberration. Given the overall poor outcome, of patients with secondary CNS disease, these results raise the question as to whether this population should be treated with Burkitt lymphoma type regimens which include intensive CNS prophylaxis.

S5.4.6 CONCLUSION

In summary, *MYC* rearrangements define a small group of patients with DLBCL, independent of COO, who are less likely to be cured with R-CHOP and may have an increased risk of CNS relapse. There are no identifiable clinical, histological, or immunophenotypic features that signal that a case of DLBCL may harbor a *MYC* aberration. Thus, all patients with DLBCL should undergo FISH analysis for assessment of the *MYC* rearrangement in addition to analysis for the t(14;18) and BCL2 expression. *MYC*+ DLBCL likely represent a distinct DLBCL subtype and regimens more in line with those used in Burkitt lymphoma may be more appropriate in this patient population.

S5.4.7 TABLES

Feature	*MYC*$^{+}$ (n = 12)	*MYC*$^{-}$ (n = 123)	*P*
Median age, y	68	61	—
Age > 60 y	8 (67)	68 (55)	.448
Male sex	9 (75)	73 (59)	.289
Stage 3 or 4	6 (50)	75 (61)	.459
B symptoms			
Extranodal any site	8 (67)	72 (58)	.584
Extranodal > 1	4 (33)	25 (20)	.295
Bone marrow	0	11 (9)	.280
Testicular	2 (17)	3 (2)	.013
Sinus	0	2 (1.6)	.280
Gastrointestinal	2 (17)	19 (15)	.911
Kidney	0	2 (1.6)	.656
Liver	0	7 (6)	.396
Bulky disease*	4 (33)	31 (26)	.575
PS > 2*	5 (42)	42 (36)	.677
LDH abnormal*	9 (75)	54 (50)	.100
LDH > 2× ULN	3 (25)	28 (26)	.930
IPI 0-2 vs 3-5	6 (50)	48 (39)	.459
Ki-67†			
More than 80%	7 (58)	27 (22.5)	.007
More than 90%	6 (50)	9 (7.5)	< .0(
More than 95%	4 (12)	8 (6.7)	.002
BCL2 protein+‡	8 (67)	86 (70)	.782
GCB phenotype§	7 (58)	61 (51)	.640

PS indicates Performance Status; LDH, lactic dehydrogenase; and ULN, upper limit of normal.* Missing data: LDH, n = 15; bulky disease, n = 3; PS, n = 5. † Failed in 3 *MYC*$^{-}$ cases. ‡ Results using the Dako antibody; not available in 1 *MYC*$^{-}$ case. § Not available in 4 *MYC*$^{-}$ cases.

Table S5.4.1 - Characteristics of *MYC*+ and *MYC*- DLBCL patients.

Risk factor	PFS *P*	OS *P*
MYC[+]	.006	.016
IPI ≥ 3	< .001	< .001
Non-GCB phenotype	.041	.058
BCL2 protein[*]	.313	.492
Ki-67		
More than 80%	.582	.418
More than 90%	.759	.751
More than 95%	.351	.642
Extranodal sites, any	.014	.034
Extranodal sites > 1	.013	.040
Bone marrow DLBCL[+]	< .001	< .001
Testicular	.124	.263
Bulky disease	.334	.841

Table S5.4.2 - Univariate analysis of risk factors for PFS and OS for DLBCL patients treated with R-CHOP

Risk factor	PFS		OS	
	Hazard ratio (CI)	***P***	**Hazard rati (CI)**	***P***
MYC^{+}	3.28 (1.49-7.21)	.003	2.98 (1.28-6.95	.011
IPI ≥ 3	2.69 (1.48-4.86)	.001	3.29 (1.68-6.46	.001
Non-GCB phenotype	1.86 (1.04-3.34)	.038	—	NS
Bone marrow DLBCL	3.74 (1.67-8.36)	.001	4.06 (1.72-9.58	.001

NS indicates not significant; and —, not applicable. * Not available in 4 *MYC*$^{-}$ cases.

Table S5.4.3 - Multivariate analysis of risk factors of PFS and OS of DLBCL patients treated with R-CHOP.

S5.4.8 FIGURES

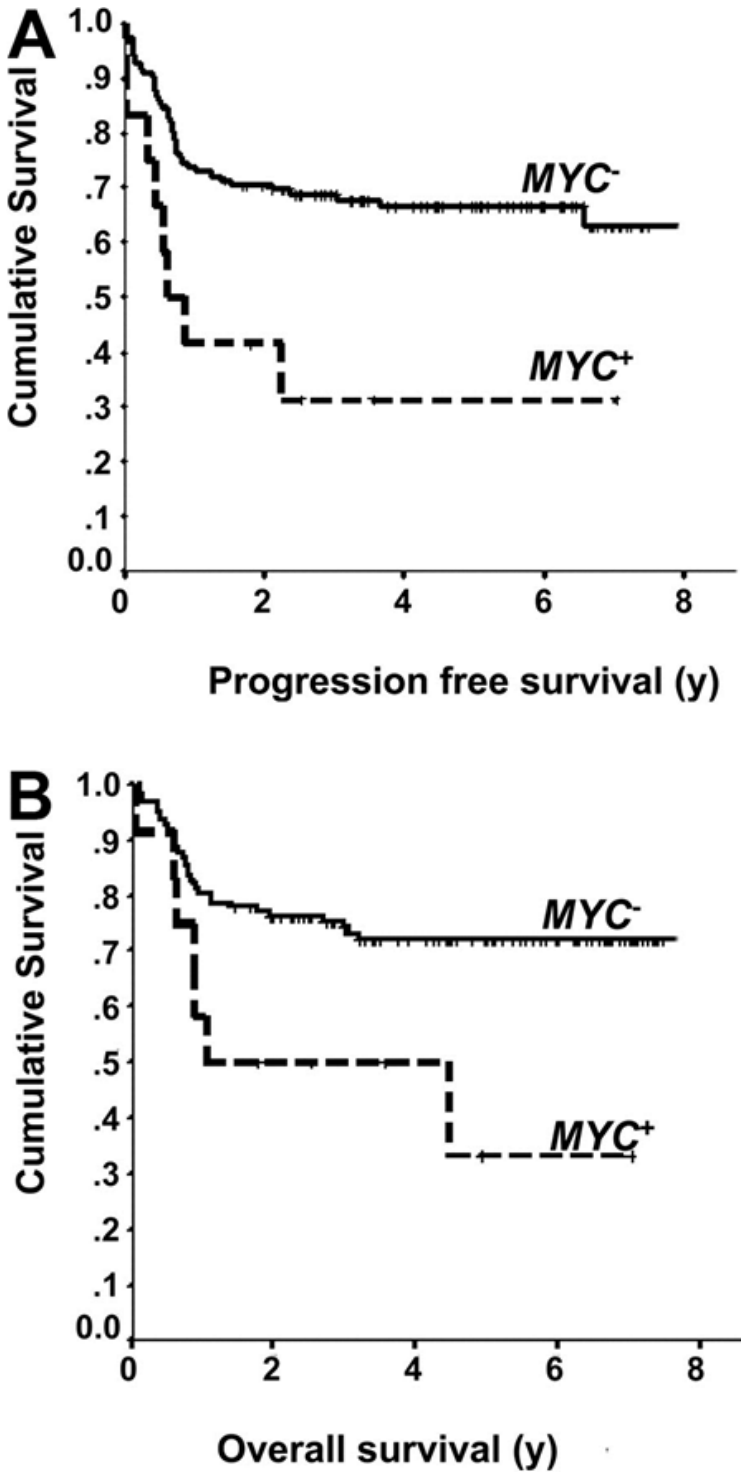

Figure S5.4.1 - Outcomes of patients with *MYC*+ DLBCL treated with R-CHOP, Progression free survival (A) and overall survival (B).

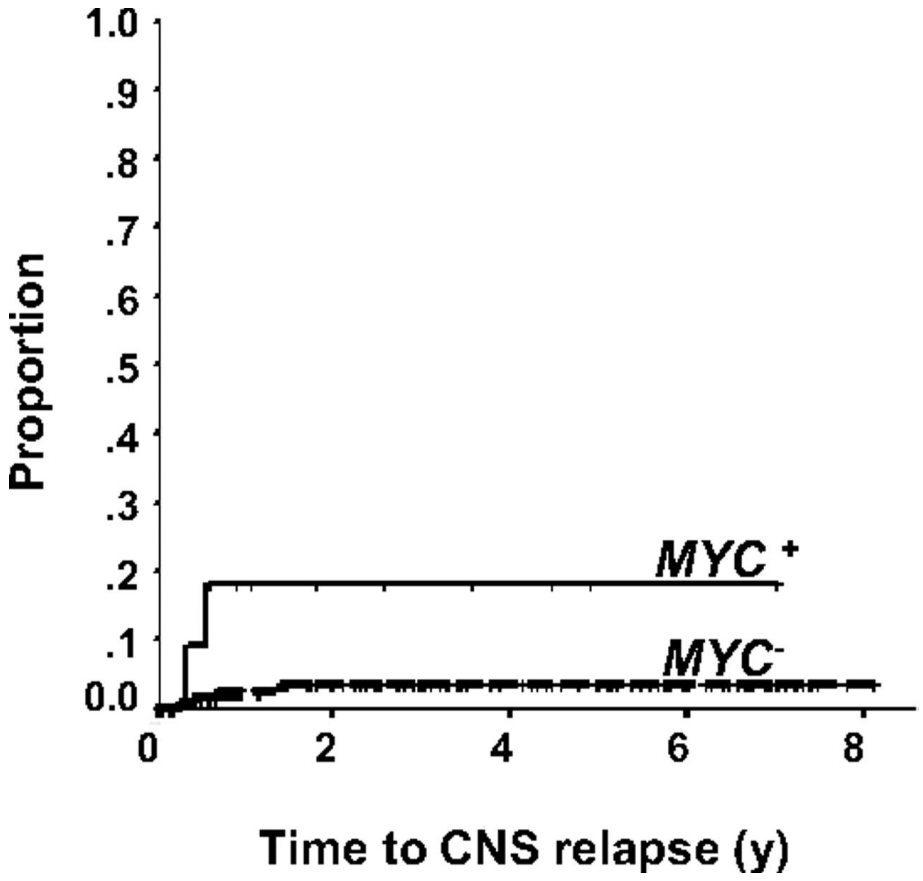

Figure S5.4.2 - Time to central nervous system relapse of *MYC*+ and *MYC*− DLBCL.

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Section 6: New Biological Predictors of Survival in Hodgkin Lymphoma: The Microenvironment

6.1 INTRODUCTION

Classical Hodgkin lymphoma (CHL) is one of the successes of recent treatment strategies in oncology. Despite great progress, about 20% of patients with advanced-stage disease still die of relapse or progressive disease while a similar proportion of patients are over-treated (1, 2), many of whom suffer from treatment-related late sequelae, such as solid tumours and end-stage organ dysfunction (3-5).

None of the clinical variables, namely the International Prognostic Score (IPS) (6), used to help guide treatment decisions can predict the majority of patients in whom standard treatment will fail to eradicate disease. New biological markers that can improve on outcome prediction will be important to advancing the field.

CHL most characteristic and defining features is the predominance of reactive cells in the tumour microenvironment, which largely outnumber the malignant cells (7). Thus, many studies have focused on the features of this prominent and abnormal immune microenvironment, exploring whether immune related cells can contribute to outcome prediction (8-11). Although some biomarkers have proven to be predictive in more than one study, most of these markers require validation in independent cohorts. Importantly, these studies must include patients treated uniformly, as heterogeneity of treatments has been recently implicated as an important factor contributing to the marked variability in published studies that have examined the role of various immune cells in the tumour microenvironment of lymphoid cancers (12).

6.2 AIM

The aim of our study was to determine cellular signatures that correlate with treatment outcome in CHL, using gene-expressing profiling (GEP) and immunohistochemical (IHC) analysis of diagnostic lymph node biopsies in CHL.

We further sought to validate any significant findings using a cohort of CHL patients uniformly treated in a single institution.

6.3 MATERIAL AND METHODS

6.3.1. Patients & Samples Selection

For GEP, we selected fresh frozen lymph node specimens, which had been obtained at the time of diagnosis from 130 patients with CHL, from the tissue archives at the BCCA and the University of Nebraska Medical Center. The criteria that we used included a primary diagnosis of CHL after central review, representative lymph node tissue (at least 1 cm^2 in tissue sections), negative status for HIV infection, and first-line treatment with ABVD chemotherapy (doxorubicin, bleomycin, vinblastine, and dacarbazine) or an ABVD-like regimen and, if indicated, radiation therapy (including wide-field radiation for patients with limited-stage disease). Primary treatment was defined as a failure if the lymphoma had progressed at any time after the initiation of therapy; treatment success was defined as the absence of progression or relapse. The median follow-up time for living patients in the treatment-success group was 3.9 years (range, 0.5 to 21.0). The GEP cohort was stratified according to treatment outcome (failure *vs*. success) in order to analyze differences between the two outcomes (Figure 6.1). Advanced-stage disease was defined with the use of Ann Arbor staging criteria (13). On the basis of the availability of formalin-fixed, paraffin-embedded tissue (FFPET) biopsies of diagnostic lymph node specimens, we also selected samples from 166 independent cases of CHL for IHC testing on a tissue microarray (TMA). To increase the statistical power for observations linked to progression-free survival (PFS) and disease-specific survival (DSS), this cohort was enriched for all available cases of treatment failure that were identified through the Centre for Lymphoid Cancer database at the BCCA. The number of cases of treatment failure was roughly matched to the number of cases of treatment success and both groups were roughly matched for clinical characteristics at diagnosis. For the independent validation cohort, we also recorded the outcome of salvage therapy delivered with curative intent in 61 patients; these secondary therapies included autologous stem-cell transplantation in 55 patients, CVPP chemotherapy (cyclophosphamide,

vinblastine, procarbazine, and prednisone) plus involved-field radiation in 5 patients, and GDP chemotherapy (gemcitabine, dexamethasone, and cisplatin) plus extended-field radiation in one patient. In this cohort, the median follow-up time was 4.0 years (range, 0.5 to 20.8). The study was approved by the institutional review boards at the University of British Columbia-BCCA & University of Nebraska Medical Center. Written consent was obtained from all patients in accordance with the principle of the Declaration of Helsinki.

6.3.2. Gene Expression Analysis

Total RNA was extracted from multiple (10 to 20) 20-µm freshly cut tissue sections after mechanical homogenization. Expression profiles were obtained with the use of GeneChip Human Genome U133 Plus 2.0 arrays containing 54,120 probe sets for genome-wide analysis of the human transcriptome (Affymetrix Inc., U.S.A). (Data are available at www.ncbi.nlm.nih.gov/geo/query/acc.cgi [accession number, GSE17920].

RNA preparation, array hybridization, and washing were performed according to routine protocol with modifications. All 130 microarray experiments met homogeneous criteria for quality control. Total RNA was extracted using Allprep RNA extraction kits (Qiagen Inc., ON, Canada). 1-8 µg of extracted RNA were subsequently used to prepare biotinylated cRNA in a one-cycle reaction following routine protocols including reverse transcription and labelling in-vitro transcription reactions. 11µg of labelled cRNA were then hybridized on the array overnight and the arrays were washed, stained and scanned using Affymetrix Fluidics Station 450 and Affymetrix GeneChip Scanner. All 130 reported microarrays passed homogeneous criteria for testing quality control including present call rates >20% and normalized unscaled standard errors <1.05 (NUSE). Thirteen microarrays were excluded from the analysis.

6.3.3. Immunohistochemical Analyses

To confirm the findings of the gene expression analysis, we performed IHC analysis using TMAs that was constructed from duplicate 1.5mm cores of 166 independent samples enriched for cases of treatment failure (Beecher Instruments, Silver Springs, MD). Antibodies used for IHC included CD3, CD20, CD30, CD68, and MMP11. IHC scoring ranged from 1 to 3 for CD68, from 1 to

4 for CD20, and from 0 to 3 for MMP11, with higher scores indicating a greater proportion of positive cells. TMA sections were evaluated by both morphology and IHC stains performed for routine diagnostic purposes. TMA sections were stained for CD68 (Dako, KP1), MMP11 (Abcam, ab52904), CD20 (Dako, L26), CD3 (polyclonal, Cell Marque) and CD30 (Dako, Ber-H2) following routine protocols for automated immunohistochemistry on the Ventana Benchmark XT (Ventana Medical Systems, Tucson, Arizona). The slides were independently scored by RDG, GH and PF. For CD68 staining, cells were scored in three representative high-power fields (tumour cell regions) and the relative percentage of CD68+ cells in relation to overall cellularity was reported as an average of both duplicate cores according to four groups: <5% IHC score 1, 5-25% IHC score 2, >25-50% IHC score 3 and > 50% IHC score 4. However, there were only eight cases with > 50% CD68+ macrophages and thus this latter group was merged with cases showing 25-50% (IHC score 3). Elevated numbers of CD68+ cells were defined as IHC scores 2 and 3 according to a threshold of 5% established on 5 benign tonsil controls and previous studies (14). Subgroup analysis of limited-stage CHL patients included a total of 41 cases. Due to the small sample size in IHC scoring groups 3 & 4 (n = 5 and n = 1, respectively), these groups were merged with IHC score group 2 (n = 17) for the purposes of DSS analysis. Discrepant cases between observers were centrally reviewed to reach consensus.

To control for case to case histologic variability, areas of sclerosis, necrosis and presence of primary or secondary lymphoid follicles, we recorded: 1) HRS cell CD20 positivity, 2) positivity of the surrounding reactive small B cells only (excluding sclerotic areas and follicles) and, 3) presence of B cell follicles. MMP11 staining was recorded as the overall cellularity of MMP11+ cells: IHC score 0 (no positivity), IHC score 1 (<1% of cells), IHC score 2 (1-10% of cells) and IHC score 3 (>10% of cells). Positive cells included HRS cells, macrophages and microvessels. Scores were stratified as high (3 or 4) or low (1 or 2) for CD20 staining.

6.3.4. Data Analysis

6.3.4.1. Predictive Models

In brief, we used the gene expression data from the 130 patients for whom all prognostic factors of the IPS were recorded and constructed a multidimensional classifier on the basis of feature selection, using sparse multinomial logistic regression (SMLR) and leave one-out cross-validation (15). Our aim was to build a robust discriminative model that was predictive of treatment failure in addition to identifying a small set of features (genes) that could be used as the basis for separating these data into the respective outcome groups (treatment success *vs.* failure). SMLR imposes a sparsity promoting prior distribution that models whether a gene should be considered in the construction of the classifier. As such, the model heavily penalizes a gene for being included in the feature set, resulting in a small set of discriminative features that are strongly supported by these data. SMLR performs feature selection and classification simultaneously (15). We trained an SMLR classifier using leave-one-out cross validation (LOOCV) analyzing pre-treatment data of the 130 patients with diagnostic lymph node biopsies. For feature selection in the training sets we also included the 7 clinical variables as defined in the IPS (age, Ann Arbor stage, sex, albumin, absolute lymphocyte count, lymphocyte percentage and hemoglobin) in addition to the 25,388 gene expression probe sets.

We measured classification accuracy using a Receiver Operator Characteristic (ROC) curve, the Area under Curve (AUC), the confusion matrix and class-specific error rates (15). Treatment failure was assigned as the positive class, and treatment success as the negative class. Thus, an accurate prediction of failure was a true-positive result, and an inaccurate prediction of failure was a false-positive result; an accurate prediction of success was a true-negative result, and an inaccurate prediction of success was a false-negative result. We compared three types of input data with respect to predictor performance: gene expression data only, clinical variables only, combination of the two. For determining relative variable importance we created Random Forests (RF) using the SMLR selected features (16). Following settings were used: 1) number of predictors considered for each node, 2) square root (number of input variables), 3) number of trees to build 10,000, class weights and 4) balanced. A ranked list of these variables (relative variable importance) was plotted using the standard method, which is based on randomizing of the variable values and measuring the resultant decline in model accuracy.

6.3.4.2. Statistical Analysis

Group comparisons were performed by means of the X^2 test and Student's t-test. For time-to-event analyses, we used two primary end points: progression-free survival (PFS - based on the time from initial diagnosis to progression at any time, relapse from complete response, or initiation of new, previously unplanned treatment) and disease specific survival (DSS - based on the time from initial diagnosis to death from lymphoma or its treatment, with data for patients who died of unrelated causes censored at the time of death). Cox-proportional-hazards models and time-to-event analyses with the use of the Kaplan–Meier method were performed with SPSS software, version 11.0 (17, 18).Two-sided P values of less than 0.05 were considered to indicate statistical significance.

6.4 RESULTS

6.4.1. Gene Expression Analysis

Unsupervised hierarchical clustering of the gene expression results did not identify clusters that were significantly associated with the outcome of treatment or any other reported clinical variable. However, using Global Test, with which we tested all pre-filtered genes, we found a significant correlation between the gene expression profile and the outcome of first-line treatment (P=0.02) (Table 6.1). Table 6.2 shows the clinical characteristics of the gene expression cohort. On the basis of supervised analyses with the data set stratified according to the failure or success of primary treatment, we identified 271 differentially expressed genes in the two outcome groups. Unsupervised hierarchical clustering of all 130 expression profiles with the use of these differentially expressed genes identified two clusters, one of which was associated with treatment success (cluster A) and the other with both success and failure (cluster B) (Figure 6.2). In the treatment-failure group, pathway analyses identified the following functions, 1) cell-mediated immune response, 2) cell-to-cell signalling and interaction, and 3) up-regulation of pathway genes involved in interleukin-12 signalling, 4) macrophages and 5) apoptosis (Table 6.3 & 6.4). Down-regulated pathways in the treatment-failure group included genes

involved in CTLA4 signalling in cytotoxic T lymphocytes and G-protein–coupled signalling (Table 6.5 & 6.6).

These results prompted an investigation of the association between microenvironment gene signatures and outcome. Using Global test, we performed associative testing to identify previously described cellular and pathway gene signatures that were differentially expressed in the two outcome groups – Figure 6.3 to 6.6 (19). In the treatment-failure group, there was over expression of gene signatures of tumour-associated macrophages (P=0.02) and monocytes (P=0.01), findings that are in agreement with the reported over expression of macrophage-signalling–associated genes according to pathway analysis (20, 21) – Table 6.1 & Figure 6.5. There was also over expression of gene signatures for angiogenic cells (P=0.04), adipocytes (P=0.01), and HRS cells (P=0.047) and under expression of a signature for germinal center B cells (P=0.01) in the treatment-failure group (22-25). Furthermore, previously described genes that are associated with an unfavorable outcome in CHL, such as lysozyme (*LYZ*) and cathepsin L1 (*CTSL1*), were over expressed in the treatment failure group (P=0.04) (26).

To test the overall power of expression profiles for outcome prediction, we constructed a classifier by means of SMLR (15). This algorithm identified 86 non-redundant annotated genes, age, and Ann Arbor stage by a cross-validation approach (Table 6.7). Figure 6.7 shows the 30 features with the highest discriminative power, as determined by a random-forest algorithm (16). Among the 27 individual genes with discriminative power exceeding that of the best clinical variable (age) was MMP11 (probe set235908_at), which was over expressed in the treatment-failure group (P=0.03 after adjustment for the false discovery rate). We selected this gene for further immunohistochemical testing, since previous studies have shown that the gene family of matrix metallopeptidases is over-expressed in patients in whom treatment has failed and that MMP11 in particular is expressed by tumour-associated macrophages (27, 28).

We compared the three data sources for feature selection and settled on a gene expression profiling model for gene expression probe sets only, a clinical model based on the International Prognostic Score only, and a combination model including both features. This comparison showed that the AUC value was

highest for the gene expression model, as compared with the clinical and combination models (0.837 vs. 0.625 and 0.821, respectively) (Figure 6.8). The differences in accuracy among the models were most prominent at low false-negative rates, with the gene expression model and the combination model yielding higher true-negative rates than the clinical model.

6.4.2 Immunohistochemical Analysis

Our gene expression study and previous studies by other investigators suggested that a predominance of tumour-infiltrating macrophages, a lack of small B cells, and over expression of matrix metallopeptidases were correlated with the failure of primary treatment. For these reasons, we selected the markers CD68 (macrophages), CD20 (B cells), and MMP11 for immunohistochemical analysis of a tissue microarray containing lymph node biopsies of 166 patients with CHL (who were unrelated to the patients in the gene expression analysis), including 79 patients in whom treatment had failed (Table 6.2). Of these markers, CD68 and CD20 antibodies are routinely used in the diagnosis of lymphoma. The TMA was also stained for CD30 (a sensitive marker for HRS cells) and CD3 , but neither the number of CD30+ cells nor the number of CD3+ cells was correlated with outcome. Of these IHC markers, CD68 stood out because of its significant correlation with primary and secondary treatment outcomes (Figure 6.9). Using univariate analysis, we found a significant correlation between the number of CD68+ lymphoma-associated macrophages (LAM) and shortened PFS (P=0.03) (Table 6.8). Patients with a high number of CD68+ cells (an IHC score of 3) had a median PFS of 2.7 years, whereas during an observation period of 16.4 years, the median survival was not reached in patients with a score of 1 (Figure 6.10). In a multivariate Cox regression model that included all 7 clinical factors of the International Prognostic Score and immunohistochemical scores for CD68, CD20, and MMP11, CD68 was not an independent factor for an association with PFS. In contrast, an increased number of CD68+ macrophages correlated with DSS in both univariate and multivariate analysis (P=0.003 for both comparisons) and outperformed the International Prognostic Score (P=0.03) (Table 6.8). The 10-year disease-specific survival rate was significantly lower among patients with a CD68 immunohistochemical score of 3 (59.6%) than among those with a score

of 2 (67.4%) or 1 (88.6%) (P=0.003 for all comparisons) (Figure 6.11). The number of LAM was also correlated with the outcome after secondary treatment. Secondary treatment that was administered with curative intent failed in only 12.5% of patients with a CD68 immunohistochemical score of 1, as compared with failure in 51.7% of those with a score of 2 and in 62.5% of those with a score of 3 (P=0.009 for all comparisons). In particular, there was a significant correlation between the failure of autologous hematopoietic stem-cell transplantation and a CD68 score of more than 1 (P=0.008). A high-risk International Prognostic Score (>3) was not significantly associated with the number of CD68+ macrophages in diagnostic biopsy samples (8.7% for a score of 1, as compared with 18.1% for a score of 2 and 22.9% for a score of 3; P=0.17 for all comparisons). When the analysis was restricted to limited stage disease, a CD68 score of 1 was associated with a long-term DSS rate of 100% (P=0.04) (Figure 6.12). In agreement with the findings of the gene expression study, MMP11 immunohistochemical staining showed a significant correlation with progression-free survival in both univariate analysis (P=0.008) and multivariate analysis (P=0.009), although not with DSS (Table 6.8). Among the cells with positive staining were HRS cells, macrophages, and endothelial cells. Using univariate analysis, we also found that an increased number of CD20+ small B cells (IHC score, >2) was significantly associated with prolonged PFS (P=0.02) and DSS (P=0.02) (Table 6.8). However, the number of CD20+ small B cells was not an independent predictor of survival and was strongly correlated with advanced stage disease (P=0.002). Neither the number of CD20+ HRS cells nor the presence or absence of primary or secondary lymphoid follicles was associated with the outcome.

6.5 VALIDATION STUDY

In a retrospective analysis of 167 adult patients with CHL, prospectively treated in a single institution, Centro Hospitalar Lisboa Central (CHLC) with Stanford V regimen, the impact of CD68+ IHC score prognostic factor was evaluated.

6.5.1. Material & Methods

From November 1996 to December 2009, 218 consecutive patients with newly diagnosed CHL at CHLC were included in a prospective study using the Stanford V regimen. The study included patients between the ages of 15 and 60 years with untreated locally extensive or advanced stage, biopsy-proven CHL, stage I to IV disease. Exclusion criteria included a positive HIV test, pregnancy, significant malignant disease other than CHL and a serum bilirubin greater than 5 mg/dL. This is a brief chemotherapy regimen given weekly for 12 weeks as follows: vinblastine 6 mg/m^2 and doxorubicin 25 mg/m^2 on weeks 1, 3, 5, 7, 9, and 11; vincristine 1.4 mg/m^2 (maximum dose, 2 mg) and bleomycin 5 units/m^2 on weeks 2, 4, 6, 8, 10, and 12; mustard 6 mg/m^2 on weeks 1, 5, and 9; and etoposide 60 mg/m^2 daily times two on weeks 3, 7, and 11. Prednisone 40 mg/m^2 was administered orally every other day on weeks 1 through 10 and tapered during weeks 11 and 12. Doses of doxorubicin, vinblastine, mustard, and etoposide were reduced to 65% if the absolute neutrophil count was less than 1,000/μL, and treatment was delayed if the absolute neutrophil count was less than 500/μL. When it became commercially available, granulocyte colony-stimulating factor (G-CSF) was incorporated after the initial dose reduction or delay. G-CSF was administered thereafter for 5 days on the odd weeks after myelosuppressive chemotherapy. Prophylactic ancillary medication included trimethoprim-sulfamethoxazole, ranitidine, and acyclovir, which were given throughout the treatment period. Initially, 36 to 44 Gy RT was delivered 2 to 4 weeks after chemotherapy to initial bulky disease sites and to residual radiographic disease. Most patients with bulky mediastinal disease received a modified mantle field that included mediastinal, bilateral hilar and bilateral lower neck irradiation but excluded axillary, occipital, and cervical irradiation.

Response and follow-up evaluation included a complete blood cell count, chemistry panel, erythrocyte sedimentation rate, chest x-ray, and plain film of the abdomen every month during treatment, at the completion of RT, and every 2 months during year 1, every 3 months during year 2, every 4 months during year 3, and every 6 months during years 4 and 5. All computed tomography scans that were abnormal at diagnosis were repeated at the conclusion of chemotherapy and RT and scans of the chest, abdomen, and pelvis were done at the end of years 1, 2, and 5 (29, 30).

Five patients were not evaluable for response and the remaining 213 were the source cohort for this study. The end date for follow-up was June 2010.

We received diagnostic lymph node FFPET biopsies of 191 patients for central review using 2008 WHO criteria (31). Standard IHC analysis for CD68 (KP1, Dako®, Glostrup, Denmark) were done on a representative whole section for each case. A total of 167 cases had the diagnosis confirmed and representative whole sections as well as interpretable IHC results were analyzed. Tumour cell regions were assessed for CD68+ cell content at low power (Olympus BX40, 40x). At least three areas representative of CD68+ cell content variability were scored at high power (400x) as the relative percentage of positive cells in relation to overall cellularity, according to similar 3 groups stratification: <5% - IHC score 1; 5 – 25% - IHC score 2 and > 25% IHC score 3. The predominant IHC score determined the final IHC score – Figure 6.13.

Data regarding patient demographics and patient outcome were obtained from the existing registry (Table 6.9). Treatment failure was defined as progression at any time after the initiation of therapy. Advanced-stage disease was defined using Ann Arbor staging criteria.

Comparisons between groups were performed by means of the X^2 test for categorical data. For time-to-event analyses, we used two primary endpoints: overall survival (OS), calculated from the date of diagnosis to the date of death or last follow-up) and disease free survival (DFS), measured from achievement of complete remission until the date of relapse, last follow-up or death). Probabilities of OS and DFS were calculated using the Kaplan-Meier method (17). Univariate comparisons were made using the log-rank test for OS and DFS. Multivariate analysis was carried out using the Cox proportional hazards model (18). Two-sided P values of less than 0.05 were considered to indicate statistical significance. Statistical analysis was performed with SPSS software, version 17.0®.

6.5.2. Results

The baseline characteristics of the 167 treated patients are listed in Table 6.6. The median age at diagnosis was 29 years (range, 15-69 years), with half of patients (51%) presented with advanced stage disease. The median follow-up

duration was 75 months (range 7 months to 14 years). All patients received at least 2 cycles of chemotherapy and no major protocol violations were observed. Complete response rate was 91% (152/167). Fifteen patients were primary refractory and the relapse rate was 22% (33/152). Treatment failure was significantly associated with male gender (P=0.017), advanced stage (P<0.001), presence of B symptoms (P=0.002), IPS>3 (P<0.001) and immunohistochemical CD68 score>1 (P<0.001). A high-risk IPS (>3) was also significantly associated with high LAM score (>1) (P=0.037).

At 10 years, OS and DFS in this cohort were respectively 86% and 74%, respectively. The 10-year OS was significantly lower among patients with an IHC score of 2 or 3 (82% and 79%) than among those with a score of 1 (100%) (P=0.001 for high score *vs.* low score comparison) (Figure 6.14). Using univariate analysis (Table 6.9), we found a significant correlation between a high CD68+ LAM IHC score (>1) and a shortened overall survival (P=0.001). Importantly, cases with limited stage disease (IA/IIA) and low LAM content (IHC score 1) had 100% OS and 97% DFS, respectively. However, in a multivariate Cox regression model, IHC LAM score was not an independent factor of OS.

The 10-year DFS was also significantly lower among patients with an IHC LAM score of 2 or 3 (68% and 69%) in comparison to those with a low score (96%) (P<0.001 for high score *vs.* low score comparison) - Figure 6.15. Both univariate and multivariate analysis (Table 6.9), demonstrated a significant correlation between high CD68+ LAM score and shortened DFS. In multivariate analysis, this factor out-performed the IPS for DFS (P=0.003 *vs.* not significant).

A total of 33 patients, 8 with primary refractory disease and 25 in relapse, received high-dose chemotherapy and autologous stem cell transplantation. The median age at diagnosis was 28 (range, 15-61 years), with 64% of patients presenting with advanced stage disease. The median follow-up for living patients was 53 months after transplantation (range, 7 months to 13 years). The response rate was 73% (24/33) and 4 patients relapsed (17%). In this subgroup, OS and DFS at 5 years were 67% and 87%, respectively. We found a trend for better OS and DFS among patients with low LAM IHC score in comparison to high LAM IHC score (80% *vs.* 64% for OS and 100% *vs.* 84% for DFS), but this did not reach statistical significance. Univariate and multivariate analysis did not allow us to identify independent risk factors for outcome after

autologous stem cell transplantation. It is likely that statistical significance could be achieved with a larger cohort of patients.

6.6 DISCUSSION

We found that the overexpression of a macrophage signature based on gene expression profiling studies of diagnostic lymph node specimens obtained from patients with CHL was associated with the failure of primary treatment. Using immunohistochemical analysis, we also found that an increased number of CD68+ cells in the diagnostic sample was associated with an inferior outcome in an independent set of samples from 166 patients. Multivariate analysis revealed that the number of CD68+ cells was also associated with the outcome of secondary treatment, independently of the IPS. We further validated the LAM IHC results using a different validation cohort of uniformly treated patients (Stanford V) in a single institution and using routine whole section diagnostic biopsies.

Three previous studies that have used expression profiles of the microenvironment in CHL have been reported (26, 27, 32). Of these, one identified a gene signature of macrophages but did not show the clinical value of LAM assessed by means of a common immunohistochemical marker (26). The association between the number of macrophages and treatment outcome has also been studied in other B cell lymphoid malignancies (14, 25, 33). Differences in survival among patients with various lymphoma subtypes, which are linked to macrophage content, might be explained by the variable presence of macrophages with M1 or M2 differentiation in biopsy samples, indicating distinct biologic features of the tumours (34). However, in these lymphoma subtypes, including Hodgkin's lymphoma, the functional link between macrophage numbers and the contribution of these cells to the treatment outcome remains unclear (7). Our gene expression classifier for the outcome of primary treatment outcome revealed *MMP11*, a gene that has been found by other investigators to be expressed in tumour-associated macrophages involved in remodelling of apoptotic lymphatic tissue (28). Using IHC analysis, we were able to confirm the correlation between the number of MMP11+ cells and PFS in an independent cohort of patients. However, MMP11 stained many different

cell types, including macrophages, and thus did not allow us to identify the particular cells that were responsible for the production of the protein.

Our findings also validate the recent report of a correlation between an increased number of small B cells and a favorable outcome (32). The recently described correlation between the number of CD20-positive B cells and survival in patients with CHL needs to be reassessed in the context of clinical studies showing successful treatment with the addition of rituximab to standard chemotherapy (35, 36).

We report and validate the clinical value of a single marker, CD68, in the identification of LAM by IHC analysis, an analytic method that can be easily incorporated into a routine diagnostic approach.

The use of such markers in combination with well-established clinical risk factors could improve on the predictive value of a single biomarker. We focused on LAM because of the strong signal from the gene expression data and the recently renewed interest in these non-neoplastic cells as major contributors to the biologic features of lymphoma and outcome prediction. The value of assessing the number of LAM as a biomarker is highlighted by the association between these cells and the outcome after secondary therapy with autologous stem-cell transplantation, a widely used salvage treatment option. Accurate prediction of the outcome after secondary treatments delivered with curative intent would provide better risk stratification for these expensive therapeutic options. Clinical predictors of the outcome after autologous stem-cell transplantation have been of limited value (37). In addition, our findings based on the TMA cohort are not restricted to advanced-stage disease alone. Thus, the IHC CD68+ macrophage content represents a biomarker with clinical applicability in all stages of CHL, both at the time of diagnosis and at the time of relapse.

In summary, our study showed the value of enumerating CD68+ macrophages in diagnostic lymph node samples for prediction of the outcome after both primary and secondary treatments (in particular, autologous stem-cell transplantation). The absence of an increased number of CD68+ cells in patients with limited stage disease defines a subgroup of patients for whom the rate of long-term disease-specific survival is 100% (and 97% in the validation set) with the use of available treatments.

Moreover, the LAM IHC score was applied and validated using routine diagnostic whole section biopsies, which not only validates the aforementioned results obtained using TMAs, but also underscores its use in daily practice. Given these results, we posit that the amount of CD68+ LAM in lymph node specimens, a routine pathology test easily applied to routine biopsy samples, is a solid predictive factor for outcome in CHL. At this time, a relevant question is whether these results will have impact in the management of HL patients. A personalized treatment strategy may be developed, identifying at diagnosis those patients with increased resistance to current therapeutic options.

6.7 TABLES

Name of signature	# of probe sets	Reference	Primary treatment outcome: success (92) vs. failure (38)	Most influential genes in signature
All genes	25388	Affymetrix UA 133 2.0 Plus	0.020	-
Comparison I: primary treatment failure vs success	315	present study	1.56×10^{-6}	DDX3Y, HRH1, PYGL, ETS2, LHFP
Microenvironment: Up-regulated in treatment failure	194	Sanchez-Aguilera et al., Blood 2006[11]	0.036	FKBP5, LYZ, ARF3, CTSL1
Resting NK cells	97	Su et al., PNAS 2004[10]	0.031	FCGR3A, FCGR3B, ADAMTS1, APOL1, LAIR1
Germinal center B cells (GCB)	497	Dave et al., NEJM 2006[12]	0.012	RGS13, CR2, ELL3, CCDC23
Erythroid progenitors	287	Su et al., PNAS 2004[10]	0.029	TGM2, GLUL, ITSN1, AQP1, FLII
Tumor-associated macrophages	117	Duff et al., J Surg Res 2007[9]	0.017	HSP90AB3P, HSP90AB1, CTSB, CFL1
Adipocyte	97	Urs et al., J Nutr 2004[8]	0.012	GLUL, MGST1, COL1A2, FABP4
Plasmacytoid dendritic cells (CD123+)	507	Lindstedt et al., J Immunol 2005[13]	0.046	SRPX, CTSB, APP
Angiogenesis	108	Lenz et al, NEJM 2008[7]	0.036	ADH1B, CD93, SRPX, PLA2G2A
HRS cells	26	Karube et al., Annals of Oncology 2006[14]	0.047	CSF1R, TNFSF12, TNFSF13, CCL26, TNFSF12
Macrophages	153	Martinez et al., J Immunology 2006[15]	0.022	CCL13, MS4A4A, CCL23, VCAN
Resting monocytes	279	Su et al., PNAS 2004[10]	0.014	F13A1, SERPINA1, LILRB2, VCAN, CD93
TGFβ	108	Kang et al., Mol Cell 2003[16]	0.046	SLC39A8, P4HB, CYP1B1, HYOU1, PEA15

Table 6.1 - Association of previously described gene signatures with treatment outcome: Results of Global Test. Signatures with $P < 0.05$ are shown.

Variable	Gene-Expression Profiling (N=130)			Immunohistochemical Analysis (N=166)		
	Treatment Success (N=92)	Treatment Failure (N=38)	P Value	Treatment Success (N=87)	Treatment Failure (N=79)	P Value
Median age (range) — yr	37 (8–80)	46 (12–84)	0.03	33 (16–80)	36 (15–82)	0.21
Male sex — %	51	68	0.08	51	53	0.74
Histologic subtype — %			0.15			0.19
Nodular sclerosis	82	63		89	80	
Mixed cellularity	12	26		6	8	
Lymphocyte-rich	2	3		0	4	
Lymphocyte- depleted	1	5		0	3	
Not classifiable	3	3		6	6	
Stage — %			0.003			0.01
I	14	8		11	3	
II	60	32		53	39	
III	16	37		20	34	
IV	10	24		16	24	
Presence of constitutional symptoms — %	36	50	0.14	40	53	0.10
Tumor size			0.39			0.27
Median (range) — cm†	6 (2–17)	7 (2–26)		6 (0–28)	7 (0–19)	
≥10 cm — %	18	26		33	30	
IPS ≥4 (high risk) — %‡	14	18	0.05	14	20	0.27
Primary treatment — %			1.00			0.32
ABVD chemotherapy with or without radiation	96	95		99	100	
Extended-field radiation alone	4	5		1	0	
Secondary treatment — %						
Autologous stem-cell transplantation	ND	ND		NA	70	
Other therapy with curative intent (CVPP or GDP plus radiation)	ND	ND		NA	8	
Palliative treatment (including single-agent chemotherapy or radiation)	ND	ND		NA	22	

* ABVD denotes doxorubicin, bleomycin, vinblastine, and dacarbazine, CVPP cyclophosphamide, vinblastine, procarbazine, and prednisone, GDP gemcitabine, dexamethasone, and cisplatin, IPS International Prognostic Score, NA not applicable, and ND not done.
† The tumor size was calculated as the longest diameter of the largest involved area.
‡ The IPS ranges from 0 to 7, with higher scores indicating increased risk.

Table 6.2 - Demographic and clinical characteristics of the two cohorts of patients.

Category	P-value	Molecules
Infectious Disease	1.67E-05 - 2.63E-02	RNPS1, IKBKG, LARS, PRNP, APP, RXRA, CALD1, NMT1, GPT2, PYGL, HLX, ERI2, RPS4Y1, SIPA1L2, ALOX5, IGHMBP2, TRIM8, PFKL, CD44, RBM25 (includes EG:58517), FBLIM1, HLA-C, EPOR, FLJ40125, WBP4, PTGS1, KPNB1, ARHGEF19, ETS2, STIP1, FXR1, HRH1, ZNF182, SSB (includes EG:6741)
Cell Death	6E-05- 4.68E-02	PDGFRB, RNPS1, IKBKG, HSP90AA1, PRNP, APP, BLVRA, TRADD, TNFSF13, ALOX5, LILRB1, BRCC3, FKBP8, MUC1, PEA15, CD44, HSPA5, CALR, HSPA1A, PTAFR, SIRT2, ARRB2, FGFR1, PTGS1, MVP, TRIM27, ETS2, PPP5C, CCT6A, APOL1, CTBP2, RASSF4, NFE2L2, TRAF2, FXR1, AP2A2
Reproductive System Disease	1.33E-04- 4.75E-02	GABARAPL1, PDGFRB, TXNIP, ATRN, SLC2A5, HSP90AA1, IQGAP1, PRNP, GNAS, RXRA, APP, TRADD, MCAM, COMT, ERI2, ALOX5, SHC1, UBE3A, MUC1, PEA15, CD44, HSPA5, NF1, MINK1, HSPA1A, MAPK7, CDC2L6, HLA-C, EPOR, PTAFR, ARRB2, MMP24, PTGS1, FGFR1, VAMP2, KCNK6, SCN1B, ETS2, CP, PPFIA1, FRMD4A, HRH1
Cell-To-Cell Signaling and Interaction	1.42E-04- 4.68E-02	JMJD6, CALR, HSPA1A, PDGFRB, MCAM, CD93, HLA-C, MUC1, CD44, APP
Cell-mediated Immune Response	1.42E-04- 4.37E-02	CALR, NF1, NFE2L1, PTAFR, HLA-C, CAPZB, ATRN, ARRB2, IQGAP1, PRNP, APP, GNAS, CALD1, JMJD6, PXK, MUC1, CD44, HRH1, WAS
Cellular Growth and Proliferation	1.42E-04- 4.59E-02	PDGFRB, SURF6, TXNIP, IKBKG, DNAJA1, AGGF1, RXRA, MCAM, TNFSF13, FKBP4, RRAD, LZTS2, LILRB1, SHC1, MUC1, CD44, WAS, HSPA5, NF1, CALR, PTAFR, EPOR, HLA-C, SIRT2, C19ORF10, ARRB2, MMP24, PTGS1, FGFR1, MVP, TRIM27, PPP5C, ETS2, PDAP1, RASSF4, UBC, CTBP2, TRAF2
Hematological System Development and Function	1.42E-04- 4.59E-02	GNAQ, CALR, HLA-C, PTAFR, EPOR, ATRN, MVP, F13A1, JMJD6, TNFSF13, LILRB1, MUC1, CD44, WAS, HSPA5
Cell Signaling	1.73E-04- 4.68E-02	GNAQ, NF1, PPP5C, MINK1, PTAFR, IKBKG, RAB7A, FGFR1, KPNB1, F13A1, APP, MAPK13
DNA Replication, Recombination, and Repair	1.73E-04- 1.19E-02	GNAQ, PTAFR, RAB7A, KPNB1, PRNP
Nucleic Acid Metabolism	1.73E-04- 3.53E-02	GNAQ, ADAP1, PTAFR, SHMT1, RAB7A, KPNB1

Table 6.3 - Ingenuity Pathway Analysis: genes high in primary treatment failures, top 10 Functions.

Canonical Pathways	-Log(P-value)	Ratio	Molecules
PPAR Signaling	4.15E00	8.05E-02	PDGFRB, MAPK7, IKBKG, HSP90AA1, TRAF2, SHC1, RXRA
Androgen Signaling	3.41E00	6.09E-02	GNAQ, CALR, MAPK7, PRKAR2A, HSP90AA1, SHC1, GNAS
PPARα/RXRα Activation	3.29E00	5.19E-02	GNAQ, MAPK7, PRKAR2A, IKBKG, HSP90AA1, SHC1, RXRA, GNAS
IL-12 Signaling and Production in Macrophages	2.79E00	5.66E-02	JMJD6, MAPK7, IKBKG, RAB7A, RXRA, MAPK13
Fructose and Mannose Metabolism	2.6E00	7.69E-02	PFKFB2, SORD, PFKL, PFKFB4
Glucocorticoid Receptor Signaling	2.58E00	3.6E-02	HSPA1A, MAPK7, FKBP4, IKBKG, HSP90AA1, TRAF2, SHC1, HSPA5, MAPK13
Amyloid Processing	2.5E00	7.69E-02	MAPK7, PRKAR2A, APP, MAPK13
Acute Phase Response Signaling	2.42E00	4.17E-02	TRADD, MAPK7, CP, IKBKG, TRAF2, SHC1, MAPK13
α-Adrenergic Signaling	2.39E00	5.56E-02	GNAQ, PYGL, MAPK7, PRKAR2A, GNAS

Table 6.4 - Ingenuity Pathway Analysis: genes high in primary treatment failures, top 10 Canonical Pathways.

Category	P-value	Molecules
Protein Synthesis	1.3E-05-3.72E-02	RPS27L (includes EG:51065), APOA1, RPL14, RPL30, RPL38 (includes EG:6169), SQSTM1, RPS4X, MMP11, RPL34, RPL31, EIF2S3, RPL22, RPS17 (includes EG:6218), UBE3A, NACA, GLMN, EIF3E
Cell-To-Cell Signaling and Interaction	1.26E-04-4.87E-02	NLGN3, NRD1, NCKIPSD, VIPR1, AGRN, CEACAM5 (includes EG:1048), BRAF, RET
Cellular Assembly and Organization	1.26E-04-3.72E-02	SRPK2, SNRPD1, SNRPD2, NLGN3, MUC5AC, STMN1, APOA1, NRD1, SMC1A, PTMS, AGRN, RET
RNA Post-Transcriptional Modification	7.14E-04-1.88E-02	PAPOLG, RPS16, SRPK2, SNRPD1, BRUNOL4, SNRPD2, RPL14, SNRPN, RPS17 (includes EG:6218), RPS15, U2AF1
Nervous System Development and Function	1.04E-03-3.72E-02	NLGN3, CHRNB2, AGRN
Genetic Disorder	5.01E-03-3.72E-02	APOA1, ATN1, ASCL1, RET, ARX, ABCB7, PRKAG2, NLGN3, FGFR2, PANK2, CD3D, UBE3A, ADRA2B, DCLRE1C
Neurological Disease	5.01E-03-4.14E-02	ATN1, ASCL1, ARX, RET, SLC1A4, ABCB7, NLGN3, GRIN1, CHRNB2, PANK2, ABAT, UBE3A, ADRA2B
Respiratory Disease	5.01E-03-5.01E-03	ASCL1, RET
Hepatic System Disease	6.79E-03-1.88E-02	GPT (includes EG:2875), PDE3B, PDE4D
Inflammatory Disease	6.79E-03-3.72E-02	FGFR2, GPT (includes EG:2875), PDE3B, PDE4D

Table 6.5 - Ingenuity Pathway Analysis: genes low in primary treatment failures, top 10 Functions.

Canonical Pathways	-Log(P-value)	Ratio	Molecules
Relaxin Signaling	2.12E00	5.65E-02	RLN1, PRKAG2, PIK3CA, PRKACB, BRAF, PDE3B, PDE4D
cAMP-mediated Signaling	2.09E00	5.06E-02	PRKACB, BRAF, PDE3B, RGS10, ADRA2B, CREM, PDE4D, AKAP7
Cardiac β-adrenergic Signaling	1.62E00	5.04E-02	PPM1L, PRKAG2, PRKACB, PDE3B, PDE4D, AKAP7
Cellular Effects of Sildenafil (Viagra)	1.47E00	4.62E-02	PRKAG2, MYL5, PRKACB, SLC4A11, PDE3B, PDE4D
Oxidative Phosphorylation	1.43E00	4.26E-02	COX7C (includes EG:1350), NDUFA7, COX11, NDUFC1, COX6C, ATP5L
G-Protein Coupled Receptor Signaling	1.42E00	3.92E-02	PRKAG2, PIK3CA, PRKACB, BRAF, PDE3B, RGS10, ADRA2B, PDE4D
Nitric Oxide Signaling in the Cardiovascular System	1.41E00	5.71E-02	PRKAG2, PIK3CA, PRKACB, PDE3B
CTLA4 Signaling in Cytotoxic T Lymphocytes	1.23E00	4.71E-02	PPM1L, TRA@, CD3D, PIK3CA
Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells	1.2E00	8E-02	TRA@, CD3D
Melanocyte Development and Pigmentation Signaling	1.13E00	4.76E-02	PRKAG2, PAX3, PIK3CA, PRKACB

Table 6.6 - Ingenuity Pathway Analysis: genes low in primary treatment failures, top 10 Canonical Pathways.

Probe set	Importance	Gene Symbol	Gene Title
214980_at	100.000	UBE3A	E6-AP isoform-III
206131_at	97.825	CLPS	colipase, pancreatic
229080_at	72.950	EMID2	EMI domain containing 2
232820_s_at	65.193	GTSF1L	gametocyte specific factor 1-like
201806_s_at	63.262	ATXN2L	ataxin 2-like
235908_at	62.537	MMP11	matrix metallopeptidase 11 (stromelysin 3)
216114_at	60.429	NCKIPSD	NCK interacting protein with SH3 domain
203112_s_at	57.592	WHSC2	Wolf-Hirschhorn syndrome candidate 2
203598_s_at	55.895	WBP4	WW domain binding protein 4 (formin binding protein 21)
227374_at	54.068	EARS2	glutamyl-tRNA synthetase 2, mitochondrial (putative)
219811_at	50.678	DGCR8	DiGeorge syndrome critical region gene 8
207783_x_at	49.571	HUWE1	HECT, UBA and WWE domain containing 1
207408_at	48.129	SLC22A14	solute carrier family 22, member 14
200635_s_at	47.423	PTPRF	protein tyrosine phosphatase, receptor type, F
239648_at	44.349	DCUN1D3	DCN1, defective in cullin neddylation 1, domain containing 3 (S. cerevisiae)
204491_at	44.145	PDE4D	phosphodiesterase 4D, cAMP-specific (phosphodiesterase E3 dunce homolog, Drosophila)
223740_at	43.933	RIPPLY2	ripply2 homolog (zebrafish)
217095_x_at	43.446	NCR1	natural cytotoxicity triggering receptor 1
219905_at	43.325	ERMAP	erythroblast membrane-associated protein (Scianna blood group)
209980_s_at	41.167	SHMT1	serine hydroxymethyltransferase 1 (soluble)
203488_at	40.265	LPHN1	latrophilin 1
207270_x_at	39.822	CD300C	CD300c molecule
242048_at	39.139	PFDN6	prefoldin subunit 6
204952_at	38.310	LYPD3	LY6/PLAUR domain containing 3
204802_at	37.767	RRAD	Ras-related associated with diabetes
218550_s_at	37.296	LRRC20	leucine rich repeat containing 20
227249_at	37.010	NDE1	NudE nuclear distribution gene E homolog 1 (A. nidulans), mRNA (cDNA clone MGC:33664 IMAGE:4828494)
age	36.769	AGE	_
204104_at	35.497	SNAPC2	small nuclear RNA activating complex, polypeptide 2, 45kDa
208384_s_at	35.170	MID2	midline 2
218865_at	34.926	MOSC1	MOCO sulphurase C-terminal domain

			containing 1
213693_s_at	34.826	MUC1	mucin 1, cell surface associated
219525_at	34.644	SLC47A1	solute carrier family 47, member 1
224839_s_at	34.635	GPT2	glutamic pyruvate transaminase (alanine aminotransferase) 2
236703_at	33.557	NT5C2	5'-nucleotidase, cytosolic II
32062_at	33.501	LRRC14	leucine rich repeat containing 14
223389_s_at	33.280	ZNF581	zinc finger protein 581
229941_at	33.255	FAM166B	family with sequence similarity 166, member B
209985_s_at	33.027	ASCL1	achaete-scute complex homolog 1 (Drosophila)
219398_at	32.904	CIDEC	cell death-inducing DFFA-like effector c
219411_at	32.718	ELMO3	engulfment and cell motility 3
221187_s_at	31.425	FUZ	fuzzy homolog (Drosophila)
223836_at	30.950	FGFBP2	fibroblast growth factor binding protein 2
228452_at	30.073	C17orf39	chromosome 17 open reading frame 39
213601_at	29.546	SLIT1	slit homolog 1 (Drosophila)
209460_at	29.416	ABAT	4-aminobutyrate aminotransferase
227440_at	29.364	ANKS1B	ankyrin repeat and sterile alpha motif domain containing 1B
214456_x_at	29.333	SAA1	serum amyloid A1 /// serum amyloid A2
219877_at	28.122	ZMAT4	zinc finger, matrin type 4
204856_at	27.277	B3GNT3	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 3
224434_s_at	26.815	MORG1	mitogen-activated protein kinase organizer 1
239975_at	26.541	HLA-DPB2	major histocompatibility complex, class II, DP beta 2 (pseudogene)
216611_s_at	25.998	SLC6A2	solute carrier family 6 (neurotransmitter transporter, noradrenalin), member 2
219224_x_at	24.651	ZNF408	zinc finger protein 408
203899_s_at	24.506	CRCP	CGRP receptor component
202790_at	22.422	CLDN7	claudin 7
1553262_a_at	22.360	UTS2R	urotensin 2 receptor
217744_s_at	22.269	PERP	PERP, TP53 apoptosis effector
207832_at	22.226	BAIAP2	BAI1-associated protein 2
225244_at	21.746	SNAP47	synaptosomal-associated protein, 47kDa
229559_at	21.063	FLJ40125	hypothetical protein FLJ40125
239159_at	20.610	GOSR2	Golgi SNARE (GS27)
219472_at	20.126	CENPO	centromere protein O
214625_s_at	19.020	MINK1	misshapen-like kinase 1 (zebrafish)
213981_at	18.956	COMT	catechol-O-methyltransferase
213102_at	18.146	ACTR3	ARP3 actin-related protein 3 homolog (yeast)
230134_s_at	18.145	RC3H2	ring finger and CCCH-type zinc finger domains 2
stage	18.126	STAGE	_
1569631_at	17.832	NMNAT1	nicotinamide nucleotide adenylyltransferase 1
223248_at	17.219	HSDL1	hydroxysteroid dehydrogenase like 1
222208_s_at	16.768	POLR2J4	polymerase (RNA) II (DNA directed) polypeptide J4, pseudogene

205706_s_at	16.644	ANKRD26	ankyrin repeat domain 26
203099_s_at	16.589	CDYL	chromodomain protein, Y-like
230807_at	16.295	CCDC151	coiled-coil domain containing 151
233550_s_at	15.995	SLC4A11	solute carrier family 4, sodium borate transporter, member 11
212688_at	15.840	PIK3CB	phosphoinositide-3-kinase, catalytic, beta polypeptide
221610_s_at	15.190	STAP2	signal transducing adaptor family member 2
217599_s_at	14.945	MDFIC	MyoD family inhibitor domain containing
241354_at	14.740	NCRNA00105	non-protein coding RNA 105
222392_x_at	13.936	PERP	PERP, TP53 apoptosis effector
239316_at	13.168	LOC751071	methyltransferase LOC751071, mitochondrial-like
223333_s_at	11.924	ANGPTL4	angiopoietin-like 4
215689_s_at	11.880	SHBG	sex hormone-binding globulin
221854_at	11.823	PKP1	plakophilin 1 (ectodermal dysplasia/skin fragility syndrome)
239012_at	10.451	RNF144B	CDNA FLJ59797 complete cds, highly similar to E3 ubiquitin ligase IBRDC2 (EC 6.3.2.-)
219198_at	9.682	GTF3C4	general transcription factor IIIC, polypeptide 4, 90kDa
213870_at	8.784	COL11A2	collagen, type XI, alpha 2

Table 6.7 - SMLR combination model features and their relative importance rankings according to Random Forests (RF) (16) – 86 annotated genes and 2 clinical variables.

Variable	Patients with Characteristic	P Value for Progression-free Survival		P Value for Disease-Specific Survival	
		Univariate Analysis	Multivariate Analysis	Univariate Analysis	Multivariate Analysis
	no. (%)				
Demographic data					
Male sex	86 (51.8)	0.90		0.63	
Age >44 yr	54 (32.5)	0.32		0.05	
Immunohistochemical data†					
≥5% CD68+ cells (IHC score, >1)	120 (72.3)	0.03		0.003	0.003
CD20+ cells					
≤10% Background B cells (IHC score, <3)	85 (51.2)	0.02		0.02	
Reed–Sternberg cells	20 (12.0)	0.95		0.59	
Lymphoid follicles	44 (26.5)	0.24		0.34	
≥1% MMP11+ (IHC score, >1)‡	65 (40.6)	0.008	0.009	0.09	
Laboratory data					
Albumin <40 g/liter	68 (41.0)	0.047		0.03	
Hemoglobin <10.5 g/dl	29 (17.5)	0.004		0.11	
White-cell count >15,000/mm^3	23 (13.9)	0.52		0.23	
Lymphocyte count <600/mm^3 or <8%	25 (15.1)	0.13		0.15	
Clinical data					
IPS >3 (high risk)§	28 (16.9)	0.38		0.004	0.03
Advanced-stage disease	125 (75.3)	0.002	0.001	0.05	
Constitutional symptoms	76 (45.8)	0.08		0.48	
Bulky tumor (≥10 cm in diameter)	53 (31.9)	0.82		0.57	

* P values are for the correlation between each factor and survival. Univariate analyses were calculated with the use of a Cox proportional-hazards regression model, and multivariate analyses were performed with a Cox proportional-hazards regression model (forward stepwise likelihood ratio).
† Immunohistochemical (IHC) scores range from 1 to 3 for CD68, from 1 to 4 for CD20, and from 0 to 3 for MMP11, with higher scores indicating a greater proportion of positive cells.
‡ Data regarding immunohistochemical staining were missing for six patients.
§ The International Prognostic Score (IPS) ranges from 0 to 7, with higher scores indicating increased risk.

Table 6.8 - Progression-free and Disease-Specific Survival in a Validation Cohort of 166 Patients.

Clinical & Pathologica characteristics		Overall Survival		Disease Free Survival	
N=167	**# (%)**	**Univariate**	**Multivariate**	**Univariate**	**Multivariate**
Male gender	83 (50)	0.155	ns	0.553	ns
Age > 44 y	32 (19)	0.541	ns	0.058	ns
Histologic subtype Nodular sclerosis Mixed cellularity Lymph.-rich Lymph.-depleted Not classifiable	 135 (82) 22 (13) 3 (2) 1 (0.6) 4 (2)	?	ns	?	ns
Stage IA/IIA IB/IIB III/IV	 82 (49) 28 (17) 57 (34)	0.010*	ns	0.031*	ns
B symptoms	56 (34)	0.088	ns	0.215	ns
Bulky disease	90 (54)	0.821	ns	0.025	
IPS ≤ 3 > 3 Missing	125 (75) 25 (15) 17 (10)	<0.001**	0.037	0.018**	ns
IHC score 1 2 3	 58 (35) 90 (54) 19 (11)	0.001***	ns	<0.001***	0.003

Legend: ns – non significant, *- early stage vs. advanced stage; ** IPS 1 & 2 vs. IPS>3; *** - IHC score 1 vs. IHC score 2 & 3

Table 6.9 – Clinical and pathological characteristics of the uniformly treated validation cohort of 167 cHL patients.

6.8 FIGURES

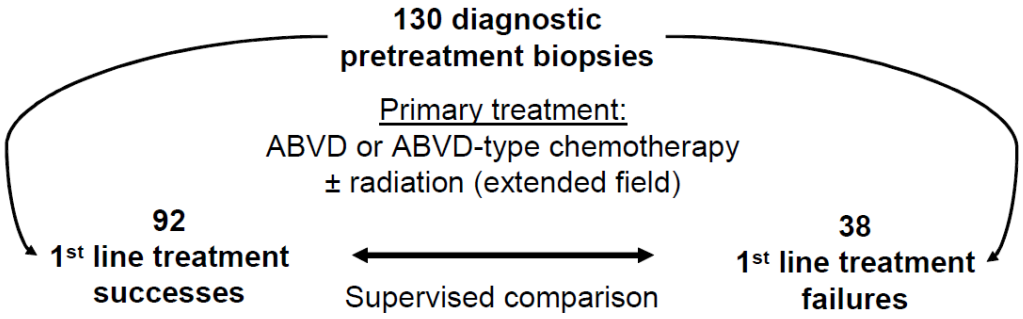


Figure 6.1 - Gene expression study cohort of 130 patients with classical Hodgkin lymphoma. Dichotomy of comparison groups according to clinical extremes: primary treatment success *vs.* failure.

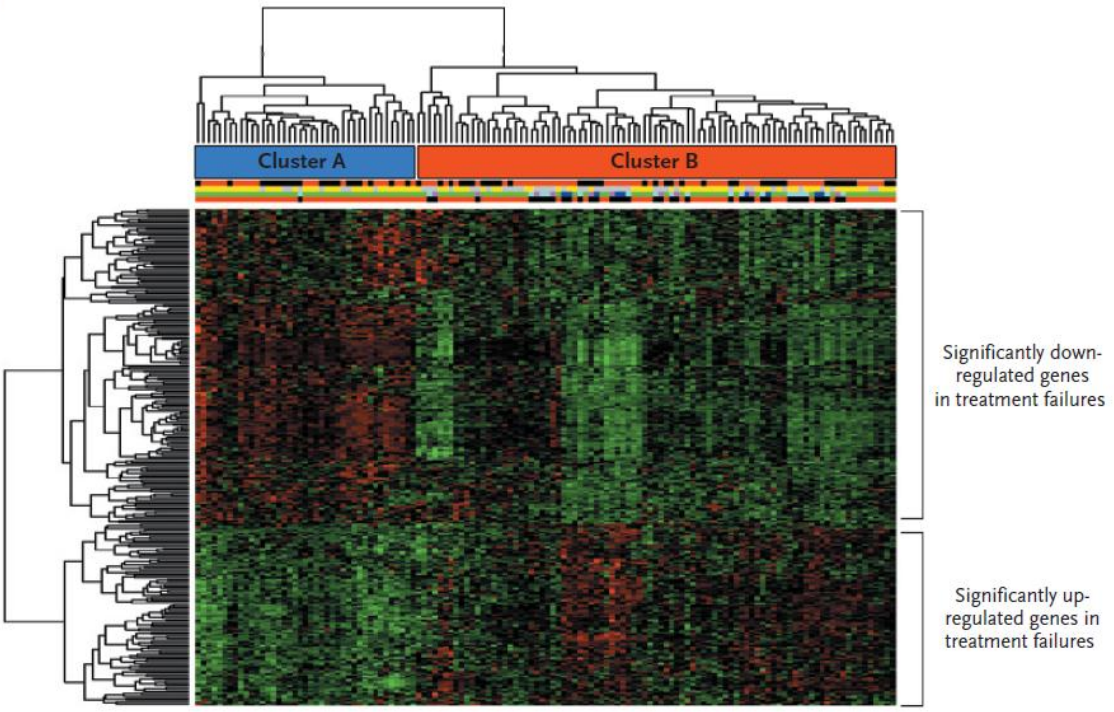


Figure 6.2 - Hierarchical clustering of 130 gene-expression profiles for patients with cHL. Cluster A has been enriched with primary treatment successes and Cluster B with both primary treatment successes and failures. Immediately below

the cluster bars, the first multicolored bar indicates sex (red for male and black for female), the second bar indicates stage (yellow for limited disease and gray for advanced disease), the third bar indicates the type of treatment failure (green for no treatment failure, purple for refractory, dark blue for early relapse, and light blue for late relapse), and the fourth bar indicates the primary treatment outcome (black for failure and red for success). (For details, see Tables 6.2 & 6.3)

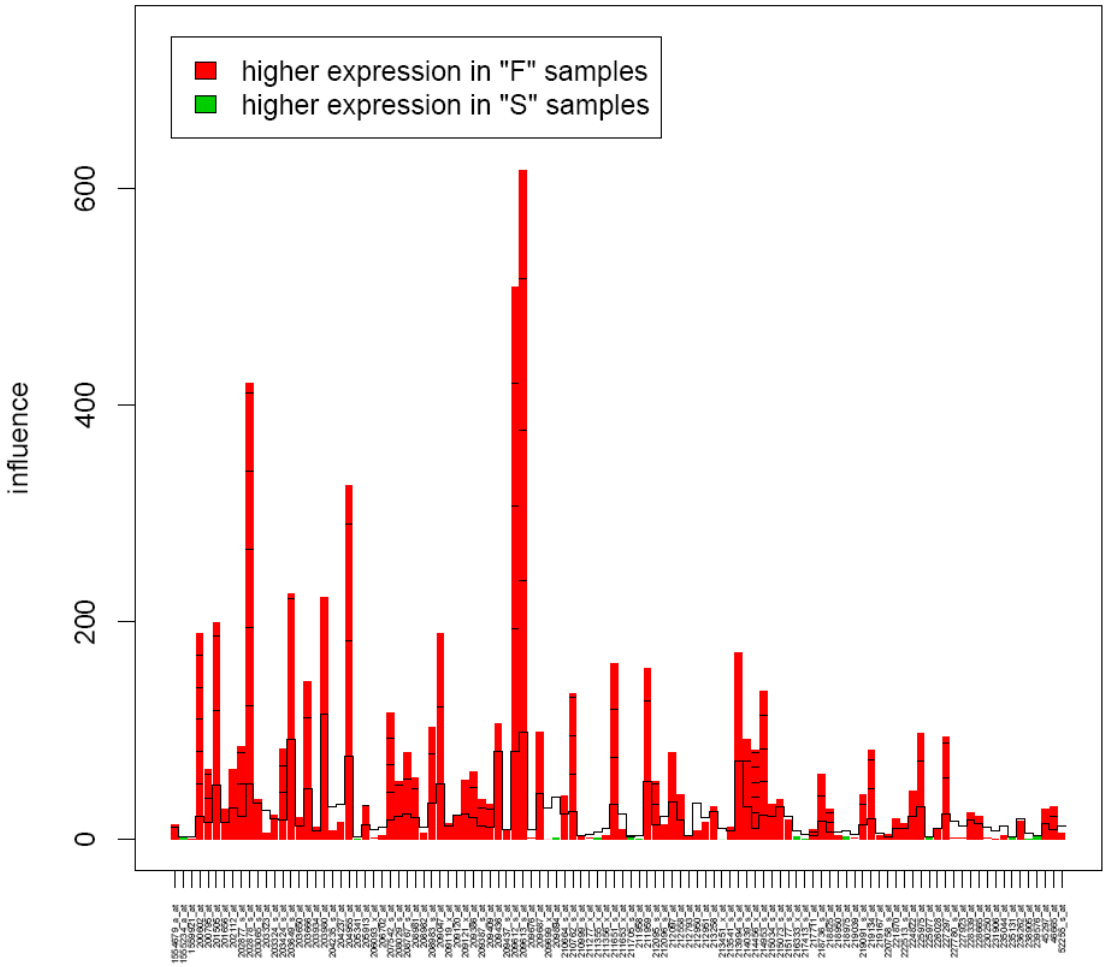


Figure 6.3 - Global test diagrams (probe set influence): Angiogenesis signature (25).

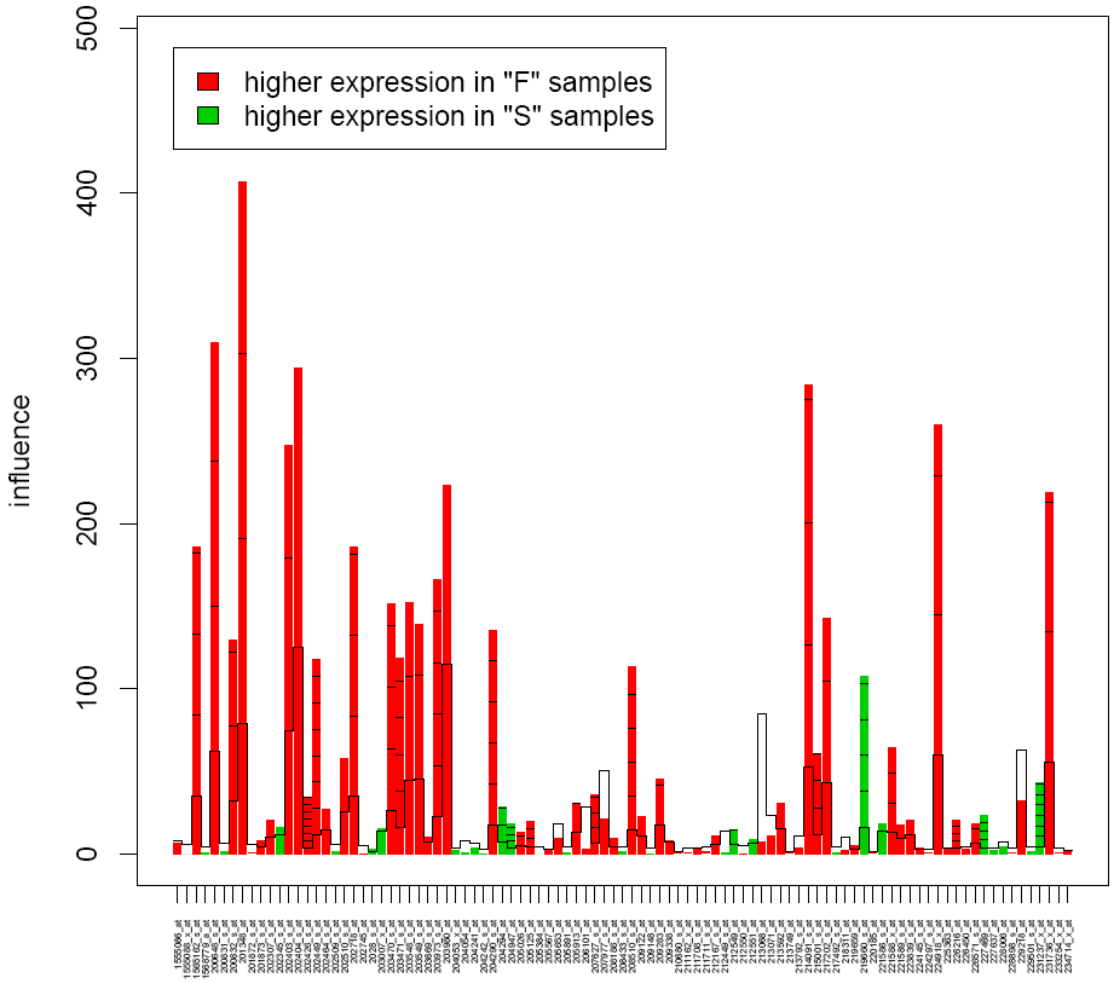


Figure 6.4 - Global test diagrams (probe set influence): Adipocyte signature (22).

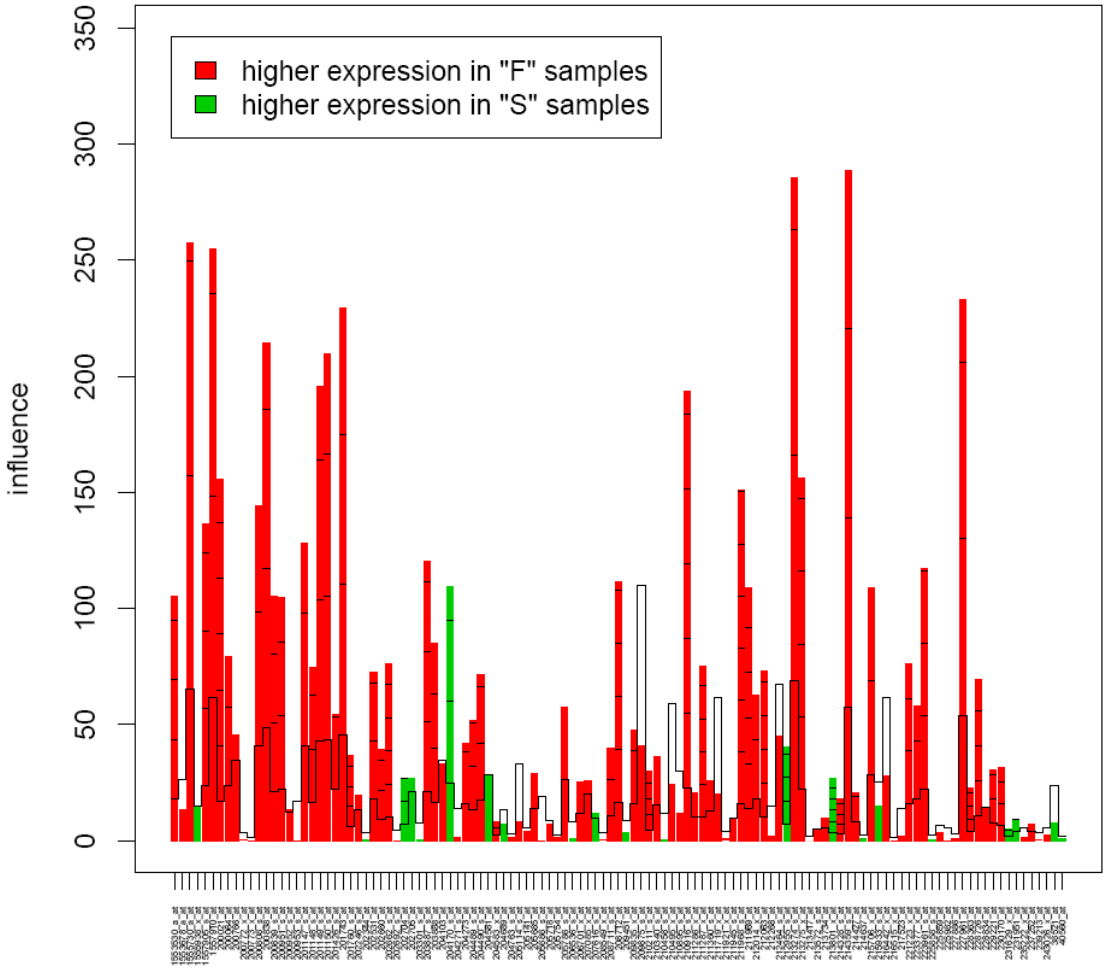


Figure 6.5 - Global test diagrams (probe set influence): Tumor-associated macrophage signature (21).

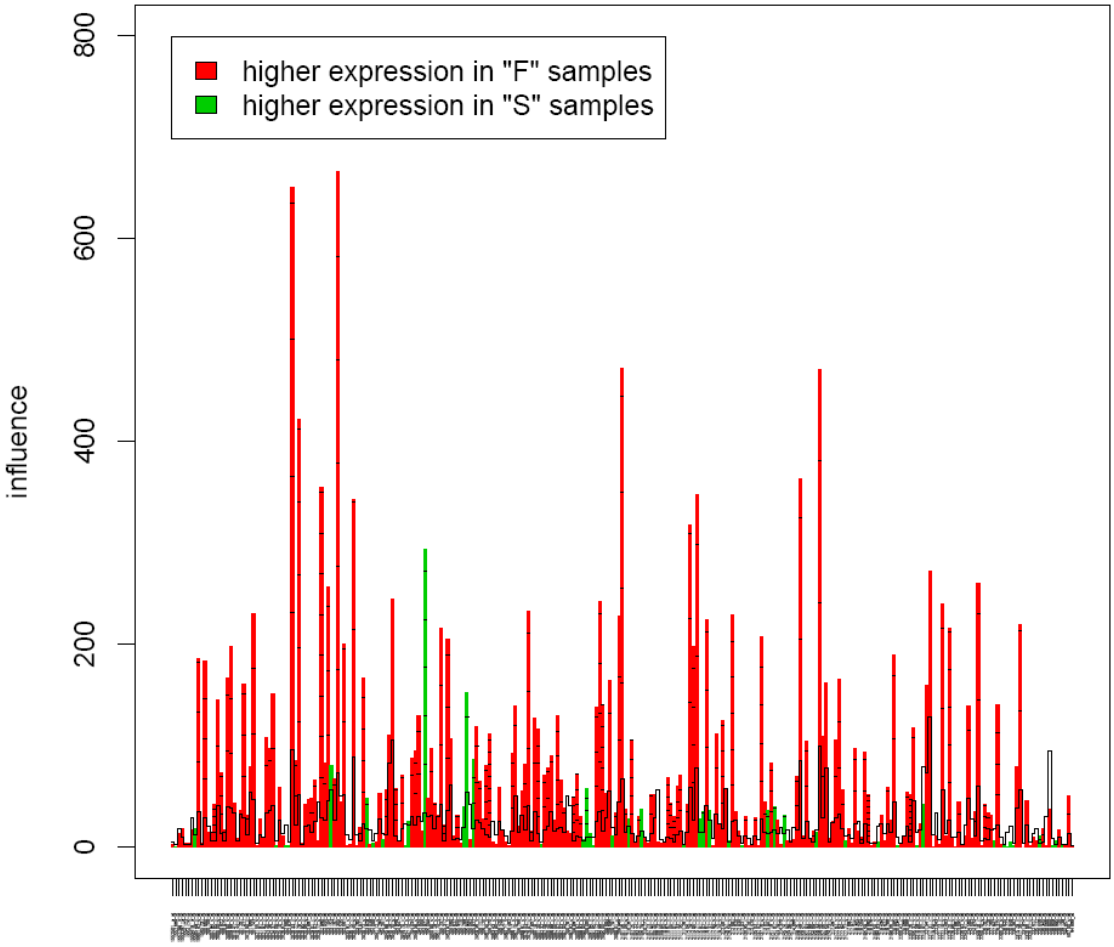


Figure 6.6 - Global test diagrams (probe set influence): Resting monocyte signature (20).

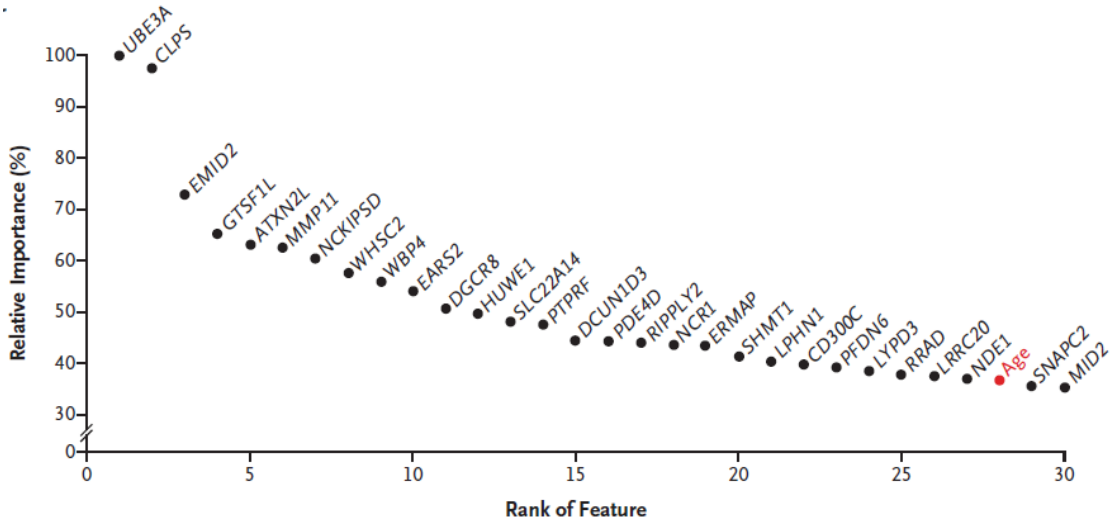


Figure 6.7 - Importance of Individual Genes for Outcome Prediction. Relative importance is shown for 30 annotated probe sets (selected with the use of sparse multinomial logistic regression) that were more influential than Ann Arbor staging. Among the 27 individual genes exceeding the importance of the best clinical variable, age (shown in red), was *MMP11*.

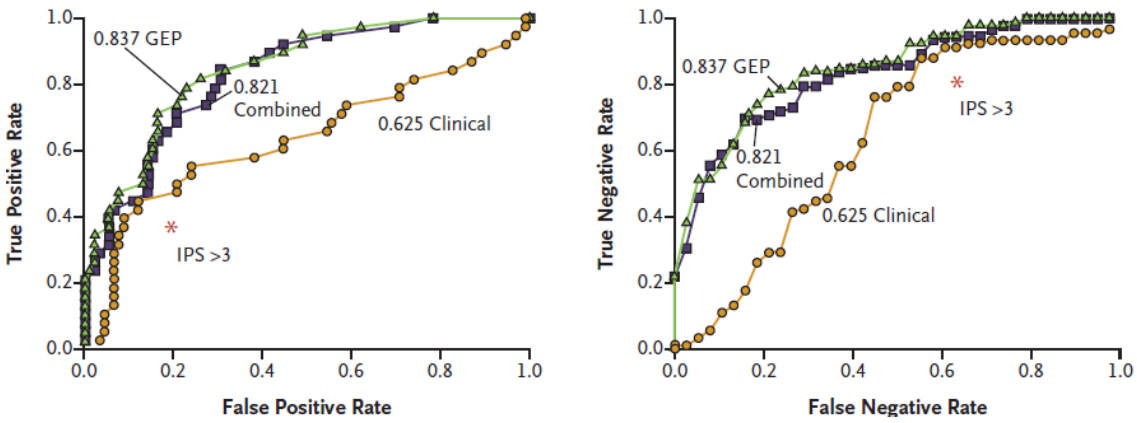


Figure 6.8 – It shows plots of true positive and false positive rates and true negative and false negative rates (receiver-operating-characteristic curves) for three models that were used for feature selection: gene-expression profiling (GEP), a model based on the International Prognostic Score (IPS) for clinical variables, and a model combining these two features. This comparison showed that the value for the area under the curve (AUC) was highest for the GEP model, as compared with the clinical and combined models (0.837 vs. 0.625 and 0.821, respectively). For comparison with the established IPS, red asterisks indicate an IPS of more than 3, as calculated with the use of IPS thresholds (6).

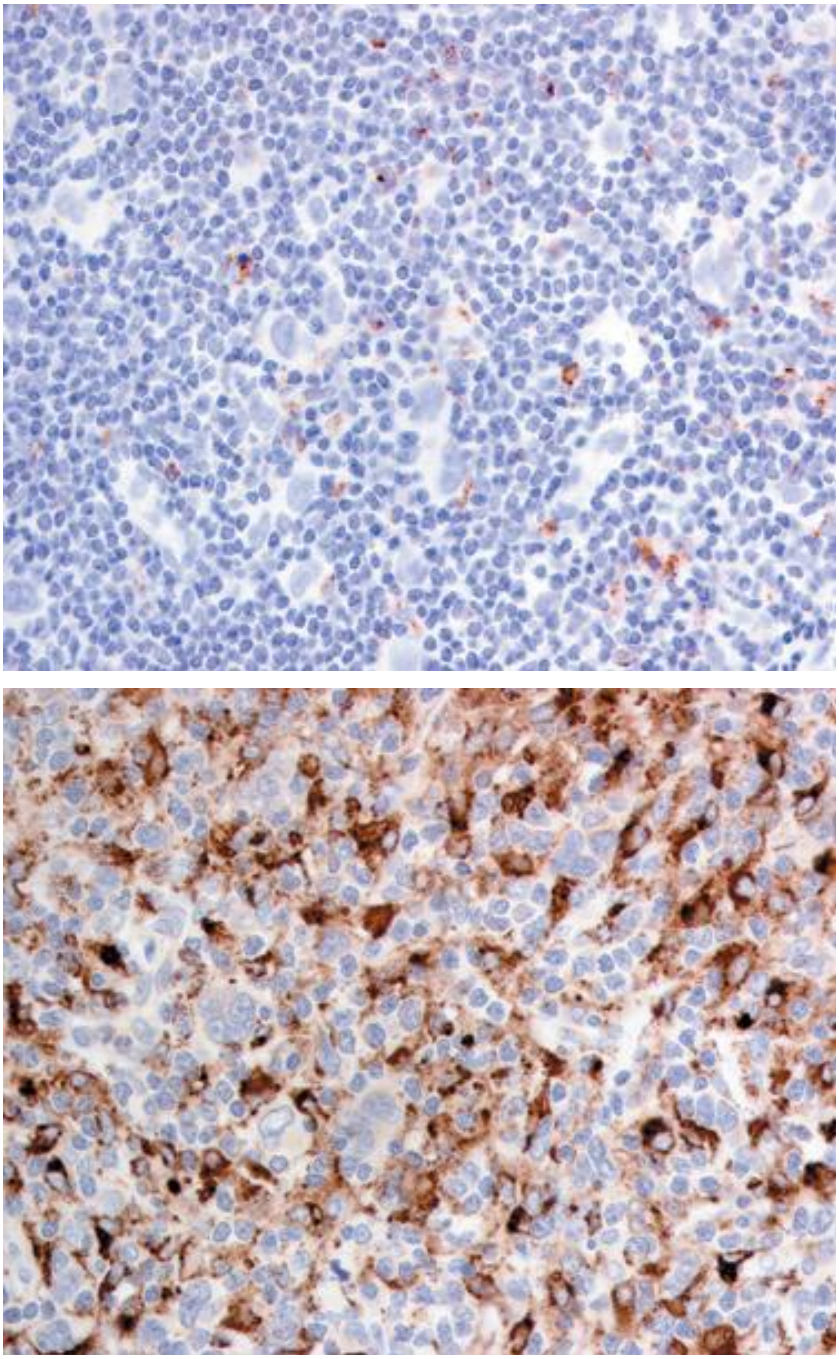
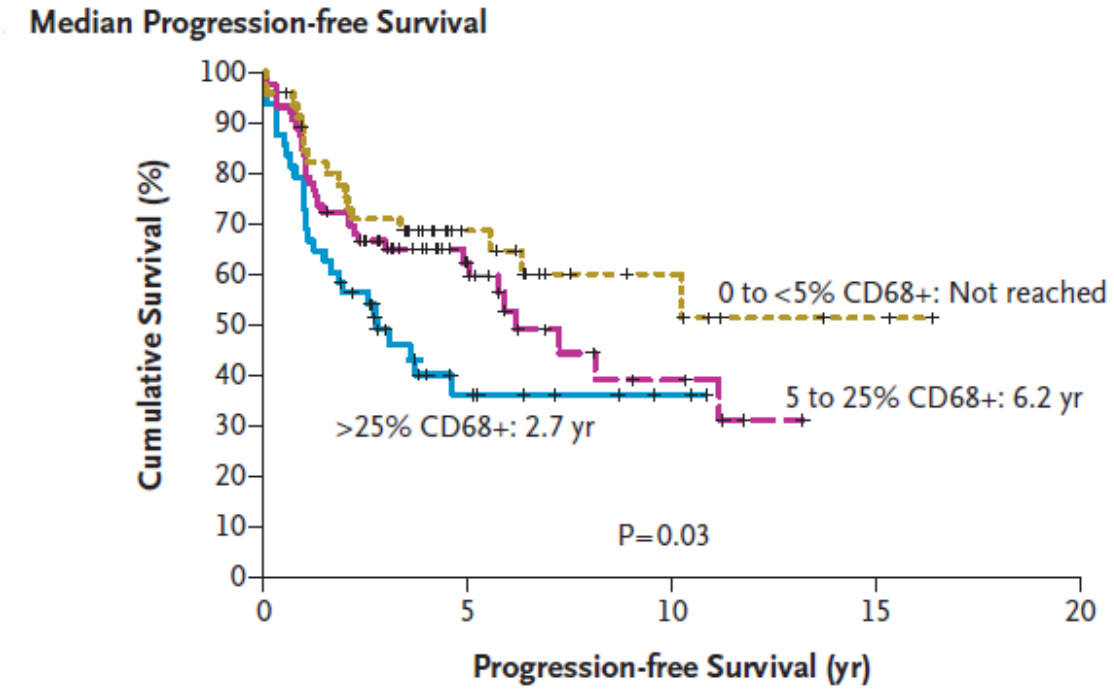
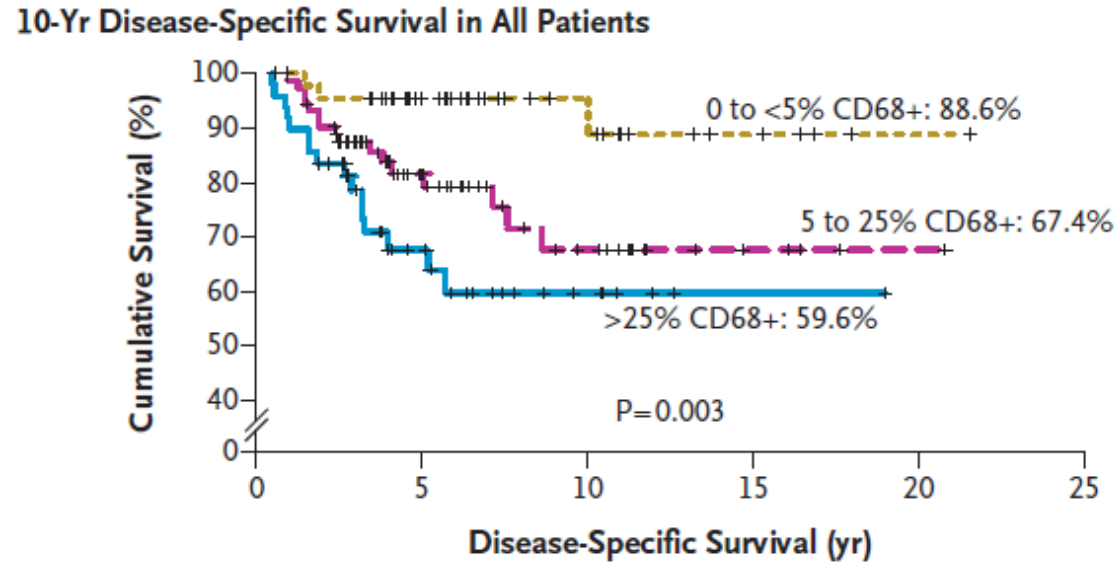


Figure 6.9 - Representative Immunohistochemical Analyses for CD68 in Lymph-Node Biopsy Samples from Two Patients. Upper image shows a sample obtained from a patient in the treatment-success group, with few CD68+ macrophages, and lower image shows a sample from a patient in the treatment-failure group, with many CD68+ macrophages. Both patients had the nodular sclerosis subtype of CHL.



No. at Risk					
0 to <5% CD68+	46	17	7	2	0
5 to 25% CD68+	72	21	6	0	0
>25% CD68+	48	9	2	0	0

Figure 6.10 - Progression-Free Survival in a validation cohort of 166 patients, according to the number of infiltrating CD68+ macrophages in pre-treatment lymph-node biopsy specimens. According to the immunohistochemical scoring system that was used, a score of 1 indicates less than 5% CD68+ cells, a score of 2 indicates 5 to 25%, and a score of 3 indicates more than 25%.



No. at Risk

0 to <5% CD68+	46	28	13	5	1	0
5 to 25% CD68+	72	33	14	4	1	0
>25% CD68+	48	19	6	1	0	0

Figure 6.11 - Disease-Specific Survival in a validation cohort of 166 patients, according to the number of infiltrating CD68+ macrophages in pre-treatment lymph-node biopsy specimens. According to the immunohistochemical scoring system that was used, a score of 1 indicates less than 5% CD68+ cells, a score of 2 indicates 5 to 25%, and a score of 3 indicates more than 25%.

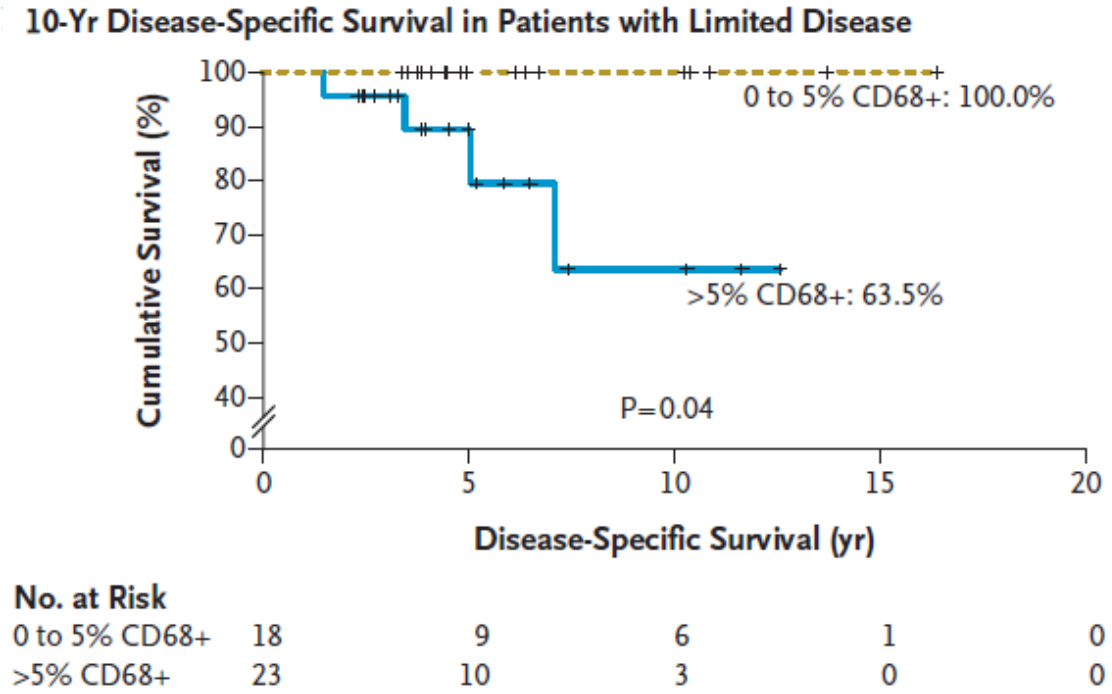


Figure 6.12 - Limited-stage disease Specific Survival in a validation cohort of 166 patients, according to the number of infiltrating CD68+ macrophages in pre-treatment lymph-node biopsy specimens. According to the immunohistochemical scoring system that was used, a score of 1 indicates less than 5% CD68+ cells, a score of 2 indicates 5 to 25%, and a score of 3 indicates more than 25%.

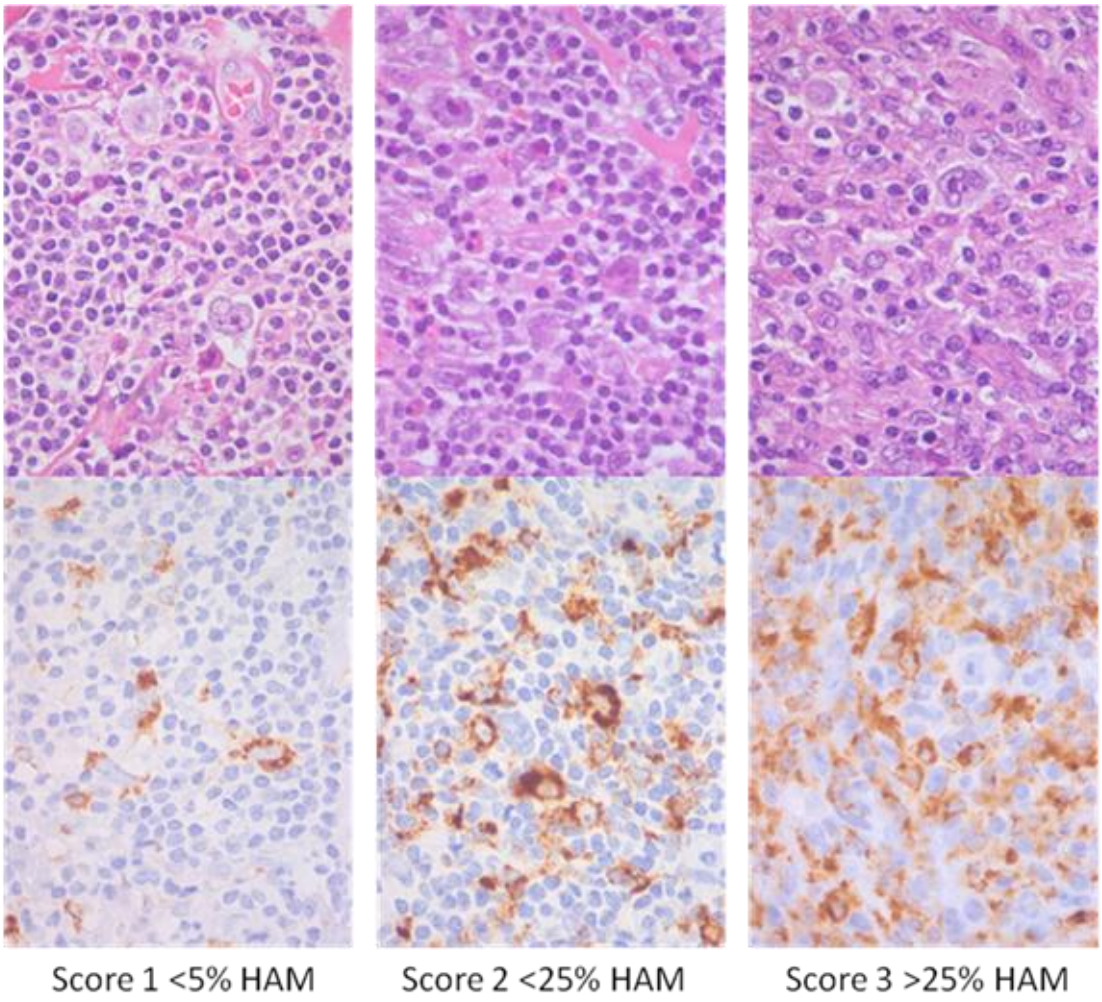


Figure 6.13 - Representative Immunohistochemical Analyses for CD68 in Lymph-Node Biopsy Samples from three Patients from CHLC. The left image shows a sample obtained from a patient in the treatment-success group, with few CD68+ macrophages, while the central and right ones show samples from patients in the treatment-failure group, with many CD68+ macrophages. All patients had the nodular sclerosis subtype of classical Hodgkin´s lymphoma.

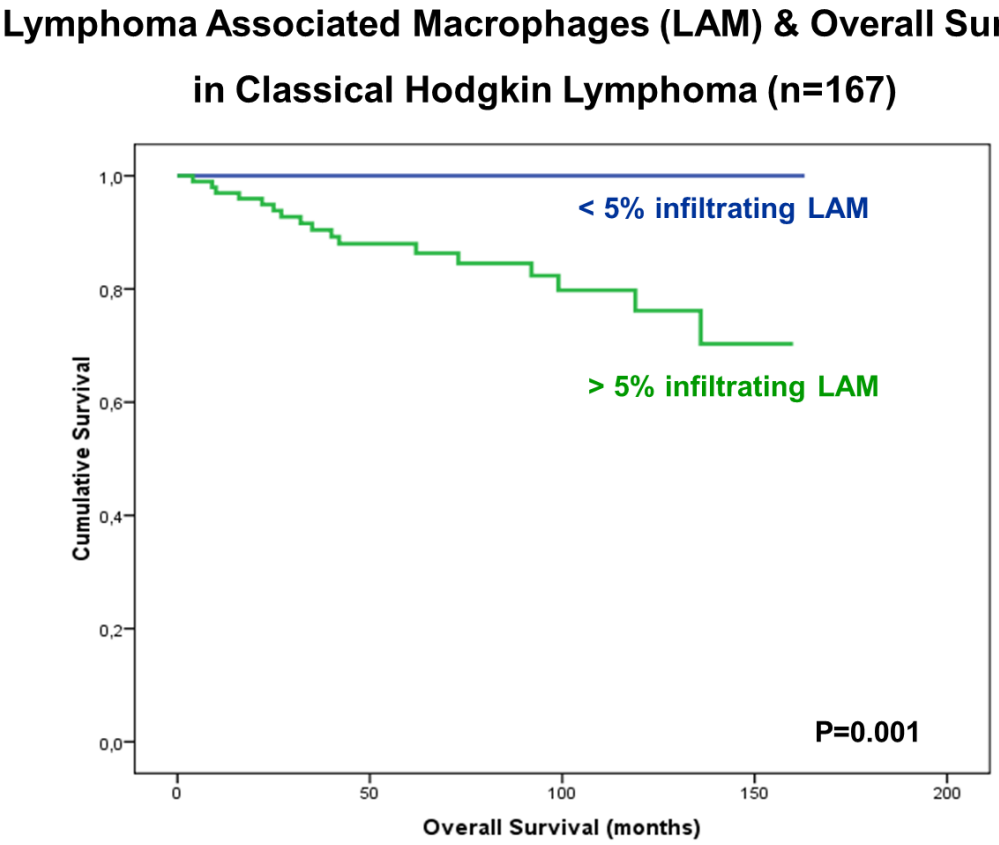


Figure 6.14 - Overall survival in a validation cohort of 167 patients, according to the number of infiltrating CD68+ macrophages in pre-treatment lymph-node biopsy specimens.

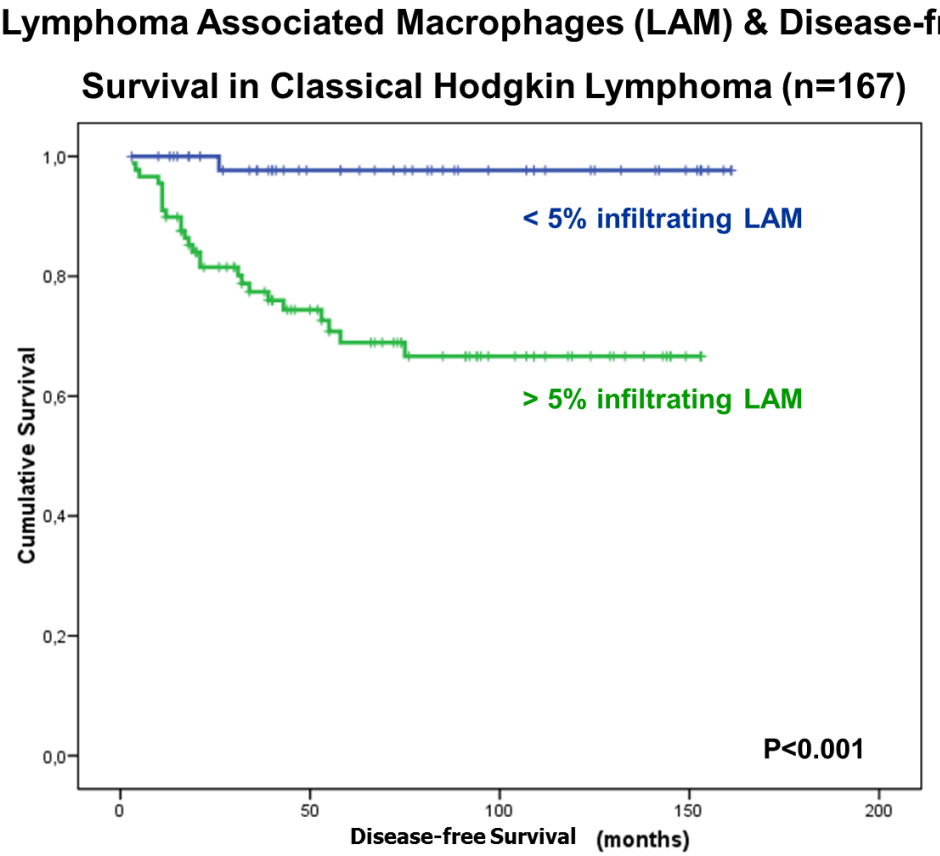


Figure 6.15 - Disease-Free Survival in a validation cohort of 167 patients, according to the number of infiltrating CD68+ macrophages in pre-treatment lymph-node biopsy specimens.

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Section 7: New Biological Predictors of Survival in Mantle Cell Lymphoma: The Microenvironment

7.1 INTRODUCTION

Mantle cell lymphoma (MCL) is a clinically aggressive small B cell lymphoma characterized by frequent relapses and short survival. Clinical behavior and response to therapy vary considerably and are often unpredictable based on clinical factors. Prior gene and protein expression studies have demonstrated proliferation and TP53 expression as biological mechanisms that strongly influence survival (1-3). Recent studies have highlighted the role of the microenvironment in follicular lymphoma (FL) demonstrating both T cell and macrophage numbers and immuno-architectural patterns that correlate with patient survival (4-6). Within most tumors, different T cell subsets co-exist, including cells with cytotoxic or regulatory functions and macrophages may display marked plasticity including an M2 phenotype promoting tumor growth and angiogenesis (7). The significance of the non-malignant cellular compartment in the prognosis of patients with MCL is unknown.

7.2 AIM

Assess the prognostic impact of different non-malignant cells in MCL patients from a single institution experience using flow cytometry (FCM), gene expression profiling (GEP), tissue microarrays (TMA) and immunohistochemistry (IHC) of diagnostic lymph node biopsies in combination with clinical correlates.

7.3 MATERIAL & METHODS

7.3.1. Patients & Samples Selection

The study includes two cohorts of patients diagnosed and treated at BCCA. The first cohort was studied based on available FCM data from the initial biopsy, while the second was organized based on diagnostic biopsies of formalin-fixed

paraffin-embedded tissue (FFPET) samples. For both series, the baseline clinical characteristics, including the international prognostic index (IPI) variables, pathology of their staging bone marrow, and clinical outcomes were recorded.

The Ethical approval to conduct this retrospective review was granted by the University of British Columbia–BCCA Research Ethics Board, and informed consent was obtained in accordance with the Declaration of Helsinki.

7.3.1.1. Cohort 1

7.3.1.1.1. Patients & Samples Selection

Firstly, patients with *de novo* MCL (122 patients), diagnosed and reviewed by BCCA hematopathologists according to the 2008 WHO criteria (8) who had FCM analysis performed using their diagnostic biopsies between 1992 and 2007. Patients were excluded if they were too frail to receive chemotherapy or if FCM was only performed on peripheral blood or bone marrow. For a subset of these patients with available diagnostic frozen tissue (28 patients) and diagnostic FFPET (53 patients), Affymetrix GEP and IHC & TMA correlative studies were performed.

7.3.1.1.2. Monoclonal Antibodies

Cell suspensions from freshly disaggregated lymph node biopsies were stained according to the manufacturer's recommendations with monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), or PE-Cy5. The routine diagnostic panel comprised the following 7 tubes. Tube 1 contained anti-CD45–FITC, anti-CD14–PE, and anti-CD19–PE-Cy5. Tube 2 contained isotype controls IgG1-FITC, IgG1/IgG2a-PE, and IgG1–PE-Cy5. Tube 3 contained anti-CD10–FITC, anti-CD11c–PE, and anti-CD20–PE-Cy5. Tube 4 contained anti-CD5–FITC, anti-CD19–PE, and anti-CD3 PE-Cy5. Tube 5 contained anti-CD7–FITC, anti-CD4–PE, and anti-CD8–PE-Cy5. Tube 6 contained anti-FMC7–FITC, anti-CD23–PE, and anti-CD19–PE-Cy5. Tube 7 contained anti–kappa-FITC, anti-lambda–PE, and anti-CD19–PE-Cy5. All antibodies were obtained from Beckman Coulter (Fullerton, CA) except CD23 and CD19–PE-Cy 5 which were obtained from Dako North America (Carpinteria, CA).

7.3.1.1.3. Cell Preparation

The cell suspensions were generated by disaggregating cells from fresh tissue and suspending them in phosphate-buffered solution (Dulbecco PBS; StemCell Technologies®, Vancouver, BC) to a lymphocyte concentration approximating 10^7/mL. Cell concentration and viability was assessed using Trypan blue exclusion dye (Invitrogen®, Carlsbad, CA). A total of 500 000 live cells were stained with the appropriate antibody combinations (see above) and incubated at 4°C for 30 minutes. Cells were treated with 250 µL Opti-Lyse C containing 1.5% formaldehyde (Beckman Coulter®) to deplete red cells and fix the lymphocytes. The remaining cells were then washed once with IsoFlow sheath fluid (Beckman Coulter) before FCM analysis.

7.3.1.1.4. FCM Analysis

Quantitative fluorescence analysis was performed using a Beckman Coulter Cytomics FC500 equipped with a single 488-nm argon laser source. FITC/PE/PE-Cy5 emission was collected in FL1/2/4 channels using 525/575/675 nm bandpass filters, respectively. Daily instrument calibration was performed using Flow-Set/Flow-Check beads (Beckman Coulter). List mode files were analyzed using FlowJo software, version 8.7.1 (TreeStar, Ashland, OR). A minimum of 5000 events was analyzed for all gated populations presented. Live cells were gated using forward and side scatter criteria. FCM data was analyzed for expression of CD3, CD4, CD8, CD19, CD20, and kappa and lambda light chains on cells gating on lymphoid populations. Estimates of the non-neoplastic B cells were derived from total CD19/CD20 positive B cells excluding the light chain restricted population.

7.3.1.1.5. Gene Expression Analysis

RNA was extracted from frozen lymph node tissue taken at diagnosis (28 samples) using Qiagen Allprep extraction kits (Qiagen Inc., ON, Canada) and hybridized to Human Genome U133A Plus 2.0 arrays (Affymetrix®, Santa Clara, USA) according to the manufacturer`s protocol with modifications. The raw expression data were background corrected, normalized and summarized using RMA procedure (9). Gene expression analysis was performed using the

statistical computing environment R (10) and Bioconductor packages (11) (http://www.bioconductor.org).

Differentially expressed genes were analyzed by Ingenuity® Pathway Analysis (IPA) (Ingenuity® Systems, www.ingenuity.com, Mountain View, CA). Fisher's exact P-values are reported for groups of overexpressed genes in the CD8-high group that could be mapped to annotated biological functions.

7.3.1.2. Cohort 2

7.3.1.2.1. Patients & Samples Selection

Patients diagnosed and treated at BCCA were included in the study if they had MCL with available diagnostic FFPET biopsy and had adequate clinical information. 185 patients treated at the BCCA between 1983 and 2004 were included. All cases were confirmed to have MCL based on FISH for the t(11;14) and/or Cyclin D1 (CCND1) IHC staining.

7.3.1.2.2. Tissue Microarray & Immunohistochemistry

A TMA was built with duplicate 0.6mm cores from each case using a tissue arrayer device (Beecher Instruments®, Silver Spring, MD). Slides from the TMA were cut at 4 µm. A haematoxylin and eosin (H&E) stain of the TMA was prepared using routine methods. The immunohistochemical stains performed, included CD20, CCND1, CD3, CD8, CD4, CD68, CD34, TIA1 (a cytotoxic marker), CD163 (monocyte & macrophage marker with M2 phenotype), FOXP3 (regulatory T cell), PD1 (immune-modulatory T cell), CD57, CD21, TP53 and KI67. Standard IHC was performed using a Dako autostainer and the EnVision polymer detection system. Antigen retrieval was used for all antibodies and included enzyme pre-digestion for CD21 and pressure cooking (5 minutes) for the remainder. A variety of buffers were used depending on the specific antibody to allow optimal detection of the antigens. The chromogen in all cases was diaminobenzidine. TP53 and Ki67 IHC were scored using image analysis (VIAS).

The immuno-stained TMA slides were scored into three expression groups for each marker in duplicate according to the percentage of positive cells: (CD3, CD4, CD8, CD57, TIA1, CD68, CD163, FOXP3, PD1, Ki67) or as either positive

or negative: (CCND1, CD21, TP53). Microvessel density as assessed by CD34 was scored similarly.

7.3.2. Statistical Analysis

Univariate survival analysis was performed using the log-rank test and Kaplan-Meier method using SPSS software, version 17 (12). The primary endpoint used was overall survival (OS) defined as the time from diagnosis to death of any cause. As initial management included observation only for several patients, event-free survival was not assessable. The Cox proportional hazard model was used to determine the relationship between survival and the known covariates in this study (13). The χ^2 test was used to compare patient and pathological characteristics. Non-parametric correlations were assessed by the Spearman Rank method. Two-sided P values less than 0.05 were considered significant.

7.4 RESULTS

7.4.1. Cohort 1

Patients & Samples Selection

The 122 patients with newly diagnosed MCL had FCM performed on their primary biopsy, of which 53 were also part of cohort 2 and were included in the tissue microarray. The median age was 67 years (range 22-94) with 69% male. 91% had advanced-stage disease with 16% having a high IPI (4/5) and 34% a high MIPI. Fifty (41%) patients received rituximab (R) as part of initial or subsequent therapy. Primary and secondary treatment regimens were markedly heterogeneous and included: observation (17 pts); single agent alkylators (48 pts); CHOP-like with R (39 pts); CHOP-like without R (42 pts); CVP-like (8); fludarabine-based (18); gemcitabine-based (9); biological agents (17 pts); autologous stem cell transplant (12 pts); allogeneic stem cell transplant (2 pts); radiation (33 pts); and therapeutic splenectomy (6 pts) (Tables 7.1 & 7.2).

The median follow up of the living patients was 30 months. The five-year OS for the group was 21%. Use of R was associated with superior survival with a median survival of 4.0 years in recipients and 1.6 years in non-recipients.

(P=0.001). The median survival by IPI group was as follows: low (39 pts) 3.9 years, intermediate (62 pts) 2.5 years and high (20 pts) 0.7 years. Median survival for 83 patients with data available for calculation of MIPI was as follows: low (31 pts) 5.9 years, intermediate (24 pts) 2.3 years and high (28 pts) 0.7 years (P<0.001).

Non-neoplastic Lymphoid Population

FCM analysis revealed considerable variation in the composition of the non-neoplastic lymphoid populations with median CD3 of 12%, (range: 0.6-69%), CD4 of 7%, (range: 0-39%), CD8 of 4% (range: 0-28%) and non-tumour B cells of 2%, (range: 0-18%) (Figure 7.1). There was no significant difference in the composition of the non-neoplastic population according to age, gender, stage, IPI, MIPI, LDH, and site of biopsy.

Increased CD8 expression by FCM is associated with inferior survival

Comparison of OS according to FCM marker expression, dichotomized using the median values, revealed an inferior outcome for patients whose biopsies contained more than 4% of CD8+ cells (Table 7.3). Further analysis revealed optimal separation of survival curves at a cut-point of 8%. Patients with ≥8% CD8+ T cells in their biopsies had a median survival of 1.4 years compared with 2.8 years for those with <8% CD8 T cells. (P=0.009) (Figure 7.2). The number of CD3, CD4 and non-clonal B cells did not correlate with survival.

MIPI, CD8 and Rituximab therapy are independent predictors of outcome

Multivariate analysis was performed to examine the relationship of tumor CD8+ T cell content with covariates known to predict outcome in MCL including IPI and prior R therapy. The association was also examined on a subset of patients (83 pts) where the MIPI could be ascertained (Table 7.4). This analysis confirmed tumor CD8+ T cell content to be a predictor of overall survival, independent of MIPI score and prior R therapy. Stratifying cases by R usage, revealed a survival benefit for the patients with low CD8+ T cells in their biopsies that received R (Figure 7.3).

The median survival of patients receiving R with low biopsy CD8+ was 4.9 years compared with a median survival between 1.3-1.8 years for those who did

not receive R or with high CD8+ T cell content (P<0.001). Correlation of tumour CD8+ T cell content with other clinical and pathological parameters did not identify any significant differences between CD8 expression groups in relation to age, gender, extent of disease, IPI and MIPI, MCL subtype, blastoid features, therapy received and best treatment response (Table 7.5). We can therefore surmise that in this cohort, CD8+ cell content is not a surrogate for other clinical or biological factors that may influence outcome.

IHC analysis confirmed CD8 outcome correlation and identified the number of TIA1+ T cells and CD163+ macrophages to be associated with inferior survival

53 of the 122 cases also belonged to cohort 2 and thus were included in the TMA and were analyzed for expression of a wider range of markers (see below) including: CD20, CCND1, CD3, CD8, CD4, CD68, CD34, TIA1, CD163, FOXP3, PD1, CD57, CD21, TP53 and KI67. Findings for CD8 by IHC were similar to those observed using FCM, including the favorable response to R in patients with few CD8 positive cells. However, CD8 expression by FCM and IHC were only moderately correlated (r=0.497, P=0.01). In addition, an increase in TIA1 positive cells was also adversely prognostic (median survival 3.7 years for lowest expressers versus 1.4 years for highest expressers, P=0.004), and correlated with expression of CD8 (r=0.523, P<0.001) suggesting that many of CD8 T cells were cytotoxic T cells. Immunohistochemical analysis also identified a poor outcome for patients with more than 5% of CD163 positive macrophages in their biopsies (P=0.008) with a median survival of less than one year. The same tendency was also noted for CD68+ cells (P=0.057). While CD68 and CD163 expression were weakly correlated (r=0.35, P=0.01) there was no significant association between macrophage markers and CD8 (r=0.23, p=0.09 for both) (Figure 7.4 & Table 7.6).

Ki-67 expression (>35%) was also adversely associated with survival (P=0.028) as described previously (14-16). However, there was no correlation between Ki67 expression and CD8 expression (P=0.565). CCND1, CD3, CD4, CD34, FOXP3, PD1, CD57, CD21 and TP53 expression did not correlate with survival.

Cases with high CD8+ T cell content exhibit increased expression of Th1 T cell and M1 macrophage genes

Differences in gene expression between cases with high and low CD8 T cell content were surveyed using cases at the extremes: low CD8 group was composed of samples with CD8 ≤2% (12 cases) and the high CD8 group was composed of samples with CD8≥9% (7 cases). Differential expression was measured by a modified t-test and genes were ranked by their P-values adjusted using Benjamini-Hochberg multiple test correction (17). Although there were no significantly differentially expressed probe sets after the adjustment, gene enrichment analysis of the top 200 overexpressed genes using Ingenuity® Pathway Analysis (IPA) (Ingenuity® Systems, www.ingenuity.com, Mountain View, CA) identified significant overrepresentation of genes involved with an inflammatory Th1 response ($P=5.7 \times 10^{-7}$), chemotaxis of T cells ($P=2.0 \times 10^{-5}$) and macrophages ($P=3.3 \times 10^{-4}$) (table 7). Moreover, global associative testing found significant association between an M1 macrophage gene signature (*CXCL9, CXCL10, CXCL11, CCL5, CCL19, CTSC, FAS, IL15RA*) (18) and increased CD8 content of the samples (P=0.023). The Th1 immune response genes (19) (*IFNG, IRF1, MT1H, GBP1, TNFSF10*) similarly showed increased expression in the CD8 high cases in comparison to the CD8 low group (P=0.006). Using the top 200 differentially expressed genes, the heatmap in Figure 7.5, shows unsupervised clustering of samples resulting in complete separation of gene expression profiles with high and low CD8 cellular content.

7.4.2. Cohort 2

Patients & Samples Selection

The median age was 68 years (range 22-91) with 67% males. 89% had advanced-stage disease with 13% having a high IPI (4/5). The IPI predicted OS (P=0.0013).

Twenty seven (15%) patients received R as part of initial or subsequent therapy.

Primary and subsequent therapy included multiple regimens including observation (22 pts); single agent alkylators (50 pts); CHOP-like with R (25 pts); CHOP-like without R (61 pts); CVP-like (11 pts); fludarabine-based (14 pts);

gemcitabine-based (6 pts); biological agents (15 pts); autologous stem cell transplant (9 pts); allogeneic stem cell transplant (1 pts); radiation (33 pts); and therapeutic splenectomy (6 pts) (Table 7.3).

The median follow-up of living patients was 5.75 years. The IPI was predictive of OS (RR=2.5, 95% CI =1.5-4.4, P=0.002).

Besides KI67 and TP53, IHC analysis showed increased density of CD68+ cells and microvessels CD34+ to be significantly associated with inferior survival

All 185 cases were either CCND1and/or t(11;14) FISH-positive. The numbers of evaluable cases varied for the different biomarkers (range 157 to 167).

Both proliferation (quartiles of $Ki67^+$ cells) and TP53 (dichotomized based on cut-off >40%) were significant predictors of OS independent of IPI (OS, P=0.0002). Cases showing more than 5% infiltrating CD68+ cells (18/185) had a 4 years OS of 5% vs. 35% (P=0.0081). These CD68+ cases significantly correlated with 12 cases showing more than 5% infiltrating CD163+ cells (x^2, P=0.001), which by itself also significantly influenced OS (P=0.008) (Figure 7.6 & Table 7.8).

Seventeen cases had increased microvessel density (MVD), defined by CD34+ cells, which correlated with inferior OS (P=0.02). The cases with increased MVD typically had >5% CD163+ cells (a M2 marker) (x^2, P=0.05), but not increased CD68+ cells.

Two T cell biomarkers, CD3 and CD8, showed significant correlation with survival limited to patients who did not received R. In line with the results presented in cohort 1, cases showing more than 5% infiltrating CD8+ cells (7/122) had an inferior outcome (P=0.006) and this finding significantly correlated with cases showing increased CD68+ and CD163+ (x^2=0.01). Cases with more than 5% infiltrating CD3+ cells (31/122) had a superior outcome (P=0.06) and significantly correlated with CD8+ infiltrating cells (P<0.0001), but not with macrophage markers. Multivariate analysis of the entire cohort 2 including IPI, TP53, Ki67, CD68, CD163, CD34, CD8 and CD3 showed IPI (RR=1.8, 95%CI=0.98-3.2, P=0.024), TP53 (RR=2.3, 95%CI=1.3-4.2, P=0.007), Ki67 (RR=2.2, 95%CI=1.4-4.1, P=0.018), CD34 MVD (RR=1.8, 95%CI=1.0-3.2,

P=0.039) and CD68 (RR=2.2, 95%CI=1.6-4.1, P=0.016) to be independent predictors of OS (Table 7.9).

In 27 patients who received R at some time during their disease course, increased $CD68^+$ cells did not significantly affect OS (P=0.1), suggesting that, similar to FL, the prognostic effect of LAM may be abolished by R.

7.5 DISCUSSION

While the influence of the tumor microenvironment has been described in other lymphoid neoplasms, there is a paucity of information in MCL, perhaps due both to the strong correlation of survival with the proliferation rate of the malignant cells and to the relative paucity of non-neoplastic immune cells observed during routine microscopic analysis of tumor biopsies. However, it is increasingly apparent that MCL are not just large collections of autonomously proliferating cells occurring in isolation, but interact with a complex microenvironment composed of immune cells, microvasculature, mesenchymal cells and other stromal elements.

The difficulty in propagation of MCL cells *ex vivo* (20), highlights the necessary requirement for interaction between tumor cells and non-neoplastic cells, all of which comprise the normal lymph node microenvironment. In this study we analysed the microenvironmental impact on MCL prognosis using two different cohorts (except for 53 patients (17%) in common to both arms of the study) ranging across different time & treatment periods; 1993 to 2007 (cohort 1) and 1983 to 2004 (cohort 2), and using different methodologies. Importantly, in both cohorts we found that patients whose tumours exhibited an inflammatory reaction characterised by increased cytotoxic T cells and macrophages had significantly shortened overall survival independent of the IPI. This represents the first study showing a significant impact of non-malignant cells present in microenvironment of MCL. The subset of patients with an adverse outcome is small, but may represent a group of patients with such a grim outcome that new therapeutic regimens are urgently needed.

A similar finding has been noted in DLBCL, with tumours exhibiting a “host response” (HR) signature characterized by increased expression of T cell and natural killer cell receptor and activation pathway components, complement

cascade members, macrophage/dendritic cell markers, and inflammatory mediators experiencing inferior outcome following CHOP chemotherapy (21).

Importantly, there were interesting differences in the study of both cohorts, with apparently conflicting results. In the FCM data, gene expression data on selected samples (19 cases) representative of cases with increased *vs*. decreased CD8+ cells shows an association between a M1 macrophage gene signature and increased CD8+ cell content suggesting that a cytotoxic and pro-inflammatory milieu would be advantageous to the MCL cells. In contrast, in the TMA & IHC study the adverse prognosis is significantly associated with increased CD68+ and CD163+ macrophage cell content, with CD163 a previously reported marker of M2 macrophages (22). Notably, the higher CD163 cell content correlates significantly with increased MVD measured by CD34, which is also associated with adverse prognosis. Functionally, M2 macrophages are known to be pro-angiogenic. Finally, in the same cohort these markers are correlated with increased CD8 cell content. Despite being unable to validate CD8 cell content as an independent marker of prognosis, these results are consistent with findings from cohort one.

The impact of tumour cytotoxic T lymphocyte (CTL) content on outcome has been previously examined in several other lymphoid neoplasms. While results vary between studies, elevated levels of CTLs have been found to be unfavorable in Hodgkin lymphoma (HL) (23-26), DLBCL (27, 28), anaplastic large cell lymphoma (24), but favorable in FL (29) and DLBCL (30, 31).

M1-type macrophages or “classically-activated” macrophages support a Th1 response while “alternatively-activated” macrophages (M2-type) support a Th2 response and/or regulatory T cell expansions. M1 macrophages are involved in inflammatory reactions whereas M2 are involved with tissue remodelling and angiogenesis (7). Macrophages in solid tumours have previously been reported to show an M2 phenotype, which supports tumour progression and metastasis (32). Yet, macrophage polarization in lymphoma is less well defined (33). In the current study, there are apparently conflicting results related to the macrophage phenotype associated with inferior prognosis between the two cohorts. Similar findings have been reported in other lymphomas subtypes, including some original work in FL. There are a few hypotheses that may explain these findings. Firstly, the methodologies used for analysis of the macrophage phenotypes are

not directly comparable, as the IHC markers used in the TMA studies may be imperfectly correlated with live gated cells obtained by FC. Moreover, the GEP study focussed on the extremes of CD8 counts and may not be representative of the entire cohort. Secondly, it has been shown that *in-situ* macrophages cannot be rigidly categorised as distinct M1 vs. M2 phenotypes, which are mostly derived based on data from *in vitro* studies. Often, these cells are neither classically nor alternatively activated, but possess aspects of both phenotypes consistent with an immune regulatory phenotype determined by tissue stimulus and phase-of-inflammation (33). Thirdly, the impact of heterogeneous treatment protocols used in these cohorts maybe an important factor explaining the divergent results. The two cohorts, organized by the availability of diagnostic tissue biopsy blocks and/or FC covers a number of different treatment eras including an increased number (34%) of patients receiving rituximab in cohort-1 in contrast to 15% in cohort-2. In FL, the favourable immune response-1 signature included genes mainly expressed by T cells and the unfavourable immune response-2 signature, mainly expressed by macrophages or follicular dendritic cells (4). Validation studies using TMA and IHC have shown increased macrophage number in FL biopsies to correlate with both favorable (29, 34), unfavorable (5, 35-37) or to have no effect on FL outcomes (38, 39). While some of this variability is due to the heterogeneity of treatment protocols included in most studies, in the few series with uniformly treated patients the use of R abrogated the negative impact of high content of LAM in FL. Indeed, studies performed in the R era suggested that biologic prognostic factors may be treatment dependent (34, 37, 40). In support of this concept, *in vitro* studies have shown that M2 macrophages phagocytize R-opsonized cells more efficiently than M1 cells (41). Thus, in the current MCL study the cases analyzed by GEP were enriched for cases treated with R, which might have modulated the macrophage phenotype differently than cases studied in cohort 2 that included fewer cases exposed to R. Furthermore, the high IFNγ microenvironment present in tumors with increased CD8 content may prevent significant Th2 T cell or M2 macrophage recruitment within the tumour (41).

Therefore, the most probable cause of the suboptimal response to R in patients with elevated tumour CD8 content could be related to the association of

elevated tumor CD8 content and an M1 macrophage gene signature. Similar to what has been described in solid tumors and FL treated before the introduction of R, the study arm including mostly cases not treated with R showed the worse prognosis associated with increased M2 macrophages.

7.6 CONCLUSION

This study provides data that inform for the first time on the prognostic impact of the non-neoplastic immune cells in the microenvironment of MCL, independent of clinical parameters, proliferation and TP53 While the non-neoplastic cellular infiltrate often constitutes only a minor fraction of the tumour mass in MCL, it appears similar to other lymphoma subtypes suggesting that the microenvironment is important in MCL biology, prognosis and response to therapy. We suggest that MCL shows two distinct microenvironments depending on the therapeutic regimen used; the first characterized by the production of IFNγ, a Th1 cellular response and M1 macrophages heralds a diminished response to R; the second being a more permissive or immunosuppressive microenvironment that permits recruitment of M2 macrophages within the tumor eventually promoting tumour growth and inducing angiogenesis. Yet, it portends a more favorable response to R (Figure 7.8). Expression of CD163, described as a marker of M2 polarized macrophages, also predicts survival and correlates with increased angiogenesis.

Further studies are required to prospectively validate these observations and define the mechanism by which the immune response negatively influences outcome. With time and improvements in our understanding of MCL biology, it is possible that treatments targeting the microenvironment may become a treatment option in MCL. Inclusion of these biomarkers (and others) into the design of prospective clinical trials in MCL would seem prudent.

7.7 TABLES

Clinical features		Cases
Age		67(22-94
Male		84(69)
Stage	Advanced	99(91)
	Limited	10(9)
	Bulk (≥10cm)	21(20)
LD>ULN		35(37)
IPI Group	Low	39(32)
	Intermediate	63(52)
	High	20(16)
MIPI	Low	31(37)
	Intermediate	24(29)
	High	28(34)
Extranodal site	0	29(24)
	1	59(48)
	2	27(22)
	3 or more	7(6)
Location	Bone Marrow	80(82)
	Peripheral blood	22(18)
	Oro/Nasopharyn	14(11)
	Lung/Pleural	7(6)
	Gastrointestinal	7(6)
	Liver	2(2)
	CNS/Ocular	3(2)
	Cutaneous	2(2)
	Other	8(7)

Table 7.1 - Clinical features of Cohort 1 MCL patients.

Therapy		Initial	Subsequen
Radiotherapy		12	21
CHOP-like		27	15
R-CHOP-like		23	4
R-CHOP then ASCT		11	1
Allogeneic SCT			2
CVP-like		3	5
R-CVP-like			2
Alkylator		22	26
Fludarabine based		4	14
Gemcitabine based			9
Biological agents			17
Splenectomy		3	3
Observation		15	2
Other		1	10
No of Therapies	1	59 (55)	
	2	21 (19)	
	3	12 (11)	
	4	7 (6)	
	5	3 (3)	
	6	3 (3)	
	7	3 (3)	
Best treatment reponse	CR	36 (30)	
	PR	38 (31)	
	SD	3 (2)	
	PD	19 (16)	
	NA	26 (21)	

Table 7.2 - Therapy regimens used in Cohort 1 MCL patients.

	Cut-point (percent)	P
CD19	84	0.516
CD20	86	0.435
CD3	12	0.256
CD4	8	0.778
CD8	4	0.015
Non-clonal B cells	2	0.662

Table 7.3 - Univariate analysis comparing survival outcomes for the highest and lowest expression groups by marker. The cut-point is the median percentage expression for each marker. Statistical analysis was performed using the log rank test.

	Univariate	Hazard Ratio (95% CI)	Multivariate
IPI group	P<0.001	1.147 (0.776-1.698)	**P=0.491**
MIPI group	P<0.001		**P<0.001**
Rituximab	P<0.001	2.860 (1.701-4.807)	**P<0.001**
CD8 <8%	**P=0.009**	**0.578 (0.339-0.986)**	**P=0.044**

Table 7.4 - Multivariate analysis by Cox Regression examining the impact of IPI score, MIPI score, rituximab therapy and CD8 content on overall survival of patients with mantle Cell Lymphoma.

Clinical features		CD8 <8 %	CD8 ≥ 8%	P
Total		95	27	
Age median (range)		66 (22-94)	66(31-89)	0.819
Male gender		69 (73)	15 (56)	0.091
IPI	Low (0/1)	33 (34)	7 (26)	0.543
	Intermediate (2/3)	50 (52)	14 (52)	
	High (4/5)	14 (14)	6 (22)	
MIPI	Low	27 (41)	4 (22)	0.347
	Intermediate	16 (25)	8 (44)	
	High	22 (34)	6 (33)	
Advanced stage		90 (95)	24 (89)	0.279
Mass > 10cm		16 (17)	5 (19)	0.933
Increased Lactate Dehydrogenase		26(31)	8(35)	0.818
Extranodal disease		70 (74)	20 (74)	0.964
BM involvement		63 (66)	17 (63)	0.746
PB involvement		24 (25)	7 (26)	0.943
Pathology features	Nodular	35 (36)	5 (19)	0.224
	Diffuse	54 (57)	19 (70)	
	Mantle Zone	8 (8)	3 (11)	
	Blastoid Features	18 (19)	6 (22)	0.670
Best treatment response	CR	27 (28)	9 (33)	0.910
	PR	31 (32)	7 (26)	
	SD	3 (3)	0 (0)	
	PD	14 (15)	5 (19)	
	NA	20 (21)	6 (22)	

Table 7.5 - Clinical and pathologic characteristics of patients with MCL biopsies according to tumor CD8 T cell content. Parentheses contain percentages except for age as indicated. Statistical analysis was performed using Pearson Chi-test, except for age which was assessed by T-test. IPI indicates International Prognostic Index; MIPI, Mantle Cell Lymphoma International Prognostic Index. BM, Bone Marrow; PB, Peripheral Blood; CR, Complete Response; PR, Partial Response; PD, Progressive Disease; SD, Stable Disease; NA, Not Available

Marker	Cut-points (%)	P
CD3	<1, 1-5, >5	0.837
CD8	<1, 1-5, >5	0.006
CD4	<1, 1-5, >5	0.774
CD68	<5, >5	0.057
CD163	<5, >5	0.008
CD34	Vessel density/core	0.669
TIA1	<5, >5	0.004
FOXP3	<5, >5	0.79
CD57	<5, >5	0.245
P53	Image analysis	0.42
Ki67	Image analysis	0.177

Table 7.6 - Univariate analysis comparing survival outcomes for the highest and lowest expression groups by biomarker. Cut-points for each marker were set based on the expression patterns observed following an initial inspection of all cases. Statistical analysis was performed using the log rank test.

Function Annotation	P-value	Molecules
Inflammatory response	5.7×10^{-7}	*AIF1, ANXA1, AOAH, C3, CASP1, CCL5, CCR5 CD28, CSF2RA, CXCL9, CXCL10, CXCL11 GNLY, IFNG, IGHG1, PLA2G7, SBNO2*
Th1 immune respons of organism	4.7×10^{-6}	*CCR5, CLEC7A, CXCL10, IFNG, TBX21*
Chemotaxis of lymphocytes	2.0×10^{-5}	*CCL5, CCR5, CXCL9, CXCL10, CXCL11, GNLY*
Trafficking o macrophages	6.1×10^{-5}	*CCL5, CCR5*
Chemotaxis o mononuclear leukocytes	1.4×10^{-4}	*ANXA1, CCL5, CCR5, CXCL9, CXCL10, CXCL11 GNLY*
Invasion o macrophages	1.8×10^{-4}	*CXCL10, IFNG*
Chemotaxis o phagocytes	3.3×10^{-4}	*CCL5, CD28, CSF2RA, CXCL9, CXCL10, IFNG*

Table 7.7 - Gene enrichment analysis of the top 200 overexpressed genes using Ingenuity® Pathway Analysis (IPA) (Ingenuity® Systems) identified significant overrepresentation of genes involved with an inflammatory Th1 and macrophages responses.

Biomarke	# Failed	# Positive (%	Comments
TP53	18	17 (10)	>40% of positive cells per core
Ki67	18	Quartiles	% of positive cells per core
CD68	28	18 (12)	>5% $CD68^{+}$ cells per core
CD163	22	12 (8)	>5% $CD163^{+}$ cells per core
CD34	20	17 (10)	>30 luminal $CD34^{+}$ structures per core

Table 7.8 - Significant biomarkers in IHC cohort 2 studied by IHC & TMA, Cut-points for each marker were set based on the expression patterns observed following an initial inspection of all cases.

Biomarker	> 5% CD163$^+$ cells (12)	< 5% CD163$^+$ cells (151)	χ^2 (P)	OS
				0.008
IPI (2-5)	66% (8)	67% (101)	0.14	**0.0013**
Ki67 (>50%)	66% (8)	34% (45)	0.06	**0.0002**
TP53 (POS	17% (2)	10% (14)	0.34	**0.0002**
CD68 (>5%)	42% (5)	9% (13)	0.006	**0.008**
CD34 (POS)	33% (4)	8% (12)	0.02	**0.02**

Table 7.9. Clinical and pathologic characteristics of patients with MCL biopsies according to tumor CD163 macrophage content. Parentheses contain number of positive cases). Statistical analysis was performed using Pearson Chi-test and survival analysis was performed using the log rank test. IPI indicates International Prognostic Index

7.8 FIGURES

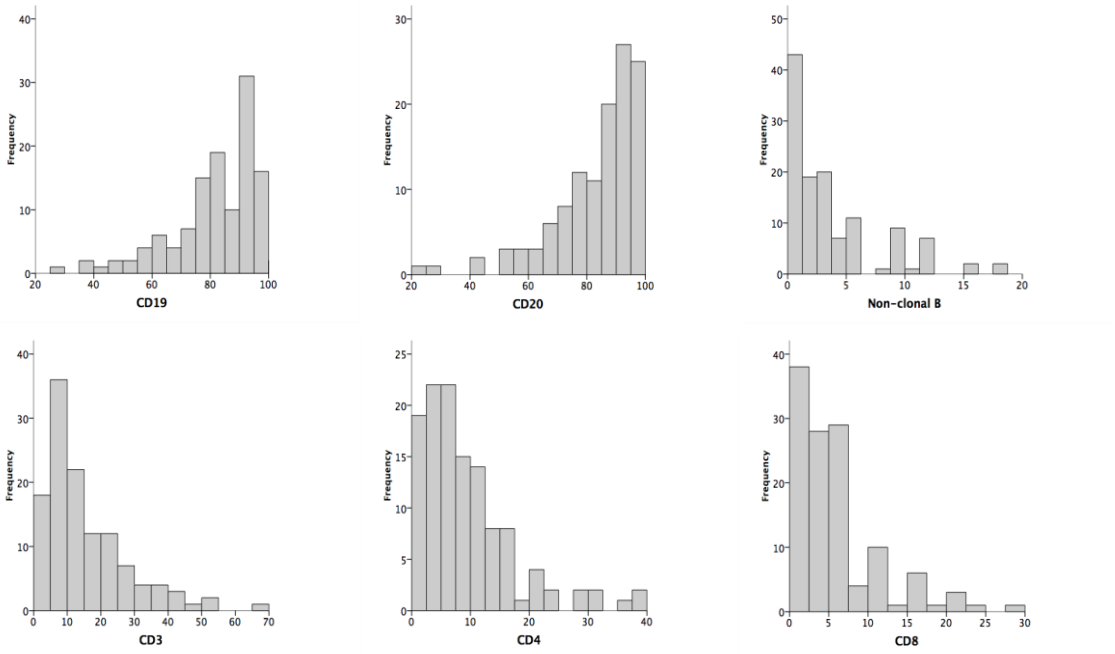

Figure 7.1 - Composition of the non-neoplastic lymphoid populations within MCL microenvironment studied by FCM analysis.

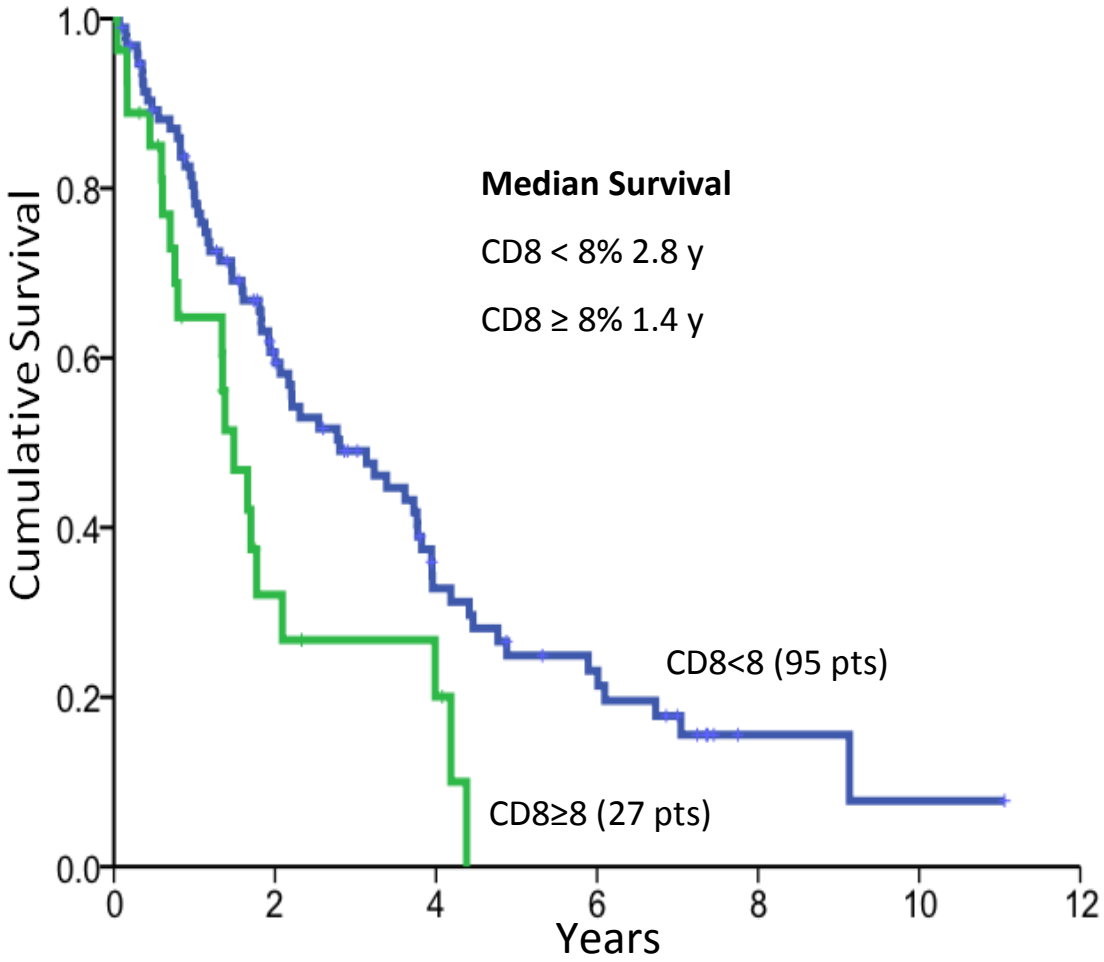

Figure 7.2 - Optimal separation of survival curves of CD8+ cells at a cut-point of 8% Patients with ≥8% CD8+ T cells in their biopsies had a median survival of 1.4 years compared with 2.8 years for those with <8% CD8 T cells.

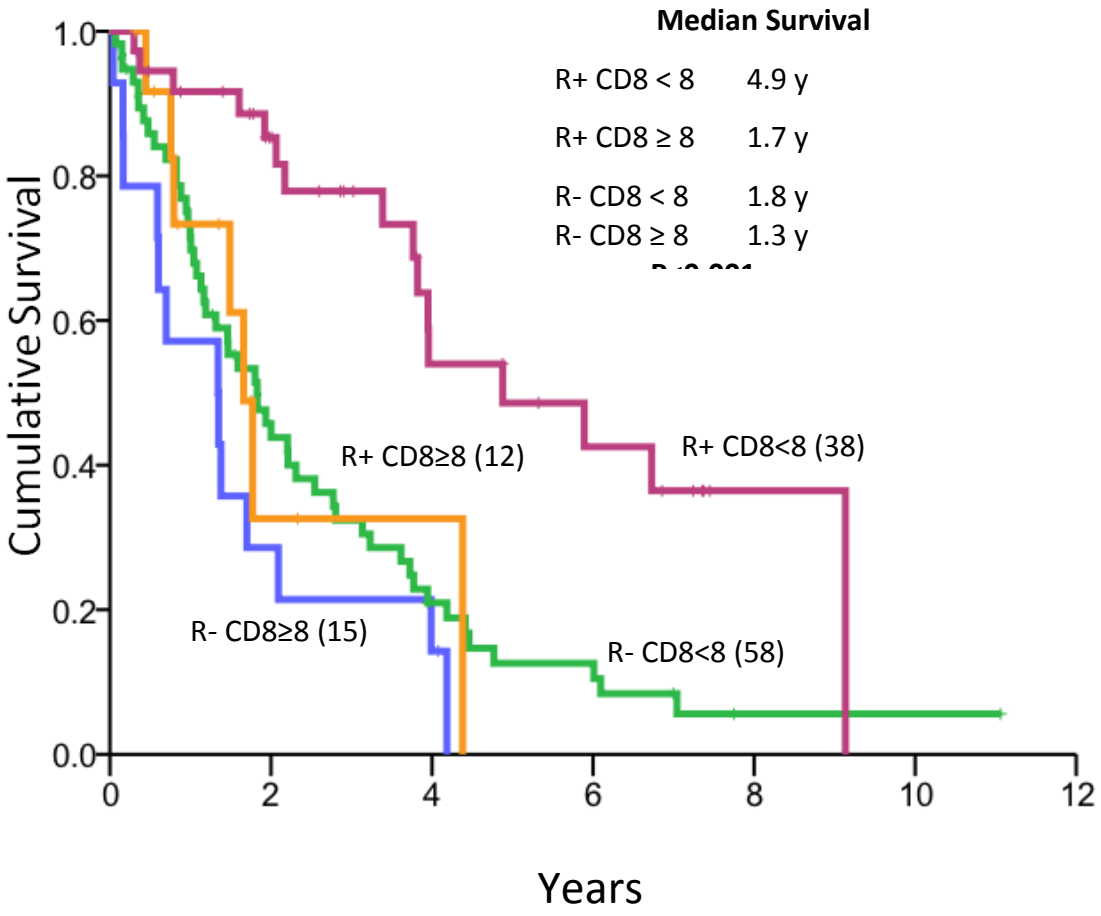

Figure 7.3 - CD8+ T cell content by FCM stratified by R usage is a predictor of OS. It shows a survival benefit for the patients with low CD8+ T cells in their biopsies that received R.

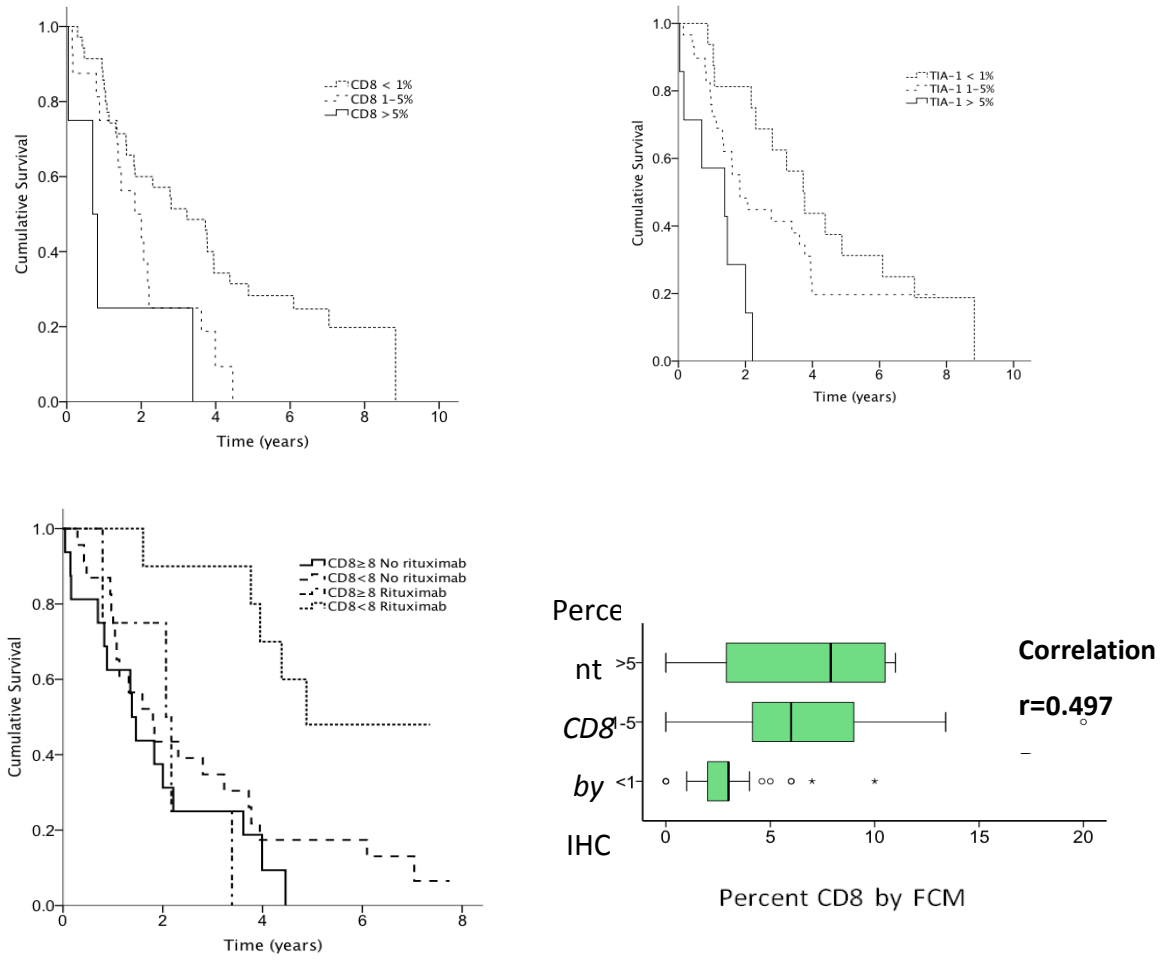

Figure 7.4 - CD8+ T cell content alone and stratified by R usage,TIA1+ T cell content analyzed by IHC & TMA are predictors of OS. There was a significant correlation between FCM and IHC & TMA assessment of CD8+ cell content (r=0.497, P=0.01for both)

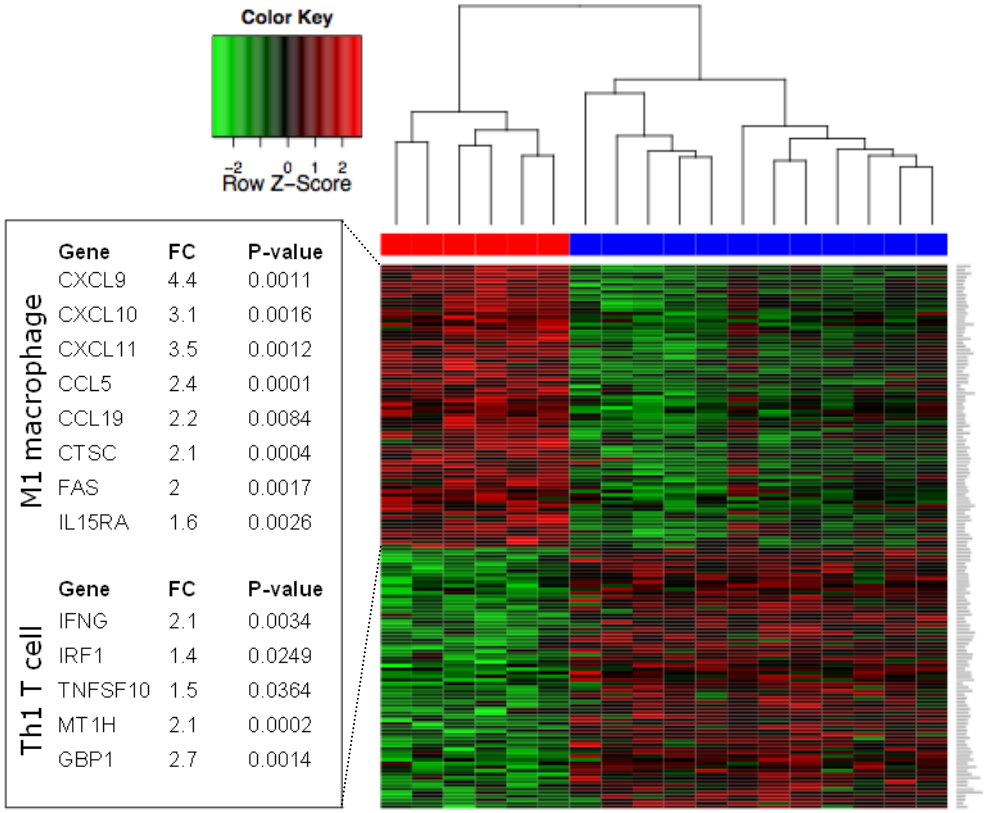

Figure 7.5 - GEP of MCL cases with high and low CD8 cellular content. Unsupervised clustering of samples using the top 200 differentially expressed genes shows resulting in complete separation of GEPs.

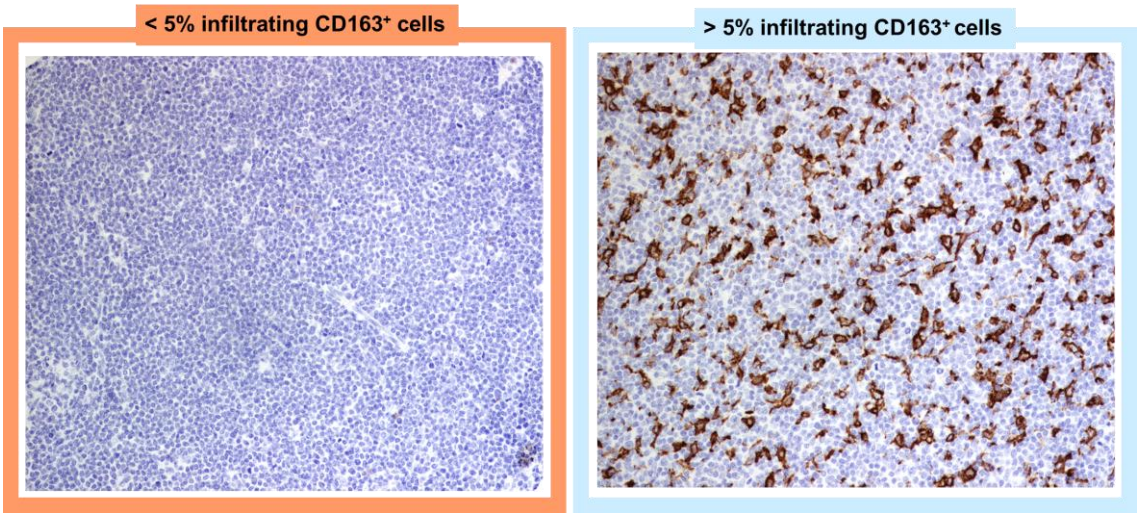

Figure 7.6 --Lymphoma-associated-macrophages infiltrating MCL (cohort 2). Most cases had very few LAMs (left image). A subset of cases showed increased LAM within the tumour (right image). (Microscope NIKON Eclipse E600; Digital Camera Dxm1200).

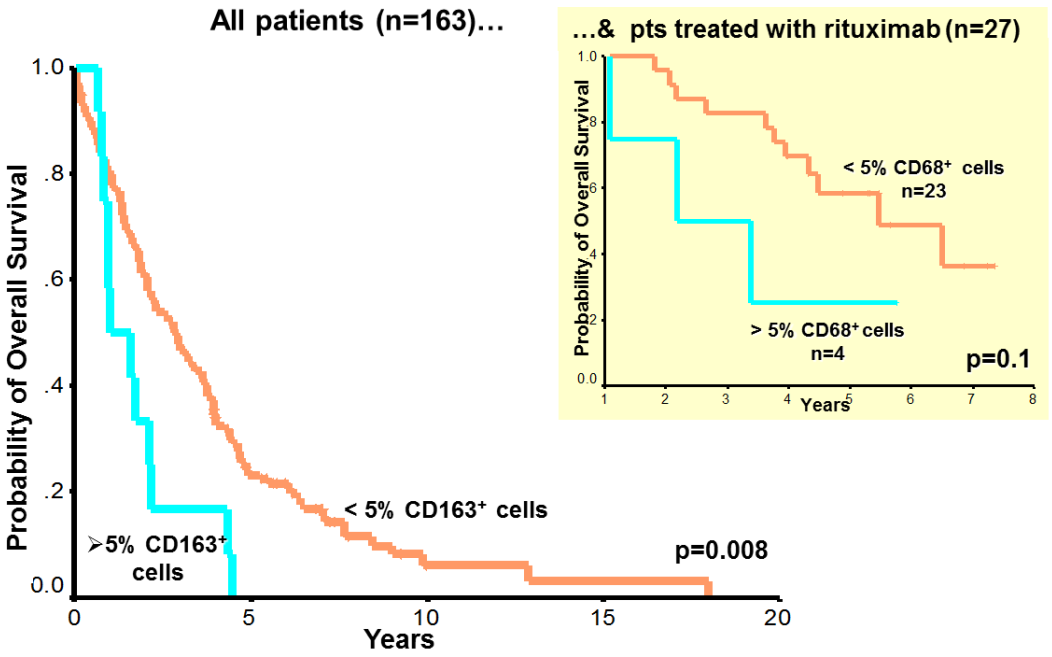

Figure 7.7 - Impact of LAM on MCL prognosis (cohort 2). Patients with >5% CD163+ cells in their biopsies had a median survival of 1.1 years compared with 4.0 years for those with <5% CD163+ cells.

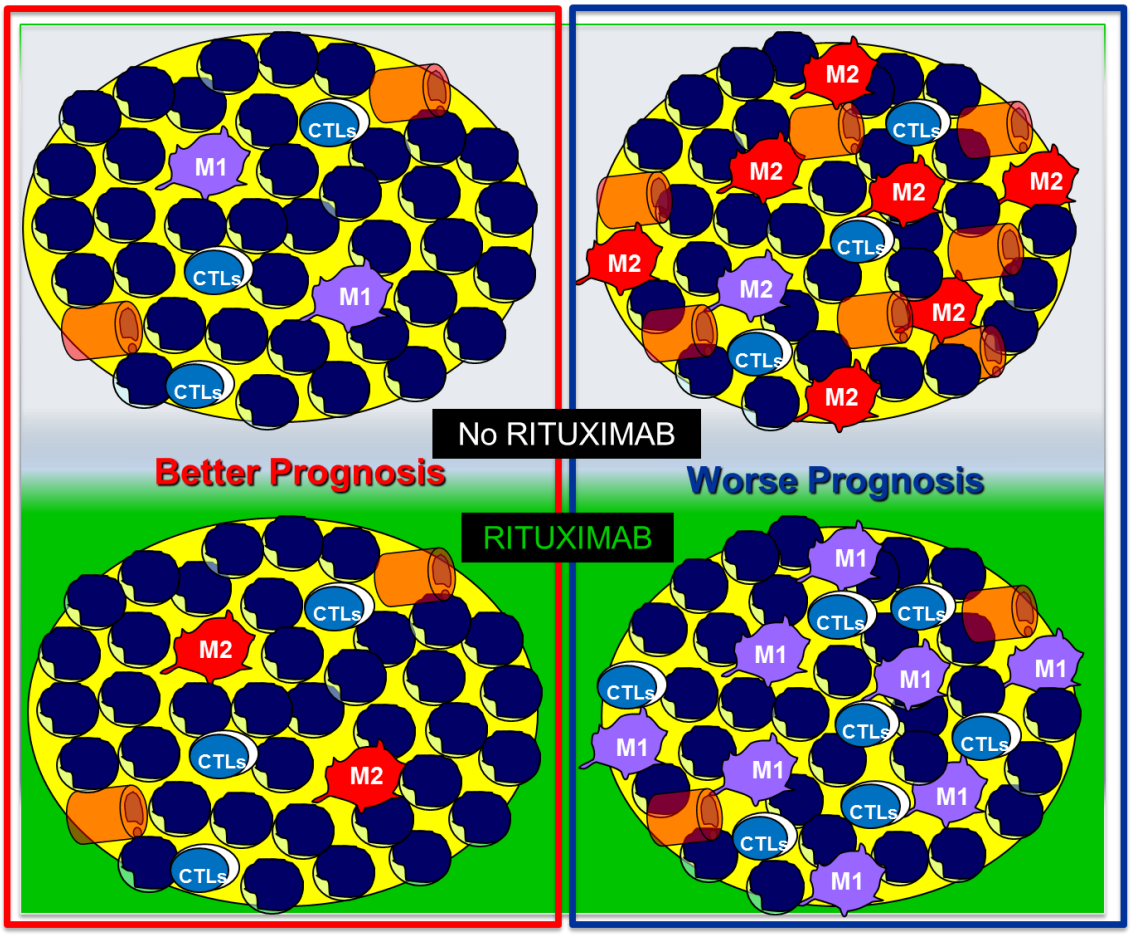

Figure 7.8 - Dual model for the role of tumor immunity in MCL and its impact on survival of patients treated with or without R. In the pre-R era increased numbers of vessels ( ) and macrophages M2 type ( ) represent cases where malignant cells edited local immunity to support tumor growth. Yet, when R is added to therapy these effects are minored and alternatively, increased cytotoxic CD8+ cells ( ) and macrophages M1-type ( ) are characteristic of cases resistant to therapy including R.

7.9 REFERENCES

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Section 8: New Biological Predictors of Survival in Primary Mediastinal Large B Cell Lymphoma: The Microenvironment

8.1 INTRODUCTION

Primary Mediastinal large B Cell Lymphoma (PMBCL) is a distinct lymphoma entity arising in the mediastinum from putative thymic B cells (1). It is composed of medium to large-sized B cells with abundant cytoplasm, ovoid nuclei and is characteristically associated with fine, background fibrosis (Figure 8.1). It accounts for 2-4% of all non-Hodgkin-lymphoma and occurs predominantly in young adults with a female predominance. It is locally aggressive, but less likely to extend beyond the mediastinum. The therapy is similar and outcome superior to nodal DLBCL in most series with a survival plateau after 2.5 years (2, 3). Yet, clinical criteria, such as the IPI, imprecisely predict the response to therapy (4).

PMBCL shares histological and gene expression features with both DLBCL and Classic Hodgkin Lymphoma (CHL) (5, 6). Like DLBCL, PMBCL may show loss of expression of Major Histocompatibility Class II (MHC II) antigens (7). The MHC II genes (HLA-DR, HLA-DQ, HLA-DP, HLA-DM and HLA-DO) are located in a single locus on chromosome 6p. Expression of all MHCII genes is regulated by the MHCII master transactivator, MHCIITA, and its expression is usually highly correlated with MHCII expression. Class II MHC antigens are constitutively and concurrently expressed in antigen-presenting cells, including monocytes, macrophages, dendritic cells and B cells responsible for presenting antigens and stimulation of T cells invoking an immune response against peptides (8).

In PMBCL and most DLBCLs, mechanisms of MHCII loss of expression are largely unknown, but loss of HLA-DR and related antigens may constitute a mechanism by which tumour cells escape immunosurveillance. Indeed, the association of loss of MHCII molecules on high-grade lymphoma cells has been described since the 1980s (9, 10) including an impact on patient prognosis (11). Recently, MHC class II genes enrich in one of the 4 gene expression signatures associated with clinical outcome in a large study of 240 DLBCLs as reported by

the Leukemia and Lymphoma Molecular Profiling (LLMPP) consortium in 2002 (12). In subsequent studies, these same authors also showed a significant reduction in the number of tumour infiltrating CD8+ lymphocytes to be associated with the lack of MHCII proteins in a small subset of DLBCLs (7). Similarly, a minority (12%) of a series of 42 PMBCLs reported by the LMMPP showing loss of MHCII gene expression had a worse survival(13).

8.2 AIM

The aim of our study was to validate HLA-DR protein expression as a prognostic biomarker in a larger cohort of PMBCLs and correlate it with the immune response using immunohistochemistry (IHC). New, easily measured biomarkers are needed to better stratify patients and to evaluate relevant candidate pathways for therapy.

8.3 MATERIALS & METHODS

8.3.1. Patient Characteristics

The BCCA is the primary referral center for patients diagnosed with lymphoid malignancies in the province of British Columbia, Canada. 103 patients diagnosed with PMBCL at the BCCA between 1980 and 2005 had available formalin-fixed paraffin-embedded tissue (FFPET) diagnostic biopsies were included. All patients were treatment-naive and therapy included multi-agent chemotherapy CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone) in 38 patients, or CHOP-like regimens (ACOP, CEOP, CVPP, ECV and VACOPB) in 65 patients. Rituximab (R) and radiation were added in 18 patients and 25 patients, respectively. 12 patients received bone marrow transplant at relapse. Patients were aged 16 to 61 years and were HIV-negative. Approval to this study was given by the University of British Columbia – BCCA Research Ethics Board. All biopsies were reviewed and classified according to the 2008 WHO Lymphoma Classification (1).

8.3.2. Tissue Microarray Construction (TMA)

Duplicate 0.6mm cores were used to construct a TMA using a tissue arrayer device (Beecher Instruments®, Silver Spring, MD) (Figure 8.2). Slides from the TMA block were cut at 3-4 microns.

8.3.3. Immunohistochemistry

An H&E stain of the TMA was prepared using routine methods. Immunohistochemistry (IHC) was performed routinely for CD20 (clone L26), HLA-DR (IgG2b), CD3 (polyclonal Cell Marque/Novo Mix), CD4 (clone 4B12), CD8 (clone C8/144B), CD57 (clone NK-1), CD68 (clone KP1), TIA1 (clone 2G9A10F5) and Granzyme B (clone GrB-7) using a Dako® autostainer and the EnVision polymer detection system. Antigen retrieval was used for all antibodies using pressure cooking (5 minutes). A variety of buffers were used depending on the specific antibody to allow optimal detection of the antigens. The chromogen in all cases was diaminobenzidine.

8.3.4. Scoring

HLA-DR expression was assessed combining both intensity (1 to 3) and percentage of positive cells (0 to 100%), using a Histoscore – (HScore) (14). All the other markers were quantified counting the number of positively-stained cells compared to the number of malignant cells per core. For each marker results were dichotomized about the median and correlated with overall survival. Further analysis revealed optimal separation of survival curves at the following cut-off points: 5% for CD4, CD57 and Granzyme B; 10% for TIA1; 25% for CD3, CD8 and CD68.

8.3.5. Statistics & Survival Analysis

Analysis was performed using Pearson Chi-test. Overall survival (OS) was defined as the interval from date of diagnosis until death from any cause. Progression free survival (PFS) was defined as the interval from date of diagnosis until death from disease Survival estimates were calculated using the Kaplan-Meier method (15) and multivariate analysis using the proportional-hazards regression Model (16)

8.4 RESULTS

There were 92 suitable cases for analysis. The median follow-up of the living patients was 10 years. Estimated 5-year OS and PFS was 85% and 73%, respectively. The IPI was significant for OS (P=0.042), but not PFS (P=0.17) (Figure 8.3). In total, 32 cases showed strong cytoplasm membrane HLA-DR staining (mean HScore of 253; range 100-300; standard deviation 80) while 60 cases showed decreased expression of the protein (mean HScore 5.5; range 0-80; standard deviation 17) (Figures 8.4 & 8.5). Patients with strong cytoplasm membrane HLA-DR staining and patients with decreased expression of HLA-DR- had 10-year OS of 86% *vs.* 61% (P=0.006) and 2-year PFS of 78% *vs.* 53% (P=0.018), respectively (Figure 8.5). The distribution of clinical and pathology variables between cases with strong HLA-DR+ and cases with decreased HLA-DR expression were evenly matched (Table 8.1). A Cox multivariate model established both HLA-DR status and IPI as independent predictors of OS (RR=0.3, 95%CI=0.12-0.75, P=0.01; RR=2.9, 95%CI=1.2-6.9, P=0.06, respectively). HLA-DR expression correlated significantly with increased content of all analyzed T cell markers, especially CD3, CD8 and TIA1 (x^2, P<0.001), but not with macrophage content (CD68) (Table 8.2). Clinical and pathological characteristics are summarized in Table 8.1 & 8.2.

Of all non-malignant markers, only TIA1+ cell content significantly correlated with survival. Of the 83 cases with interpretable staining, 43 had more than 10% of infiltrating TIA1+ cells and 40 had less, with 10-year OS of 83% vs. 57% (P=0.0014) and 2-year PFS of 76% vs. 50% (P=0.014) (Figures 8.6 & 8.7), respectively. In multivariate analysis, including IPI and HLA-DR and cytotoxic markers, only TIA1 status was an independent predictor of OS (RR=0.3, 95%CI=0.11-0.63, P=0.003).

8.5 DISCUSSION

The impact of tumour immunity in both low and high-grade B cell lymphomas and the relationship to prognosis has been recently highlighted with an ever increasing number of reports using both gene and protein expression profiling

(17-19). MHCII loci are one of the key elements defining immune reactions including immunosurveillance and anti-tumoral immune responses. Thus, the association with prognosis has been recognized early on in cancer research (9, 10). HLA-DR is the member of the MHCII family whose protein expression has been used in most studies (11). It is one of the mostly highly expressed MHCII proteins and IHC antibodies have been widely used for years in routine practice in different areas of Haematopathology. Easily measured and robust biomarkers are essential so that their use can be incorporated into routine practice. Moreover, tumour immunity pathways are increasingly relevant not only in lymphoma, but also in solid tumors (20) as this may become an important pathway to target by therapy.

In this study we validated the impact of HLA-DR expression in PMBCL using FFPET diagnostic biopsies of patients treated in a single institution. HLA-DR loss is a common event in DLBCL involving immune-privileged sites such as testis and the central nervous system (21). In these anatomic sites, MHCII loss has been reported in more than a half of the cases and it is frequently associated with deletions in the MHCII loci involving chromosome 6p. In non-immune-privileged DLBCL, loss of MHCII has been variably reported, ranging, from 10% to more than 50% of cases, depending on the techniques used (7, 11, 13, 22, 23) Most recent gene and protein expression studies report the loss of HLA-DR to occur in approximately 10% of the cases. Yet, in both non-immune-privileged DLBCL and PMBCL, the mechanism of reduced MHCII expression remains unclear. Genetic deletions in the MHCII locus or of its transactivator, MHCIITA, as well epigenetic alterations, such as hypermethylation of its regulators, do not seem to account for the loss of expression (7, 24). In PMBCL, one interesting candidate mechanism relates to the common high expression of PDL1 and PDL2 (programmed cell death 1 & 2 ligands) by the tumour cells (6). Both genes are located at 9p24 in a region often amplified in PMBCL. These genes encode members of the B7 family, ligands to PD1 receptor on T cells. They have been reported to have regulatory effects on T cell responses, including inhibition of tumour immunity (25). Recently, our group has demonstrated one prominent mechanism responsible for this finding as genomic *CIITA* breaks are highly recurrent in PMBCL (38%), and although the fusion partners are promiscuous, 50% involve in-frame gene fusions with PDL1

and PDL2.(26). Functionally *CIITA* gene fusions promote HLAII down-regulation and over-expression of PDL1 and PDL2 and were shown to be associated with inferior prognosis.

Loss of HLA-DR expression has been associated with decreased survival in cohorts of treated patients pre and post addition of R to chemotherapy (11, 13, 27). Although in the most recent reports the complete loss of expression defined cases with markedly inferior prognosis, even when the whole cohort was divided in quartiles, the impact on survival was still significant (7). This suggests that a decrease in HLA-DR expression is associated with survival as a continuous variable. Similarly, in PMBCL, only a minority of the cases showed complete loss of MHCII expression and the overall survival of this subset of patients was poor. In agreement with our findings, poor patient survival correlated with incremental decreases in MHCII expression in a series of 40 patients (13).

Thus in the current study we used a method of protein expression assessment that combines both intensity and percentage of positive cells, such as the Histoscore (HScore) (14). Applied on a TMA section it allows the comparison of a large series of cases to be assessed using a consistent immunohistochemistry technique. The HScore enabled us to distinguish cases with HLA-DR bright cytoplasm membrane expression in the majority of cells from those cases with only a minor subset of HLA-DR+ tumor cells as well as cases with diminished expression of the marker. These latter cases were not only significantly associated with worse survival (independent of the IPI), but also significantly correlated with decreased T cells assessed using different markers. Moreover, CTL content, measured by the expression of TIA1 antibody was also significantly and independently associated with prognosis in this series. These results strongly suggest in PMBCL that decreased expression of HLA-DR is an immune editing mechanism allowing tumor cells to escape T cell surveillance and concurs with novel mechanisms recently described (26).

The analysis of prognosis using patients with heterogeneous treatments has been suggested to be responsible for the controversy in studies that address the role of the microenvironment impact on prognosis (28). In this series, spanning almost two decades, several chemotherapy protocols were used. Given that PMBCL is a rare disease, it would be difficult to achieve statistical

significance using uniformly treated patients. In addition to chemotherapy, radiation was included in the treatment package in 27 cases and 18 patients also received R. Interestingly, for those patients receiving radiation (despite the low number) both HLA-DR and TIA1 markers did not impact significantly on survival (P=0.28 & P=0.19, respectively) when compared with the patients that did not receive radiation (P=0.0074 for both). Radiation affects not only tumoral cells, but also impacts the microenvironment. In murine models, radiation decreases the numbers of all lymphocyte subsets, but differentially affects some T cell subsets, as immunosuppressive regulatory T cells (Tregs) have been shown to be more resistant to radiation than other T cell subtypes (29). In human lymphoma, Tregs are increased in number and associated with suppression of anti-tumoral immunity promoting tumoral growth (30, 31). Yet, in PMBCL Treg cell content assessed by the expression of its master transcription factor, FOXP3, did not have an impact on prognosis (unpublished observation, data not shown).

8.6 CONCLUSION

In this study, we validated the negative prognostic impact of loss of HLA-DR expression by neoplastic B cells in PMBCL patients treated with multi-agent chemotherapy in a single institution experience. Loss of HLA-DR expression correlated with decreased numbers of infiltrating benign T cell populations, especially CD8+ and TIA1+ cells. Significantly, decreased CTL content correlated independently with inferior survival (Figure 8.8). This study shows loss of immunogenicity and cultivating immune privilege are key oncogenic mechanisms in PMBCL and suggests that specific therapies focused on this pathway may benefit patients. Yet, validation of both markers in a large cohort of patients receiving uniform, standard of care therapy is needed.

8.7 TABLES

Feature	Strong HLA-DR # (%)	Decreased HLA-DR # (%)	Total (%)	p-value (χ2)
Number	32 (35)	60 (65)	92 (100)	-
Clinical Features				
Median age (y)	34	36	-	**0.2**
Female (%)	56	48	-	**0.5**
Median follow-up (y)	12.5	9.2	-	**0.5**
IPI Group 1 (0/1)	15 (47)	25 (42)	40 (44)	
Group 2 (2/3)	15 (47)	27 (45)	42 (46)	**0.6**
Group 3 (4/5)	2 (6)	8 (13)	10 (11)	
Treatment regimen				
CHOP	7 (22)	21 (35)	28 (30)	**0.5**
CHOP-like	25 (78)	39 (65)	64 (70)	
With rituximab®	4 (13)	13 (28)	17 (19)	**0.4**
With Radiation	10 (31)	15 (25)	25 (27)	**0.6**
Treatment Response				
CR	30 (94)	45 (75)	75 (82)	
PR	1(3)	8 (13)	9 (10)	**0.09**
NR	1 (3)	7 (12)	8 (9)	
Pathology Features				
Non-malignant cell content				
↑CD3(%)	19 (59)	14 (24)	33 (36)	**0.001**
↑CD4(%)	14 (45)	10 (17)	24 (27)	**0.006**
↑CD8(%)	15 (48)	4 (7)	19 (21)	**<0.001**
↑CD57(%)	17 (55)	11 (19)	28 (32)	**0.001**
↑TIA1(%)	23 (77)	20 (38)	43 (52)	**0.001**
↑GZB(%)	13 (43)	11 (19)	24 (27)	**0.023**
↑CD68((%)	**12 (38)**	**15 (25)**	**27 (29)**	**0.24**

Table 8.1 - Distribution of clinical and pathology variables between cases with strong HLA-DR+ and cases with decreased HLA-DR expression. IPI = International Prognostic Index score, CR = complete remission, PR = partial remission, NR = no response.

HLA-DR expression	POS	NEG	p (χ2)	OS (p)	PFS (p)
				0.006	0.018
No patients (#)	32	60	-	-	-
↑ CD3 (%)	59	24	0.001	0.14	0.2
↑ CD4 (%)	45	17	0.006	0.2	0.2
↑ CD8 (%)	48	7	<0.001	0.19	0.2
↑ CD57 (%)	55	19	0.001	0.17	0.3
↑ TIA1 (%)	77	38	0.001	0.0014	0.014
↑ GzB (%)	43	19	0.023	0.6	0.5

Table 8.2 - Distribution of different T cell subsets between cases with strong HLA-DR+ and cases with decreased HLA-DR expression. Statistical analysis was performed using Pearson Chi-test and survival analysis was performed using the log rank test.

TIA1+ cells	>10%	<10%	p (χ2)	OS (p)	PFS (p)
				0.0014	0.014
No patients (#)	43	40	-	-	-
Median age (y)	34	38	0.13	0.045	0.033
Female (%)	47	58	0.31	0.6	0.9
Median follow-up of living patients (y)	10	11	0.5	-	-
IPI Group (% IPI 2/5)	49	62	0.3	0.05	0.21
HLA-DR (% POS)	54	18	0.001	0.0065	0.018
↑ Macrophages (%)	91	60	0.001	0.9	0.5

Table 8.3 - Distribution of clinical and pathology variables between cases with cases with increased TIA1+ cells (>10%) and cases with decreased TIA1+ cells (<10%). Statistical analysis was performed using Pearson Chi-test and survival analysis was performed using the log rank test. IPI indicates International Prognostic Index.

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8.8 FIGURES

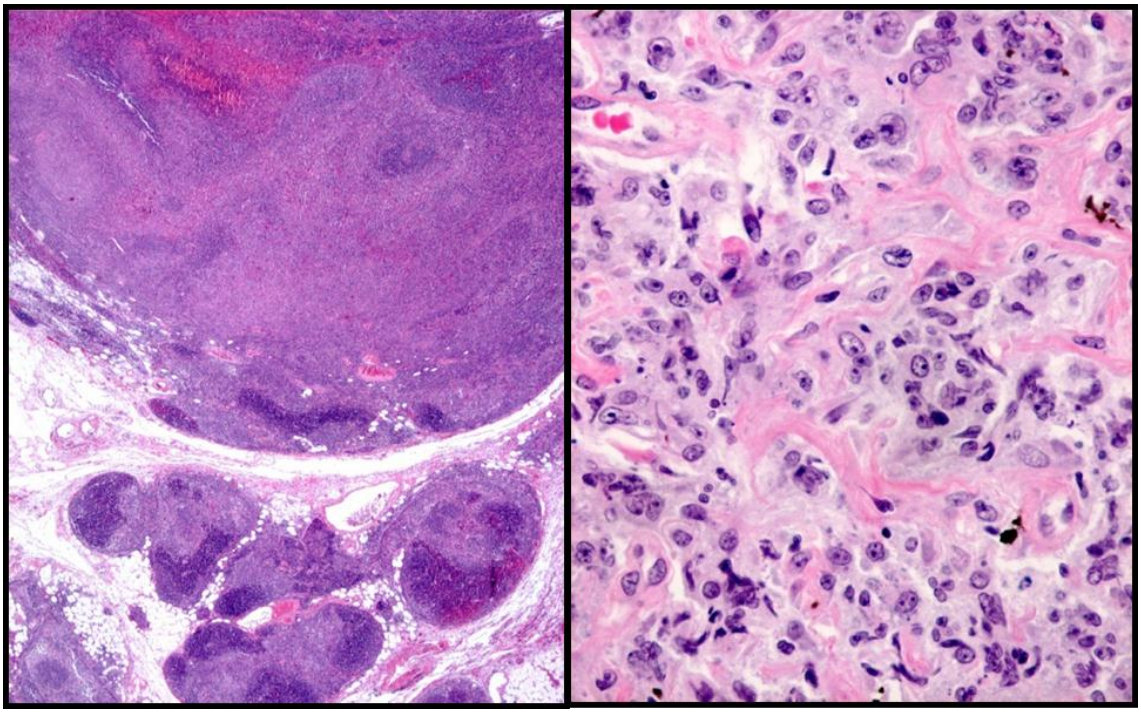

Figure 8.1- Primary Mediastinal B-Cell Lymphoma (PMBCL) infiltrating the thymus (left image). It is composed of medium to large-sized cells with abundant cytoplasm, ovoid nuclei and is characteristically associated with background fibrosis (right image). Olympus BX40 / Nikon Elipse®; Digital Camera Dxm1200; 10x (left) & 400x (right).

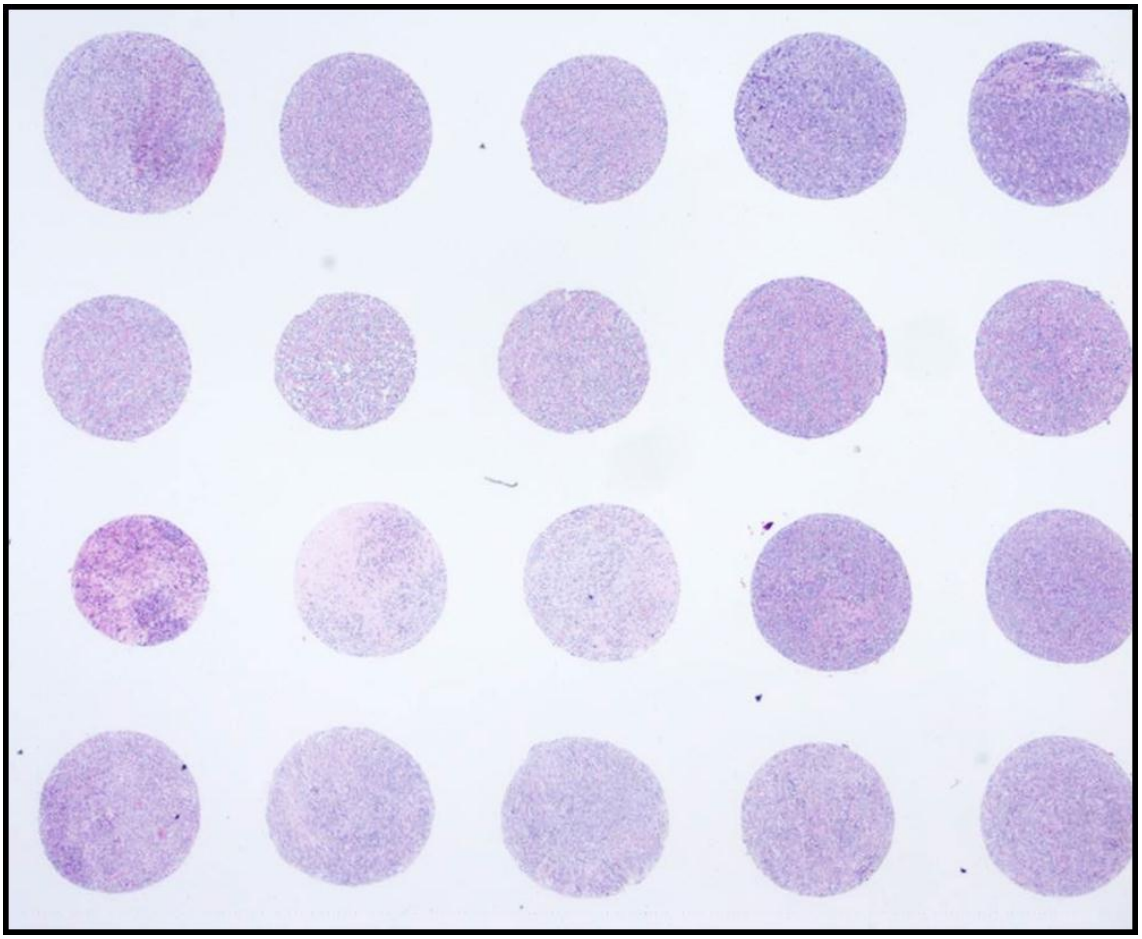

Figure 8.2 - Primary Mediastinal B-Cell Lymphoma (PMBCL) Tissue Microarray duplicate 0.6mm cores of paraffin embedded diagnostic biopsies. Olympus BX40 / Nikon Elipse®; Digital Camera Dxm1200; 40x.

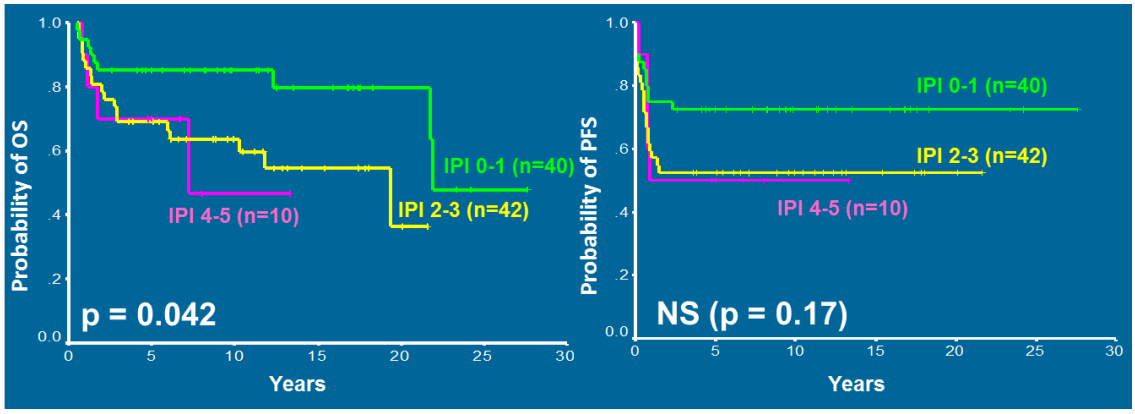

Figure 8.3. - Overall Survival (left) and Progression Free Survival (right) based on IPI groups. IPI: International Prognostic Index.

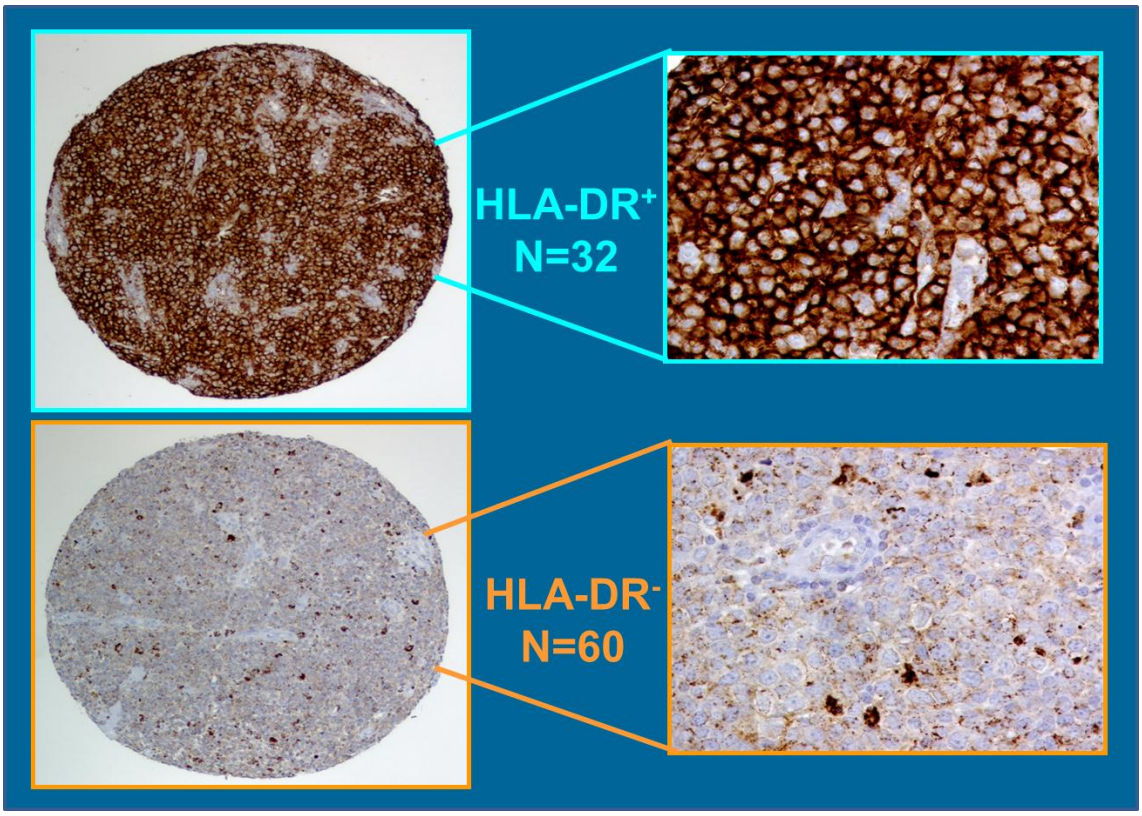

Figure 8.4 - Representative immunohistochemical images from two patients for HLA-DR expression. The top images show a sample with strong cytoplasmic membrane HLA-DR expression and the bottom ones show loss of HLA-DR expression by the malignant cells. Olympus BX40 / Nikon Elipse®; Digital Camera Dxm1200; 40x & 400x..

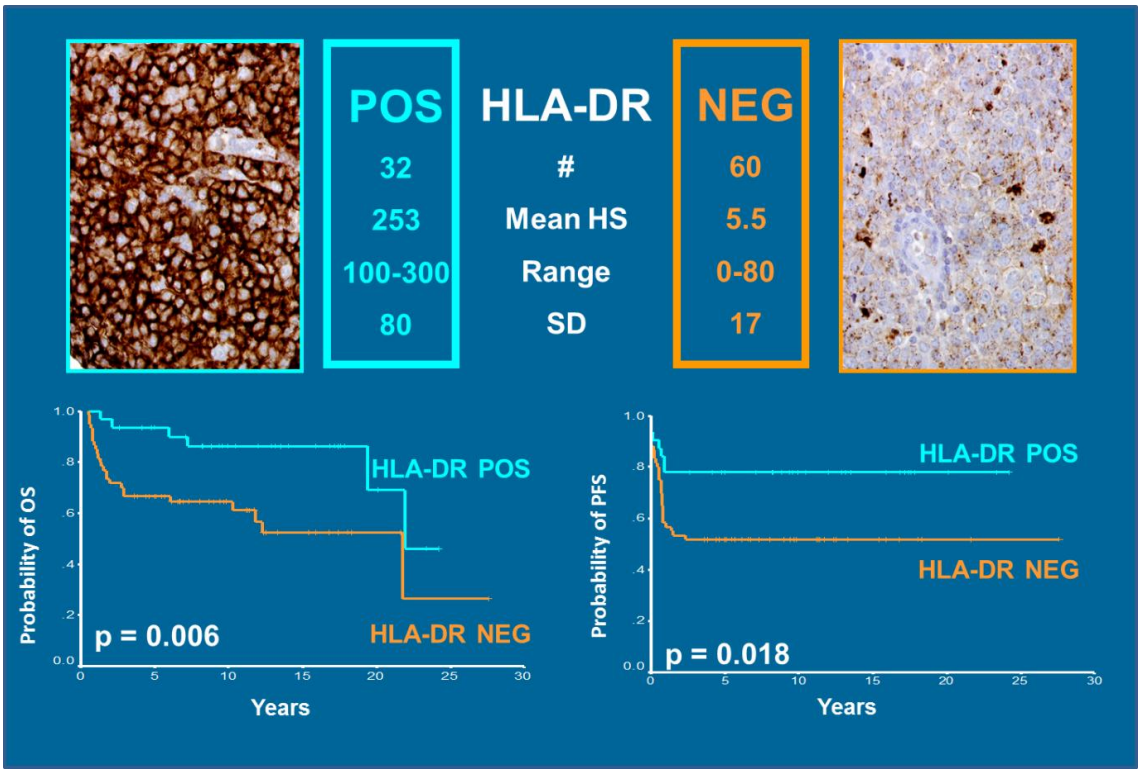

Figure 8.5 - Overall Survival (left) and Progression Free Survival (right) based on strong cytoplasmic membrane HLA-DR expression (HLA-DR POS) *vs.* decreased of HLA-DR expression by the malignant cells (HLA-DR NEG). Olympus BX40 / Nikon Elipse®; Digital Camera Dxm1200; 40x.

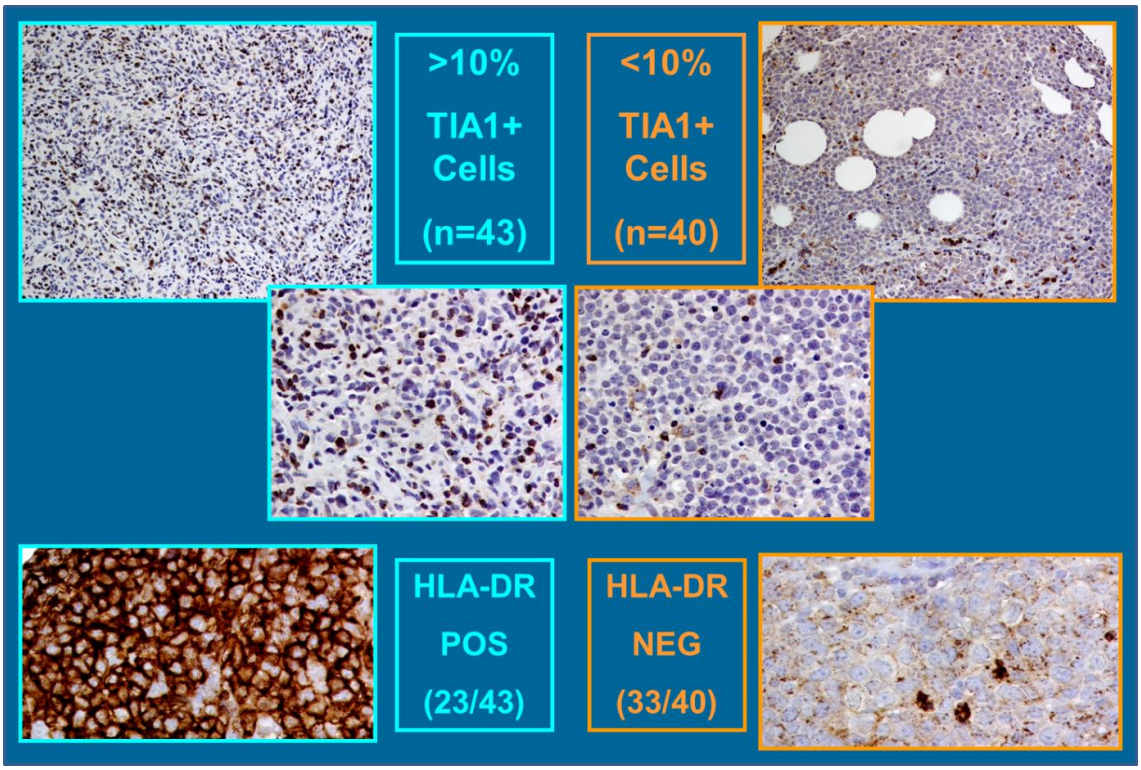

Figure 8.6 - Representative immunohistochemical images from two patients (left and right images) for TIA1+ cell content and HLA-DR expression. On the left is shown increased TIA1+ cell infiltration (top and middle images) associated with strong cytoplasmic membrane HLA-DR expression (bottom). On the right, there is low TIA1+cells infiltration (top and middle images) and loss of HLA-DR expression by the malignant cells (bottom). Olympus BX40 / Nikon Elipse®; Digital Camera Dxm1200; (top images 40x; middle & bottom images 400x).

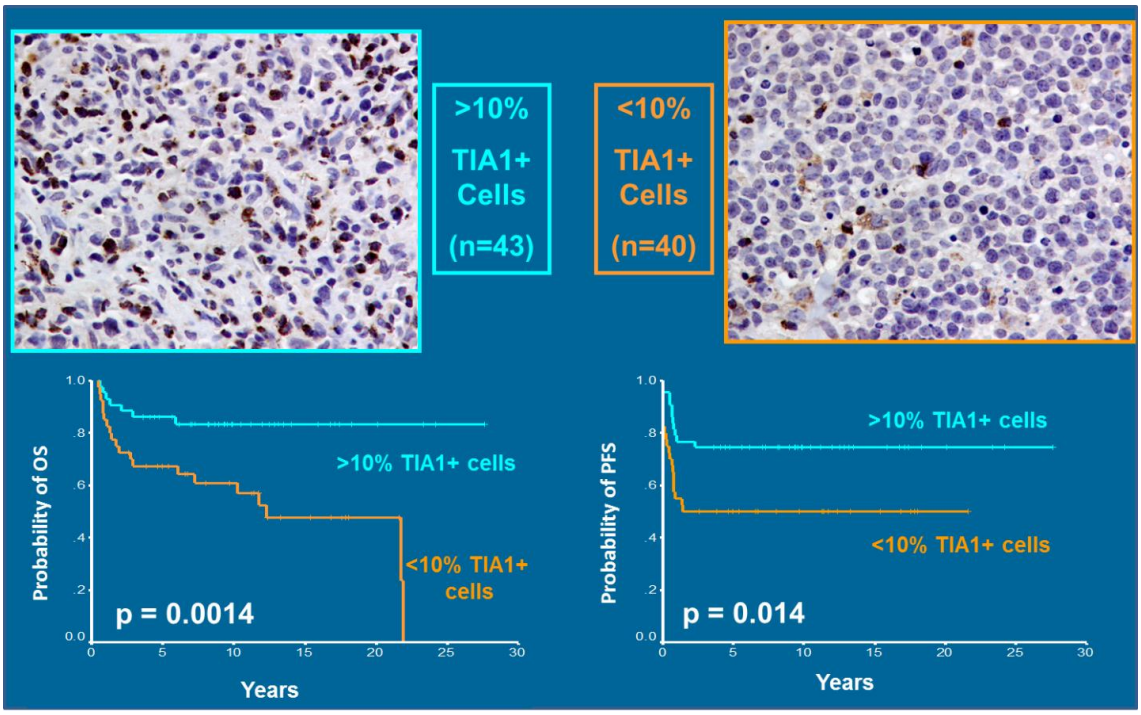

Figure 8.7 - Overall Survival (left) and Progression Free Survival (right) based on increased infiltration of TIA1+ cells vs. decreased infiltration of TIA+ cells (cut off value 10%). Olympus BX40 / Nikon Elipse®; Digital Camera Dxm1200; 400x.

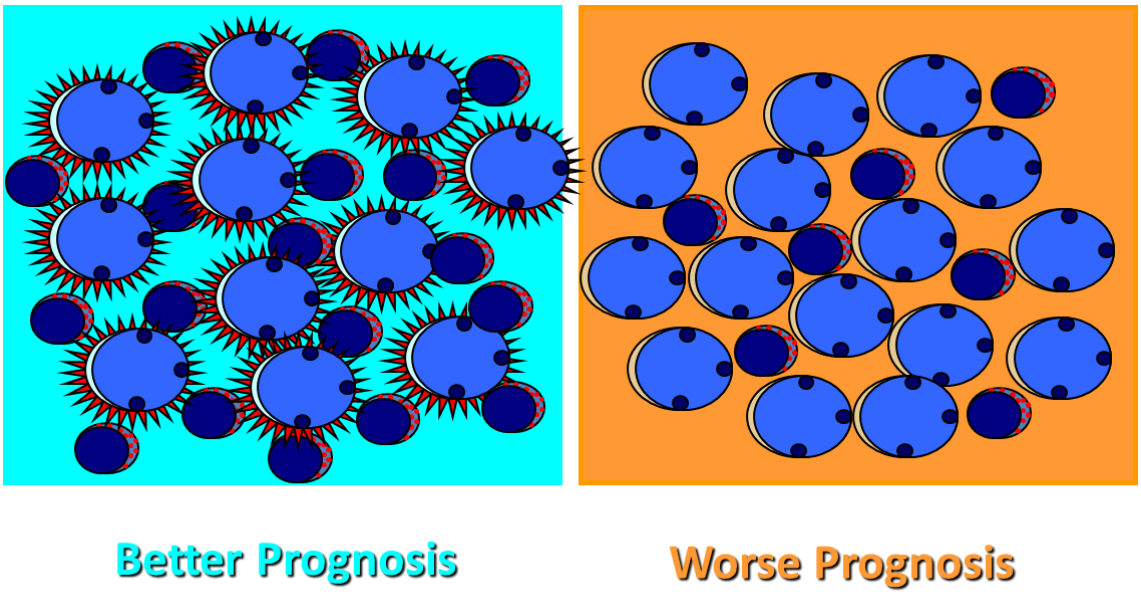

Figure 8.8 - Immuno-surveillance in PMBCL. This study suggests loss of immunogenicity and immune-surveillance are key mechanisms in the response to treatment of PMBCL patients. Decreased HLA-DR expression correlated with decreased numbers of infiltrating benign T cell populations, especially cytotoxic TIA1+ cells, and both features correlated independently of clinical parameters with inferior survival.

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Section 9: Conclusions

9.1 SUMMARY

Human B cell lymphomas comprise over 85% of lymphoid neoplasms and contrary to most other cancers, the incidence of lymphoma is increasing worldwide. Non-Hodgkin lymphomas (NHL) are made up of many different subtypes having heterogeneous clinical behaviour, but in most cases clinical outcome is insufficiently predicted using the currently available clinical prognostic indices used to risk-stratify patients (1). Prognostic and in particular predictive biomarkers are needed for risk stratification and planning more targeted therapies. The identification of novel biomarkers will be required to personalize treatments in NHL and identify critical targets for innovative novel therapies. Prior to the routine use of new therapies, companion diagnostics will be required at diagnosis to identify at-risk patients who will require alternative, more targeted therapy.

FL is the second most common lymphoma worldwide and the most frequent indolent small B cell lymphoma. The clinical course if highly variable and there are no agreed upon standard treatment approaches. Histologic transformation represents a dominant clinical event in the course of disease, heralding aggressive behaviour and shortened survival. Thus, the identification of clinically useful biomarkers that predict both overall survival and risk of transformation are needed in order to identify at-risk patients who might benefit from alternative treatment approaches. At the initiation of this thesis work, there were no consistent clinical markers that could predict with accuracy the likelihood of survival and the probability of transformation. New genome-wide analysis platforms were just introduced including gene expression profiling and aCGH (2). We hypothesized that these unbiased genome-wide platforms might be of value to identify novel biomarkers, including candidate oncogenes and tumour suppressor genes that might be contained within genomic gains and losses, respectively. Moreover, the use of these analysis tools might identify candidate loci wherein important driver mutations might be found. The planned analyses in this thesis held the promise of improving our understanding of lymphomagenesis and at the same time allowing the identification of clinically

useful biomarkers that might inform on survival and transformation risk in FL. Ultimately we hoped that novel insights into FL pathogenesis would facilitate the development and implementation of new targeted therapies.

As I started my PhD thesis, tissue microarrays (TMA) were just beginning to be used and offered an inexpensive and ideal platform for both discovery and validation of large cohorts of clinical cases, while reducing experimental noise and reagent consumption. TMAs allowed for the analysis of biomarkers using diagnostic FFPET samples based on IHC, FISH and ISH techniques. TMA technology opened up the possibility of surveying large numbers of samples from pathology archives of diagnostic FFPET and is the platform that was ideally suited to test and validate new biomarkers related to lymphoma prognosis. This innovation happened about the same time as insights into the role of the tumoral microenvironment were being recognized (3). A gene expression profiling study was just published in FL, requiring that these gene expression changes be validated using other orthogonal technologies such as TMA and IHC. Furthermore, these translational research techniques offered the possibility to determine the cells responsible for specific gene expression and the real possibility of extending these studies to several other common lymphoma subtypes.

9.2 DISCUSSION & CONCLUSIONS

9.2.1. Genomic Mechanisms of FL Transformation into DLBCL

Chapter 2 details the search for biological mechanisms underlying the transformation of FL. The comparison of biopsy material from the same individual pre and post-transformation was defined as the prerequisite for clear understanding the molecular mechanisms driving the event. Until recently, most studies have revealed a number of recurring cytogenetic abnormalities (4-11) each one occurring in a small subset of cases, highlighting the molecular diversity of transformation. The combination of global gene expression analysis supplemented with genomic copy-numbered analysis may provide new and unbiased insights into the mechanisms of FL transformation.

In the current study we used a combination of genome-wide microarray techniques including different commercially available expression microarrays platforms as well as the Vancouver BCCRC arrayCGH based platform (SMRT arrays, aCGH). We studied histologic transformation using two different strategies: (1) a series of paired fresh snap frozen lymph node biopsies retrieved pre and post the transformation and, (2) a cohort of FFPET composite lymphomas with FL and DLBCL diagnosed in the some lymph node representing clonally related early transformation events.

In the first study we found using GEP a short list of 18 new genes not previously reported in association with transformation. Importantly, the correlation between these GEP results with aCGH was complex and favoured that mechanisms other than copy number alterations were responsible for deregulated gene expression in FL transformation. Interestingly, genomic copy number complexity was not always associated with the more aggressive disease favoring sub-clone selection and evolution from a common malignant ancestral clone in contrast to clonal evolution resulting from stochastic genetic hits accumulated over time (12, 13). Furthermore, given this heterogeneity and the complex relationship between genomic aberrations and GEP, this small study indicated a much larger number of cases will need to be studied to fully appreciate the specific genetic events responsible for transformation, especially if using whole-genome analysis.

The second study validated the recently described methodology of extracting RNA of sufficient quantity and quality from FFPET diagnostic samples producing successful and meaningful GEP results (14). Using composite lymphomas, we showed three potential different mechanisms were present as early events of FL transformation. One distinct pattern was associated with a proliferation signature as previously reported (15). Despite the low number of genes, distinctive gene sets characterizes the other two clusters with several good candidate genes for diagnostic and eventual therapeutic use. Further cases are needed and mandatory to validate these results.

9.2.2. Biological Prognostic Markers in Lymphoma

The importance of microenvironment and non-malignant cells within human tumours is not a recent finding. Rudolf Virchow first suggested it more than one

hundred years ago (16, 17) and Paul Erlich proposed the concept of tumoral immunosurveilance (18). Since then, tumoral immunity has been shown to have different contexts. Along with the better studied anti-tumoral response there are several different mechanisms by which tumoral immunity promotes tumoral growth, so called cancer immunoediting, as the tumour sculpts its own immunity promoting its growth and progression (19).

In lymphoma, the microenvironment has well been recognized since the early descriptions of these diseases, such as Hodgkin lymphoma, where it is a crucial & required feature for its diagnosis. Similarly, FL lymphoma retains many of the morphological features of the normal follicle suggesting intense cross talk between malignant B cells and non-malignant immune cells, including T cells, Follicular Dendritic Cells (FDCs), macrophages, mast cells and endothelial cells. Important survival and trophic signals from FDCs/macrophages and T cells reach the neoplastic B cells, which are highly dependent on these signals. These data in-part help explain the difficulty of establishing FL cell lines without cytokine stimulation or feeder layers (20).

In chapter 3 we used diagnostic lymph nodes of FL patients treated uniformly at BCCA and tested the impact of morphological features of microenvironment, such as architectural distribution and cell content of non-malignant cells. We used routine FL diagnostic lymph node biopsies arrayed in TMA and stained with different antibodies. We provided compelling new data that supported a role for benign macrophages, microvessels and Tregs in predicting outcome in aggressively treated FL patients and further validated the recent gene expression profiling data highlighting the importance of non-neoplastic cells in determining prognosis in FL. Notably and for the first time we showed two biomarkers, the architectural pattern of Tregs and the microvessel density as measured by TVD90 to significantly predict risk of transformation. This cohort comprised two very important and rare advantages; the patients were all treated uniformly and had very long follow-up. In a disease with many different treatment options used in clinical practice, most reports in the literature of FL show heterogeneous treatment modalities within and between different series. This has been considered responsible for the controversy in the published literature as to the prognostic significance of a number of biomarkers (21-31).

Following these encouraging results in FL we decided to expand the study of the microenvironmental impact on prognosis to a number of other lymphoma types.

Classical Hodgkin lymphoma (CHL) was one ideal candidate as its most characteristic and defining features is the predominance of reactive cells in the tumour microenvironment, which largely outnumber the malignant ones (32). CHL is one of the success stories of oncology as roughly 80% of all patients are currently cured; yet about 20% of patients with advanced-stage disease are still dying of relapse or progressive disease while a similar proportion of patients are likely over-treated (33, 34), many of whom suffer from treatment-related sequelae such as solid tumours and end-stage organ dysfunction (35-37). None of the clinical variables used to help guide treatment decisions can predict the majority of patients in whom standard treatment will fail to eradicate disease.

In chapter 6, we aimed to determine cellular signatures that correlate with treatment outcome in classical HL, using gene expressing profiling and immunohistochemical analysis of diagnostic lymph node biopsies. We further validated these significant findings using a cohort of CHLs patients uniformly treated in a single institution. We demonstrated that CD68+ macrophage content measured as an IHC score in diagnostic lymph node samples was a strong and independent predictor of outcome after primary treatment and even secondary treatment. If absent in patients with limited stage disease, this finding defined a subgroup of patients with 100% (and 97% in the validation set) long-term disease-specific survival. Moreover, the IHC score was applied and validated to routine whole section diagnostic biopsies, which not only validates the aforementioned results obtained in tissue microarrays but also facilitates its use in daily practice. Given these results, enumeration of CD68+ LAM in lymph node specimens is a strong candidate marker to become a routine pathology test in CHL. It may be used to develop personalized treatment strategies in CHL, identifying at diagnosis those patients with increased resistance to current therapeutic options.

MCL is another important NHL subtype with few candidate biomarkers. MCL is a clinically aggressive lymphoma characterized by frequent relapses and very short overall survival. The clinical behavior and response to therapy in MCL

vary considerably and are often unpredictable based on clinical factors. Proliferative rate and TP53 mutations are biological factors that strongly influence survival (38-40), but the significance of the non-malignant cellular components impacting the prognosis of patients with MCL is unknown and has never been studied using diagnostic samples from clinical material. In chapter 7, we assessed the prognostic impact of microenvironment in MCL patients using FCM, GEP and IHC using TMAs of diagnostic lymph node biopsies. Although the non-neoplastic cellular infiltrate constitutes only a minor fraction of the tumor mass in MCL, we demonstrated and validated that microenvironmental factors are important in MCL biology, prognosis and response to therapy. We showed that the number of lymphoma-associated macrophages and MVD are important prognostic factors in MCL, independent of clinical parameters, proliferation and TP53 expression. Interestingly, expression of CD163, described as a marker of M2 polarized macrophages, also predicts survival and correlates with increased angiogenesis. Importantly, a distinct inflammatory and cytotoxic microenvironment characterized by the production of IFNγ, a Th1 (CD8-rich) cellular response and M1 macrophages were associated with diminished response to rituximab.

Primary mediastinal large B cell lymphoma (PMBCL) was studied in chapter 8. It shares histological and gene expression features with both DLBCL and CHL (41, 42). These findings suggest that the microenvironment might represent a relevant feature in PMBCL. This disease frequently shows decreased expression of Major Histocompatibility Class II (MHC II) antigens (43). When we started this study, the mechanisms underlying loss of MHCII expression were largely unknown. We hypothesized that the lack of expression of MHCII may constitute a mechanism by which tumor cells escape immunosurveillance after chemotherapy. In this study, we aimed to validate HLA-DR protein expression as a prognostic biomarker in a larger cohort of PMBCLs and correlate it with the immune response using Immunohistochemistry (IHC). We demonstrated a negative prognostic impact of loss of HLA-DR expression by neoplastic B cells in patients treated with multi-agent chemotherapy in a single institution experience. Loss of HLA-DR expression correlated with decreased numbers of

infiltrating benign T cell populations, especially cytotoxic CD8+ and TIA1+ cells. Significantly, decreased cytotoxic T cell content correlated independently with inferior survival. This study shows loss of immunogenicity and immune surveillance are key mechanisms affecting the response to treatment of PMBCL patients. These results proved to be important in promoting further evaluation by our group of the mechanisms responsible for HLA-DR loss in the malignant cells. Genomic breaks in the master controller of MHC Class II expression, the transactivator *CIITA* that maps to chromosome 16p, were found to be highly recurrent in PMBCL. The gene fusion partners were promiscuous, with 50% of the fusion partners accounted for by translocations involving the PD1 ligands PDL1 and PDL2, mapping to chromosome 9p24 (44). These genes encode ligands to PD1 receptor on T cells and have been reported to have regulatory effects on T cell responses, including inhibition of tumour immunity (45). Importantly, cases harbouring *CIITA* breaks had a negative impact on survival (44).

Finally, in chapter 5 we focused on DLBCL, the most common lymphoma type, accounting for 30-40% of newly-diagnosed cases of NHL (46). Although curable in a sizable proportion of patients with CHOP chemotherapy, approximately 60% of patients with DLBCL will relapse after standard first-line therapy (47, 48). This variability in clinical outcome likely relates to genetic heterogeneity within DLBCL, reflected in a wide array of cytogenetic and molecular abnormalities. Several other biologic factors have been proposed as prognostically significant, including *TP53* mutations, TP53 and/or BCL2 protein expression and BCL6 and FOXP1 expression (49-55). Moreover, landmark DLBCL studies using microarray gene expression techniques defined two major biological categories of DLBCL with clinical and prognostic differences based on cell of origin distinctions; including germinal centre B cell (GCB) and activated B cell (ABC) subtypes (56). Currently, the addition of rituximab (R) to CHOP chemotherapy (R-CHOP) represents the standard of care and has improved significantly the survival of DLBCL patients. However, the addition of R-CHOP has diminished the significance of established prognostic biomarkers including BCL2 and BCL6 protein expression (57, 58). The underlying mechanism(s) responsible for this effect is/are largely unknown. Thus, in chapter 5 we

assessed the prognostic impact of several candidate biomarkers in the current era of immunochemotherapy.

Using TMAs, IHC, microarray mutational analysis and FISH we were able to establish the following biomarkers as significant prognostic factors in DLBCL treated with R-CHOP:

***TP53* Mutations and Protein Expression:** *TP53* is the most extensively studied tumor suppressor gene and a key regulator of cell homeostasis. In cancer, TP53 function is lost due to mutation of the gene in about half of all human tumors and there are many reports including lymphoma, correlating the presence of mutant *TP53* and more advanced cancer and worse prognosis (59, 60). In this study we showed strong TP53 protein expression to be an easy biomarker to assess in routine FFPET diagnostic biopsies of DLBCL patients and it correlated almost universally with *TP53* missense mutations also studied in FFPET using a AmpliChip p53 microarray. Importantly, strong TP53 protein expression was an independent prognostic factor for patients with DLBCL even when treated with R-CHOP. Interestingly, its prognostic impact is superior within the GCB subgroup. This was one of the first significant biomarkers of R-CHOP, identifying a subgroup of patients with inferior survival deserving alternate therapies.

Rituximab Impact on COO Subgroup Survival: COO distinctions in DLBCL are well established, since the initial studies using gene expression profiling, having identified two distinct subgroups of DLBCL, namely GCB and ABC with different clinical outcomes (56). Using TMA and immunohistochemical staining we have shown in a collaborative study that COO subtyping correlates closely with the results of gene expression profiling in predicting outcome (51). We also showed that non-GCB cases do not harbor the t(14;18), more commonly have isolated *BCL2* gene copy number gain and have a higher percentage of cytogenetically “normal” BCL2+ cases. The latter finding suggests a prominent role for transcriptional up-regulation resulting from constitutive activation of NFκB. DLBCL cases with a t(14;18) are virtually always GCB and never show isolated *BCL2* gene copy number gains, suggesting that these two events are mutually exclusive (61). In the current era of R-CHOP, rituximab was show to preferentially prevent chemotherapy failures in DLBCLs that express BCL2 (57).

As BCL2 is differentially expressed in COO subgroups it is expected that the effect of the introduction of rituximab is greater in the non-GCB subtype. In this study we confirmed that R-CHOP is associated with better OS in DLBCL due largely to its effect on the non-GCB subgroup. Moreover, the OS of BCL2+ DLBCL patients is significantly improved by the addition of rituximab. These results provided insight into the possible mechanisms by which rituximab exerts its beneficial therapeutic effect.

Prognostic Significance of BCL6 Rearrangements in DLBCL: Rearrangement of the *BCL6* proto-oncogene located at chromosome band 3q27 is the most frequent cytogenetic abnormality in DLBCL, occurring in up to 35% of cases (54, 62, 63). In this study we compared for the first time the impact of *BCL6* gene rearrangement on survival in DLBCL patients treated with CHOP and R-CHOP and secondly determined the relationship between *BCL6* rearrangement and other clinical and biological prognostic variables in DLBCL, including COO phenotype. Our data suggested an association between *BCL6* rearrangement and inferior outcome in patients treated with R-CHOP, but not in patients treated with CHOP chemotherapy alone. However, *BCL6* rearrangement also correlated with the presence of high-risk clinical features and did not demonstrate prognostic significance independent of the International Prognostic Index (IPI) score. *BCL6* rearrangement was also correlated strongly with a non-GCB immunophenotype.

***MYC* Genomic Breaks in DLBCL:** Approximately 5-10% of DLBCLs harbor a *MYC* rearrangement (64). Although approximately 20% of these cases may also harbor a concurrent *IGH-BCL2* translocation t(14;18), the so-called ‘double-hit’ or dual translocation lymphomas (65), which is known to be associated with a poor outcome, there is very little information of the prognostic importance of isolated *MYC* aberrations in DLBCL. In the current study we studied using FISH an unselected series of patients with DLBCL for *MYC* aberrations to determine the frequency of this occurrence and any associated pathologic or clinical defining features as well as to assess the prognostic impact of *MYC* aberrations in DLBCL patients treated with R-CHOP chemotherapy.

MYC gene rearrangements defined a small group of patients with DLBCL, independent of COO, who had an adverse outcome with R-CHOP and may have an increased risk of CNS relapse (66). There are no identifiable clinical,

histological, or immunophenotypic features that signal that a case of DLBCL may harbour a *MYC* aberration. Thus, it is recommended that all patients with DLBCL should undergo FISH analysis for assessment of the *MYC* rearrangement in addition to analysis of the t(14;18) if BCL2 expression is found. *MYC*+ DLBCL likely represent a distinct DLBCL subtype and regimens more in line with those used in Burkitt lymphoma may be more appropriate in this patient population.

Finally, in chapter 3 we describe a new biomarker in FL, NOXA, which is expressed by malignant B cells. NOXA is a BCL2 homology 3-only member of the BCL2 family, also called apoptosis "sensitizer / derepressor" as it promotes apoptosis. This gene has been previously reported in FL transformation GEP studies (67). In this study we showed NOXA protein expression (NPE) significantly correlated with a favorable OS in FL. In this uniformly treated cohort from the BCCA, NOXA was an independent predictor of outcome. This is the first pro-apoptotic member of the BCL2 family to impact FL prognosis. It is a *TP53* target gene that is physiologically up-regulated in cell stress and damage responses (68, 69). In accordance, in our study NOXA expression correlated with increasing histological grade and proliferation index (Ki67). This significant result in FL may open new therapeutic options for this lymphoma, as intracellular levels of NOXA can be increased in malignant cells by therapeutic agents such as proteasome inhibitors (Bortezomib) leading to NOXA-mediated apoptosis (70, 71).

9.2.3. Phenotype & Genotype Assessment of LAMs

In this thesis we demonstrated that LAM content is an independent adverse prognostic factor in advanced-stage FL patients treated uniformly with chemotherapy and radiation (21). This finding suggests LAMs may be recruited and "educated" by tumours using a range of growth factors and chemokines, so that they adopt a "trophic" role that facilitates matrix breakdown, tumour-cell motility and angiogenesis (72). Recent data suggests there is more plasticity between the B cell and myeloid lineages than previously thought (73). This plasticity is associated with changes in transcription factor expression and/or

activity that induce the transdifferentiation of committed B lymphocytes into functional macrophages that still retain immunoglobulin gene rearrangements as evidence of their B cell origin (74).

The favoured hypothesis regarding the pathogenesis of FL suggests that B cells harbouring a t(14;18) arise at the pre-B stage in the marrow and occurs as a result of an error during *VDJ* rearrangement. The translocation is insufficient but necessary for the malignant transformation (75). These cells eventually enter the circulation, home to the lymph node and seed the follicles. The t(14;18) has been found frequently in the blood of healthy blood donors as well as in germinal centers of patients who are asymptomatic or only develop FL several years later (76, 77). Interestingly, it has been shown that in FL, a subpopulation of endothelial cells show the characteristic t(14;18) translocation of FL.(78).

In chapter 4 we aimed to verify if intratumoural monocytes & macrophages in FL patients were clonally related with the malignant B cell clone. Using FICTION we found a significant proportion of macrophages with *BCL2* abnormalities in patients harbouring the translocation, but not in reactive lymph nodes or lymphomas with no evidence of *BCL2* breaks. Despite many open questions, our findings are provocative as may indicate neoplastic precursor cell plasticity and the ability of neoplastic B cells to transdifferentiate into mature-like monocytes & macrophages, providing the neoplastic B cells with a trophic / pro-survival microenvironment. Further validation is mandatory to convincingly demonstrate these findings.

9.3 FUTURE DIRECTIONS

Currently, all these significant biomarkers related with malignant or non-malignant cells remain interesting clinic-pathological findings established using retrospective patient cohorts. Many of them, especially those related to the microenvironment still represent functionally untested hypotheses. The difficulty in establishing *in vivo* models of FL hampers their study. Cell sorting strategies to study specific cell types in isolation are underway, and may in time further improve the understanding of the interactions between non-neoplastic macrophages, T cells, microvessels with neoplastic B cells. Similarly, cell purification techniques would also help validate the findings that suggest a

clonal relation between neoplastic B cells and a minor subset of macrophages in FL.

Hopefully, the range and complexity of genomic and phenotypic studies done using routine FFPET biopsies will expand and accelerate the use of these and newly identified biomarkers into routine clinical practice.

Moving forward, it will be important to test all potentially useful biomarkers in prospective clinical trials that might expedite their validation and their eventual use in clinical practice. Ultimately, it may be possible to manipulate the lymphoma microenvironment in favour of the host and turn off tumoral immune-suppression and trophic survival signals and fuel the immunosurveillance and modulatory signals derived from non-neoplastic B cells.

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Resumo Alargado

Os linfomas de células B correspondem a 85% das neoplasias linfóides e contrariamente à maioria dos outros cancros, a sua incidência está a aumentar globalmente. Os linfomas dividem-se em vários subtipos com comportamentos clínicos heterogéneos, e na maioria dos casos, os índices de prognósticos clínicos utilizados actualmente são insuficientes (1). São necessários novos biomarcadores que permitam: uma melhor estratificação de prognóstico e de resposta à terapêutica; o planeamento de terapias mais personalizadas e a identificação de novos alvos terapêuticos. O LF é o segundo linfoma mais comum globalmente e o linfoma indolente mais frequente. Tem um comportamento clínico variável e uma abordagem terapêutica heterogénea. A transformação histológica num linfoma de alto grau representa um evento clínico determinante, correspondendo a comportamentos clínicos mais agressivos e a sobrevidas mais curtas. Assim, a identificação de biomarcadores preditivos de sobrevida global e risco de transformação são mandatórios a fim de identificar doentes de risco que poderão beneficiar de abordagens alternativas de tratamento. Nesta tese, utilizamos plataformas de análise global do genoma, através de perfis de expressão genómica e de alterações quantitativas do genoma, recentemente disponíveis no início dos trabalhos (2). A hipótese principal é que através destas novas tecnologias poderemos identificar novos biomarcadores, nomeadamente oncogenes e genes supressores que possam estar contidos dentro de segmentos do genoma ganhos ou perdidos nas células tumorais. Além disso, a utilização dessas ferramentas de análise pode identificar loci que contenham mutações determinantes à transformação do LF. Finalmente, estes novos marcadores poderão facilitar o desenvolvimento e implementação de novas terapias direccionadas.

Também no início desta tese, o uso de “tissue microarray” (TMA) era recente e ofereceu uma plataforma de baixo custo e ideal para a descoberta e validação de hipóteses utilizando grandes coortes de casos, todos submetidos às mesmas condições técnicas e reduzindo drasticamente o consumo de reagentes. Os TMAs permitem a análise de biomarcadores utilizando amostras de diagnóstico fixadas em formol e parafina (FFPET). Permitem igualmente

aplicar várias técnicas como, imunohistoquímica (IHC) e fluorescência e hibridização in situ (FISH & ISH). Nesta tese, utilizamos os TMAs para examinar um grande número de amostras de diagnóstico de linfoma presente no arquivo do departamento de Patologia do BCCA e validar novos biomarcadores relacionados com o prognóstico do LF, sobretudo relacionados com o papel do microambiente tumoral (3). Em 2004 apenas um estudo usando "gene expression profilling" (GEP) tinha sido publicado realçando o impacto do microambiente no LF. O uso de TMAs & IHC permitiram validar estes resultados, e expandir a pesquisa translacional a outras áreas relacionadas com o LF e outros subtipos de linfomas. O principal objectivo foi validar resultados dos estudos das técnicas recentes do "genomics" utilizando técnicas que permitam o seu uso imediato na prática clínica destas doenças.

Mecanismos biológicos envolvidos na transformação do LF em LDGCB.

O capítulo 2 centra-se no estudo dos mecanismos biológicos envolvidos na transformação do LF em LDGCB. Neste estudo, a comparação de material do mesmo doente, pré e pós-transformação, é um pré-requisito para a compreensão clara dos mecanismos moleculares da transformação. A maioria dos estudos publicados revelou uma série de alterações citogenéticas recorrentes (4-11) cada uma ocorrendo apenas em reduzidos números de casos, realçando a diversidade molecular da transformação. A combinação da análise global da GEP complementados com a análise das alterações quantitativas do genoma ("copy-number") poderão fornecer novos "insights" sobre os mecanismos de transformação. No presente estudo foi utilizada uma combinação de técnicas de microarrays do genoma, incluindo diferentes plataformas de microarrays de GEP disponíveis comercialmente, bem como a plataforma de arrayCGH construída em Vancouver, no centro associado ao BCCA, o BCCRC (SMRT aCGH). Estudámos a transformação histológica do LF utilizando duas estratégias diferentes: (1) uma série de pares de biopsias de gânglios linfáticos congelados a fresco pré e pós a transformação e, (2) um grupo de linfomas compostos com LF e LDGCB diagnosticados simultaneamente em biopsias FFPET. No primeiro estudo encontramos por GEP uma lista de 18 novos genes não associados previamente com a transformação. É importante sublinhar que a correlação entre esses resultados

GEP com aCGH foi complexa, tecnicamente e na análise bioinformática, tendo os resultados sugerido que outros mecanismos para além das alterações no número de cópias são responsáveis pela expressão destes genes na transformação do FL. Curiosamente, a complexidade genómica nem sempre está associada com a transformação em doença mais agressiva, favorecendo a selecção e evolução de sub-clones a partir de um clone maligno ancestral comum, em contraste com a evolução clonal resultante da acumulação estocástica de mutações genómicas acumulados ao longo do tempo (12, 13). Além disso, dada a heterogeneidade e a complexa relação entre as aberrações cromossómicas e GEP, este estudo está limitado pelo reduzido número de amostras, obrigando a análise de um número muito maior de casos para determinar as alterações genéticas específicos responsáveis pela transformação, especialmente quando utilizamos técnicas de estudo global do genoma. O segundo estudo validou a metodologia recentemente descrita de extracção de RNA a partir de amostras de diagnóstico em FFPET, que permitem obter resultados significativos de GEP (14). Usando uma série de 14 linfomas compostos, evidenciamos três potenciais mecanismos distintos de transformação, já presentes nas fases iniciais de transformação de FL em LDGCB. Um destes caracteriza-se por uma assinatura genómica de proliferação como anteriormente descrito (15). Dos conjuntos de genes característicos dos outros dois grupos, e apesar do seu reduzido número, distinguem-se alguns como eventuais candidatos para diagnóstico e eventual utilização terapêutica. Novamente, dado o reduzido número de amostras, casos adicionais são necessários e obrigatórios para validar estes resultados.

Marcadores biológicos de prognósticos em linfoma.

Os capítulos 3 e 5 a 8 centram-se na identificação e validação de biomarcadores relacionados com microambiente tumoral. A importância do microambiente e das células não-malignas presentes nos tumores humanos não é uma descoberta recente. Rudolf Virchow foi o primeiro a sugeri-la, há mais de cem anos (16, 17) e Paul Erlich propôs em 1909 o conceito de "immunosurveilance" tumoral (18).

Desde então, demonstrou-se que a imunidade tumoral tem diferentes contextos. Juntamente com a mais bem estudada resposta anti-tumoral, existem vários mecanismos diferentes, através dos quais a imunidade tumoral promove o crescimento tumoral, esculpindo a sua própria imunidade como factor indutor do seu crescimento e progressão (19). No linfoma, o microambiente também tem sido reconhecido, desde as primeiras descrições destas doenças, tais como no linfoma de Hodgkin, onde é uma característica fundamental e necessária para o seu diagnóstico. Da mesma forma, o LF mantém muitas das características morfológicas do folículo normal, sugerindo uma intensa interacção entre as células B malignas e as células imunes não-malignas, que incluem as células T, células dendríticas foliculares (FDC), macrófagos, mastócitos e células endoteliais. A sobrevivência das células B neoplásicas está totalmente dependente dos sinais tróficos destas células como é evidenciado na dificuldade de estabelecer culturas celulares de LF sem estimulação por citocinas (20).

Linfoma Folicular

No capítulo 3 foram utilizadas biopsias ganglionares de diagnóstico de doentes com LF tratados uniformemente no BCCA e foi testado o impacto das características morfológicas do microambiente, nomeadamente a arquitectura e conteúdo das células não-malignas. Utilizaram-se TMA corados por IHC com diferentes anticorpos e demonstraram-se, nesta análise, o impacto significativo dos macrófagos, microvasos e células T reguladoras (Tregs) no prognóstico destes doentes. Notavelmente e pela primeira vez, foi possível demonstrar que dois biomarcadores, o padrão arquitectural de Tregs e a densidade de microvasos, correlacionam-se significativamente com o risco de transformação. Este coorte, quando comparado com outros estudos de LF, caracteriza-se por duas grandes vantagens, o tratamento uniforme dos doentes e a duração alargada de seguimento deste grupo de doentes.

Numa doença caracterizada por modalidades terapêuticas muito heterogéneas, a maioria dos estudos publicados incluem séries de doentes tratados com esquemas diferentes entre diferentes estudos e mesmo dentro de cada série. Esta heterogeneidade tem sido considerada como o principal factor para a obtenção de resultados controversos dos biomarcadores associados ao

microambiente em LF (21-31). Após os resultados obtidos no LF, decidimos expandir o nosso estudo destes biomarcadores a outros tipos de linfomas.

Linfoma de Hodgkin Clássico

O linfoma de Hodgkin clássico (CHL) é um dos candidatos ideais, pois tem como principal característica o predomínio das células reactivas no microambiente tumoral, correspondendo as células neoplásicas a menos de 1% das células tumorais (32). O CHL é uma das histórias de sucesso de oncologia com taxas de cura muito próximas dos 80%. Contudo, cerca de 20% dos doentes com estadio avançado ainda morrem de recidiva ou progressão da doença, enquanto uma proporção similar de doentes sofre de morbilidade associada a sobre-tratamento (33, 34), tais como tumores sólidos ou hematológicos ou insuficiências crónicas de vários órgãos (35-37). Nenhuma das variáveis clínicas usadas actualmente no processo de planeamento de tratamento consegue prever a erradicação da doença na maioria dos doentes que sofrem progressão/ recidiva. No capítulo 6, foram estudados em doentes com CHL, perfis de expressão genómica global (GEP) obtidos de biopsias diagnósticas congeladas a fresco, complementados com a análise de biomarcadores por IHC em FFPET, e correlacionados com prognóstico e resposta terapêutica. Os resultados significativos foram posteriormente validados num coorte de doentes uniformemente tratados numa única instituição. Neste estudo, demonstra-se que o conteúdo de macrófagos CD68+ (LAM) no microambiente do CHL é um biomarcador independente e preditivo da resposta à terapêutica primária e até mesmo do tratamento secundário. Em doentes com doença limitada, a presença de apenas escassas destas células caracteriza um grupo de doentes com 100% (e 97% na série de validação) de sobrevivência a longo prazo. Além disso, no estudo de validação, adaptamos a avaliação dos LAMs na totalidade das secções das biopsias de diagnóstico usadas na rotina, o que não só valida os resultados acima obtidos em TMA, mas também facilita a sua utilização na prática diária. Assim, a enumeração de células CD68 + em amostras de diagnóstico torna este marcador um forte candidato a tornar-se um teste de rotina em CHLs. Pode ser utilizado para desenvolver estratégias de tratamento personalizado no CHL, identificando no diagnóstico os doentes com resistência aumentada às actuais opções terapêuticas.

Linfoma do Manto

MCL é um linfoma de células B, indolente mas agressivo, clinicamente caracterizado por surtos frequentes e uma curta sobrevida global. O comportamento clínico e resposta à terapêutica em MCL variam consideravelmente e são muitas vezes imprevisíveis com base em factores clínicos. O índice proliferativo e as mutações de *TP53* são factores biológicos que influenciam fortemente a sobrevida (38-40), mas o significado das características do microambiente tumoral, nomeadamente das células imunes não-neoplásicas, no prognóstico dos doentes com MCL é desconhecido e nunca foi estudado. Utilizando amostras de diagnóstico FFPET usadas na rotina, avaliou-se no capítulo 7, o impacto no prognóstico do microambiente em pacientes MCL utilizando FCM, GEP e IHQ em TMAs de biópsias ganglionares de diagnóstico. Apesar de estas células corresponderem habitualmente a uma pequena fracção da massa tumoral em MCL, demonstramos e validamos que factores microambientais são importantes na biologia MCL, no prognóstico e na resposta à terapêutica. O número de macrófagos LAM e a densidade de microvasos (MVD) são importantes factores de prognóstico em MCL, independentes de parâmetros clínicos, da proliferação e da expressão do gene *TP53*. Curiosamente, a expressão de CD163, descrito como um marcador de macrófagos polarizados M2, correlaciona-se com o aumento da angiogénese e tem impacto no prognóstico. De realçar que o microambiente com características inflamatórias e citotóxicas caracterizado pela produção de IFNγ, com uma resposta celular com predomínio Th1 (rico em CD8+) e de macrófagos M1 associou-se significativamente à diminuição da resposta ao rituximab.

Linfoma de Grandes Células B Primário do Mediastino

O PMBCL partilha aspectos histológicos, fenotípicos e genómicos com os linfomas LDGCB e CHL (41, 42). Estes achados sugerem que o microambiente possa representar um aspecto relevante nos PMBCL. Esta doença apresenta frequentemente uma expressão diminuída de antigénios do complexo Major de Histocompatibilidade Classe II (MHC II) (43). Quando começamos este estudo, os mecanismos subjacentes à perda de expressão MHCII eram em grande parte desconhecidos. Neste estudo, colocamos a hipótese de que a reduzida

expressão de MHCII pudesse constituir um mecanismo pelo qual as células tumorais escapam à imunovigilância após a quimioterapia. Neste estudo, validamos a expressão da proteína HLA-DR como um biomarcador de prognóstico num coorte de mais de 100 doentes com PMBCL. Demonstramos um impacto negativo no prognóstico relacionado com a perda de expressão de HLA-DR pelas células B neoplásicas em doentes tratados com quimioterapia, numa única instituição. Além disso, a perda de HLA-DR correlacionou-se com a diminuição do número de células T reactivas, em especial as células citotóxicas CD8+ e TIA1+. A diminuição de células T citotóxicas correlaciou-se de forma independente com uma sobrevida inferior. A perda da imunogenicidade e da vigilância imunológica são mecanismos importantes que afectam a resposta ao tratamento de pacientes PMBCL. Estes resultados levaram o nosso grupo a estudar possíveis mecanismos responsáveis pela perda de HLA-DR nas células malignas. Foram identificadas quebras genómicas recorrentes no gene CIITA, no cromossoma 16, o controlador principal da expressão de proteínas MHC Classe II. Os parceiros de fusão deste gene foram vários, com 50% dos parceiros de fusão explicada por translocações que envolvem os ligandos de PD1, PDL1 e PDL2, ambos localizados em 9p24 (44). Estes genes codificam ligandos do receptor PD1 nas células T e têm sido descritos como reguladores das respostas de células T, incluindo a inibição da imunidade tumoral (45). É importante realçar que a presença destas quebras em CIITA tem um impacto negativo na sobrevida dos doentes com PMBCL (44).

Linfoma Difuso de Grandes Células B

Finalmente, no capítulo 5 estudamos LDGCBs, o linfoma mais comum e responsável por 30-40% dos casos recém-diagnosticados de NHL (46). Embora curável numa proporção considerável de doentes sujeitos a quimioterapia CHOP ou CHOP-like, aproximadamente 60% dos doentes com LDGCB sofrem recaídas após a terapêutica de primeira linha (47, 48). Essa variabilidade da evolução clínica provavelmente correlaciona-se com a heterogeneidade genética existente nos LDGCB, reflectida numa grande variedade de alterações moleculares e citogenéticas. Múltiplos factores biológicos de prognóstico têm sido propostos como significativos, incluindo mutações do *TP53* e/ou expressão da proteína TP53, expressões de BCL2, BCL6 e FOXP1 (49-55). Além disso, estudos utilizando técnicas GEP em

microarray definiram duas grandes categorias biológicas de LDGCB com diferenças biológicas, clínicas e de prognóstico baseado na distinção da célula de origem, em linfomas relacionáveis com células B do centro germinativo (GCB) ou células B activadas (ABC) (56). Actualmente, a introdução da imunoterapia, rituximab (R) à quimioterapia CHOP (R-CHOP) representa a terapêutica padrão, esta veio melhorar significativamente a sobrevida destes doentes. No entanto, a adição de R-CHOP diminuiu o impacto de biomarcadores definidos na época pré-R, incluindo a expressão de BCL2 e BCL6 (57, 58). Os mecanismos subjacentes, responsáveis por este efeito, são em grande parte desconhecidos. Assim, no capítulo 5 avaliou-se o impacto de prognóstico de vários biomarcadores na era actual de imunoquimioterapia. Usando TMAs, IHC, análise mutacional em microarray e FISH, pudemos estabelecer os seguintes biomarcadores como importantes factores de prognóstico em doentes com LDGCB tratados com R-CHOP: Mutações e expressão da proteína *TP53*; impacto de R nos grupos definidos por COO e rearranjos de *BCL6* e *MYC*.

1. O *TP53* é o gene supressor de tumor mais extensivamente estudado e um gene regulador chave da homeostase celular. Em cerca de metade de todos os tumores humanos, a função do *TP53* é perdida devido à mutação do gene, mais frequentemente associada a neoplasias, incluindo os linfomas, em estádios avançados e com pior prognóstico (59, 60). Neste estudo, demostramos que a expressão intensa da proteína TP53 é um biomarcador facilmente avaliado por IHC em biópsias FFPET de diagnóstico de doentes com LDGCB e correlaciona-se com a presença de mutações "missense" do *TP53,* também estudados em FFPET usando um microarray p53 AmpliChip. É importante realçar que a expressão intensa da proteína TP53 é um factor independente de prognóstico mesmo para doentes tratados com R-CHOP. Curiosamente, o seu impacto no prognóstico é superior dentro do subgrupo GCB dos LBDGC. Este foi um dos primeiros biomarcadores a identificar um subgrupo de doentes com sobrevida inferior após a introdução de R, que possam eventualmente beneficiar de outras terapias alternativas.

2. Impacto do rituximab na sobrevida dos subgrupos de LDGCB definidos por COO. Os dois subgrupos, GCB e ABC, caracterizam-se não só por diferentes alterações moleculares e fenotípicas, mas também por prognósticos clínicos distintos (56). Previamente, num estudo cooperativo usando TMA e IHC, validamos o impacto de COO no prognóstico inicialmente publicado em estudos usando GEP (51). Nesta tese, demonstramos que os casos não-GCB não têm t(14;18) com rearranjo do *BCL2*, sendo contudo mais frequente o ganho de número de cópias de *BCL2,* e tem uma maior percentagem de casos sem alterações do gene BCL2 mas com sobre-expressão da proteína BCL2. O último achado sugere um papel proeminente da activação constitutiva da via NFkB. Os casos com t(14;18) são quase sempre GCB e nunca mostram ganhos isolados de cópias de *BCL2*, sugerindo que esses dois eventos são mutuamente exclusivos (61). Na era actual de R-CHOP, o benefício de sobrevida ganho com rituximab relaciona-se com a diminuição de resistência à quimioterapia em LGCBD que expressam BCL2 (57). Como BCL2 é expresso diferentemente nos subgrupos COO espera-se que o efeito da introdução do rituximab será maior no subtipo não-GCB. Neste estudo, confirmou que R-CHOP está associado com melhor OS em LDGCB em grande parte devido ao seu efeito sobre o subgrupo não-GCB. Além disso, o prognóstico dos doentes com linfomas BCL2+ é significativamente melhorado pela adição de rituximab. Estes resultados sugerem possíveis mecanismos pelos quais rituximab exerce o seu efeito terapêutico.
3. O rearranjo do proto-oncogene *BCL6* localizado em 3q27 é a alteração citogenética mais frequente em LDGCB, ocorrendo em cerca 35% dos casos (54, 62, 63). Neste estudo, comparamos, pela primeira vez, o impacto do rearranjo de *BCL6* na sobrevivência de doentes tratados com CHOP e R-CHOP e em segundo determinamos a relação entre o rearranjo de *BCL6* e outras variáveis clínicas e biológicas prognósticas em LDGCB, incluindo o fenótipo COO. Os nossos resultados sugerem uma associação entre o rearranjo *BCL6* e a sobrevida inferior em doentes tratados com R-CHOP, mas não em doentes tratados apenas com quimioterapia CHOP. No entanto, o rearranjo *BCL6* também se

correlacionou com a presença de características de alto risco clínico e não demonstrou significado prognóstico independente do IPI. Finalmente, o rearranjo *BCL6* também se associou significativamente com o fenótipo não-GCB.

4. Aproximadamente 5-10% dos LGCBD tem rearranjo *MYC* (64). Cerca de 20% destes casos tem concomitantemente a translocação t(14;18), envolvendo IGH e BCL2, também chamados linfomas com translocação dupla ou "double-hit" (65), característica associada com um mau prognóstico. Contudo, a informação sobre a importância prognóstica de aberrações isoladas *MYC* em LDGCB é desconhecida. No presente estudo foram estudadas por FISH alterações do *MYC*, numa série não seleccionada de pacientes com LDGCB. Determinou-se a frequência desta ocorrência, quais as alterações patológicas ou clínicas associadas, bem como o impacto prognóstico das aberrações *MYC* em doentes tratados com R-CHOP. O rearranjo do *MYC* está presente num pequeno grupo de doentes com LDGCB, é independente de COO.Estes doentes apresentam uma resposta inferior à terapêutica a R-CHOP e um risco aumentado de recaída no CNS (66). Não se documentaram características clínicas, histológicas, ou imunofenotípicas associadas à presença uma aberração *MYC*. Assim, recomenda-se que todos os pacientes com LDGCB deverão ser submetidos a análise FISH para a avaliação do rearranjo *MYC*, além de análise da t(14;18), se BCL2+. Os LDGCB *MYC*+, provavelmente representam um subtipo distinto de LDGCB, cujos doentes poderão beneficiar de esquemas terapêuticos semelhantes aos utilizados no linfoma de Burkitt.

Finalmente, no capítulo 3, descrevemos um novo biomarcador em LF, a expressão de NOXA pelas células B malignas. *NOXA* é um membro da família *BCL2*, "BCL2 homology 3-only", com funções de "sensibilizador / derepressor" da apoptose. Este gene foi previamente descrito associado a transformação de LF, em estudos de GEP (67). Neste estudo demonstramos que a expressão proteica de NOXA (NPE) está significativamente correlacionada com o prognóstico do LF. No coorte de doentes uniformemente tratados no BCCA, NOXA foi um marcador independente de prognóstico. Trata-se do primeiro membro pró-apoptótico

da família BCL2 descrito com impacto de prognóstico do LF. É um gene alvo do *TP53* que é fisiologicamente sobre-expresso em situações de stress celular (68, 69). Em conformidade, no nosso estudo, NOXA correlacionou-se com o aumento do grau histológico e do índice de proliferação (Ki67). Este resultado significativo no LF pode abrir novas opções terapêuticas para este linfoma, através do aumento dos níveis intracelulares de NOXA nas células malignas por agentes terapêuticos, tais como inibidores de proteosoma (bortezomib) que induzam apoptose mediada por NOXA (70, 71).

Fenótipo & Genótipo dos Macrófagos-Associados-Linfoma (LAMs)

Nesta tese, demonstrou-se que o conteúdo LAM é um factor independente de prognóstico em doentes com LF em estadios avançados e tratados uniformemente com quimioterapia e radiação (21). Este achado sugere que estas células podem ser recrutadas e "educadas" pelos tumores, utilizando factores de crescimento e quimocinas, tornando-se "tróficas" e favorecendo a degradação da matriz, a motilidade das células tumorais e a angiogénese (72). Dados recentes sugerem que há maior plasticidade entre as células B e células das linhagens mielóides do que se pensava anteriormente (73). Esta plasticidade está associada a alterações na expressão de factores de transcrição que induzem a transdiferenciação de linfócitos B em macrófagos funcionais que ainda mantêm rearranjos do gene da imunoglobulina como evidência da sua origem nos linfócitos B (74). A hipótese mais provável da patogenia inicial do LF, sugere que as células B portadoras de t(14;18) surgem na fase de célula pré-B na medula óssea e ocorrem como um resultado de um erro durante o rearranjo *VDJ*. A translocação é insuficiente por si só, mas necessária para a transformação maligna (75). Estas células entram na circulação geral e são recrutadas para os folículos linfóides, onde poderão acumular mais alterações genómicas que facilitem a linfomagénese. A t(14;18) é detectada frequentemente no sangue de dadores de sangue saudáveis bem como em centros germinativos com morfologia conservada de indivíduos sem doença ou que apenas desenvolvem LF vários anos mais tarde (76, 77). Curiosamente, foi descrito no LF uma subpopulação de células endoteliais com a mesma translocação t(14;18) das células neoplásicas (78). O capítulo 4 teve

como objetivo verificar se os monócitos e macrófagos intra-tumorais de doentes com LF estão clonalmente relacionados com o clone maligno de células B. Usando a técnica de FICTION, descobrimos que uma proporção significativa de macrófagos contém rearranjos do *BCL2*, em doentes com LF portadores da translocação, mas não em gânglios linfáticos reactivos ou linfomas, sem evidência de rearranjo do *BCL2*. Estes resultados sugerem plasticidade das células neoplásicas precursoras e a capacidade das células B neoplásicas poderem se transdiferenciar em monócitos e macrófagos, fornecendo às células B neoplásicas um microambiente pró-linfoma. A validação adicional é mandatória para demonstrar de forma convincente estes achados.

Direcções Futuras

Presentemente, todos os biomarcadores significativos relacionados com células malignas ou não-malignas, descritos nesta tese, permanecem como interessantes associações clínico-patológicas estabelecidas usando coortes retrospectivas. Muitos deles, especialmente aqueles relacionados ao microambiente, representam ainda hipóteses funcionalmente não testadas. A dificuldade em estabelecer modelos *in vivo* de LF dificulta o seu estudo. Várias estratégias de "cell sorting" estão em curso para estudar os diferentes tipos de células específicas e assim obter uma melhor compreensão das interacções entre os macrófagos, células T, microvasos e as células B neoplásicas. Da mesma forma, técnicas de purificação de células poderão também ajudar a validar os resultados que sugerem uma relação clonal entre as células B neoplásicas e uma população de macrófagos intra-LF. Felizmente, a variedade e complexidade de estudos genómicos e fenotípicos, que já se podem realizar em biópsias FFPET de rotina, irão acelerar o uso destes biomarcadores na prática clínica. Será muito importante testar todos os biomarcadores em ensaios clínicos prospectivos que possam acelerar a sua validação e a sua eventual utilização na prática clínica. Em última análise, pode ser possível manipular o microambiente do linfoma em favor do hospedeiro e abolir os sinais de sobrevida tumoral e de imunosupressão induzidos pelo linfoma.

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Appendices

Major contributions already published in different Sections are appended:

Section 1

Relander T, Johnson NA, Farinha P, Connors JM, Sehn LH, Gascoyne RD. Prognostic Factors in Follicular Lymphoma. J Clin Oncol. 2010 Jun 10;28(17):2902-13. Epub 2010 Apr 12. Review. PMID: 20385990

Section 3

Farinha P, Masoudi H, Skinnider BF, Shumansky K, Spinelli JJ, Gill K, et al. Analysis of multiple biomarkers shows that lymphoma-associated macrophage (LAM) content is an independent predictor of survival in follicular lymphoma (FL). Blood 2005; 106:2169-2174. PMID: 15933054

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Section 5

Savage K, Johnson N, Ben-Nerian S, Connors J, Sehn L, Farinha P, Horsman D, Gascoyne R. *MYC* gene re-arrangements are associated with a poor prognosis in diffuse large B cell lymphoma patients treated with R-CHOP chemotherapy. Blood. 2009;114(17): 3533-7. PMID: 19704118

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Section 6

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REVIEW ARTICLE

Prognostic Factors in Follicular Lymphoma

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ABSTRACT

Follicular lymphoma (FL) is one of the most common types of non-Hodgkin's lymphoma. It is usually diagnosed at an advanced stage, for which many treatment options exist, however, no curative standard therapy has been identified. The outcome is highly variable with a median survival of approximately 10 years. The life expectancy of patients with FL has been extended with the use of rituximab, a monoclonal antibody targeting the CD20 antigen on FL cells, but there remains a group of patients who fail to respond to chemoimmunotherapy and die early of their disease. Transformation of FL to an aggressive histology is an important event with high morbidity and mortality. The Follicular Lymphoma International Prognostic Index has become the clinically useful prognostic tool, but gives only a rough estimate of expected outcome. There is a need for useful biomarkers for prediction of the disease course of single patients to individualize therapy, especially in the new era of chemoimmunotherapy.

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INTRODUCTION

Follicular lymphoma (FL) constitutes approximately 20% of all newly diagnosed lymphoma cases,[1] making it the second most common subtype of non-Hodgkin's lymphoma worldwide and the most common subtype seen in North America.[2] It is characterized by an indolent clinical course, typical morphology, and the presence of a chromosomal translocation, t(14;18)(q32;q21) or variant in 85% of patients. This chromosomal aberration results in the juxtaposition of the immunoglobulin heavy chain gene (*IGH*) on chromosome 14 with the *BCL2* oncogene on chromosome 18 leading to constitutive, and therefore inappropriate, expression of the BCL2 protein. Overexpression of *BCL2* confers relative resistance to apoptosis, thus giving the cells a survival advantage that may facilitate the acquisition and retention of secondary genetic abnormalities.

FL is a heterogeneous entity with some patients developing progressive or transformed disease early and 15% dying within 2 years from diagnosis, while others remain alive for decades without need for treatment. This variability in outcome underscores the necessity to gain further insight into the biology and clinical behavior of the disease to enable individualized therapy.

TREATMENT AND CLINICAL COURSE

The median age at diagnosis of FL is 59 years with a male to female ratio of 1:1.7.[3] FL is typically diagnosed in the advanced stages, with only 26% to 33% of patients presenting with stage I to II disease.[3,4] With conventional chemotherapy, even if combined with radiotherapy, advanced-stage FL is incurable. It is characterized by an indolent course with patients developing slowly progressive lymphadenopathy over many years, with or without constitutional B symptoms (eg, fever, weight loss > 10%, drenching night sweats). Temporary, spontaneous regressions occur in 20% of patients managed without initial therapy.[5] Historically, median survival has ranged from 6 to 10 years.[4-7]

Transformation to an aggressive lymphoma occurs at a rate of 3% per year and is associated with substantial morbidity and mortality.[8,9] Transformation is defined as the development of a more aggressive histology lymphoma, most commonly diffuse large B-cell lymphoma (DLBCL), that is thought to be clonally related to the original FL.

Limited-stage FL treated with external-beam radiotherapy results in prolonged remission in 30% to 50% of patients and an apparent plateau on the disease-free survival curve after 15 to 20 years.[10,11] Two randomized studies showed that deferred initial treatment in asymptomatic advanced-stage patients does not compromise long-term outcome or risk of transformation.[7,12] FL is highly sensitive to antineoplastic agents early, but grows increasingly resistant with successive lines of therapy.[4]

The chimeric anti-CD20 monoclonal antibody rituximab has significant activity alone[13] and in combination with chemotherapy.[14] The addition of

rituximab to chemotherapy has resulted in a higher rate of complete remission (CR) and prolonged remission duration without increasing clinically relevant toxicity.[15] Several studies have confirmed an improvement in overall survival (OS) with immunochemotherapy as the initial treatment for FL.[15-17] Furthermore, rituximab as maintenance therapy after induction therapy for relapse improves progression-free survival (PFS) and OS.[18] However, an improved survival for patients diagnosed with FL has been noted over the past 25 years,[19] an effect at least in part predating the introduction of rituximab, likely explained by improved diagnosis and supportive care.

CLINICAL PARAMETERS

Multiparameter Indices

The International Prognostic Index (IPI), originally developed for aggressive lymphoma, identifies four risk groups based on age, tumor stage, serum lactate dehydrogenase (LDH) level, performance status, and number of extranodal sites of disease.[20] The IPI also reliably identifies risk groups among FL[21-23]; however, it only classifies a small proportion of patients into the highest-risk category.

Three prognostic indices have been developed specifically for FL. The Italian Lymphoma Intergroup (ILI) index identifies three risk groups with 10-year survival rates of 65%, 54%, and 11% based on six clinical parameters (advanced age, male sex, number of extranodal sites of disease, B symptoms, serum LDH level, and erythrocyte sedimentation rate).[24]

In 2004, the Follicular Lymphoma International Prognostic Index (FLIPI) was published resulting from a multicenter effort.[25] It includes five parameters: age (> 60 *v* ≤ 60 years), stage (III-IV *v* I-II), anemia (hemoglobin < 120 *v* ≥ 120 g/L), number of involved nodal areas (> 4 *v* ≤ 4), and serum LDH (elevated *v* normal), and classifies patients into three groups with 10-year OS rates of 71%, 51%, and 36%, respectively. The FLIPI is predictive in patients treated with immunochemotherapy,[26] can be applied in first relapse,[27] and predicts transformation.[28] The IPI, FLIPI, and ILI indices have been directly compared, all identifying somewhat different risk groups.[29] Recently, the FLIPI-2 index was published, incorporating beta-2 microglobulin, lymph node size larger than 6 cm, bone marrow involvement, anemia, and age older than 60 years (Table 1).[25,30,36] All of these clinical indices are robust, easy to use in clinical practice, and of value for stratification in clinical trials; however, marked variations in outcome remain within each risk group.

Biomarkers in Blood and Bone Marrow

There are conflicting reports regarding the prognostic impact of bone marrow (BM) involvement by FL. BM involvement was associated with decreased survival in several investigations including those of the ILI and the FLIPI.[24,25,32,33] In contrast, others have not found BM involvement in itself to affect survival,[34] but rather the histologic pattern[35] or degree of marrow involvement.[36] Importantly, BM status at the time of diagnosis has been included in the FLIPI-2.[30]

Table 1. Adverse Factors in the FLIPI and the FLIPI2 Indices

FLIPI[25]	FLIPI2[30]
Age > 60 years	Age > 60 years
Stage III-IV	Bone marrow involvement
Anemia (Hb < 120g/L)	Anemia (Hb < 120 g/L)
Number of involved nodal areas > 4	Nodes > 6 cm
LDH > ULN	β2-microglobulin > ULN

Abbreviations: FLIPI, Follicular Lymphoma International Prognostic Index; Hb, hemoglobin; LDH, lactate dehydrogenase; ULN, upper level of normal.

The characteristic t(14;18)(q32;q21) generates a *BCL2-IGH* fusion gene, which can be used as a marker of disease detectable by polymerase chain reaction (PCR).[37] This technique is highly sensitive and can routinely detect one translocation-positive cell in 10^6 cells in peripheral blood (PB) or BM. PCR has been used for detecting minimal residual disease (MRD) after therapy.[38] Three different break points within the *BCL2* gene have been identified in FL.[39-41] However, the value of molecular monitoring in FL has not yet been established. Despite attaining a CR after chemotherapy, the BM remained positive by PCR in all or most patients without clear prognostic impact in some reports,[42-44] whereas others have found molecular CRs to be common.[45] Rambaldi et al[46] reported that the degree of BM involvement by lymphoma by quantitative real-time PCR before rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) therapy was predictive of outcome and that patients attaining molecular CR experienced a prolonged freedom from relapse.

Currently, the implication of the presence of t(14;18)-positive cells detected by PCR remains unknown. Not all PCR-positive patients relapse, and some convert to PCR negativity without therapy.[47] Furthermore, t(14;18)-positive cells have been found in patients in long-term remission after therapy for FL[48,49] and *BCL2* translocations have been detected by PCR in PB from healthy individuals and from autopsies from patients without lymphoma.[50,51]

Other Biomarkers in Blood

Biomarkers readily measurable from the PB that have been correlated with outcome in FL are summarized in Table 2.[52-67] Beta-2 microglobulin has reached wide acceptance as a prognostic marker and is included in FLIPI-2,[31] but is not universally available in retrospective cohorts.[24,25] LDH is a robust prognostic factor in several types of lymphoma; however, in FL it is elevated in only 20% of patients.[24,25] Serum angiogenic factors have been analyzed in heterogeneous groups of patients with lymphoma with partly conflicting results.[59,60,61,63,68] Interestingly, polymorphisms leading to high expression of tumor necrosis factor may influence outcome in non-Hodgkin's lymphoma, pointing to the importance of host factors.[62,64-66]

PATHOLOGIC PARAMETERS

FL is a neoplasm of germinal center B cells that mimic the architecture of normal secondary lymphoid follicles. Benign and malignant follicles contain a heterogeneous mixture of non-neoplastic cells including T cells, benign B cells, follicular dendritic cells (FDC), and macrophages. The majority of patients with FL manifest two major types of neoplastic B cells: small centrocytes and larger centroblasts. Histologic grading of FL is based on the relative proportions of these two cell types.

Histologic Grade

The current WHO classification recognizes three histologic grades (grades 1 to 3) of FL.[69] Grading of FL is poorly reproducible

Table 2. Peripheral Blood Biomarkers and Prognosis in FL

Biomarker	Effect on OS	Other Effects	Comment
Absolute lymphocyte count	Longer if ↑ [52]		
Beta-2 microglobulin		Poor response and short TTF if ↑ [15,53]	
	Shorter if ↑ [54]		
	Shorter if ↑ [55]		FL grade 3
	Shorter if ↑ [56]	Shorter FFP if ↑	FL grade 3
		Shorter FFP in first relapse if ↑ [57]	
LDH	Shorter if ↑ [24,25,33]		
	No effect[58]		Stage I-II
Albumin			
Serum	Shorter if ↓ [24,25]		
Urine		Poor response and short PFS if ↑ [58]	
VEGF	Shorter if ↑ [59]	Associated with other poor prognosis factors	Only 13% FL
FGF	Shorter if ↑ [60]	Strong prognostic factor	Only 14% FL
VEGF + FGF	Shorter if ↑ [61]	Combination independent of IPI	Only 14% FL
	No effect[62]		30% FL
Endostatin	Shorter if ↑ [63]	Associated with VEGF ↑	Only 16% FL
TNF	Shorter if ↑ [64,65]	Shorter PFS if ↑	Combined with receptor levels; 40% indolent lymphoma
TNF + sCD23		Poor response if ↑ [66]	Indolent lymphoma
sICAM-1	Shorter if ↑ [67]	Correlated to advanced disease and B-symptoms	Minority FL

Abbreviations: FL, follicular lymphoma; OS, overall survival; TTF, time-to-treatment failure; FFP, freedom from progression; LDH, lactate dehydrogenase; PFS, progression-free survival; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; IPI, International Prognostic Index; TNF, tumor necrosis factor; sICAM-1, soluble inter-cellular adhesion molecule-1.

among pathologists, calling into question studies that report prognostic impact based solely on this variable. Similarly, subtle architectural variations, such as the presence of diffuse areas, have not been well annotated in most FL studies. Immunostaining for FDCs has been infrequently performed, making it difficult to accurately determine true follicle formation in most reported studies.[70,71] Several studies have suggested a correlation between grade and survival in FL.[23,69,72,73] Although there is no OS difference between patients with grade 1 or 2 FL, there remains controversy about an inferior OS correlating with FL grade 3. This may reflect that grade 3 FL itself is heterogeneous. Anthracycline-based therapy has been claimed to be beneficial when treating patients with grade 3 FL[69] and to have a potential for cure; however, this remains controversial.[23,55,56,74,75] Miller et al[76] found no plateau in the survival curve or difference in OS between patients receiving aggressive therapy for FL of different grades. In a retrospective analysis, Chau et al[73] found no difference in survival among FL grades 1 to 3. Patients with FL grade 3 were similarly at risk for late relapses as for grades 1 and 2.

The WHO classification subdivides FL grade 3 into FL grade 3A, made up of a mixture of centroblasts and centrocytes, and FL grade 3B, in which the centroblasts are distributed in confluent sheets.[69] Potentially, FL grade 3A may lie within the spectrum of disease with FL grades 1 to 2, while FL grade 3B may behave similarly to de novo DLBCL. Similar to FL grades 1 to 2, FL grade 3A is more likely to be CD10 positive and t(14;18) positive compared with FL grade 3B.[71] Furthermore, chromosomal aberrations involving 3q27 (*BCL6*) occur in a similar frequency in FL grade 3B and DLBCL.[71,77] However, in one report comprising 190 patients with FL grade 3, no difference in event-free survival or OS was found between patients classified as grade 3A and grade 3B,[70] which is supported by Chau et al.[73] Thus, the relevance of distinguishing between FL grade 3A and 3B remains unclear. Identifying areas of diffuse disease,[70] likely a harbinger of transformation, may be more clinically relevant.

Immunophenotype: Neoplastic Cells

Proliferation index. The proliferation index has prognostic value in FL.[23,78] It correlates with the number of large cells and thus with histologic grade and with OS in univariate analysis but not in multivariate analysis.[79]

BCL2 and BCL2 family proteins. The family of BCL2-related proteins plays a central role in the surveillance of mitochondrial integrity by balancing between pro- and antiapoptotic members. Only a few of these proteins have been studied in relationship to prognosis in FL and no consistent correlation has been found.[80] As a possible exception, a high number of MCL1-positive centroblasts has been correlated with poor OS.[81] Similarly, expression of the long RNA isoform of $BCLX_L$ has been correlated with prognosis in FL,[82] however, expression of the protein did not.[83]

Using reverse-phase protein microarrays, when microdissected follicles from reactive lymph nodes were compared with follicles with FL, most proapoptotic and antiapoptotic proteins except for BCL2 were present at comparable levels in BCL2-positive and BCL2-negative tumors with activation of the AKT/BAD signaling pathway.[84] Gulmann et al[85] also used a proteomic approach to analyze apoptotic pathways in FL. They found phosphorylation of AKT to be frequent in FL suggesting that it may act as an antiapoptotic agent along with BCL2. High ratios of BCL2/BAK and BCL2/BAX were associated with early death from disease. The expression patterns of other proapoptotic members of the BCL2 family have largely unknown clinical impact.[86] Future studies on the role of BCL2 family proteins in FL are

needed as they may identify patients who will benefit from novel BH3 mimetics, small molecules that specifically target BCL2 proteins.[87,88]

Germinal center–related markers. *BCL6* encodes a zinc finger transcription factor involved in germinal center formation. CD10 is a membrane metalloendopeptidase expressed in the germinal center. In FL, these two markers of germinal center origin are expressed in more than 95% and 75% of cases, respectively.[89] Although their prognostic significance is established in DLBCL,[90] their relevance in FL is less clear. Using immunohistochemical scoring systems to combine the percentage of positive malignant cells and their intensity of expression, the expression level of BCL6 has been correlated with favorable prognosis in FL.[91] PU.1 is an ETS-domain transcription factor essential for the development of myeloid and B lymphoid cells. Constitutive PU.1 expression inhibits the earliest B-cell development, and low levels of PU.1 expression in hematopoietic progenitor cells are instrumental in promoting B-cell fate determination.[92] Torlakovic et al[93] studied the clinical impact of proteins associated with the germinal center in FL and found PU.1 protein expression, but not CD10 or BCL6, to be a favorable marker of OS independent of the FLIPI.

Cell cycle regulators. *TP53* mutations have been reported in FL at diagnosis and are associated with an inferior survival.[94] The presence of mutations leading to overexpression of the TP53 protein has been associated with transformation.[95,96] Expression of TP53 protein is only moderately correlated with the presence of mutations (64%) and similarly with the expression of key regulators of TP53, such as CDKN1A or MDM2.[97] The latter have also been shown to correlate with transformation.[97,98] A gene expression profiling study of 57 patients with FL established a set of 14 genes that were highly expressed in patients with a favorable response to CHOP; however, only expression of *CYCLIN B1* mRNA and protein level had prognostic impact independent of the FLIPI.[99]

Others. SOCS3 is a cytokine suppressor that inhibits cytokine signaling by Janus kinase (ie, the JAK-STAT pathway). Overexpression of SOCS3 has been reported to be an independent unfavorable prognostic factor in FL.[100] Overexpression of Ying-Yang 1, a zinc-finger protein regulating *IL4* gene expression, has also been linked with shorter survival in FL.[101]

Cytogenetics and Molecular Genetics

Classical karyotyping. Cytogenetic analysis of FL reveals a wide range of recurrent, nonrandom chromosomal alterations. FL is characterized by the overexpression of the antiapoptotic protein BCL2 as a result of the t(14;18)(q32;q21) or its rare variants t(2;18) and t(18;22). t(14;18) is present in approximately 85% of grade 1 and 2 FL and although it is considered insufficient on its own to cause FL, it provides these B cells with a survival advantage leaving them prone to accumulate additional genomic abnormalities.[102] However, in a study of FL cases lacking the t(14;18), Horsman et al[103] found distinct patterns of recurrent chromosomal alterations. In another report, FLs lacking the t(14;18) were less likely to express CD10 or BCL2, were more likely to arise at extranodal sites and had a better OS. Recently, Katzenberger et al[104] described t(14;18)-negative FL characterized by a predominantly diffuse growth pattern, presenting clinically with large but localized inguinal tumors. The majority of FL patients without a t(14;18) have a deletion in 1p36.

The malignant cells in FL have an average of four to six different cytogenetic changes in addition to the *BCL2* translocation.[105,106] Only 5% of FL have t(14;18) as the sole detectable abnormality. The most common abnormalities are break points in chromosome 1, deletions in the long arm of chromosome 6 (6q−), trisomy 7 (+7), trisomy 12 (+12), presence of a derivative of chromosome 18 (der(18), and duplication of X (+X).[106]

Early studies suggested that the number of abnormalities and presence of certain alterations (6q−, +7, +X, +21) were associated with an inferior survival.[107] However, as karyotypic complexity increases after therapy,[108] there is an inherent selection bias if biopsies

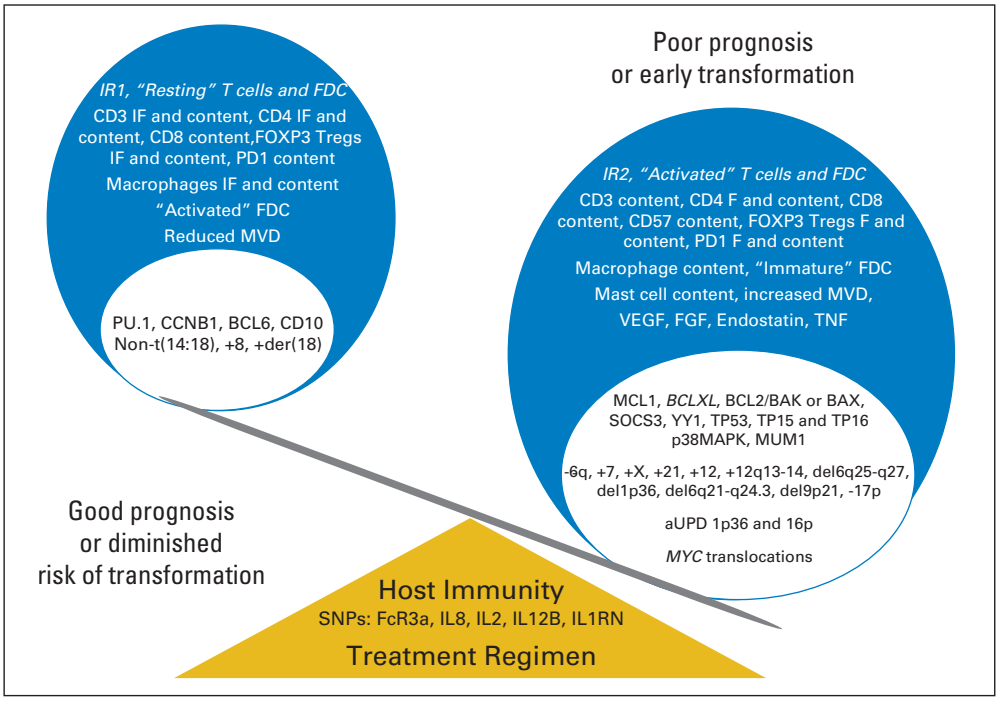

Fig 1. Biomarkers impacting survival or transformation in follicular lymphoma (FL). Some biomarkers are controversial, resulting from treatment heterogeneity. Larger circle: features of nonmalignant cells. Inner circle: tumor cells. IR, immune response; FDC, follicular dendritic cells; IF, interfollicular; F, follicular; MVD, microvessel density; VEGF, vascular endothelial growth factor; EGF, endothelial growth factor; TNF, tumor necrosis factor; aUPD, acquired uniparental disomy; SNPs, single nucleotide polymorphisms.

taken at the time of progression or relapse are used for analysis. Tilly et al[109] reviewed 66 FL biopsies taken at the time of diagnosis and found deletion 6q to be associated with an inferior survival. Höglund et al[110] reviewed the karyotypes of 336 cases of FL and, using principle components analysis, postulated distinct pathways of clonal evolution of chromosomal changes arising in a temporal order with early changes being 1q+, +7, +8,+12, and +der(18) and later 2p−, 10p−, −15, 17p−, and 17q−. Based on the clinical data on 165 patients, +12 and 17p− were correlated with an adverse outcome. In contrast, we recently reviewed 210 karyotypes from FL biopsies taken at diagnosis and found no association between the number or type of cytogenetic abnormalities with clinical outcome.[111]

Comparative genomic hybridization. Comparative genomic hybridization (CGH) can be performed by hybridizing tumor DNA against normal chromosomes (chromosomal CGH) or by using microarray platforms with spotted DNA probes (array CGH).[112] Viardot and colleagues[113] analyzed 124 samples of FL using chromosomal CGH and reported a number of recurrent alterations. In a subset analysis of 82 patients, loss of chromosomal material at 6q25-27 was a strong independent predictor of inferior survival. Cheung et al[114] identified two genomic regions, deletions in 1p36.22-p36.33 and 6q21-q24.3, to be highly associated with transformation and inferior OS in patients with FL using array CGH. Recently, Schwaenen et al[115] reported a large number of recurring genomic aberrations in FL as analyzed by array CGH, of which deletions in 9p21 (*CDKN2A/B*), 6q25, and 6q26 were associated with inferior survival.

Single nucleotide polymorphism arrays. Copy-neutral loss of heterozygosity, undetectable by previously available methods, can be identified using single nucleotide polymorphism (SNP) arrays. This phenomenon, termed acquired uniparental disomy (aUPD), results from mitotic recombination or nondisjunction and has been described in FL.[112,116] In a recent report, a number of recurring aUPDs were described in FL, of which aUPD on 1p36 correlated with shortened OS and aUPD on chromosome 16 was predictive of transformation.[117]

Host Constitutional Genetics

SNPs are changes in the DNA sequence by one base pair. Recent studies have suggested that SNPs in the *FcγR* genes may significantly alter the binding affinity between the Fc portion of rituximab and the Fc receptors on macrophages.[118,119] Cartron et al[120] found a correlation between the *FcRIIIA* genotype and response to single-agent rituximab in untreated FL. Patients homozygous for −158VV *FcRIIIA* polymorphism did significantly better than the heterozygotes −158VF. In a study of recurrent FL, Weng and

Table 3. Morphologic, Immunophenotypic, and Gene Expression Biomarkers in FL

Biomarker	Impact on Survival	Risk of Transformation
Morphology		
Tumor cells		
Histologic grade[70,74]	Therapy-dependent, increasing grade associated with worse OS; effect possibly mitigated by doxorubicin-containing regimens	No effect
Grade 3A *v* 3B[22,55,70,71,76,77]	No effect	Typically already present in grade 3B
Architecture (diffuse)[26,71]	Controversial	Increased
Proliferative rate[22,78,79,133,174,175]	Controversial	No effect
Microenvironment		
Microvessel density[129-131]	Controversial	Not studied
Macrophages[83,127,135-137]	Unfavorable	No effect
FDC[102,135,176]	Unfavorable if immature phenotype of FDCs	Increased; associated with FDC disruption
$CD4^+$ T cells[83,102,136,140,142,149,177-179]	Controversial	No effect
$CD8^+$ T cells[136,142,146,147]	Controversial	Controversial
Regulatory T cells[136,140-142,177]	Controversial	Not studied
Single gene (RNA and/or protein)		
BCL6 and *CD10*[91]	Favorable	Not studied
BCL2 and *BCL2/BAX* or *BAK*[80,86]	High ratio favoring *BCL-2* associated with early death	Not studied
MCL1[81]	High *MCL-1* associated with worse outcome	Not studied
$BCLX_L$[82]	High $BCL\text{-}X_L$ associated with inferior survival	Not studied
MUM1[180,181]	Expression of *MUM1* may be associated with inferior survival	Not studied
PU.1[93]	Favorable	Not studied
SOCS3[100]	Unfavorable	Not studied
YY.1[101]	Unfavorable	Not studied
Multigene		
IR-1 and *IR-2*[127]	Variable; *IR-1* associated with favorable OS, while *IR-2* predicts for inferior OS	Not studied
81-gene predictor[128]	No effect; strongly predictive of immediate clinical behavior	No effect

Abbreviations: FL, follicular lymphoma; OS, overall survival; FDC, follicular dendritic cells; IR, immune response signature.

colleagues[121] confirmed these findings and identified a second polymorphic site related to the duration of response (*FcRIIA* 131 histidine/arginine). Similarly, Ghielmini et al[122] reported that *FcRIIIA* V/V was a predictive factor for event-free survival in rituximab monotherapy in FL. In contrast, Maloney et al and others[123,124] did not find that *FcRIIIA* or *RIIA* polymorphisms correlated with outcome in a study of R-CHOP.

Recently, Cerhan et al[125] analyzed the impact of immune response SNPs in FL. Germline DNA was analyzed from patients with FL and a final set of four prognostically relevant immune response SNPs was identified (interleukin [*IL*] *-8*, *IL2*, *IL12B,* and *IL1RN*). An outcome predictor was built using clinical and demographic factors combined with the four deleterious SNPs, which identified three risk groups with 5-year OS estimates of 96%, 72%, and 58%, respectively. These patients were treated in an era before the use of rituximab. Although these four genes strongly predict outcome in patients with FL, none of them has been shown to be associated with the risk of developing FL.[125,126] Together with the gene expression profiling (GEP) data below,[127] these results suggest that the composition and functional status of the immune cells in the tumor microenvironment of FL may largely be driven by the genetics of the host.

Non-Neoplastic Cells of the Microenvironment

Lymph nodes involved with FL contain an admixture of non-neoplastic T cells, FDCs, macrophages, and other cells. A renewed interest in the microenvironment in FL followed the Leukemia Lymphoma Molecular Profiling Project (LLMPP) study in 2004[127] in which GEP of whole-section frozen lymph nodes from 191 cases of FL demonstrated that the tumor microenvironment was the most important predictor of patient outcome. Two signatures of gene expression were identified that best correlated with survival prediction. The immune-response 1 (IR-1) signature included genes encoding for T-cell markers and some genes that are highly expressed in monocytes/macrophages, and predicted a favorable outcome. The IR-2 signature included genes preferentially expressed in macrophages or dendritic cells, and predicted an unfavorable outcome. When patients were grouped into quartiles based on their survival-predictor scores, median survival rates ranged from 3.9 years to 13.6 years. These data highlighted the dominant prognostic role of the microenvironment in FL and suggested that the critical determinants of outcome might already be present at diagnosis.

After the LLMPP study, Glas et al[128] performed GEP in a series of patients with FL who were grouped according to outcome and transformation. They defined an 81-gene predictor that correlated with immediate clinical behavior but not with long-term survival or risk of transformation. Interestingly, the genetic profile of patients with early transformation exhibited an activated status of T cells, FDCs, and macrophages characterized by genes that were present in the favorable IR-1 signature of the LLMPP study. Differences in experimental design, patient selection, gene expression platforms, and data analysis may explain the different conclusions between these two studies.

Microvessel density. Despite evidence for a vital role of angiogenesis in supporting tumorigenesis,[129] some reports have suggested that increased vessel density correlates with better prognosis in FL.[130] Intriguing results by Streubel et al[131] demonstrated that endothelial cells of the microvasculature share the *BCL2* rearrangement characteristic of FL, implying an intimate relationship between the two.

FDC meshwork "immaturity." In addition to presenting antigens, FDCs comprise the scaffolding of both benign and malignant follicles, their dendritic processes providing structural support for B cells and non-neoplastic cells. An immature FDC phenotype is typified by expression of low-affinity nerve growth factor and CNA.42, accompanied by absence of mature FDC markers such as CD21, CD35, CD23, and CXCL13. This phenotype typifies undifferentiated fibroblastic cells and has been reported to correlate with progression

Table 4. Molecular Genetic Biomarkers in FL

Gene	Impact on Survival	Risk of Transformation
BCL2[165,182-186]	No clear survival difference between t(14;18)-positive FL and t(14;18) negative; suggestion that *BCL2* breakpoint may affect survival, but unconfirmed by later studies	Somatic mutations of *BCL2* gene on the translocated allele may rarely underlie transformation
MYC[161-163,170]	Translocations of *MYC* rarely encountered at diagnosis; tend to confer an inferior survival	Infrequently associated with transformation, but when found they are associated with markedly inferior post-transformation survival
CDKN2A/B[160,187]	Tumor suppressor genes on chromosome 9p21; only rarely found at diagnosis and not definitively associated with survival	Paired sample studies clearly show loss of *CDKN2A/B* through deletion, mutation, or hypermethylation is associated with histologic transformation
TP53[95-97]	Loss of *TP53* tumor suppressor only rarely seen at diagnosis; mutation is closely correlated with protein expression in FL	Loss of *TP53* was the first gene implicated in transformation of FL; LOH not typically associated with 17p13 deletion
MDM2[97,99]	None	Expression correlated with transformation, but not obviously correlated with TP53 gene status; found within the amplified region of chromosome 12q13-14 frequently associated with transformation
BCL6[164,184,188]	No clear affect on survival in FL	Postulated to increase risk of transformation, but studies documenting the presence of translocations were based exclusively on inverse LD-PCR
CCNB1[99]	Increased expression of cyclin B1 associated with improved survival after CHOP chemotherapy	

Abbreviations: FL, follicular lymphoma; LOH, loss of heterozygosity; LD, long-distance; PCR, polymerase chain reaction; CHOP, cyclophosphamide, doxorubicin, vincristine, and prednisone.

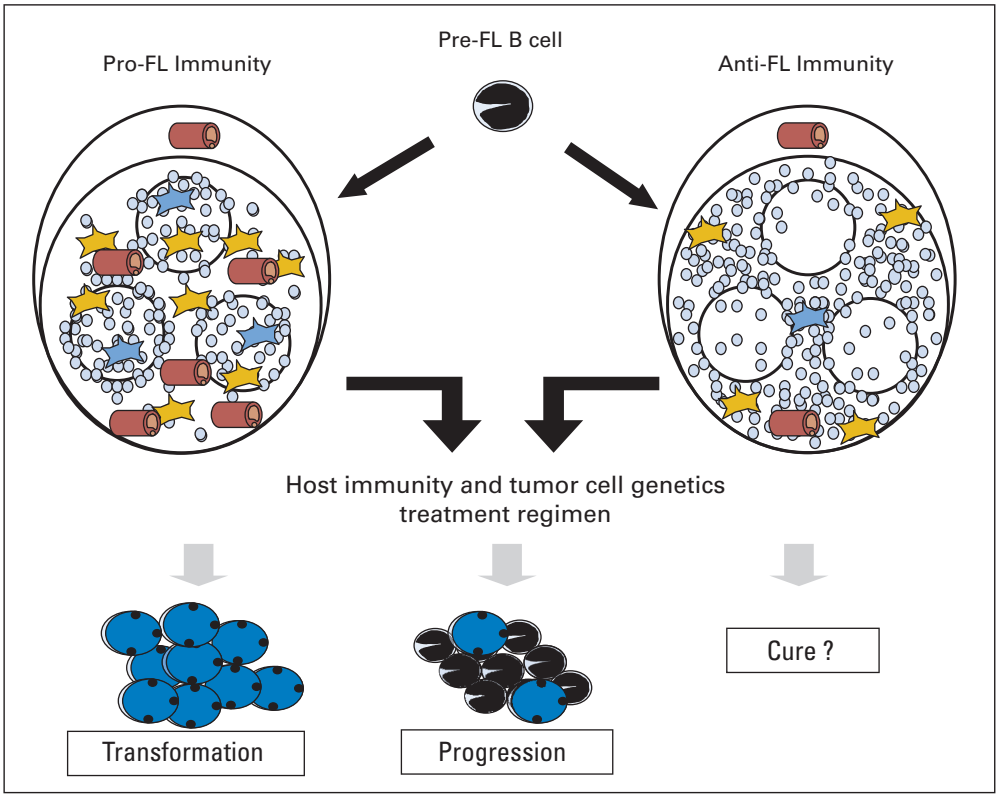

Fig 2. Model of how non-neoplastic cells in the microenvironment might impact survival and transformation in follicular lymphoma (FL). Left: follicular polarization of CD4+ cells, especially forkhead box protein P3–positive regulatory T cells (blue circles), increased numbers of vessels (red tubes), mast cells (blue stars), or macrophages (gold stars): local immunity supports tumor growth. Right: an antilymphoma immune response.

and/or transformation,[132] but not in all studies.[83] These changes correlate with loss of normal T-cell infiltration within follicles and might reflect a reduced cross-talk between these two cell types. Disruption of the tight FDC meshwork that characterizes FL has been associated with early transformation.[133]

Macrophages. Tumor-associated macrophages have been described in a number of cancers, with increased numbers of benign macrophages typically associated with inferior survival. Farinha and colleagues[83] demonstrated that the small subset of FL cases with high macrophage content experienced markedly decreased OS and PFS independent of the IPI. These data appear consistent with the LLMPP GEP study, with high macrophage content being a surrogate for the IR-2 signature.[127] Alvaro et al[134] reported opposite results finding that increased macrophages were associated with indolent clinical behavior. In a related study, these same authors found that 30% of macrophages express STAT1 protein and that presence of this subset of cells was associated with inferior survival.[135] In two recent trials, a high macrophage content was associated with poor survival after chemotherapy, but not if combined with rituximab.[136,137]

Macrophage plasticity has been well described, as these cells can be broadly separated into helper (M1) and healer (M2 or activated) cell types.[138] In most tumors, tumor-associated macrophages are polarized to a M2 phenotype and appear to create a trophic environment that favors the tumor cells.[139] A pan-macrophage marker (CD68) cannot capture these distinctions and may explain the discordant results.

T-cell subsets. T cells comprise a majority of the non-neoplastic cells in FL biopsies and play an important role in FL. Subsets include helper CD4-positive T cells, cytotoxic CD8-positive T cells, CD57-positive follicular helper T cells, and immunosuppressive regulatory T cells (Treg), but the exact role played by these subsets is not completely understood. Studies investigating the roles of CD4-positive and CD8-positive T cells in FL utilize different methodologies and have conflicting results.[133,134,140-144] Tregs are a subset mostly with a CD4-positive CD25-positive forkhead box protein P3-positive[145] immunophenotype that serve a critical role in regulating CD4 and CD8 effector functions by suppressing proliferation and cytokine production of these cells.[146] In epithelial malignancies, Tregs have been implicated in creating an immunosuppressed microenvironment that allows the tumor cells to escape the host immune response.[147] Some investigators have reported that an increased number of Tregs was associated with favorable clinical behavior in FL,[134,141] while others claim that the T-cell distribution may be more relevant.[143,148] Recently, a low number of tumor-infiltrating programmed cell death 1–positive was associated with transformation and with inferior survival.[149] Still, the precise role of T-cell subsets in FL biology remains unclear. The clinical impact of these cells in the microenvironment may be largely influenced by the characteristics of the patient and the treatments received.[145] Specific therapies may have different effects on neoplastic cells versus cells within the microenvironment. Moreover, the role of host genetics influencing the immune microenvironment has only recently been explored.[125]

Transformation

Histologic transformation is often heralded by a sudden change in clinical behavior, such as a rise in serum LDH or development of unusual extranodal sites of disease. It is a dominant clinical event, associated with shortened survival and relative resistance to therapy. The reported frequency varies dramatically ranging from 5% to 60%

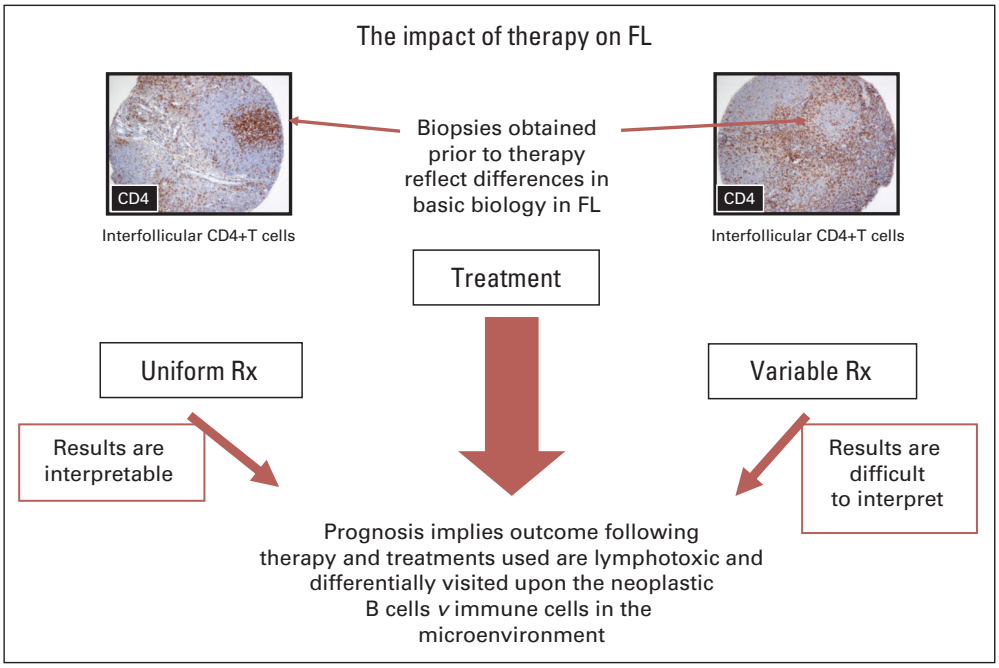

Fig 3. Treatment influences prognostic markers. Biopsies stained for CD4. Left, T cells within neoplastic follicles; right, in the interfollicular region. Importantly, treatments differentially visit toxicity on tumor cells versus cells in the microenvironment. The prognostic role of any factor must be interpreted with caution if treatment varies. FL, follicular lymphoma; Rx, therapeutic regimens.

of patients with FL.[5,8,150-156] Since risk of transformation is time dependent, approximately 3% per year, this variation probably reflects differing durations of follow-up. The length of time that patients are followed and the rigor with which biopsies are obtained at progression heavily affect the reported frequency. Biomarkers that predict for survival do not necessarily correlate with those that predict risk of transformation.

An association between increased FLIPI score and frequency of transformation has been noted.[9,28] Morphologic features associated with transformation risk include the presence of diffuse areas and disruption of the FDC meshwork.[132,157] The presence of grade 3B FL and increased intrafollicular CD4-positive T cells have also been linked to early transformation.[133]

Studies using paired samples (FL and subsequent DLBCL) have demonstrated that transformation is a molecularly heterogeneous event, including loss of *TP53*, loss of the *CDKN2A* tumor suppressor, or acquisition of *MYC* translocations, leading to upregulation of *MYC* or its target genes.[95,96,98,158-163] Other molecular alterations include mutations of the coding region of *BCL2* and translocations involving *BCL6*.[164,165] Cytogenetic studies of paired samples have revealed candidate chromosomal alterations, including +7, +12q13-14, and −6q16-21.[166-170] Lastly, a number of small studies using gene expression profiling of paired samples have been published.[160,170-173] These demonstrate the molecular heterogeneity underlying transformation, implicating upregulation of *p38 MAP KINASE*, upregulation of *MYC* and its target genes, and a generic increase in the mitotic machinery.

Recent data suggest that FL may comprise two major patient subgroups; those destined to develop transformation and those who will not.[9] Distinguishing between these two clinical risk groups might have major implications for therapy.

Translation Into the Clinic

A summary of factors associated with outcome in FL is shown in Figure 1. As presented in Tables 2, 3, and 4,[174-188] consensus on which biomarkers to use in clinical practice and how these might impact treatment decisions is still lacking. Most prognostic markers were studied before the introduction of rituximab and require validation in the current era of chemoimmunotherapy. At this time, disease behavior and robust indices, such as the FLIPI and FLIPI-2, remain the basis for clinical decision making. The key elements in the interaction of host immunogenetics, tumor microenvironment, and therapy in FL are still not well understood (Fig 2). The inclusion of correlative science into clinical trials should help to determine the precise role of biomarkers in predicting survival and transformation risk in FL. Prospective randomized clinical trials will be instrumental in defining future prognostic factors in FL, as uniform treatment groups can be compared (Fig 3). If patients who respond poorly to standard therapy can be identified and the mechanism(s) underlying their inferior outcome determined, these patients could reasonably be considered candidates for novel therapies with the potential for improved outcome avoiding toxicity from ineffective therapy.

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CORRECTIONS

Author Corrections

The January 10, 2010, article by Oktay et al, entitled, "Association of *BRCA1* Mutations With Occult Primary Ovarian Insufficiency: A Possible Explanation for the Link Between Infertility and Breast/Ovarian Cancer Risks" (J Clin Oncol 28: 240-244, 2010), contained an error.

The primary institutional affiliation for David Barad was inadvertently omitted and should have been listed as The Center for Human Reproduction, New York, NY.

The authors apologize to the readers for the mistake.

DOI: 10.1200/JCO.2010.32.8278

The July 10, 2010, article by Sargent et al, entitled, "Defective Mismatch Repair As a Predictive Marker for Lack of Efficacy of Fluorouracil-Based Adjuvant Therapy in Colon Cancer" (J Clin Oncol 28:3219-3226, 2010), contained errors.

In Table 2 and Table 4, the univariate and multivariate results (HR, 95% CI, and *P* values) for DFS and OS were presented for dMMR and pMMR patients who were not treated, whereas they should have been given for patients who were treated.

The authors apologize to the readers for the mistakes.

DOI: 10.1200/JCO.2010.32.8286

Journal Corrections

The June 10, 2010, article by Thurlow et al, entitled, "Spectral Clustering of Microarray Data Elucidates the Roles of Microenvironment Remodeling and Immune Responses in Survival of Head and Neck Squamous Cell Carcinoma" (J Clin Oncol 28:2881-2888, 2010), contained an error.

In Table 1, under Primary Tumors, the value for FA (KEGG) in the column "*P* Reduced Multivariate" was inadvertently omitted and should have been $< .001$.

Journal of Clinical Oncology apologizes to the authors and readers for the mistake.

DOI: 10.1200/JCO.2010.32.8252

The June 10, 2010, review article by Relander et al, entitled, "Prognostic Factors in Follicular Lymphoma" (J Clin Oncol 28:2902-2913, 2010), contained an error.

The left-hand image in Figure 3 was identified as "Interfollicular CD4+T cells," whereas it should have been "Intrafollicular CD4+T cells."

Journal of Clinical Oncology apologizes to the authors and readers for the mistake.

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Analysis of multiple biomarkers shows that lymphoma-associated macrophage (LAM) content is an independent predictor of survival in follicular lymphoma (FL)

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We studied the role of multiple biomarkers in determining outcome in follicular lymphoma (FL), concentrating in particular on the role of benign macrophages. The study group consisted of uniformly staged and treated patients with FL enrolled in a phase 2 trial between 1987 and 1993. All patients were younger than 61 years of age, had advanced-stage FL, and were treated with a multiagent chemotherapy regimen, BP-VACOP (bleomycin, cisplatin, etoposide, doxorubicin, cyclophosphamide, vincristine, and prednisone), followed by involved region radiation. The median follow-up of living patients was 12.5 years, and the median survival was 16.3 years. The International Prognostic Index (IPI) was predictive of overall survival (OS) ($P = .003$). Biopsy specimens from all cases were stained with an anti-CD68 antibody. Of the 99 evaluable patients with FL, 87 had less than 15 CD68$^+$ macrophages/high-power field (hpf) (median, 7; range, 1-14) and 12 had more than 15 CD68$^+$ macrophages/hpf (median, 20; range, 16-25) with a median OS of 16.3 vs 5.0 years, respectively ($P < .001$). A multivariate Cox model that included the IPI score, the histologic grade, and the lymphoma-associated macrophage (LAM) score, showed IPI and LAM to be independent predictors of OS ($P = .009$ and $P = .004$, respectively). The LAM content of FL predicts survival, and these data support a prominent role for nonneoplastic immune cells in the biology of FL. (Blood. 2005;106:2169-2174)

Introduction

Follicular lymphoma (FL) is the second most common non-Hodgkin lymphoma (NHL) subtype, surpassed only by diffuse large B-cell lymphoma (DLBCL).[1] The typical survival curve in most studies attests to the marked clinical heterogeneity that characterizes FL. With median survivals of 8 to 10 years, approximately 15% of newly diagnosed patients will die of their disease in the first 2 years, while others are still alive more than 20 years later.[2] In addition, interpretation of clinical trials of FL is made difficult by the fact that events unfold over many years.

A number of clinical prognostic variables have been described in FL but a recent international effort has culminated in a scoring system with a high likelihood of universal acceptance. The Follicular Lymphoma International Prognostic Index, or FLIPI,[3] measures 5 variables including age, Ann Arbor stage, hemoglobin level, number of nodal sites, and serum lactate dehydrogenase (LDH) level. Although clinical variables, which are surrogate markers for underlying biology, can provide useful prognostic information, there is a clear need to develop biomarkers in FL that are predictive of outcome that could then be used to supplement indices such as the FLIPI index.[4] However, few biologic markers have been developed that can be shown to have independent prognostic impact in patients with FL. A possible impact on survival has been described for several pathologic variables, although the results are inconsistent at best. The list of candidate biomarkers includes morphologic features (histologic grade, proliferation rate, presence of marginal zone differentiation, diffuse areas, small vessel content), molecular alterations (loss of *p53*, loss of *p16*, acquisition of *CMYC* alterations), cytogenetic abnormalities (del6q, −17p13, +18, +12q13-14) and altered protein expression (Bcl-2 and Bcl-6 proteins).[5-28] Problems with patient selection and inclusion bias, lack of large patient cohorts treated with uniform therapy, inconsistent interobserver reproducibility of histologic/immunohistochemical features, and low frequency of several of these molecular events hamper the clinical translation of these biomarkers.

The presence of leukocytes in human tumors was first noted in neoplastic tissues by Rudolf Virchow in 1863.[29,30] He suggested that the "lymphoreticular infiltrate" reflected an important interaction between cancer cells and cells involved in chronic inflammation. Over the past 10 years our understanding of the inflammatory microenvironment of tumors has provided support for Virchow's original hypothesis. Tumor-associated macrophages (TAMs) have been the subject of investigation in a number of different tumors, including breast, prostate, ovarian, and cervical cancers, where a clear correlation with inferior survival has been seen with increased TAM content.[31] Curiously, in other tumors such as gastric and lung cancer, the results have been contradictory.[32] These disparate findings may be due to specific functional characteristics of

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macrophages in some tumors and may be context dependent. Nonetheless, in the majority of studies increased TAM density has been associated with both metastatic spread and diminished survival. We are unaware of any previous literature implicating macrophages in the prognosis of FL.

Recent work by the Leukemia/Lymphoma Molecular Profiling Project (LLMPP) has shown that gene expression profiling can be used to establish a molecular subclassification of FL, and, importantly, was used to develop a powerful outcome predictor.[33] In a study of 191 patients using pretreatment biopsies, it was revealed that nonneoplastic immune cells were predominantly responsible for the gene expression signatures contributing most to the outcome predictor. One signature, referred to as immune response-1 (IR-1), appeared to be derived from reactive T cells in the lymph node biopsies and conferred a favorable outcome. The other signature, referred to as immune response-2 (IR-2), revealed a gene expression pattern most reminiscent of macrophages and/or follicular dendritic cells. This IR-2 signature conferred an inferior survival on patients with FL. Treatment in this study was not uniform; nonetheless, these data allowed the development of a molecular predictor score with widely divergent survivals. Moreover, these results clearly demonstrated that molecular characteristics of the tumors that impact upon survival were present in original diagnostic biopsies, suggesting that ongoing stochastic events may not be as important for determining survival in FL. The character of the IR-2 gene list (eg, complement genes, toll-like receptors, Fc receptors, etc) suggested that macrophages might be playing an important role in the biology and survival of patients with FL. On the strength of these observations, we studied the clinical impact of the content and morphologic patterns of follicular dendritic cells (FDCs), T-cell subsets, and lymphoma-associated macrophage (LAM) content in patients with FL who had been uniformly staged and treated at our own institution.

Patients, materials, and methods

Patient characteristics

The British Columbia Cancer Agency (BCCA) is the primary referral center for patients diagnosed with lymphoid malignancies in the province of British Columbia, Canada. It serves a catchment area of approximately 4 million people. Consecutive patients seen at the BCCA between July 1987 and May 1993 were offered enrolment in the BP-VACOP program consisting of chemotherapy (bleomycin, cisplatin, etoposide, doxorubicin, cyclophosphamide, vincristine, and prednisone) followed by involved field irradiation to sites of original nodal involvement. Patients were deemed eligible for enrollment if they were aged 16 to 61 years with newly diagnosed, treatment-naive, advanced-stage indolent non-Hodgkin lymphoma. Advanced stage disease was defined as Ann Arbor stage III or IV, or stage II with B symptoms, nonradioencompassable disease, or bulk of 10 cm or greater in maximum diameter at any individual tumor site. Patients were otherwise unselected other than to exclude those with independent organ dysfunction that would have made administration of the chemotherapy unacceptably toxic. Approval to review, analyze, and publish the data in this study was given by the University of British Columbia–British Columbia Cancer Agency Research Ethics Board. Informed consent was provided according to the Declaration of Helsinki. The source of pathology specimens for diagnosis included only lymph node biopsies. All patients had indolent lymphoma confirmed by standard histologic, immunohistochemical, and immunophenotypic and cytogenetic methods. Pathology was reviewed after enrollment but was not an inclusion criterion. A single pathologist (R.D.G.) classified all cases as indolent according to the Working Formulation for Clinical Usage and later updated them according to the World Health Organization (WHO) classification.[34] For this study of tumor-associated macrophages, 2 pathologists (R.D.G. and B.F.S.) re-reviewed all of the FL cases, graded them according to the WHO criteria, and recorded those with evidence of marginal zone differentiation.[34] The histologic diagnoses of the other cases, not included in this study, were small lymphocytic lymphoma, lymphoplasmacytic lymphoma, diffuse follicle-center lymphoma, mantle-cell lymphoma, and marginal-zone lymphoma.

Table 1. Antibodies used in the study

Antibody	Clone	Source	Dilution
Bcl-2	124	DAKO	1:20
Bcl-X_L	2H12	ZYMED	1:50
Bcl-6	PG-B6p	DAKO	1:10
CD3	—	Cell Marque/Novocastra	1:100
CD4	4B12	Novocastra	1:50
CD7	CD7-272	Novocastra	1:30
CD8	C8/144B	DAKO	1:50
CD10	56C6	Novocastra	1:50
CD20	L26	DAKO	1:500
Ki67	MIB1	DAKO	1:100
CD21	1F8	DAKO	1:30
CD57	NK-1	Novocastra	1:30
CD68	KP1	DAKO	1:2000
TIA-1	2G9A10F5	Beckman	1:250

ZYMED (Invitrogen, Carlsbad, CA); Cell Marque (Hot Springs, AR); Novocastra (Newcastle upon Tyne, United Kingdom).

— indicates CD3 clone PS1 (Ultra Marque CD3).

Tissue microarray construction

A tissue microarray (TMA) was constructed using a tissue arrayer device (Beecher Instruments, Silver Spring, MD). Only those FL cases with adequate biopsy material and available blocks could be included. Of the 126 cases of FL, paraffin blocks were available for 110 patients. Of these, 105 had adequate material remaining in the block to be used for the TMA. Duplicate 1.0-mm cores were used to construct the TMA. Slides from the TMA block were cut at 4 microns.

Histology and immunohistochemistry

A hematoxylin and eosin (H&E) stain of the TMA was prepared using routine methods. All of the cores contained neoplastic follicles, and no discrepant results were seen between duplicate cores. A battery of immunohistochemical stains were performed, including CD20 (L26), CD3, CD4, CD7, CD8, CD10, CD21, CD57, MIB-1, T-cell intracellular antigen-1 (TIA-1), Bcl-2, Bcl-6, Bcl-X_L, and CD68 (Table 1). Immunostaining was performed using a Dako autostainer and the EnVision polymer detection system, except for CD10 detection in formalin fixation, which employs the PowerVision system (all from DakoCytomation, Glostrup, Denmark). Antigen retrieval was used for all antibodies and included enzyme predigestion for CD21 and pressure-cooking (5 minutes) for the remainder. A variety of buffers were used depending on the specific antibody to allow optimal detection of the antigens. The chromogen in all cases was diaminobenzidine.

The immunostained TMA slides were scored both for architectural pattern and the number of positively stained cells. In total, 14 biomarkers were analyzed in this study. For CD10, CD20, Bcl-2, Bcl-6 and Bcl-X_L, the cases were scored qualitatively, as duplicate cores were either completely positive or negative. In addition to the qualitative CD10 scoring, the presence or absence of interfollicular $CD10^+$ neoplastic cells was noted. The proliferation rate of the neoplastic cells was scored based on the percentage of the MIB-1^+ cells within neoplastic follicles (MIB-$1 \leq 10\% = 1$; MIB-$1 > 10$ and $< 50\% = 2$; MIB-$1 > 50\% = 3$). T-cell markers (CD3, CD4, CD7, CD8, and CD57) were evaluated both for their distribution in relation to the neoplastic follicles (interfollicular or perifollicular compartments), the predominance of CD4 versus CD8 cells, and counting the mean number of the $CD57^+$ cells. Cytotoxic granule TIA-1^+ cells were scored qualitatively, with cases called positive in the presence of

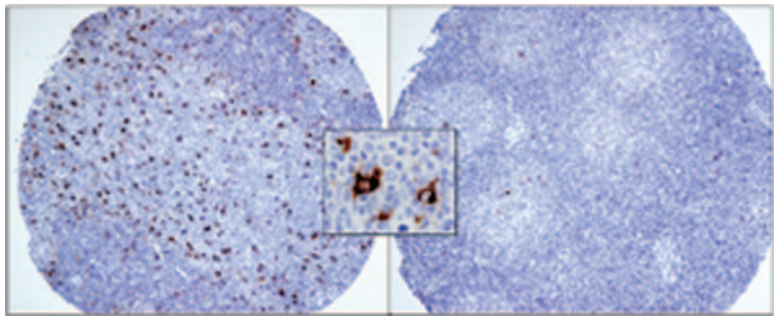

Figure 1. Representative tissue microarray cores of patients with follicular lymphoma. The image on the left shows the uncommon finding of large numbers of macrophages both within and surrounding neoplastic follicles (n = 12). The image on the right shows the more common finding of very few macrophages (n = 87). The insert shows the typical strong cytoplasmic staining of reactive macrophages with anti-CD68 antibody. Images were acquired using a Nikon Eclipse E600 clinical microscope and Dxm1200 digital camera and software (Nikon, Tokyo, Japan). Original magnification × 100 (objective, 10 ×/0.3 NA) for the larger panels and × 1000 (objective, 100 ×/1.3 NA) for the insert.

more than 10% of positive T cells. Follicular dendritic-cell (FDC) meshworks ($CD21^+$) were morphologically classified either as follicular pattern (tight concentric FDC cytoplasmic processes) or expanded when there was dissolution of the normal pattern and coalescence of FDC processes in a loose arrangement between follicles.

$CD68^+$ macrophages were first evaluated for their consistency between duplicate cores and then qualitatively. The initial analysis of CD68 staining revealed that macrophage content could be divided into 2 groups, cases with either none or few cells positive versus many positive cells. To make the determination of macrophage numbers more objective, we counted cells using high-power magnification (× 1000 oil lens). This high magnification was used to avoid counting neutrophils and intercellular debris occasionally stained with this antibody. Five representative fields per case were counted. We chose to count in areas where the staining was the strongest and most uniform. In virtually all of the cases scoring was made easier by the fact that cases were either clearly positive or negative (Figure 1). Neither FDCs nor neoplastic B cells stained with anti-CD68 in any case.

Statistics and survival analysis

Progression-free survival (PFS) was defined as the interval between diagnosis and death or lymphoma progression, whichever came first; disease-specific survival (DSS) was defined as the interval from diagnosis until death when the underlying cause of death was lymphoma or treatment toxicity, with all other causes of death censored; and overall survival (OS) was defined as the interval from date of diagnosis until death from any cause. International Prognostic Index (IPI) score originally developed for large-cell lymphomas, histologic grade, the presence of marginal zone differentiation, age, sex, and each of the 14 biomarkers (Table 2) including LAM were evaluated for prognostic significance. The relationships between LAM and other potential prognostic factors were assessed by the Pearson χ^2 test. Survival estimates were calculated using the Kaplan-Meier method.[35] Significance levels, estimates of hazard ratios (HR), and their 95% confidence intervals (CIs) were calculated using the proportional-hazards regression model.[36]

Results

Clinical characteristics and outcome

A total of 99 patients were included in this study, representing the patients with FL from the original clinical cohort (n = 126) with available blocks, sufficient tissue remaining in the blocks, and successful TMA construction and interpretation. The median age of the evaluable patients was 44 years (range, 19-61 years). There were 49 women and 50 men. The distribution of IPI score included 58 patients with IPI scores of 0/1 (group 1), 40 with IPI scores of 2/3 (group 2), and only a single patient with an IPI score of 4/5 (group 3). For analysis purposes, the single patient in IPI group 3 was included with IPI group 2 in order to eliminate a clinical group with only 1 patient. The median follow-up of living patients (n = 55) was 12.5 years. The median OS was 16.5 years. The IPI was highly significant as a predictor of OS, disease-specific survival (DSS) and progression-free survival (PFS) (P = .001, .003, and .007, respectively).

Pathology variables

Histologic grading of FL subtypes according to the WHO classification included 77 with grade 1, 15 with grade 2, and 7 with grade 3a. A total of 15 patients were determined to have

Table 2. Biomarkers in follicular lymphoma

Biomarker	No. failed	No. positive (%)	Morphologic pattern	OS *P*	Comments
Bcl-2	2	92 (89)	—	NS	
Bcl-X_L	1	15 (14)	—	NS	> 10% lymphoma cells
Bcl-6	1	81 (78)	—	NS	
CD10	2	94 (91)	—	NS	
Interfollicular CD10	2	78 (76)	—	NS	Presence of interfollicular $CD10^+$ neoplastic cells
Ki-67 (MIB-1)	5	62 (62)	—	NS	> 50% neoplastic nuclei
CD4	2	78 (76)	—	NS	Majority of T cells
CD7 pattern	1	73 (70)	Interfollicular	NS	Interfollicular CD7 predominance
CD4/CD8 pattern	5	71 (71)	Follicular	NS	Intrafollicular CD4 and perifollicular CD8
FDC (CD21) pattern	19	27 (31)	Expanded follicles	NS	Coalescence of FDC meshwork
CD57	1	67 (64)	—	NS	> 10% of T cells
CD57 pattern	13	68 (63)	Follicular	NS	Intrafollicular CD57 predominance
TIA-1	1	47 (45)	—	NS	> 10% T cells
$CD68^+$ macrophages	6	12 (12)	—	< .001	> 15 macrophages/hpf (× 1 000)

— indicates not evaluated; NS, not significant.

Table 3. Distribution of clinical and pathologic variables between high LAM and low LAM cases

Feature	No. patients with high LAM (%)	No. patients with low LAM (%)	Total (%)	*P*
No.	12 (12)	87 (88)	99 (100)	—
Clinical features				.058*
IPI Group 1 (0/1)	4 (33)	54 (62)	58 (59)	
Group 2 (2/3)	8 (66)	32 (37)	40 (40)	
Group 3 (4/5)	0 (0)	1 (1)	1 (1)	
BM involvement	6 (50)	44 (51)	50 (51)	
Treatment response				NS†
CR	6 (50)	48 (55)	54 (55)	
PR	6 (50)	36 (41)	42 (42)	
NR	0 (0)	3 (3)	3 (3)	
Pathology features				.046‡
FL grade				
Grade 1	6 (50)	67 (77)	73 (74)	
Grade 2	4 (33)	16 (18)	20 (20)	
Grade 3	2 (17)	4 (5)	6 (61)	
MZ differentiation	3 (25)	22 (25)	25 (25)	NS
Transformation	0 (0)	8 (9)	8 (8)	NS

IPI indicates International Prognostic Index score; BM, bone marrow; CR, complete remission; PR, partial remission; NR, no response; NS, not significant; —, ____; and MZ, presence of marginal zone differentiation.

*IPI group 1 versus 2/3.

†CR versus PR/NR.

‡Grade 1 versus 2/3.

marginal zone differentiation, as defined by a zone of pale cells at least 3 cells in thickness, expressing CD20 and surrounding neoplastic follicles. Neither histologic grade nor the presence of marginal zone differentiation had an effect on OS, DSS, or PFS (data not shown).

Some cores were lost during the preparation of the sections or were uninterruptible due to poor fixation and/or inadequate staining (varies with biomarker). The percentage of informative individual cores was 96%, which varied with the different biomarkers (1%-18%; Table 2). Results of the biomarkers are summarized in Table 2. The percentage of the positive cases for Bcl-2, Bcl-6, and CD10 were in line with previous reports. In total, 16% of the $CD10^+$ FL had no or minimal interfollicular involvement by $CD10^+$ neoplastic cells. Only a small number of cases (14%) had more than 10% Bcl-X_L^+ cells. There was a correlation between the proliferative rate (MIB-1) and the histologic grade, at least for the small number of grade 3a cases. The majority (83%) of grade 3a FL cases had a proliferative rate greater than 50%. Grade 2 FL cases showed more heterogeneity, with 62% having a proliferative rate between 10% and 50%, while the bulk of the remaining cases had more than 50% MIB-1^+ cells. Only 50% of the grade 1 FL cases revealed less than 10% MIB-1^+ cells, with the majority of the remaining cases demonstrating proliferative rates between 10% and 50%. These data indicate the imperfect relationship between histologic grade and proliferative rate. Importantly, neither grade nor MIB-1 score were predictive of outcome (data not shown).

The FDC pattern (CD21) was disrupted with expansion of the meshwork and fusion of the follicles in 27% of the cases. The reactive T cells showed a predominantly perifollicular distribution in most cases (70%), as well as a CD4 phenotype (76%). In 71% of the cases there was a unique follicular pattern, with CD4 cells inside the follicles surrounded by CD8 positive cells outside the follicles, whereas in other cases the distribution was diffuse for both subsets. $CD57^+$ cells represented more than 10% of the T cells in most cases (64%) and they had a clear follicular pattern in 68%, with most of the positive cells localized inside the follicles. In 82% of these cases the CD57 follicular pattern correlated with the CD4/CD8 follicular pattern and both CD57 and CD4 cells had an equal distribution within the follicles. Cases with more than 10% cytotoxic TIA-1 granule$^+$ cells were found in 45% of the cases. The numbers of $CD68^+$ macrophages (LAMs) were initially determined using low-power magnification. None of the cases showed staining of neoplastic B cells with anti-CD68 antibody. It was clear that most cases had few or no macrophages, while a small number had many (Figure 1). The distribution of these $CD68^+$ cells was uneven throughout cores with both intrafollicular and perifollicular macrophages observed. We then used high-power magnification ($\times$ 1000 oil lens) to count the $CD68^+$ macrophages, which ranged between 1 and 25 cells per high-power field (hpf). Because many of the cases had no or few macrophages, we established a cut-off of 15 cells/hpf for analysis purposes. Importantly, most of the negative cases had less than 10 cells (82%), and the positive cases had more than 20 cells (58%) per hpf, respectively. Table 3 shows the distribution of clinical and pathologic variables for the FL cases with high LAM (n = 12) versus those with low LAM content (n = 87). Only histologic grade was unevenly distributed between these 2 groups and thus was included in a multivariate model along with the IPI. All biomarker determinations were measured without knowledge of the clinical outcome. Except for the LAM score, none of the other markers showed a significant impact on outcome. Cases with less and more than 15 $CD68^+$ macrophages/hpf had median OSs of 16.3 and 5.0 years, respectively ($P < .001$; Figure 2). Figure 3 shows the progression-free survival curve. Cases with less and more than 15 $CD68^+$ macrophages/hpf had a median PFS of 7.05 and 1.69 years, respectively ($P = .001$). A multivariate proportional hazards model that included the LAM content, the IPI score, and histologic grade showed that LAM is an independent predictor of OS (HR = 2.4, 95%CI = 1.3-4.5, $P = .004$). The inferior PFS and OS of the patients with a high LAM score cannot be explained by a lower response rate to treatment (Table 3), nor an increased risk of histologic transformation (0 of 12 developed transformed disease) nor lack of appropriate treatment for relapse (all patients with relapse were treated with systemic chemotherapy, 3 with high-dose chemotherapy and hematopoietic stem cell transplantation). All but 1 of the 10 deaths among the patients with high LAM scores resulted from progressive lymphoma.

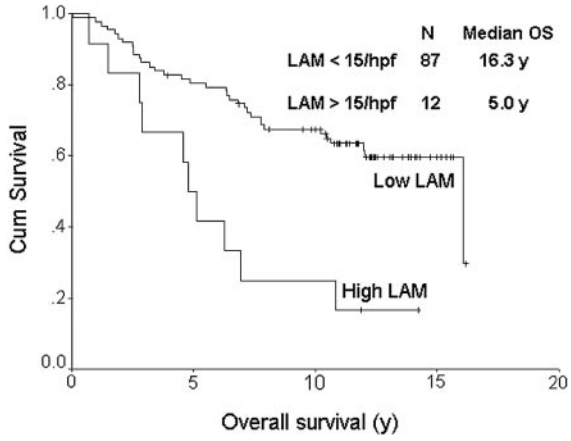

Figure 2. Overall survival curve based on lymphoma associated macrophage (LAM) content. The top curve represents cases with less than 15 $CD68^+$ macrophages/hpf; the bottom curve, those cases with more than 15 $CD68^+$ macrophages/hpf. The median OSs were 16.3 and 5.0 years, respectively ($P < .001$).

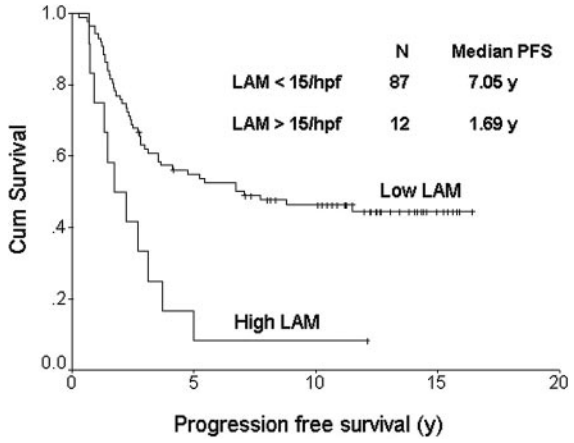

Figure 3. Progression-free survival curve based on lymphoma-associated macrophage (LAM) content. The top curve represents cases with less than 15 $CD68^+$ macrophages/hpf; the bottom curve, those cases with more than 15 $CD68^+$ macrophages/hpf. The median PFSs were 7.05 and 1.69 years, respectively ($P = .001$).

Discussion

FL represents a lymphoma subtype characterized by a significant degree of clinical heterogeneity.[2] The recent development of a clinical prognostic model (FLIPI) may help to define those patients at risk of failing current therapies, but the degree to which this index will be helpful in planning initial therapy for newly diagnosed patients remains undefined.[3] Importantly, this model includes clinical surrogates for the underlying molecular genetic alterations and thus will not help to identify novel targets for therapy. An improved understanding of the molecular abnormalities that underlie the survival differences among patients with FL may lead to improvements in risk stratification, but more importantly would offer the possibility of identifying new targets for rationale design of future treatments. To a large extent, the future of tailored or personalized therapies in cancer will depend on this very hypothesis.

Most published data for FL suggest that the molecular alterations that characterize this lymphoma result from a combination of abnormalities present at the time of diagnosis and ongoing stochastic events that occur in association with clonal evolution.[5,8,11,13,33] Because FL is a germinal center derived neoplasm and frequently reveals intraclonal heterogeneity at the level of the immunoglobulin heavy chain, there is a belief that additional molecular alterations are occurring over time and contribute to disease progression. However, a number of other possibilities exist to explain disease progression in FL, including the outgrowth of subclones that may have been present as minor populations at diagnosis.[24,37] Whatever the relative contribution of these 2 processes, there is little doubt that the molecular signature present in initial diagnostic biopsies plays a prominent role in predicting outcome in FL. The recent gene expression profiling studies of the LLMPP have clearly shown that the signatures from coordinately expressed genes play a dominant role in affecting survival, as evidenced by widely divergent survivals.[33] The most important gene expression signatures in the recently developed molecular predictor were not derived from neoplastic B cells but instead were expressed by nonneoplastic immune response cells. One of these signatures, called IR-2, included a number of genes that appeared to correspond to the repertoire expressed by macrophages and was associated with inferior survival. These results suggest that benign macrophages play a role in the biology of FL. Data supporting such a role for reactive macrophages as biomarkers in cancer have been described in a number of different tumors including breast, prostate, ovarian, and cervical cancer.[31] A suspected role in lymphoma was untested until recently conducted studies by the LLMPP consortium.

The current study clearly shows that LAM content is an independent predictor of survival in uniformly treated FL. Not unexpectedly, the IPI strongly predicted survival in this study ($P = .003$). The content of LAM was, however, also predictive of survival, including OS ($P = .003$), disease-specific ($P < .001$), and progression-free ($P = .001$) survivals, and remained an independent variable distinct from the IPI in a Cox multivariate model ($P = .004$). These data agree with similar observations made in epithelial cancers that increased numbers of macrophages portend a worse outcome.[31,32] Moreover, these results also agree with the recent gene expression studies and provide indirect validation at the protein level for the role of IR-2. Ongoing investigations of the biologic functions of macrophages in FL continue, as do studies to validate gene expression in FL by studying the expression of other proteins using paraffin-embedded material.

The precise biologic function of tissue-based macrophages in this setting remains undefined, but they may be providing important growth signals to the FL cells in the form of cytokine stimulation or specific chemokine production. Macrophage heterogeneity is well studied in the mouse, but less is known about human macrophages.[31,38,39] The plasticity of macrophage function is evident in the diverse range of functions of these cells, including inflammatory responses, immune reactions, tissue remodeling, and morphogenesis. Depending on the microenvironment, macrophages appear to be either "classic" M1 (inflammatory responses induced by interleukin-12 [IL-12] and tumor necrosis factor-α [TNF-α]) or "alternative" M2 (tissue remodeling and morphogenesis induced by IL-4, IL-13, and IL-10) subtypes, referred to as polarized macrophages. These 2 types differ in their receptor expression and cytokine/chemokine profiles.[39]

In advance of this study we studied macrophage content and distribution in a series of reactive lymph nodes (n = 10; data not shown). Virtually all cases showed large numbers of tissue-based macrophages both within and between reactive lymphoid follicles. Thus, we were surprised to find that most cases of FL in this study had no or very few macrophages. We hypothesize that in the minority of cases of FL with large numbers of macrophages the neoplastic B cells display signals that attract and/or retain predominantly M2-type macrophages. Alternatively, an inflammatory microenvironment might be created by the antitumor-responsive T cells that attract or retain the macrophages. Once in the lymph node, these cells might provide the neoplastic B cells with trophic/survival signals, facilitate tumoral invasion by disrupting basement membranes, promote angiogenesis by secreting angiogenic factors, alter the phenotype and/or function of antigen-presenting dendritic cells, or amplify regulatory T cells, leading to an immunosuppressive intratumoral environment.[40] At present, these remain untested hypotheses. Nonetheless, an improved understanding of the interactions between nonneoplastic macrophages and neoplastic B cells in FL should provide important insights into the biology of this common tumor. Ultimately it may be possible to

manipulate the microenvironment of the lymph node in favor of the host and turn off any trophic survival signals derived from nonneoplastic tissue-based macrophages. Finally, this study provides compelling data that support a role for benign macrophages in predicting outcome in aggressively treated patients with FL and further substantiate the recent gene expression profiling data highlighting the importance of nonneoplastic cells in determining prognosis in FL.

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The architectural pattern of FOXP3-positive T cells in follicular lymphoma is an independent predictor of survival and histologic transformation

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Previous studies of follicular lymphoma (FL) patients treated heterogeneously have suggested that decreased numbers of regulatory T cells correlates with improved survival. We studied advanced-stage FL patients from a single institution phase 2 trial. All patients were treated uniformly with multiagent chemotherapy and radiation. Tissue microarrays were constructed using diagnostic biopsies available in 105 patients and stained with CD4, CD8, CD25, and forkhead/winged helix transcription factor 3 (FOXP3) antibodies. Both cell content and cell distribution were evaluated. For all antibodies, there were cases with a predominant intrafollicular or perifollicular localization of cells (follicular pattern) while others displayed a diffuse pattern. The median follow-up of living patients was 17.1 years. The International Prognostic Index score predicted overall survival (OS; $P = .004$) but not risk of transformation (RT). Cell content did not impact survival, while immunoarchitectural patterns of CD4/CD8 were significant for progression-free survival (PFS; $P = .056$), CD25 for both PFS and OS ($P = .002$ and $P = .024$, respectively), and FOXP3$^+$ predicted PFS, OS, and RT ($P = .001$, $P < .001$ and $p = .002$, respectively). A Cox multivariate model showed both International Prognostic Index score and FOXP3$^+$ pattern were independent predictors of OS ($P = .008$ and $P < .001$, respectively), while only FOXP3$^+$ pattern predicted RT ($P = .004$). We conclude that FOXP3$^+$ cell distribution significantly predicts survival and RT in FL. (Blood. 2010;115: 289-295)

Introduction

Follicular lymphoma (FL) is the most common indolent non-Hodgkin lymphoma worldwide.[1] It is characterized by a significant degree of clinical heterogeneity.[2] Transformation into aggressive lymphoma, usually diffuse large B cell lymphoma is a dominant clinical event frequently associated with inferior survival.[3] Clinical prognostic variable scoring systems such as the International Prognostic Index (IPI) can be used to assess patient risk and guide therapeutic decisions.[4] However, such clinical variables represent only surrogate markers for the underlying biology. Although desirable, no consistent biologic markers have been identified that predict survival or, equally importantly, risk of transformation. The majority of candidate biomarkers studied in the past have focused on the malignant cells, including morphologic features, cytogenetic abnormalities, molecular aberrations, and altered protein expression. However, the lack of large, uniformly treated patient cohorts and the low frequency of several of these molecular events have hampered their translation into clinical practice.

Recent gene expression studies have revealed that both survival and progression of disease were significantly associated with non-neoplastic immune cell gene signatures.[5,6] On the strength of these observations we have shown in FL treated uniformly with multiagent chemotherapy (bleomycin, cisplatin, etoposide, doxorubicin, cyclophosphamide, vincristine, and prednisone [BP-VACOP]) and involved region radiotherapy, lymphoma-associated macrophage (LAM) content significantly predicted survival. Using tissue microarrays and immunohistochemistry, we found that in a minority of tumors (12%) a high density of macrophages infiltrating the tumor correlated with inferior survival.[7] Several other subsequent studies have found similar results.[8-11]

Regulatory T cells (Tregs) have the important role of suppressing effector T cells and preventing reactivity to self-antigens.[12] These cells recognize specific tumor antigens and differentiate into cells capable of suppressing naive and CD4$^+$ Th1 antitumor effector cells.[13] It has been shown that tumor immunosurveillance is more efficient when Tregs are depleted.[14,15] In human cancer, effective antitumor responses are also affected by the presence of Tregs. These cells have been identified in increased frequency in the peripheral blood of patients with several different tumor types.[16-20] Furthermore, increased density of Tregs within carcinoma biopsies is predictive of poor survival.[20]

Tregs differ from other lymphocytes in origin, phenotype, and mode of action. In humans, they constitute 5% to 10% of CD4$^+$ T cells and arise as a distinct lineage within the thymus, so-called "natural" Tregs, but regulatory function can also be acquired peripherally when uncommitted CD4$^+$ cells receive antigenic stimulation.[12] These cells also express other surface molecules associated with activated/memory cells such as CD25, glucocorticoid-inducible tumor necrosis factor receptor (GITR), CD62L, and cytotoxic T lymphocyte–associated antigen 4, and these cells can secrete transforming growth factor-beta (TGFβ) and interleuking-10 (IL-10). However, none of these proteins is unique to these cells. The forkhead/winged helix transcription factor 3 (FOXP3) is a transcriptional factor

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shown to be the key control gene in the development and function of Tregs both in mice and humans.[21-23] Although multiple T-cell subsets (eg, Tr1, Th3, Th1, Th17, Tfh) have been shown to exert negative immunoregulatory effects by producing immunomodulatory cytokines, such as TGFβ and IL-10, FOXP3$^+$ T cells represent the major Treg population critical for immune homeostasis.

In lymphoma, FOXP3 is expressed by a subset of adult T-cell leukemia/lymphoma cases that tend to have a worse prognosis.[24] In FL, FOXP3 expression is only detected in the background reactive T cells present at variable frequency. Importantly, intratumoral FOXP3$^+$ T cells have been shown to migrate or be induced in response to chemokines produced by malignant B cells and they display the ability to suppress the function of other infiltrating T cells.[25-29] The mechanisms by which Tregs exert their suppressive function are likely multiple[30] but not fully elucidated in FL.

In retrospective studies of FL, increased numbers of Tregs expressing FOXP3 have been associated with improved overall survival, a finding that is contradictory to nonhematopoietic cancers.[8,31] However, these series are limited by the marked heterogeneity of treatments used, thereby confounding straightforward interpretation of these data. Treatment heterogeneity has been shown to correlate with different, sometimes contradictory results regarding the clinical impact associated with different cells of the microenvironment in FL.[9,11,32] We hypothesize that these markedly different treatments are differentially visited on the malignant B cells versus the nonneoplastic immune cells in the tumor microenvironment. These observations and hypotheses prompted us to analyze the role of Tregs as a prognostic marker in FL, both in terms of their numbers and their distribution in the tumoral microenvironment, in a series of uniformly treated patients with long follow-up.

Methods

Patient characteristics

The British Columbia Cancer Agency is the primary referral center for patients diagnosed with lymphoid malignancies in the province of British Columbia. Consecutive patients seen at the British Columbia Cancer Agency between July 1987 and May 1993 were offered enrolment in a phase 2 trial consisting of multiagent chemotherapy (BP-VACOP) followed by involved field irradiation to sites of original nodal involvement. Patients were eligible for enrollment if they were aged 16 to 61 years, were newly diagnosed, treatment-naive, HIV-negative, and had advanced-stage indolent non-Hodgkin lymphoma. Advanced-stage disease was defined as Ann Arbor stage III or IV, or stage II with B symptoms, nonradioencompassable disease, or bulk 10 cm or larger in maximum diameter at any individual tumor site. Approval to review, analyze, and publish the data in this study was given by the University of British Columbia–British Columbia Cancer Agency Research Ethics Board. The source of pathology specimens for diagnosis included only lymph node biopsies. One pathologist (R.D.G.) classified all cases as indolent according to the Working Formulation for Clinical Usage and later updated them according to the World Health Organization (WHO) classification.[33] For this study, 2 pathologists (R.D.G. and P.F.) re-reviewed all of the FL cases, grading them according to the 2008 WHO criteria. Transformation was defined as biopsy proven or clinically diagnosed diffuse large B cell lymphoma or other aggressive lymphoma as described previously.[3]

TMA

A tissue microarray (TMA) was constructed using a tissue arrayer device (Beecher Instruments). Of the 126 cases of FL treated with BP-VACOP and radiation, 105 had paraffin blocks with adequate material remaining in the block to be used for the TMA. Duplicate 1.0-mm cores were used to construct 2 TMAs, including both B5 and formalin-fixed cases. The 2 TMAs shared 40% overlapping cases, which provided a valuable internal control. Slides from the TMA block were cut at 4 μm.

Table 1. Antibodies used in the study

Antibody	Clone	Source	Dilution
CD4	4B12	Novocastra	1:50
CD8	C8/144B	Dako	1:50
CD20	L26	Dako	1:500
CD21	1F8	Dako	1:30
CD25	4C9	Novocastra	1:100
FOXP3	236A/E7	Abcam	1:2

Histology and immunohistochemistry

A hematoxylin and eosin stain of the TMA was prepared using routine methods. All cases contained neoplastic follicles (average of 3 follicles per core). A battery of immunohistochemical stains were performed including CD20, CD21, CD4, CD8, CD25, and FOXP3 (see Table 1). Immunostaining was performed using a Dako autostainer and the EnVision polymer detection system. Pressure cooking antigen retrieval was used for all antibodies. A variety of buffers were used depending on the specific antibody to allow optimal detection of the antigens. The chromogen in all cases was diaminobenzidine.

The immunostained TMA slides were screened with CD20 to ensure tumor cell content. For the different T-cell markers (CD4, CD8, CD25, and FOXP3), the numbers of positively stained cells and their patterns of distribution were evaluated. Before counting, the morphologic patterns and cell content were first evaluated for their consistency between duplicate cores and between TMAs (B5 vs formalin).

The total number of positive cells (FOXP3 and CD25) was counted per core (× 1000 magnification). Immunoarchitectural patterns of distribution of the different T-cell markers were determined in relation to the neoplastic follicles. The "follicular" pattern was characterized by an intrafollicular and perifollicular predominance of positive cells, whereas in the "diffuse" pattern the cells were diffusely distributed with no clear relationship to the follicles. To better classify these patterns a follicular dendritic cell meshwork stain (CD21) was used. Whenever the majority of positive cells were within the follicles in each core, cases were labeled as having a follicular pattern. From the remaining cases with predominance of positive cells outside the follicles, those with a predominant perifollicular rim of positive cells were labeled perifollicular. For purposes of analysis, both groups, those with a follicular distribution of positive cells and those with a perifollicular distribution, were grouped together as having a follicular pattern. Assignment of the CD4 pattern and the combined CD4 versus CD8 pattern was done similarly to our previous study.[7] Both were scored semiquantitatively and in the CD4 versus CD8 pattern determination, both stains were evaluated using sequential sections by overlaying both slides on the microscope. Cases with a follicular predominance of CD4 versus CD8 cells were identified as having a follicular pattern, whereas cases with a similar distribution of CD4 versus CD8 cells were defined as "diffuse."

Statistics and survival analysis

Progression-free survival (PFS) was defined as the interval between diagnosis and progression of lymphoma or death due to lymphoma or toxicity of treatment. Overall survival (OS) was defined as the interval from date of diagnosis until death from any cause, and risk of transformation was defined as the interval from diagnosis until clinical or biopsy-confirmed transformation into aggressive disease. The variables IPI score, histologic grade, age, gender, and each of the 4 new biomarkers (see Table 2) were evaluated for prognostic significance. Survival estimates were calculated using the Kaplan-Meier method.[34] Significance levels, estimates of hazard ratios (HR), and their 95% confidence intervals (CI) were calculated using the proportional hazards regression model.[35]

Table 2. Biomarkers in follicular lymphoma

Biomarker	No. failed	No. positive (%)	Morphological pattern	OS *P*	PFS *P*	Comments
CD4$^+$ cells	2	78 (76)		NS	NS	Quantity of T cells (+/++/+++)
CD4/CD8 pattern	5	71 (71)	Follicular	NS	.056	Preferential intrafollicular CD4 and perifollicular CD8 localization
CD25$^+$ cells	6	51 (52)		NS	NS	> 200 cells/core (100X)
CD25 pattern	6	40 (40)	Follicular	.024	.002	Preferential intrafollicular or/and perifollicular localization
FOXP3$^+$ cells	3	34 (33)		NS	NS	> 300 cells/core (100x)
FOXP3 pattern	3	38 (36)	Follicular	< .001	.001	Preferential intrafollicular or/and perifollicular localization

OS indicates overall survival; PFS, progression-free survival; and NS, not significant.

Results

Clinical characteristics and outcome

A total of 102 patients were included in this study, representing the FL patients from the original clinical cohort (n = 126) with available blocks, sufficient tissue remaining in the blocks, and successful TMA construction and interpretation (FOXP3). The median age of the evaluable patients was 45 years (range, 19-61 years). There were 50 women and 52 men. The distribution of IPI scores included 62 patients with IPI scores of 0/1 (group 1), 39 with IPI score of 2/3 (group 2), and only 1 patient with an IPI score of 4/5 (group 3). For analysis purposes, the single patient in IPI group 3 was included with IPI group 2 to eliminate a clinical group with only 1 patient. The median follow-up of living patients (n = 51) was 17.1 years. The median OS was 15.5 years. The IPI was highly significant as a predictor of OS (HR = 2.4, 95% CI = 1.4-4.2, $P = .001$) and PFS (HR = 2.0, 95% CI = 1.2-3.3, $P = .006$), but not risk of transformation.

Pathology variables

Some cores (< 6%, variable by biomarker) were lost during the preparation of the sections or were not interpretable due to poor fixation and/or inadequate staining. Histologic grading of FL subtypes according to the WHO classification included 80 grade 1, 16 grade 2, and 6 grade 3A FL. Histologic grade, the presence of marginal zone differentiation, the proliferation rate (Ki-67), and the patterns of T cells (total CD3, total CD7, CD4 vs CD8 predominance, CD57 content, and architectural distribution) and follicular dendritic cell patterns had no effect on survival (data previously published).[7] Results of the new biomarkers (CD4 vs CD8 distribution, CD25, and FOXP3) are summarized in Table 2.

The total number of any individual T-cell population analyzed as well as the CD4 pattern showed no impact on survival. Follicular versus diffuse pattern of distribution of CD4 versus CD8$^+$ cells was associated with PFS only (HR = 0.54, 95% CI = 0.29-1.0, $P = .05$), and CD25 pattern was significant for both PFS (HR = 2.4, 95% CI = 1.4-3.9, $P = .001$) and OS (HR = 1.8, 95% CI = 1.1-3.1, $P = .024$) but not risk of transformation. Only the FOXP3 score had a significant impact on PFS, OS, and risk of transformation (Table 2). As described in "Methods," cases with either a follicular pattern (n = 20) or a perifollicular pattern (n = 18) were grouped together as Follicular FOXP3, and the remaining cases were classified as Diffuse (n = 64; see Figure 1). Cases with a follicular versus diffuse FOXP3$^+$ pattern of distribution had a median OS of 7.13 years and not reached, respectively (HR = 3.1, 95% CI = 1.8-5.4, $P < .001$; Figure 2), and a median PFS of 2.2 and 8.8 years, respectively (HR = 2.7, 95% CI = 1.6-4.6, $P = .001$). Finally, cases with follicular versus diffuse patterns had median risks of transformation of 13.3 years and not reached, respectively (HR = 3.7, 95% CI = 1.5-8.9, $P = .004$; Figure 3). Histologic grade, IPI, and bone marrow involvement were evenly distributed between these 2 groups (Table 3). Interestingly, all but 1 case with a high content of LAM had a FOXP3$^+$ follicular pattern and a follicular pattern of CD25$^+$ cells. The pattern of CD25$^+$ and FOXP3$^+$ cells was significantly correlated (Table 3). A multivariate proportional hazards model that included the FOXP3$^+$ immunoarchitectural pattern, the IPI, and the LAM content showed that

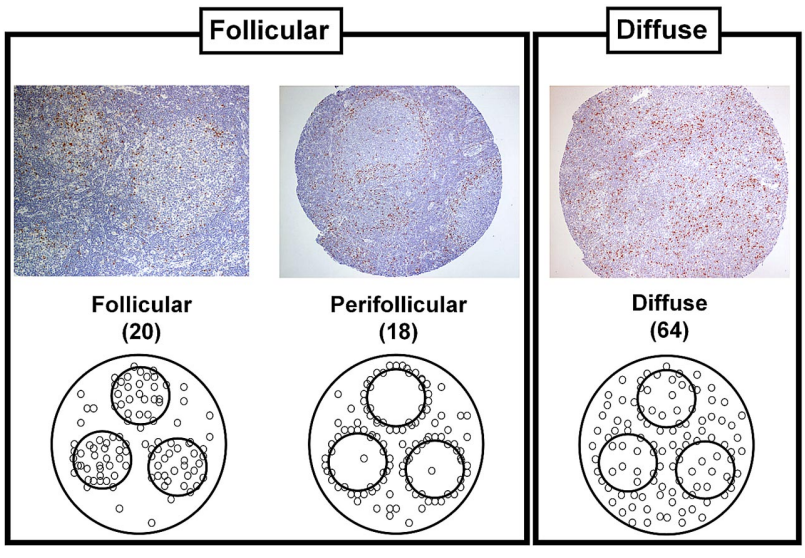

Figure 1. Representative tissue microarray cores of follicular lymphoma stained for FOXP3. The images on the left and center show the follicle-based patterns with FOXP3 cells present mostly within the follicle or around the follicle and in the mantle zone, respectively (n = 38). On the right, the image shows a diffuse case with no follicle-centered pattern defined (n = 64). Microscope: Nikon Eclipse E600; digital camera: Dxm1200.

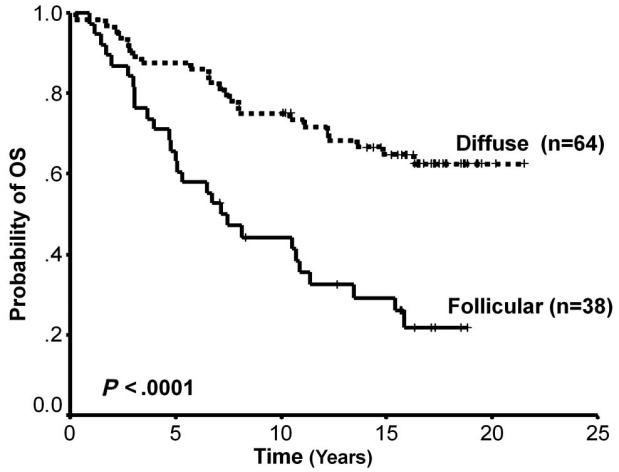

Figure 2. Overall survival curve based on FOXP3 immunoarchitectural patterns. The top curve represents cases with a diffuse pattern and the bottom curve those cases with a follicular pattern.

FOXP3$^+$ pattern and IPI are independent predictors of OS (HR = 2.9, 95% CI = 1.7-5.1, $P = .001$ and HR = 2.1, 95% CI = 1.1-3.7, $P = .008$, respectively). Interestingly, in the multivariate Cox model for risk of transformation only FOXP3 positivity showed significance (HR = 3.9, 95% CI = 1.5-9.9, $P = .004$).

Discussion

It has been almost a century since immunosurveillance was proposed as a mechanism responsible for the repression of cancer growth.[36] Since then, our understanding of tumor immunobiology has increased indicating that immunosurveillance represents 1 dimension of the complex relationship between the immune system and cancer.[37] It is now a widely held idea that the growth of tumors is influenced by nonneoplastic cells of the immune system. These cells are thought to exert selective pressure on the malignant cells. Ultimately, the malignant cells escape immune recognition and destruction and much like tolerance to self-antigens, tolerance to tumor-associated antigens may arise and permit or even promote disease progression.[37]

Tregs were initially defined as a subset of suppressor T cells that mediate immune tolerance by inhibiting autoreactive T cells.[38] Naturally occurring Tregs play a vital role in maintenance of tolerance to self-antigens and immunologic homeostasis through the antigen-specific suppression of effector CD4$^+$ and CD8$^+$ T cells.[12] However, mechanisms whereby Tregs, including peripherally induced cells, bring about the inhibition of other T-cell subtypes are still not well understood.[30] They include direct suppression by cell contact and/or cytokine production, as well as inhibition of antigen-presenting cell function. The T-cell receptor repertoire of Treg cells is as broad and diverse as that of other CD4$^+$ T cells, but it is skewed toward recognizing complexes of self-peptides and major histocompatibility complex.

Many of the tumor-associated antigens recognized by autologous T cells in cancer are antigenically normal self constituents. This suggests that Tregs normally engaged in the maintenance of self-tolerance may also suppress immunosurveillance against autologous tumor cells. Depletion of these Treg cells before a tumor challenge encourages effective immune responses to tumors in mouse models.[39] In mice with advanced tumors, including lymphoma, the administration of anti-CD25 or intratumoral agonistic anti-GITR antibodies reduces tumor-infiltrating Foxp3$^+$CD25$^+$CD4$^+$ Tregs and results in enhanced antitumor immunity with a significant decrease of the tumor burden without causing autoimmune disease.[40,41] In humans, T cells reactive to tumor-associated antigens can expand and become detectable in the peripheral blood when Tregs are first depleted.[13,42] Tregs accumulate within different types of tumor-draining lymph nodes and the peripheral blood.[16-20] FOXP3$^+$ T cells present in the tumor microenvironment contribute to the growth of the tumor, and their presence is associated with unfavorable prognosis in patients with ovarian carcinoma.[20] This study by Curiel et al highlights the importance of the chemokine network in the recruitment and function of these cells. Decreased CCL22 produced by the tumor cells and/or tumor-associated macrophages reduced the recruitment of the Tregs that characteristically express the CCL22 cognate receptor CCR4.

In reactive lymphoid tissues, FOXP3$^+$ Tregs are found mostly at the borders between T and B cells and within germinal centers where they suppress T cells as well as B-cell responses through both inhibition of immunoglobulin class switch recombination[43] or generation of "Treg epitopes" identified in the Fc region of the immunoglobulin molecule itself.[44]

Intratumoral CD4$^+$ T cells (CD25$^+$ or CD25$^-$) expressing FOXP3 are increased in FL compared with reactive lymph nodes.[25] These FOXP3$^+$ cells are either attracted (CCL22) or induced locally by lymphoma cells through cell contact without stimulation of TCR but by expressing CD70.[25,28,45] These FOXP3$^+$ cells suppress intratumoral CD4$^+$ or CD8$^+$ T cells either through TCR activation, cell contact, or by producing TGFβ.[25,27,28] In a small series of FL cases purified using fluorescence-activated cell sorting, we found that CCL22 was one of the chemokine genes most highly expressed by the malignant B cells (R.D.G., unpublished observation, 2008). Moreover, Tregs have also been shown to induce alternatively activated macrophages, the so-called M2 type,[46] which are also associated with protumoral immunity. Finally, in combination with CpG vaccination, antibody-mediated T cell modulation, mostly by depletion of Tregs through antifolate receptor 4 antibodies or functionally blocking them using anti-GITR, anti–cytotoxic T lymphocyte–associated antigen 4, or antiprogrammed death-ligand 1 antibodies, correlates with a significant reduction of

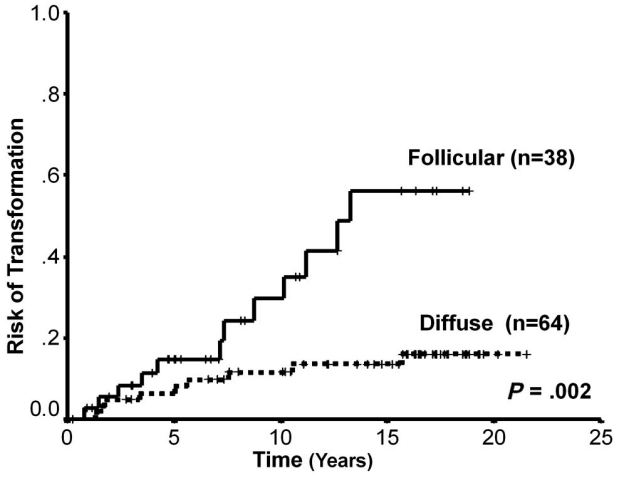

Figure 3. Risk of transformation curve based on FOXP3 immunoarchitectural patterns. The top curve represents cases with a follicular pattern and the bottom curve those cases with a diffuse pattern.

Table 3. Distribution of clinical and pathology variables between follicular and diffuse pattern of FOXP3$^+$ cells

Feature	No. patients with follicular FOXP3$^+$ cells, (%)	No. patients with diffuse FOXP3$^+$ cells (%)	Total (%)	*P*
No.	38 (37)	64 (63)	102 (100)	
Clinical features				
Median age (y)	45	45		.9
Female (%)	15 (40)	37 (58)		.6
Median follow-up (y)	17	16		.011
IPI				
Group 1 (0/1)	19 50)	43 (67)	62 (61)	
Group 2 (2/3)	19 (50)	20 (32)	39 (38)	
Group 3 (4/5)	0 (0)	1 (1)	1 (1)	.14
BM involvement	22 (60)	29 (45)	52 (51)	.3
Treatment response				
CR	18 (47)	40 (63)	58 (57)	
PR	19 50)	22 (34)	41 (40)	
NR	1 (3)	2 (3)	3 (3)	.3
Pathology features				
FL Grade				
Grade 1	29 (76)	51 (80)	80 (78)	
Grade 2	6 16)	10 (16)	16 (16)	
Grade 3	3 (8)	3 (4)	6 (6)	.8
FOXP3 cells, MD (SD)	312 (145)	200 (125)		< .001
LAM > 15 cells HPF	11	1	12 (12)	< .001
CD4/8 pattern (F)	29	40	69 (68)	.2
CD25 pattern (F)	31	7	38 (37)	< .001
Transformation	12 (32)	9 (14)	20 (20)	.04

BM indicates bone marrow; CR, complete remission; PR, partial remission; NR, no response; MD, median; IPI, International Prognostic Index; LAM, lymphoma-associated macrophages; HPF, high-power field; and F, follicular pattern.

*IPI group 1 versus 2/3.

†CR versus PR/NR.

‡Grade 1 versus 2/3.

tumor burden both in mouse models and a phase 2 clinical trial in patients with recurrent FL.[47,48]

In concordance with most published data, including mouse models and several different primary human tumors as well as in vitro and functional data in FL, where Tregs are associated with a protumoral immunity, we found a follicular pattern of FOXP3$^+$ Tregs to be associated with inferior survival in FL. Importantly, this follicular pattern also predicted an increased risk of transformation. Superficially, this finding appears to contradict previous retrospective studies of the survival impact of Tregs in FL using TMAs and immunohistochemistry.[8,31] However, in contrast to those studies, our analysis found the distribution of Tregs to be more important than their numbers. In addition, our patients received uniform therapy, thus holding this important variable constant. Recent data convincingly demonstrate the dramatically variable impacts that different therapies have on the nonneoplastic immune cells in the microenvironment.[7,9,11,32] Indeed, studies of the microenvironment in FL become nearly impossible to interpret when patients receive very heterogeneous treatments, because the specific chemotherapeutic agents, radiation, and biologic agents differentially impact immune cells. As was shown in several recent series studying the impact of LAM in FL, the specific therapeutic regimens used decisively influenced the prognostic impact of nonneoplastic cells in the microenvironment.[7,9-11,32]

In FL, where the content and distribution of various immune-related cells is markedly heterogeneous, we reasoned that the topographic distribution of Tregs relative to the neoplastic follicle might reflect their function and thus their clinical impact. Qualitative (architectural patterns) or semiquantitative biomarkers (cell counts) such as those measured in this study are difficult to define precisely and may be difficult to reproduce.[49] However, the use of TMAs and immunohistochemistry at least partially overcomes this difficulty and has been shown to provide a reliable strategy to assess large numbers of clinical samples and validate novel biomarkers discovered by gene expression profiling.[7,50]

In this study we analyzed Tregs within FL biopsies using 3 markers known to be characteristically expressed by Tregs: CD4, CD25, and FOXP3. As previously described, Tregs belong to the CD4$^+$CD25$^+$ T-cell subset. However, Tregs can be CD25$^-$, and CD8$^+$CD25$^+$ T cells can exhibit regulatory activity.[51] FOXP3 is the single best marker of activation of Tregs.[21] Simple enumeration of cells identified using these markers showed no impact on survival or risk of transformation. However, we found that a follicular distribution of these CD25$^+$ or/and FOXP3$^+$ cells strongly correlates with outcome (Table 3). Interestingly, the survival associations in our cohort followed a pattern linked to specificity for the regulatory phenotype: CD4/8 pattern had an impact just on PFS, CD25 showed significance for PFS and OS, and FOXP3 had an impact on PFS, OS, and, importantly, risk of transformation.

This impact of either follicular or perifollicular FOXP3$^+$ cells suggests a functional suppression of immunosurveillance within the tumor microenvironment that is present at the time of diagnosis. The malignant cells may have the capacity to "recruit" or "skew" Treg activity toward the suppression of antitumoral T cells, thus allowing the malignant cells to "escape" from the regulatory activity.[37] Interestingly, 11 of 12 cases with a high content of LAMs in this series had a follicular FOXP3 pattern. Both Tregs and macrophages may cooperate, in a subset of cases, providing the neoplastic B cells with trophic and survival signals that promote tumor progression, the accumulation of further genetic damage, and transformation.

In summary, the immunoarchitectural pattern of Tregs within lymph nodes involved with follicular lymphoma predicts both

survival and risk of transformation in patients treated uniformly with an aggressive combination regimen including multiagent chemotherapy (BP-VACOP) and involved region radiation. These findings require validation in other patient cohorts, but they will likely only be meaningful if treatment is held constant. Previous publications analyzing patients treated with heterogeneous regimens may have shown discrepant results because of the differential effect of these therapies on neoplastic B cells versus nonneoplastic immune cells in the microenvironment. Moreover, these findings will need to be validated in the current era of immunochemotherapy, again requiring uniform treatment and lengthy follow-up best performed in the context of phase 3 clinical trials.

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Authorship

Contribution: P.F. designed and performed research, analyzed data, and wrote the paper.; A.A.-T., K.G., and R.K. designed and performed research; and J.M.C. and R.D.G. designed and performed research and wrote the paper.

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Vascularization predicts overall survival and risk of transformation in follicular lymphoma

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ABSTRACT

Follicular lymphoma patients display heterogeneous overall survival and variable risk of transformation. Recent studies have highlighted the role of the microenvironment. The contribution of microvessel density to follicular lymphoma survival remains controversial. We used a quantitative tumor mapping approach to determine whether the degree of vascularization correlated with outcome in a uniformly treated cohort. Whole-tissue sections of diagnostic biopsies from 84 cases were stained for CD34 and tumor-to-vessel-distance that encompassed 90% of the tumor (TVD_{90}) was determined using image analysis. Twenty-one cases with lower TVD_{90} showed inferior overall survival (P=0.0001) and high risk of transformation (P=0.01). These cases significantly correlated with increased Lymphoma-Associated Macrophages (χ^2=0.025). In multivariate analysis macrophages content, IPI and TVD_{90} were independent predictors of overall survival (P=0.05, P=0.001 and P=0.01, respectively) and IPI and TVD_{90} predicted risk of transformation (P=0.008 and P=0.08, respectively). Increased angiogenesis is an independent marker of inferior survival and may promote transformation.

Key words: follicular lymphoma, vascularization, prognostication.

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Introduction

The clinical course of follicular lymphoma (FL) is unpredictable, with median survivals in the range of ten years. Histological transformation, usually into diffuse large B-cell lymphoma (DLBCL), heralds aggressive clinical behavior and is typically associated with inferior survival. A recent population based analysis from the British Columbia Cancer Agency (BCCA) including 600 newly diagnosed FL patients showed an annual risk of transformation (RT) of 3% continuing beyond 15 years of follow up.[1] The treatment of FL patients is markedly heterogeneous, as no optimal treatment approach has yet been established. The number of prior treatments, tumor burden, advanced-stage and high-risk FLIPI or IPI are the only established clinical features associated with RT.[2] Importantly, none of the clinical variables consistently predict RT. Thus, biological predictors of transformation are needed to individualize therapy and better assess risk. Angiogenesis plays a crucial role in oncogenesis, promoting growth and progression of both solid and hematologic tumors.[3,4] The acquisition of an angiogenic phenotype, referred to as the "angiogenic switch", allows the formation of neovessels that are vital for tumor growth.[5] This "angiogenic switch" results not only from interactions between vessels and cancer cells, but also involves non-neoplastic cells in the microenvironment, including macrophages. Microvessel density (MVD) has, in some tumor types, shown a correlation with survival. Yet, in FL the impact of MVD on prognosis is controversial.[6,7] In most series the number of cases studied is small and/or the therapies markedly heterogeneous, both of which preclude definitive conclusions. In this study, we report the clinical significance of MVD in uniformly treated FL patients using a quantitative tumor mapping approach.

Design and Methods

Patients

Between July 1987 and May 1993, patients with FL were enrolled in a single institution phase II trial using BP-VACOP chemotherapy (bleomycin, cisplatin, etoposide, doxorubicin, cyclophosphamide, vincristine and prednisone) followed by involved field irradiation to sites of original nodal involvement. Patients aged 16 to 61 years, with newly diagnosed, treatment-naïve and advanced-stage disease, defined as Ann Arbor stage III or IV, or stage II with B symptoms, non-

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radioencompassable disease, or bulk of 10 cm or more in maximum diameter at any individual tumor site were included. Approval to this study was given by the University of British Columbia BCCA Research Ethics Board. All biopsies were reviewed and classified according to the 2008 WHO classification.[8] Transformation was defined as biopsy proven or clinically diagnosed aggressive lymphoma as described previously.[1]

Histology and immunohistochemistry

Of the 126 cases of FL, 84 had formalin-fixed paraffin blocks with adequate material remaining in the block to be used for whole sections. Immunohistochemistry was performed routinely for CD20 (L26, dilution 1:800, Dako®, Carpentaria, California, USA) and CD34 (QBEend10, dilution 1:30, Dako®, Carpentaria, California, USA) using a Dako® autostainer and the EnVision polymer detection system. $CD68^+$ cell content biomarker, Lymphoma-Associated Macrophages (LAM) data were based on a previous study.[9]

Microvessel density and follicle size

Images of whole sections were stained for CD34 and captured using a cooled CCD camera, a motorized stage and customized NIH software.[10] Thresholding was applied to identify CD34 positive objects. The motorized stage allowed for tiling of adjacent microscopic fields, thereby allowing reassembly of the entire tumour section at high resolution (Figure 1). High-resolution images of tumor vasculature over the entire whole-sections of each case defined "distance maps". The distance from each tumor cell in the tissue to the nearest $CD34^+$ pixel was measured automatically and the mean of distances of all cells to the nearest vessel in the whole section was used to calculate the tumour-to-vessel distance that encompassed 90% of the tumor (TVD_{90}). Average follicle size per case was visually ranked according to the following groups: 0 - loss of follicular pattern; 1 - small follicles (dense staining), and 2 - large follicles (sparse staining).

Statistics and survival analysis

Overall survival (OS) was defined as the interval from date of diagnosis until death from any cause. Survival estimates were calculated using the Kaplan-Meier method[11] and multivariate analysis using the proportional-hazards regression model.[12]

Results and Discussion

There were 84 suitable cases for analysis. The median follow up of the living patients was 14.3 years and 20 patients underwent transformation. The IPI was significant for both OS and risk of transformation (RT) in univariate analysis (P=0.0001 and P=0.01, respectively). Estimated 10-year OS and RT for all patients were 65% and 20%, respectively. TVD_{90} ranged from 50.3 to 144 microns (median 78.4) and these data were divided into quartiles. In univariate analysis the quartile (21 cases) with low TVD_{90} (i.e. high MVD) showed inferior OS (P=0.0001) and an increased RT (P=0.01) (Figure 2). These 21 cases are defined as Low TVD_{90} while the three other quartiles (63 cases) are defined as High TVD_{90}. Clinical characteristics are summarized in Table 1. As most cases showed a distinct perifollicular pattern, we correlated TVD_{90} with follicle size in each case. There was a significant association between low TVD_{90} and small follicle size (χ^2, P=0.01). Low TVD_{90} also correlated with younger age (χ^2, P=0.034), increased IPI (χ^2, P=0.01) and increased $CD68^+$ macrophages (χ^2, P=0.025). In univariate analysis, TVD_{90} and IPI are significant for both OS and RT, but not age or follicle size. In this cohort, $CD68^+$ macrophages predicted OS (P=0.0004), but not RT (P=0.38). In a Cox's model including TVD_{90}, $CD68^+$ and IPI, all three markers were independent variables for OS, TVD_{90} (RR=2.5, 95% CI=1.1-5.0, P=0.01), $CD68^+$ (RR=2.2, 95% CI=1.0-5.0, P=0.05) and IPI (RR=2.9, 95% CI=1.5-5.5, P=0.001). Finally, in a Cox's model for RT, only IPI significantly pre-

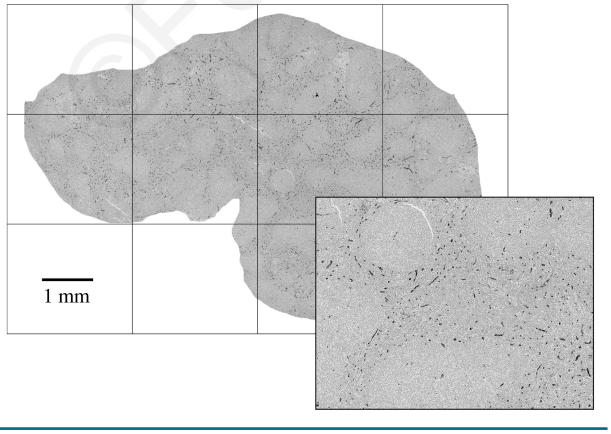

Figure 1. High-resolution image of a CD34 immunostained section allowing the identification of tumor vasculature across the entire section.

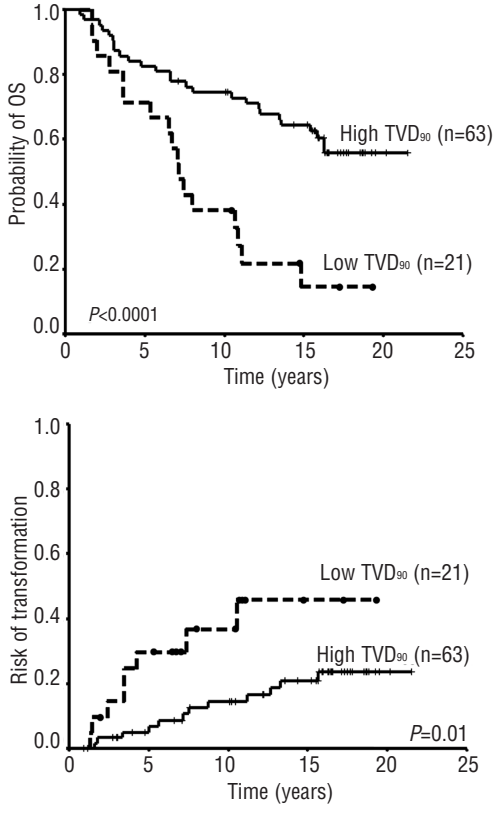

Figure 2. Tumor-to-vessel distance including 90% of malignant cells showing Overall Survival (OS) and Risk of Transformation (RT).

dicted RT (P=0.008), while TVD_{90} was of borderline significance (P=0.08). Angiogenesis plays a crucial role in the growth and progression of human solid tumors.[13] In most tumor types, increased MVD correlates with increased disease progression and decreased survival. Similar results have been reported in hematopoietic tumors, including multiple myeloma and lymphoma.[3,4,14,15] In murine lymphoma models and in lymphoma patients, circulating endothelial cells and serum vascular endothelial growth factor (VEGF) levels appear to correlate with lymphoma volume and increased angiogenesis.[16,17] In contrast, previous studies of MVD in FL have resulted in conflicting results. Koster *et al.* showed increased MVD to be associated with a more favorable OS in a series of 36 uniformly treated patients given CVP and interferon (IFN) α2b followed by IFN maintenance.[6] In a later report, Jorgensen *et al.* analyzed 107 FL cases with heterogeneous treatments and found increased interfollicular MVD predicted inferior OS and increased transformation to DLBCL.[7] In both studies, MVD was calculated using quantification of focal "vessel hot spots" within the tumor. In FL, however, the vessels show a heterogeneous distribution. Therefore, in order to avoid a scoring bias, we studied whole sections of FL biopsies and quantified automatically the average distance from CD34 stained vessel that included 90% of tumor cells: TVD_{90}. We believe this is a more accurate measurement of vessel density. Because of vessel predominance within the interfollicular and perifollicular areas, TVD_{90} was correlated with the average size of the follicles in each case. Interestingly, cases with low TVD_{90} correlated with small sized follicles although follicle size itself did not affect survival or risk of transformation.

In the current study, all 84 patients were treated uniformly with multi-agent chemotherapy and radiation, while in the series by Koster *et al.* all 36 patients were treated with CVP chemotherapy and IFN followed by IFN maintenance. In addition to the difference in cohort size, different therapeutic regimens may explain the contradictory results. IFN has both immunomodulatory and anti-angiogenic effects and thus may have been more effective in tumors with increased MVD.[18] Radiation has been shown to induce tumor cells to secrete cytokines capable of inhibiting apoptosis in endothelial cells, thereby diminishing treatment response.[19,20] It remains possible that this treatment modality influenced survival and transformation risk in our study.

It is well known that angiogenesis in cancer is critically influenced by the local tumor microenvironment.[21] Using the same uniformly treated cohort, we previously showed that increased numbers of Lymphoma-Associated Macrophages (LAM) is associated with adverse outcome.[9] Similar to solid tumor-associated macrophages, these LAM, possibly originating from bone marrow derived myeloid cells, may be attracted by hypoxia and tumor-derived chemotactic factors and show a distinct phenotype that promotes angiogenesis.[22-24] Consistent with this hypothesis, we show a significant association between TVD_{90} and LAM.

In summary, this study confirms the clinical relevance of increased angiogenesis affecting both FL survival and transformation risk in a series of advanced-stage FL patients uniformly treated with chemotherapy and radiotherapy. These findings suggest MVD as a useful biomarker in initial therapeutic decisions of patients with FL, and thus may provide a rationale for trials of anti-angiogenic therapy in FL patients with increased MVD.

Table 1. Distribution of clinical and pathology variables between high TVD90 and low TVD90 cases.

Feature	# Patients with Low TVD 90 (%)	# Patients with High TVD 90 (%)	Total (%)	*P* value (χ^2)
Number	21 (25)	63 (75)	84 (100)	-
Clinical Features				
Median age (y)	39	46	-	0.034
Female (%)	52	48	-	0.63
Median follow up (y)	13.9	14.3	-	0.83
IPI Group 1 (0/1)	8 (38)	45 (71)	62 (61)	0.01*
Group 2 (2/3)	13 (62)	17 (27)	39 (38)	
Group 3 (4/5)	0 (0)	1 (2)	1 (1)	
BM Involvement (%)	14 (66)	28 (44)	42 (50)	0.13
Treatment Response				
CR	8 (38)	39 (62)	47 (56)	0.08**
PR	13 (62)	23 (36)	36 (43)	
NR	0 (0)	1 (2)	1 (1)	
Pathology Features				
FL Grade				
Grade 1	17 (80)	49 (78)	66 (78)	0.76***
Grade 2	4 (20)	10 (16)	14 (17)	
Grade 3	0 (0)	4 (6)	4 (5)	
Large follicles (%)	1 (5)	20 (32)	21 (25)	0.01
Increased CD68+ cells (%)	5 (24)	4 (7)	9	0.025
Transformation (%)	8 (38)	12 (19)	20(24)	0.076

*IPI: International Prognostic Index score, BM: bone marrow, CR: complete remission, PR: partial remission, NR: no response; *IPI group 1 vs. 2/3 **CR vs. PR/NR ***Grade 1 vs. 2/3A.*

Authorship and Disclosures

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Correlations between *BCL6* rearrangement and outcome in patients with diffuse large B-cell lymphoma treated with CHOP or R-CHOP

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ABSTRACT

Background

BCL6 gene rearrangement is the most frequent chromosomal abnormality in diffuse large B-cell lymphoma, a malignancy characterized by genetic heterogeneity and wide variability in clinical outcome. The prognostic significance of *BCL6* rearrangement has not been evaluated in the context of rituximab therapy for diffuse large B-cell lymphoma. We analyzed the effect of the *BCL6* rearrangement on survival in patients with diffuse large B-cell lymphoma treated with CHOP and CHOP plus rituximab (R-CHOP).

Design and Methods

BCL6 rearrangement status was analyzed by fluorescence *in situ* hybridization with break-apart probes in 164 patients with diffuse large B-cell lymphoma treated with CHOP (n=65) or R-CHOP (n=99). Cell-of-origin immunophenotype including BCL6 protein expression were determined by immunohistochemistry on a tissue microarray.

Results

BCL6 rearrangement was detected in 19.5% of cases. The presence of the gene rearrangement was associated with a non-germinal center B-cell immunophenotype (P=0.006), and showed no correlation with BCL6 protein expression. A trend toward inferior overall survival was observed in association with the *BCL6* rearrangement among patients treated with R-CHOP (P=0.08), but not among patients treated with CHOP (P=0.64). However, *BCL6* rearrangement also correlated with a high International Prognostic Index score (P=0.02), and did not demonstrate independent prognostic value by multivariate analysis.

Conclusions

The introduction of rituximab may have altered the prognostic impact of *BCL6* gene rearrangement in patients with diffuse large B-cell lymphoma. However, prospective analysis within large randomized clinical trials will be needed to clarify the prognostic significance of this biomarker in the rituximab era.

Key words: diffuse large B-cell lymphoma, biomarkers, *BCL6*, FISH, rituximab.

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Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin's lymphoma, accounting for 30-40% of newly-diagnosed cases.[1] Although curable in the majority of cases with anthracycline-based combination chemotherapy and the monoclonal antibody rituximab, approximately 30-40% of patients with DLBCL will relapse after standard first-line therapy.[2,3] This variability in clinical outcome likely relates to genetic heterogeneity within DLBCL, reflected in a wide array of cytogenetic and molecular abnormalities. Recently, microarray gene expression studies have identified multiple genes of potential prognostic significance in DLBCL, and have led to the subdivision of DLBCL into two major biological categories based on their presumed cell of origin: germinal center B-cell (GCB), and activated B-cell (ABC).[4] However, the prognostic value of genetic markers in DLBCL remains controversial, and these have not yet been incorporated into routine clinical practice.[5]

Rearrangement of the *BCL6* proto-oncogene at chromosome band 3q27 is the most frequent cytogenetic abnormality in DLBCL, occurring in up to 35% of cases.[6-8] The *BCL6* gene, a zinc-finger transcription factor, may be translocated with diverse partners in DLBCL, including both immunoglobulin (*IGH*, *IGK*, *IGL*) and non-*IG* loci.[9,10] Clinical studies investigating the prognostic impact of *BCL6* rearrangement in DLBCL have yielded contradictory results, variably demonstrating favorable,[6] intermediate,[7,8,11] and adverse outcomes[12,13] in association with this abnormality.

An association has recently been reported between *BCL6* rearrangement and ABC phenotype.[14] This cell-of-origin profile was initially demonstrated as an adverse biological marker in DLBCL patients treated with CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone), and has been shown to retain its predictive impact in patients treated with CHOP plus rituximab (R-CHOP).[15]

The introduction of rituximab to standard first-line therapy has significantly improved clinical outcome in DLBCL, and may alter the prognostic impact of both clinical and biological markers in this disease.[2,3] The prognostic significance of *BCL6* rearrangement has not been re-evaluated since the introduction of rituximab into DLBCL therapy. In this study we used tissue microarray-based fluorescence *in situ* hybridization (FISH) to analyze *BCL6* rearrangement status in a retrospective cohort of patients with DLBCL. The objectives of this study were to: (i) compare the effect of *BCL6* rearrangement on survival in DLBCL patients treated with CHOP and R-CHOP, and (ii) evaluate the relationship between *BCL6* rearrangement and other clinical and biological prognostic variables in this disease, including cell-of-origin phenotype.

Design and Methods

Patients' samples

We included all patients identified through the Centre for Lymphoid Cancer database of the British Columbia Cancer Agency (BCCA) who met the following criteria: (i) confirmed diagnosis of DLBCL (excluding primary mediastinal B-cell lymphoma) following a pathology review; (ii) treated with either CHOP alone or in combination with rituximab immunotherapy (R-CHOP); (iii) available diagnostic paraffin material on a tissue microarray; (iv) negative for human immunodeficiency virus; (v) treated at the BCCA between 1999 and 2007. Ethical approval for this study was obtained from the University of British Columbia – BCCA Research Ethics Board.

Fluorescence in situ *hybridization and immunohistochemistry on tissue microarrays*

Archived, formalin-fixed, paraffin-embedded, diagnostic biopsy specimens were selected for construction of the tissue microarrays and 0.6 mm duplicate cores were obtained from representative areas containing large B cells with typical morphology. FISH was performed according to a standard protocol for paraffin-embedded material, described elsewhere,[14,16] using commercially available Vysis LSI *BCL6* Dual Color, Break-Apart Rearrangement Probes (Abbott Molecular, IL, USA). Cases were recorded as having *BCL6* rearrangement (*BCL6*$^+$) if break aparts occurred in at least one signal of more than 5% of nuclei. All other signal constellations were regarded as negative (*BCL6*$^-$). Immunohistochemical analysis was performed using monoclonal antibodies for BCL2 (Dako, clone 124, Denmark), BCL6 (Ventana Medical Systems, Tucson, Arizona), CD10 (Ventana Medical Systems, Tucson, Arizona), and MUM1 (Dako, Denmark), following routine protocols for automated immunohistochemistry on the Ventana Benchmark XT (Ventana Medical Systems, Tucson, Arizona). Cases were then categorized as GCB-like or non-GCB using a standard algorithm.[17] Accordingly, for each case, the core with the highest percentage of tumor cells stained was used for analysis. Cases were considered positive if 30% or more of the tumor cells were stained with an antibody.

Statistical analysis

Group comparisons were performed by means of χ^2 and Student's *t* tests. For time to event analyses we used SPSS software version 11.0.0, applying Kaplan-Meier survival estimates and univariate and multivariate Cox proportional hazard models with the end-point of overall survival, defined as the time from initial diagnosis to death from any cause. *P* values less than 0.05 were considered statistically significant.

Results

Patients' characteristics

A total of 174 patients with DLBCL who met all inclusion criteria were identified. FISH was successfully performed in 164 out of these 174 cases (94.3%). *BCL6* rearrangement was detected in 32 out of the 164 cases (19.5%). Representative *BCL6*$^+$ FISH images of the tissue microarray sections are shown in Figure 1A.

The baseline clinical characteristics of the patients, divided according to *BCL6* rearrangement status, are listed in Table 1. There were no significant differences in initial treatment regimen between the two subgroups, with similar proportions of *BCL6*$^+$ (15/32) and *BCL6*$^-$ (84/132) patients receiving rituximab as part of their initial therapy. A higher proportion of patients in the *BCL6*$^+$ subgroup had a high-intermediate or high-risk International Prognostic Index (IPI) score (IPI 3-5) (62.5% *versus* 40.2%, *P*=0.02). Stage IE/IIE cases were designated as 'primary extranodal

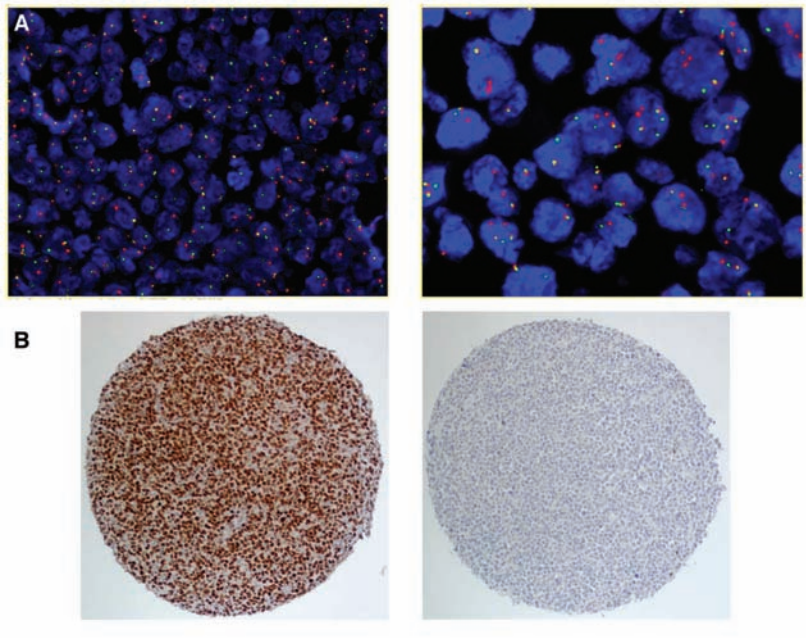

Figure 1. (A) Fluorescence *in situ* hybridization on formalin-fixed, paraffin-embedded tissue sections on a tissue microarray. Left panel: interphase nuclei with *BCL6* break apart (*BCL6*$^+$). The most common signal pattern is one fusion signal (yellow) and two split signals (1 red, 1 green) visualized at 200x magnification. Right panel: *BCL6*$^+$ interphase nuclei with break apart (split signals) and polyploidy (multiple fused signals) of the *BCL6* locus (high power field). **(B)** Immunohistochemistry for BCL-6 on tissue microarray cores. Left panel: positive staining in virtually all large B cells. Right panel: negative staining.

disease', while all other cases were considered as 'primary nodal disease'. The frequency of primary extranodal disease was not significantly different between the *BCL6*$^+$ and *BCL6*$^-$ subgroups (13% *versus* 25%, *P*=NS).

Table 2 shows the patients' immunohistochemical characteristics according to *BCL6* rearrangement status. BCL6 staining and cell-of-origin classification[17] was successfully performed in 162 of the 164 patients. Representative BCL6 staining results are shown in Figure 1B. Results of BCL2 staining were available for 115 of the 164 (70%) patients. The presence of *BCL6* rearrangement was significantly correlated with cell-of-origin; 68% of *BCL6*$^+$ patients had a non-GCB subtype, as compared with 48% of *BCL6*$^-$ patients (*P*=0.006). No association was found between *BCL6* rearrangement and either BCL6 (*P*=0.78) or BCL2 (*P*=0.95) protein expression.

Survival

The median duration of follow-up was 2.9 years (range, 0.3-8.7). Rituximab use was associated with a significant survival improvement among *BCL6*$^-$ patients (5-year overall survival, 70.1% *versus* 45.0%, *P*=0.009), but not among *BCL6*$^+$ patients (5-year overall survival, 45.3% *versus* 41.2%, *P*=0.70). Kaplan-Meier analysis of the entire cohort revealed a trend toward decreased overall survival in patients with a *BCL6* rearrangement, though this did not reach statistical significance (5-year overall survival, 59.4% *versus* 43.4%, *P*=0.07). Within treatment groups, *BCL6* rearrangement was associated with a trend toward inferior overall survival among patients treated with R-CHOP (5-year overall survival, 45.3% *versus* 70.1%; *P*=0.08), but not among patients treated with CHOP (5-year overall survival, 41.2% *versus* 45.0%; *P*=0.64) (Figure 2). No significant association was found between BCL6 protein expression and overall survival in the whole cohort (5-year overall survival, BCL6$^+$ *versus* BCL6$^-$, 52.2% *versus* 31.8%, *P*=0.11).[18]

Subgroup analysis of overall survival was performed according to the site of presentation of disease and the patients' age. Survival analysis limited to patients with primary nodal disease showed that the 5-year overall survival among *BCL6*$^+$ patients was 39.7% compared with 54.7% in *BCL6*$^-$ patients (*P*=0.09). Among patients who presented with primary extranodal disease, no difference in overall survival was found between *BCL6*$^+$ and *BCL6*$^-$ patients (*P*=0.95). Subgroup analysis by patients' age did not reveal statistically significant associations between gene rearrangement status and overall survival in patients under 65 (*P*=0.38) or those over 65 years of age (*P*=0.24).

In R-CHOP-treated patients, high IPI score (IPI 3-5) predicted a significantly worse overall survival (5-year overall survival, 46.1% *versus* 77.8%, *P*=0.002), while non-GCB immunohistochemical profile was associated with a trend toward inferior outcome (5-year overall survival, 50.8% *versus* 76.8%, *P*=0.07). A Cox regression analysis was performed for the R-CHOP-treated cohort using the following four variables: IPI score, cell-of-origin immunophenotype, BCL2 protein expression, and *BCL6* gene rearrangement status. In multivariate analysis, only high IPI score was found to be an independent adverse prognostic factor for overall survival (*P*=0.002).

Discussion

This study analyzed the clinical impact of *BCL6* gene rearrangement in a retrospective cohort of DLBCL patients, treated in both the pre- and post-rituximab eras. Our data suggest an association between *BCL6* rearrangement and inferior outcome in patients treated with R-CHOP, but not in patients treated with CHOP. However, *BCL6* rearrangement also correlated with the presence of high-risk clinical features and was not demonstrated to have a prognostic significance independent of the IPI score.

The effect of the *BCL6* rearrangement on outcome in DLBCL has remained uncertain despite previous clinical analyses. In a seminal study by Offit *et al.*, *BCL6* rearrangement was found to be a favorable prognostic marker in DLBCL, possibly due to an association with limited extranodal disease.[6] Most subsequent investigators failed to demonstrate any influence of *BCL6* rearrangement on prognosis in DLBCL, and similarly our analysis did not find that this marker had predictive value in patients treated with CHOP alone.[7,8,11,14] A more recent analysis, restricted to DLBCL patients with primary nodal disease, reported inferior survival in patients with *BCL6* rearrangement, as well as a correlation between this marker and non-GCB immunophenotype.[12] In agreement with our results, Vitolo *et al.* reported a correlation between *BCL6* rearrangement and adverse clinical features,[11] although this has not been a uniform finding in previous studies. These discrepancies may be explained in part by heterogeneity between study populations, due to concomitant genetic alterations, selection biases, and differences in molecular diagnostic techniques. Specifically, rearrangement of the *MYC* oncogene has been found by several authors to impart an inferior prognosis in CHOP-treated DLBCL patients, although this abnormality occurs in only 5-10% of patients[19,20] and *MYC* and *BCL6* rearrangement rarely coexist.[6,8,11]

The addition of rituximab to first-line combination therapy has led to a significant improvement in survival in patients with DLBCL, requiring a re-evaluation of established prognostic markers.[3] Biological prognostic markers in particular may be influenced by changes in standard therapy, because of the differential effects of new agents within biological subgroups. It has recently been reported that BCL2 protein expression, an adverse prognostic marker in CHOP-treated patients, loses its prognostic effect in the rituximab era, due to a disproportionate benefit from rituximab in BCL2$^+$ cases.[21] In our analysis, the addition of rituximab appeared primarily to benefit patients without *BCL6* rearrangement, and was associated with significantly prolonged overall survival in this subgroup. In contrast, little improvement in outcome was observed with the addition of rituximab among *BCL6*$^+$ patients, in whom long-term survival remained less than 50% even with R-CHOP. These data suggest a potential value for *BCL6* rearrangement as an adverse predictive biomarker in DLBCL in the rituximab era.

The IPI remains the most successful clinical prognostic instrument in DLBCL.[22] However, the IPI score appears to lose discriminatory power in R-CHOP-treated patients, and in a recent analysis was unable to identify a subgroup with a less than 50% chance of long-term survival.[3] Biological markers may help to refine risk-stratification in DLBCL, and to identify high-risk subgroups of patients who might benefit from intensified or novel therapies. However, to be clinically useful these markers must provide prognostic information complementary to that provided by the IPI. In our study, the presence of a *BCL6* gene rearrangement correlated with high IPI score, and in multivariate analysis this biomarker did not show independent prognostic significance. *BCL6* rearrangement has also shown an association with non-GCB immunophenotype, previously demonstrated to be an unfavorable prognostic marker in CHOP-treated patients with DLBCL.[17] However, in our cohort of R-CHOP-treated patients, cell-of-origin phenotype did not emerge as an independent prognostic variable in multivariate analysis.

The clinical impact of oncogene rearrangements may also depend on the site of disease presentation. DLBCL arising from extranodal sites differs from nodal DLBCL in terms of pathogenesis and cell of origin, and several distinct subtypes of extranodal DLBCL are recognized in the

Table 1. Clinical characteristics of 164 DLBCL patients, by *BCL6* rearrangement status.

	BCL6$^{+\ ‡}$ (n=32)	*BCL6*– (n=132)	*P* value
Age (years), median (range, sd)	65.5 (35-85, 14.1)	63 (16-92, 14.8)	*P*=NS
Gender (%)			
Male	63	61	*P*=NS
Female	37	39	
Mass size (cm), median	10	6	*P*=NS
(range, sd)	(3-20, 5.4)	(1-25, 5.0)	
Lactate dehydrogenase (%)			
Normal	25	51	*P*=0.007
Elevated	75	49	
Extranodal sites (%)			
0-1	68	75	*P*=NS
>1	32	25	
Site of presentation(%)			
Primary nodal	87	75	*P*=NS
Primary extranodal	13	25	
Stage, Ann Arbor (%)			
I-II	31	43	*P*=NS
III-IV	69	57	
IPI score (%)			
0-2	38	60	*P*=0.022
3-5	62	40	

‡*BCL6+: BCL6 gene rearrangement present; BCL6-: BCL6 gene rearrangement absent; sd: standard deviation.*

Table 2. Immunophenotypic characteristics of 164 DLBCL patients, by *BCL6* rearrangement status.

	BCL6+ (n=32)	*BCL6*$^-$ (n=132)	*P* value
BCL2 protein expression (%)			
Positive	78	71	*P*=NS
Negative	22	29	
BCL6 protein expression (%)			
Positive	69	71	*P*=NS
Negative	31	29	
Cell-of-origin* (%)			
GCB	32	59	*P*=0.006
Non-GCB	68	41	

**According to Hans* et al.[17]

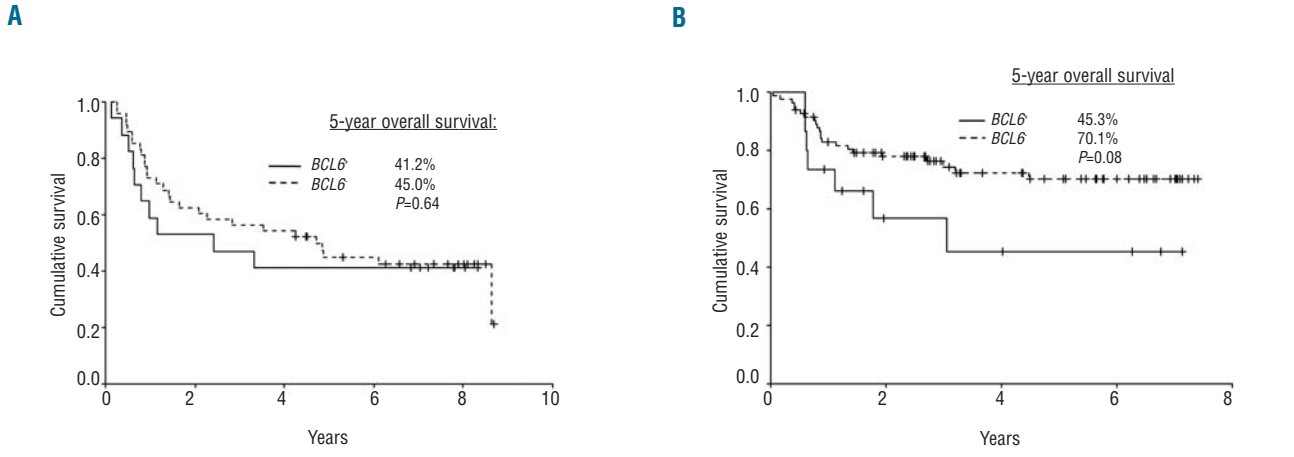

Figure 2. Overall survival according to *BCL6* rearrangement status. (A) CHOP-treated patients (n=65). (B) R-CHOP-treated patients (n=99).

2008 revision of the WHO classification of lymphoid malignancies.[1] Unlike previous groups, we found no correlation between *BCL6* rearrangement and extranodal presentation,[6,8] and no statistically significant difference in overall survival between $BCL6^+$ and $BCL6^-$ patients was demonstrated in either the primary nodal or extranodal subgroup, although this analysis was limited by the small numbers in the two groups.

The *BCL6* proto-oncogene encodes a transcriptional repressor essential for normal germinal center formation. Its deregulated expression has been implicated as an important pathway in lymphomagenesis.[23] BCL6 protein expression has been identified as a hallmark of GCB derivation in DLBCL, and has been associated with a favorable clinical outcome.[17,24] However, in keeping with prior studies, we did not find a correlation between *BCL6* rearrangement and BCL6 protein expression, but demonstrated a relationship between *BCL6* rearrangement and non-GCB phenotype.[12,14] This seemingly paradoxical finding may be related to several factors. First, mechanisms other than gene rearrangement have been shown to deregulate *BCL6* expression in DLBCL, including targeted mutations which may interrupt negative gene autoregulation.[23] Second, the functional effect of *BCL6* rearrangement may vary depending on other biological factors, including the chromosomal translocation partner and cellular context. One group of investigators demonstrated significantly lower BCL6 expression with non-*IGH/BCL6* than with *IGH/BCL6* rearrangements, and also reported inferior survival in DLBCL patients with *non-IGH/BCL6* translocations.[13,25] A more recent study by the Leukemia/Lymphoma Molecular Profiling Project examined the effect of *BCL6* rearrangement on gene expression in patients with both GCB- and ABC-subtype DLBCL.[14] In this analysis, *BCL6* rearrangement was associated with increased *BCL6* mRNA levels in patients with ABC-subtype DLBCL, but not in patients with GCB-subtype DLBCL. The clinical significance of these biological subdivisions remains to be clarified.

In summary, the introduction of rituximab may have altered the prognostic impact of *BCL6* gene rearrangement in patients with DLBCL. However, to be incorporated into clinical practice as a useful prognostic biomarker, *BCL6* rearrangement must be shown to provide prognostic information additional to that afforded by the IPI. *BCL6* rearrangement also correlates strongly with non-GCB immunophenotype, and the interdependence of these two prognostic variables requires further study. Correlative biomarker analysis within prospective randomized clinical trials will be needed to determine the prognostic value of *BCL6* rearrangement in the rituximab era.

Authorship and Disclosures

JS wrote the manuscript, GH and PF performed experiments and analyzed results, NAJ, JMC, LHS and DEH designed the research and approved the manuscript, SB analyzed and reviewed results, RDG and CS analyzed and reviewed results, designed the research and wrote the paper.

The authors reported no potential conflicts of interest.

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MYC gene rearrangements are associated with a poor prognosis in diffuse large B-cell lymphoma patients treated with R-CHOP chemotherapy

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Approximately 5% to 10% of diffuse large B-cell lymphomas (DLBCLs) harbor an *MYC* oncogene rearrangement (*MYC*$^+$). The prognostic significance of *MYC*$^+$ DLBCL was determined in an unselected population of patients with newly diagnosed DLBCL treated with rituximab in combination with cyclophosphamide, doxorubicin, vincristine, and prednisone chemotherapy (R-CHOP). Using a Vysis break-apart fluorescence in situ hybridization probe, 12 of 135 (8.8%) cases of *MYC*$^+$ DLBCL were identified that had no defining high-risk features. *MYC*$^+$ DLBCL was associated with an inferior 5-year progression-free survival (66% vs 31%, *P* = .006) and overall survival (72% vs 33%, *P* = .016). Multivariate analysis confirmed the prognostic importance of *MYC* for both progression-free survival (hazard ratio = 3.28; 95% confidence interval, 1.49-7.21, *P* = .003) and overall survival (hazard ratio = 2.98; 95% confidence interval, 1.28-6.95, *P* = .011). Cases of *MYC*$^+$ DLBCL also had a higher risk of central nervous system relapse (*P* = .023), independent of other risk factors. The diagnosis of *MYC*$^+$ DLBCL is likely underappreciated; and given the lack of defining risk factors, fluorescence in situ hybridization for *MYC* rearrangements should be performed in all patients with DLBCL. In the R-CHOP treatment era, *MYC*$^+$ DLBCLs have an inferior prognosis. Treatment regimens similar to those used in Burkitt lymphoma may be more appropriate in this patient population and need to be prospectively tested. (Blood. 2009;114: 3533-3537)

Introduction

Diffuse large B-cell lymphomas (DLBCLs) are recognized to be a heterogeneous group of diseases with clinical, morphologic, immunohistochemical, and molecular subtypes defined in the updated World Health Organization (WHO) classification.[1] Further, a new category has been created defined as "borderline cases," which are considered B-cell lymphomas, unclassifiable, with features intermediate between DLBCL and Burkitt lymphoma.[2] Morphologically, these tumors typically have a mixture of medium- to large-sized cells, a high proliferation rate, and 35% to 50% of cases have an 8q24/*MYC* translocation.[2] However, approximately 5% to 10% of DLBCLs with typical morphology also harbor an *MYC* rearrangement (herein after referred to as *MYC*$^+$), and these cases are considered in the category of DLBCL, not otherwise specified, in the updated WHO classification.[3]

There is very little information regarding the prognostic importance of an isolated *MYC* rearrangement in DLBCL using modern diagnostic criteria. A recent study suggested that *MYC* gene rearrangements identified by fluorescence in situ hybridization (FISH) in pathologically defined DLBCL patients treated with cyclophosphamide, doxorubicin, vincristine, prednisone (CHOP)–like chemotherapy are associated with an inferior prognosis.[4] However, it is unclear whether there are identifiable clinical or pathologic characteristics that suggest that a case may harbor an *MYC* rearrangement to prompt evaluation. Further, prior studies evaluating the prognostic implications of *MYC* in DLBCL have been performed before the use of rituximab. With studies showing improved outcome using rituximab in combination with CHOP (R-CHOP) or CHOP-like therapies in the treatment of DLBCL,[5-8] the importance of *MYC* rearrangement status in this population must be reestablished.

The purpose of this study was 2-fold: (1) to screen an unselected series of patients with DLBCL for *MYC* rearrangements to determine the frequency of this occurrence and whether there were any pathologic or clinical defining features in the *MYC*$^+$ group and (2) to assess the prognostic impact of *MYC* gene rearrangements in DLBCL patients treated with R-CHOP chemotherapy.

Methods

Patient identification

The British Columbia Cancer Agency (BCCA) Lymphoid Cancer Database was screened to identify adult patients (> 15 years of age) with newly diagnosed DLBCL treated with curative intent with CHOP in combination with rituximab (R-CHOP). Patients who were HIV$^+$ were excluded. Those cases with available paraffin-embedded tissue blocks from the diagnostic biopsy were used to construct a tissue microarray (TMA). For this analysis, only those cases considered to be DLBCL, not otherwise specified, by the recently updated WHO classification of lymphomas recently published were included.[3] Clinical information, including baseline characteristics for the calculation of the International Prognostic Index (IPI), was determined.[9] This study was approved by the BCCA Research Ethics Board.

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TMA

Three independent TMAs were constructed using duplicate 0.6-mm cores from formalin-fixed, paraffin-embedded tissue derived from a total of 137 samples of newly diagnosed cases of DLBCL according to the WHO classification.[3]

Immunohistochemistry

Immunohistochemistry (IHC) was performed on archived paraffin-embedded tissue using CD20 (L26; Dako North America), CD3 (Dako North America), CD10 (clone 56C6; Vector Laboratories), Ki-67 (Dako North America), BCL6 (clone PG-B6p; Dako North America), MUM1 (clone MUM1p),[10] and BCL2 (clone 124; Dako North America). The TMAs were stained using automated IHC on a Ventana Benchmark using standard protocols. The Ki-67 antibody MIB1 was used to determine the proliferation rate, and more than 80% was defined as "high proliferation" as previously described.[11] For all other immunostains, a cutoff of more than 30% was used in accordance with other studies.[12]

Determination of DLBCL cell of origin subtypes

For 76 patients, frozen tissue was available for gene expression profiling (GEP) analysis. RNA was extracted using the ALL PREP kit (QIAGEN), and after reverse transcription it was hybridized to the U133-2 Plus arrays (Affymetrix) according to the manufacturer's protocol. CEL files were normalized using robust multichip analysis.[13] The cell of origin (COO) phenotype (germinal center B-cell type [GCB], activated B-cell type [ABC], or unclassified) was determined using the 185 gene list and model scores determined by the Bayesian formula as previously described.[14] For the remaining patients, the COO was determined to be GCB or non-GCB using IHC according to the Hans algorithm.[12] Cases that were unclassified by the Bayesian formula were assigned using the Hans criteria.

Cytogenetic analysis

All cases were screened for an *MYC* rearrangement using a commercial Vysis dual-color FISH break-apart probe (Abbott Molecular) applied to the TMA. Cases harboring an *MYC* rearrangement were also screened for the presence of a t(14;18) rearrangement using the LSI *IGH/BCL2* dual-color, dual-fusion translocation probe (Abbott Molecular) on isolated cells from the original paraffin-embedded tissue block and/or frozen or methanol/acetic acid-fixed cell pellets if available.

Statistical analysis

Progression-free survival (PFS) was determined from the date of the pathologic lymphoma diagnosis to the date of relapse, progression or death resulting from lymphoma, or treatment toxicity. Overall survival (OS) was determined from the date of diagnosis to the date of death of any cause. The time to central nervous system (CNS) relapse was defined from the date of diagnosis to the date of documented relapse in the CNS. The χ^2 test was used to compare baseline characteristics between MYC^+ and MYC^- cases. Survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test.[15] The Cox proportional hazards model was used, including factors with a P less than .1 in univariate analysis, to determine the impact of multiple factors on PFS, OS, and time to CNS relapse.[16] All statistical analyses were performed using SPSS software, Version 11.5.

Results

A total of 137 R-CHOP–treated cases of DLBCLs had tissue available for the TMA (61% nodal and 39% extranodal biopsy specimens), and FISH analysis was successful in 135 cases at defining the presence or absence of an *MYC* rearrangement, with 2 technical failures. In total, 12 of 135 (8.8%) cases of DLBCL were positive for an *MYC* rearrangement in this series. Three of the MYC^+ cases had a concurrent t(14;18) chromosome, so-called dual translocations or "double-hit."

Table 1. Characteristics of *MYC*$^+$ and *MYC*$^-$ DLBCL patients

Feature	*MYC*$^+$, n (%) (n = 12)	*MYC*$^-$, n (%) (n = 123)	*P*
Median age, y	68	61	—
Age > 60 y	8 (67)	68 (55)	.448
Male sex	9 (75)	73 (59)	.289
Stage 3 or 4	6 (50)	75 (61)	.459
B symptoms			
Extranodal any site	8 (67)	72 (58)	.584
Extranodal > 1	4 (33)	25 (20)	.295
Bone marrow	0	11 (9)	.280
Testicular	2 (17)	3 (2)	.013
Sinus	0	2 (1.6)	.280
Gastrointestinal	2 (17)	19 (15)	.911
Kidney	0	2 (1.6)	.656
Liver	0	7 (6)	.396
Bulky disease*	4 (33)	31 (26)	.575
PS > 2*	5 (42)	42 (36)	.677
LDH abnormal*	9 (75)	54 (50)	.100
LDH > 2× ULN	3 (25)	28 (26)	.930
IPI 0-2 vs 3-5	6 (50)	48 (39)	.459
Ki-67†			
More than 80%	7 (58)	27 (22.5)	.007
More than 90%	6 (50)	9 (7.5)	< .001
More than 95%	4 (12)	8 (6.7)	.002
BCL2 protein$^+$‡	8 (67)	86 (70)	.782
GCB phenotype§	7 (58)	61 (51)	.640

PS indicates Performance Status; LDH, lactic dehydrogenase; and ULN, upper limit of normal.

*Missing data: LDH, n = 15; bulky disease, n = 3; PS, n = 5.

†Failed in 3 *MYC*$^-$ cases.

‡Results using the Dako antibody; not available in 1 *MYC*$^-$ case.

§Not available in 4 *MYC*$^-$ cases.

Patients who had MYC^+ DLBCL were predominantly male (75%) with a median age of 69 years (range, 22-85 years) with no particular high risk defining clinical features other than a higher incidence of testicular involvement at disease presentation, compared with MYC^- cases (Table 1). Half were early stage and were in a favorable risk group by the IPI (Table 1). There was no difference in the frequency of extranodal versus nodal primary biopsy sites in the MYC^+ and MYC^- groups ($P = .301$).

IHC and cell of origin

MYC^+ cases were more likely to have a high proliferation rate (Table 1). Eight cases (67%) of the MYC^+ DLBCL were $BCL2^+$ using the commercially available Dako antibody with no difference in frequency observed compared with the MYC^- cases (Table 1). Of interest, 2 out of 3 of the MYC^+ cases that were also $t(14;18)^+$ were BCL2 $protein^-$ by IHC using the Dako antibody. Given this finding, these 2 cases were subsequently analyzed using an alternate antibody, clone E17 (Epitomics), which targets amino acids 60 to 80 compared with the Dako BCL2 clone 124, which targets amino acids 41 to 54. Using the E17 antibody, there was nearly 100% staining for BCL2. In one of these cases, subsequent sequencing of the BCL2 gene detected mutations in the flexible loop domain but not the BH3 domain,[17] thus interfering with binding of the Dako antibody. However, the clinical significance of this finding is unknown.

The updated WHO classification of lymphomas recognizes 2 molecular subtypes of DLBCL based on the COO phenotype as GCB and ABC[3,14] or by IHC as GCB and non-GCB. The COO

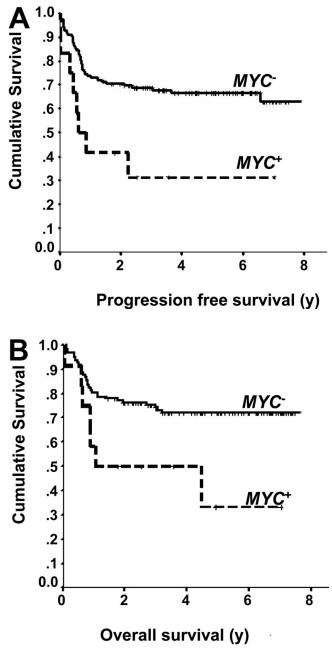

Figure 1. Outcomes of patients with *MYC*+ DLBCL treated with R-CHOP. (A) Progression-free survival of *MYC*+ and *MYC*− DLBCL. (B) Overall survival of *MYC*+ and *MYC*− DLBCL.

phenotype was available for 131 cases (97%; 4 missing in the *MYC*− group); 62 were assigned by GEP[14] and 69, including 14 unclassified cases by GEP, using IHC by the Hans algorithm.[12] Of note, there were 58 cases in which we had both GEP and IHC results that were obtained in a blinded fashion, to evaluate the concordance of the 2 approaches at assigning the COO status. Of the 46 cases with a definitive COO assignment by GEP (12 were unclassified), 35 were concordant with the Hans COO designation for an overall agreement rate of 85%, consistent with prior studies.[12]

For the purpose of the COO assignment in our study, 2 groups were considered: GCB and non-GCB (the latter including those assigned as ABC by GEP). Cases that were unclassifiable by GEP were assigned by the Hans criteria. A GCB phenotype was observed in 68 (51%) cases. There was no difference in the frequency of GCB versus non-GCB between the *MYC*+ and *MYC*− cases (Table 1). Not surprisingly, the 3 cases with dual translocations had a GCB phenotype.

Impact on survival of *MYC* rearrangements in R-CHOP–treated patients

The 5-year PFS (66% vs 31%, $P = .006$) and OS (72% vs 33%, $P = .016$) were inferior in cases of DLBCL treated with R-CHOP that harbored an *MYC* rearrangement (Figure 1). Within the favorable GCB subgroup (n = 68), cases that harbored an *MYC* translocation had an inferior PFS ($P = .049$) and OS ($P = .014$). However, within the non-GCB subgroup (n = 63), there was an inferior PFS ($P = .033$) but not OS ($P = .303$). In univariate analysis, in addition to the presence of an *MYC* rearrangement, the IPI, a non-GCB phenotype, extranodal involvement and bone marrow involvement with DLBCL were all associated with an inferior PFS (Table 2). Similar results were observed for OS, although only a trend for an inferior outcome was observed with a non-GCB phenotype. BCL2 protein expression and a high proliferation rate, including variable cutoffs for Ki-67 (≥ 80%, ≥ 90%, or ≥ 95%) were not prognostic for PFS or OS (Table 2). Multivariate analysis using a Cox proportional hazard model confirmed that the presence of an *MYC* rearrangement remained a significant factor for both PFS (hazard ratio [HR] = 3.28; 95% confidence interval [CI], 1.49-7.21, $P = .003$) and OS (HR = 2.98; 95% confidence interval, 1.28-6.95, $P = .011$; Table 3). Of note, if the *MYC*+ cases with a dual translocation are removed and the analysis is repeated, *MYC*+ status retains prognostic significance for both PFS ($P = .009$) and OS ($P = .036$; results not shown).

Table 2. Univariate analysis of risk factors for PFS and OS for DLBCL patients treated with R-CHOP

Risk factor	PFS *P*	OS *P*
MYC+	.006	.016
IPI ≥ 3	< .001	< .001
Non-GCB phenotype	.041	.058
BCL2 protein*	.313	.492
Ki-67		
More than 80%	.582	.418
More than 90%	.759	.751
More than 95%	.351	.642
Extranodal sites, any	.014	.034
Extranodal sites > 1	.013	.040
Bone marrow DLBCL+	< .001	< .001
Testicular	.124	.263
Bulky disease	.334	.841

*Results using the Dako antibody; not available in 1 *MYC*− case.

Risk of CNS relapse in R-CHOP–treated patients with an *MYC* rearrangement

Given that CNS relapse is a known consequence in Burkitt lymphoma, we evaluated whether there was an increased risk of CNS relapse in cases of DLBCL that harbor an *MYC* rearrangement. Only one patient with testicular involvement received intrathecal prophylaxis at the time of primary therapy; otherwise, CNS prophylaxis was not used in the primary therapy. In total, there were 6 CNS relapses (2 of 12, 17% MYC+ vs 4 of 123, 3% MYC−). Neither of the cases of CNS relapse in the *MYC*+ group had testicular involvement. Using the time to CNS relapse as the endpoint, the presence of an *MYC* rearrangement ($P = .018$) was predictive of a CNS relapse in R-CHOP–treated patients (Figure 2). In multivariate analysis, the presence of an *MYC* rearrangement (HR = 8.0; 95% CI, 1.33-48.03, $P = .023$) and kidney involvement (HR = 25.28; 95% CI, 2.60-245.86, $P = .005$) remained significant in the Cox proportional hazards model after adjusting for the IPI, extranodal sites more than 1, or other high-risk extranodal sites (bone marrow, testicular involvement, and sinus involvement). Of interest, a high proliferation rate was not predictive of an increased risk of CNS relapse (results not shown).

Table 3. Multivariate analysis of risk factors of PFS and OS of DLBCL patients treated with R-CHOP

	PFS		OS	
Risk factor	Hazard ratio (CI)	*P*	Hazard ratio (CI)	*P*
MYC+	3.28 (1.49-7.21)	.003	2.98 (1.28-6.95)	.011
IPI ≥ 3	2.69 (1.48-4.86)	.001	3.29 (1.68-6.46)	.001
Non-GCB phenotype*	1.86 (1.04-3.34)	.038	—	NS
Bone marrow DLBCL+	3.74 (1.67-8.36)	.001	4.06 (1.72-9.58)	.001

NS indicates not significant; and —, not applicable.
*Not available in 4 *MYC*− cases.

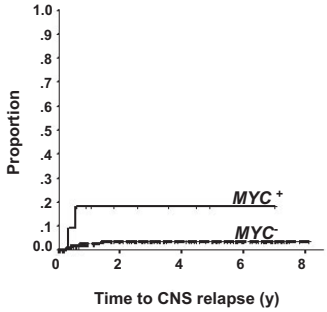

Figure 2. Time to central nervous system relapse of *MYC*$^+$ and *MYC*$^-$ DLBCL.

Discussion

A limited number of studies have evaluated the prognostic importance of *MYC* status in DLBCL patients treated with CHOP-like regimens.[4,18-20] In the large GEP effort by the Molecular Mechanisms in Malignant Lymphomas Network project, *MYC*$^+$ aggressive lymphomas with a "nonmolecular Burkitt lymphoma" or "intermediate" gene expression signature profile had an inferior prognosis compared with those that were *MYC*$^-$. However, not all of these cases were morphologically DLBCL, and most were treated with CHOP-like chemotherapy without rituximab.[19] Interestingly, 11 cases of DLBCL in this study were determined to have a molecular signature of Burkitt lymphoma, but only 8 of these cases harbored an *MYC* translocation, suggesting that other genetic alterations can lead to *MYC* deregulation, such as copy number changes. The outcome of these patients is not reported; thus, it is unknown whether they may have benefitted with a more dose-intensive approach. The German High-Grade Non-Hodgkin Lymphoma Study Group recently evaluated 177 patients who were treated in the BH1[21] and BH2[22] clinical trials, which compared CHOP-like regimens in patients with aggressive B-cell lymphomas.[4] Confining the analysis to DLBCL, cases were selected if they had tissue available for the construction of a TMA to assess for the presence of an *MYC* gene rearrangement. In this comprehensive analysis, the presence of an *MYC* rearrangement was associated with an inferior OS ($P = .047$), and there was a trend to a reduction in EFS ($P = .062$).[4] It is unclear from this analysis what proportion of cases had a concurrent t(14;18) translocation. In a study by Niitsu et al, 11% of cases of DLBCL were found to harbor an *MYC* translocation; however, the cases evaluated were those that had abnormal karyotypes, which may have been selected based on high-risk features, which is also reflected by the high proportion of cases with a concurrent *BCL2* translocation.[20] The presence of an *MYC* translocation was associated with an inferior prognosis; however, this was largely driven by the presence of a concurrent t(14;18). In contrast, one study found that the presence of an *MYC* rearrangement did not predict for a worse outcome in DLBCL; however, the treatment received was not detailed, and it is possible that patients may have received more dose-intensive regimens.[23]

Importantly, all of the prior studies were performed before the routine use of rituximab-containing anthracycline-based regimens, which is the accepted standard of care in DLBCL based on established superiority in several randomized controlled trials.[5,7,8,24] The present study is the first published to evaluate whether the presence of an *MYC* gene rearrangement is still of clinical relevance in patients treated in the R-CHOP era. In this "unselected" population of DLBCL, the frequency of *MYC* rearrangements was 8.8%, which is comparable with the German study (7.9%). Similar to the German study, we did not identify any high-risk clinical features at presentation in the *MYC*$^+$ group; and contrary to prior studies, there was no difference in the frequency of extranodal disease in *MYC*$^+$ patients.[23] Although there was a tendency for the tumors in our study to have a high Ki-67 score, a range was seen, and this feature cannot be relied on to identify patients at a higher risk of harboring an *MYC* rearrangement. Furthermore, our results are in contrast to the German High-Grade Non-Hodgkin Lymphoma Study Group study in which no correlation between a high proliferation rate and the presence of an *MYC* rearrangement was found, which may reflect the high variability and poor reproducibility of this IHC marker.[25] Nevertheless, a high proliferation rate was also not associated with outcome in this analysis. In contrast, the presence of an *MYC* rearrangement retained prognostic significance in R-CHOP–treated patients in multivariate analysis for both PFS and OS; thus, it is independent of clinical risk factors and COO phenotype.

CNS relapse is a known risk in patients with Burkitt lymphoma; and as a result, chemoprophylaxis is incorporated into treatment regimens for this disease. Although there was a small number of CNS relapse cases overall, we did find that there was an increased risk of CNS relapse in cases of DLBCL treated with R-CHOP that harbored an *MYC* rearrangement adjusting for other high-risk factors. Given the overall poor outcome of patients with secondary CNS disease, these results raise the question as to whether this population should be treated with Burkitt lymphoma–type regimens, which integrates intensive CNS prophylaxis. This approach has been explored in patients with aggressive B-cell lymphomas with a high Ki-67 fraction ($\geq$ 95%) who received dose-modified CODOX (cyclophosphamide, doxorubicin, vincristine, methotrexate)–M/IVAC (ifosfamide, Ara-C, carboplatin). The authors concluded that the regimen did not improve outcome in patients with DLBCL[26]; however, the overall 2-year PFS for the DLBCL patients in this study was 55%, which appears to be more favorable than estimates with CHOP-like chemotherapy. Furthermore, this study did not provide information on the outcome of patients with MYC$^+$DLBCL in isolation and was performed in the prerituximab treatment era. Thus, the efficacy of this regimen and other dose-intensive therapies, in combination with rituximab, in *MYC*$^+$ DLBCL requires further study.

In conclusion, *MYC* gene rearrangements define a small group of patients with DLBCL who are less probable to be cured with R-CHOP and may have an increased risk of CNS relapse. There are no identifiable clinical, histologic, or immunophenotypic features signaling that a case of DLBCL may harbor an *MYC* rearrangement. Thus, all patients with DLBCL should undergo FISH or karyotype analysis for assessment of *MYC* rearrangement status in addition to analysis for the t(14;18) and BCL2 protein expression. *MYC*$^+$ DLBCL may represent a distinct DLBCL subtype, and regimens more in line with those used in Burkitt lymphoma may be more appropriate and require further study in this patient population.

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Authorship

Contribution: K.J.S. designed and performed the research, contributed to the data collection, analyzed the data, and wrote the manuscript; N.A.J. contributed to the data collection and analyzed the data; R.D.G. performed the pathologic review, designed the research, contributed to the data collection, and analyzed the data; D.E.H. performed the FISH experiments, contributed to the data collection, and analyzed the data; S.B.-N. performed the FISH experiments; P.F. performed the pathologic review and analyzed the data; and L.H.S. and J.M.C. contributed to the data collection and analyzed the data.

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Tumor-Associated Macrophages and Survival in Classic Hodgkin's Lymphoma

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ABSTRACT

BACKGROUND

Despite advances in treatments for Hodgkin's lymphoma, about 20% of patients still die from progressive disease. Current prognostic models predict the outcome of treatment with imperfect accuracy, and clinically relevant biomarkers have not been established to improve on the International Prognostic Score.

METHODS

Using gene-expression profiling, we analyzed 130 frozen samples obtained from patients with classic Hodgkin's lymphoma during diagnostic lymph-node biopsy to determine which cellular signatures were correlated with treatment outcome. We confirmed our findings in an independent cohort of 166 patients, using immunohistochemical analysis.

RESULTS

Gene-expression profiling identified a gene signature of tumor-associated macrophages that was significantly associated with primary treatment failure ($P=0.02$). In an independent cohort of patients, we found that an increased number of CD68+ macrophages was correlated with a shortened progression-free survival ($P=0.03$) and with an increased likelihood of relapse after autologous hematopoietic stem-cell transplantation ($P=0.008$), resulting in shortened disease-specific survival ($P=0.003$). In multivariate analysis, this adverse prognostic factor outperformed the International Prognostic Score for disease-specific survival ($P=0.003$ vs. $P=0.03$). The absence of an elevated number of CD68+ cells in patients with limited-stage disease defined a subgroup of patients with a long-term disease-specific survival of 100% with the use of current treatment strategies.

CONCLUSIONS

An increased number of tumor-associated macrophages was strongly associated with shortened survival in patients with classic Hodgkin's lymphoma and provides a new biomarker for risk stratification.

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Current therapies do not cure at least 20% of patients with classic Hodgkin's lymphoma, and a similar proportion of patients are overtreated.[1] It remains a challenge to identify patients whose disease will not be eradicated by standard therapies. Currently, most patients receive at least four cycles of polychemotherapy and, if indicated, radiotherapy.[2] Autologous hematopoietic stem-cell transplantation can rescue about 50% of patients in whom primary therapy has failed.

Initial clinical decisions and risk stratification for patients with Hodgkin's lymphoma are largely based on clinical variables that distinguish those who are at high risk from those at standard risk. Classic Hodgkin's lymphoma, which constitutes about 95% of all cases of Hodgkin's lymphoma and is distinguished by the presence of Reed–Sternberg cells with characteristic features, is usually managed on the basis of Ann Arbor staging as follows: limited disease (stages I and IIA without constitutional symptoms) or advanced disease (stages IB and IIB with bulky disease [largest deposit, ≥10 cm in diameter] and stages III and IV either A or B [with or without constitutional symptoms]).[3] The International Prognostic Score (on a scale of 0 to 7, with higher scores indicating increased risk) is the standard that is used for risk stratification of advanced-stage Hodgkin's lymphoma,[4] but it does not apply to limited stages, and none of the published prognostic-factor systems can reliably identify patients in whom treatment is likely to fail. Moreover, neither the International Prognostic Score nor its individual clinical components are suitable for accurately predicting the outcome of autologous hematopoietic stem-cell transplantation in patients with Hodgkin's lymphoma. For these reasons, reliable biomarkers for predicting long-term survival at diagnosis are needed for such patients.

In Hodgkin's lymphoma, unlike most other cancers, the malignant Reed–Sternberg cells are outnumbered by non-neoplastic cells in the microenvironment of the tumor. The frequency and distribution of these cellular components and Reed–Sternberg cells vary considerably among individual patients and among subtypes of Hodgkin's lymphoma.[5] Several studies have focused on the prediction of outcomes by means of markers expressed predominantly by Reed–Sternberg cells[6-9] or the microenvironment.[10-13] However, most of these markers require validation in independent cohorts.

Methods

Samples from Patients

For gene-expression profiling, we selected fresh-frozen lymph-node specimens, which had been obtained at the time of diagnosis from 130 patients with Hodgkin's lymphoma, from the tissue archives at the British Columbia Cancer Agency (BCCA) and the University of Nebraska Medical Center. The criteria that we used included a primary diagnosis of classic Hodgkin's lymphoma after central review, representative lymph-node tissue (at least 1 cm^2 in tissue sections), negative status for human immunodeficiency virus infection, and first-line treatment with ABVD chemotherapy (doxorubicin, bleomycin, vinblastine, and dacarbazine) or an ABVD-like regimen and, if indicated, radiation therapy (including wide-field radiation for patients with limited-stage disease).

Primary treatment was defined as a failure if the lymphoma had progressed at any time after the initiation of therapy; treatment success was defined as the absence of progression or relapse. The median follow-up time for living patients in the treatment-success group was 3.9 years (range, 0.5 to 21.0). The gene-expression cohort was stratified according to treatment outcome (failure or success) in order to analyze differences between the two outcomes (see Fig. S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org). Advanced-stage disease was defined with the use of Ann Arbor staging criteria.[3]

On the basis of the availability of formalin-fixed, paraffin-embedded diagnostic lymph-node specimens, we also selected samples from 166 independent cases of classic Hodgkin's lymphoma for immunohistochemical testing on a tissue microarray. To increase the statistical power for observations linked to progression-free and disease-specific survival, this cohort was enriched for all available cases of treatment failure that were identified through the Centre for Lymphoid Cancer database at the BCCA. The number of

cases of treatment failure was roughly matched to the number of cases of treatment success. For the independent cohort, we also recorded the outcome of secondary therapy delivered with curative intent in 61 patients; these secondary therapies included autologous stem-cell transplantation in 55 patients, CVPP chemotherapy (cyclophosphamide, vinblastine, procarbazine, and prednisone) plus involved-field radiation in 5 patients, and GDP chemotherapy (gemcitabine, dexamethasone, and cisplatin) plus extended-field radiation in 1 patient. In this cohort, the median follow-up time was 4.0 years (range, 0.5 to 20.8).

The study was approved by institutional review boards at the University of British Columbia–BCCA and the University of Nebraska Medical Center. Written informed consent was obtained from all patients in accordance with the principles of the Declaration of Helsinki.

GENE-EXPRESSION ANALYSIS

Total RNA was extracted from multiple (10 to 20) 20-μm freshly cut tissue sections after mechanical homogenization. Expression profiles were obtained with the use of GeneChip Human Genome U133 Plus 2.0 arrays (Affymetrix). (Data are available at www.ncbi.nlm.nih.gov/geo/query/acc.cgi [accession number, GSE17920].) RNA preparation, array hybridization, and washing were performed according to routine protocol with modifications. All 130 microarrays met homogeneous criteria for quality control (for details on data processing and statistical analysis of array data, see the Supplementary Appendix).

IMMUNOHISTOCHEMICAL ANALYSIS

To confirm the findings of the gene-expression analysis, we performed immunohistochemical analysis on a tissue microarray that was constructed from duplicate 1.5-mm cores of 166 independent samples enriched for cases of treatment failure. Markers that were used included CD3, CD20, CD30, CD68, and MMP11. Immunohistochemical scoring ranged from 1 to 3 for CD68, from 1 to 4 for CD20, and from 0 to 3 for MMP11, with higher scores indicating a greater proportion of positive cells (for details, see the Supplementary Appendix). Scores were stratified as high (3 or 4) or low (1 or 2) for CD20 staining.

PREDICTIVE MODELS

In brief, we used the gene-expression data from the 130 patients for whom all prognostic factors of the International Prognostic Score were recorded and constructed a multidimensional classifier on the basis of feature selection, using sparse multinomial logistic regression (SMLR) and leave-one-out cross-validation.[14] Our aim was to build a robust discriminative model that was predictive of treatment failure in addition to identifying a small set of features (genes) that could be used as the basis for separating these data into the respective outcome groups. We determined the relative importance of variables by means of a decision-tree–based algorithm (random forest),[15] using the SMLR selected features. The relative importance of variables was defined with the use of a standard method, which is based on randomization of the variable values and measurement of the resultant decline in the accuracy of the model. We measured classification accuracy using a receiver-operating-characteristic (ROC) curve and the area under the curve (AUC).

Treatment failure was assigned as the positive class, and treatment success as the negative class. Thus, an accurate prediction of failure was a true positive result, and an inaccurate prediction of failure was a false positive result; an accurate prediction of success was a true negative result, and an inaccurate prediction of success was a false negative result. (For additional details on the predictive models, see the Supplementary Appendix.)

STATISTICAL ANALYSIS

Group comparisons were performed by means of the chi-square test and Student's t-test. For time-to-event analyses, we used two primary end points: progression-free survival (based on the time from initial diagnosis to progression at any time, relapse from complete response, or initiation of new, previously unplanned treatment) and disease-specific survival (based on the time from initial diagnosis to death from lymphoma or its treatment, with data for patients who died of unrelated causes censored at the time of death). Cox proportional-hazards models and time-to-event analyses with the use of the Kaplan–Meier method were performed with SPSS software, version 11.0. Two-sided P values of less than 0.05 were considered to indicate statistical significance.

RESULTS

GENE-EXPRESSION ANALYSIS

Unsupervised hierarchical clustering of the gene-expression results did not identify clusters that were significantly associated with the outcome of treatment or any other reported clinical variable (data not shown). However, using Globaltest, with which we tested all prefiltered genes, we found a significant correlation between the gene-expression profile and the outcome of first-line treatment (P=0.02) (Table S1 in the Supplementary Appendix).

Table 1 shows the clinical characteristics of the gene-expression cohort. On the basis of supervised analyses with the data set stratified according to the failure or success of primary treatment (Fig. S1 in the Supplementary Appendix), we identified 271 differentially expressed genes in the two outcome groups (Tables S2 and S3 in the Supplementary Appendix). Unsupervised hierarchical clustering of all 130 expression profiles with the use of these differentially expressed genes identified two clusters, one of which was associated with treatment success (cluster A) and the other with both success and failure (cluster B) (Fig. 1A). In the treatment-failure group, pathway analyses identified the functions of cell-mediated immune response, cell-to-cell signaling and interaction, and up-regulation of pathway genes involved in interleukin-12 signaling and production in macrophages and apoptosis (Table S4 in the Supplementary Appendix). Down-regulated pathways in the treatment-failure group included genes involved in CTLA4 signaling in cytotoxic T lymphocytes and G-protein–coupled signaling.

These results prompted an investigation of the association between microenvironment gene signatures and outcome. Using Globaltest, we performed associative testing to identify previously described cellular and pathway gene signatures that were differentially expressed in the two outcome groups.[16] In the treatment-failure group, there was overexpression of gene signatures of tumor-associated macrophages (P=0.02)[17] and monocytes (P=0.01),[18] findings that are in agreement with the reported overexpression of macrophage-signaling–associated genes according to pathway analysis (Table S1 and Fig. S2 in the Supplementary Appendix). There was also overexpression of gene signatures for angiogenic cells (P=0.04),[19] adipocytes (P=0.01),[20] and Reed–Sternberg cells (P=0.047)[21] and underexpression of a signature for germinal center B cells (P=0.01)[22] in the treatment-failure group (Table S1 in the Supplementary Appendix). Furthermore, previously described genes that are associated with an unfavorable outcome in Hodgkin's lymphoma,[23] such as lysozyme (*LYZ*) and cathepsin L1 (*CTSL1*), were overexpressed in the treatment-failure group (P=0.04).

To test the overall power of expression profiles for outcome prediction, we constructed a classifier by means of sparse multinomial logistic regression.[14] This algorithm identified 86 nonredundant annotated genes (Table S5 in the Supplementary Appendix), age, and Ann Arbor stage by a cross-validation approach. Figure 1C shows the 30 features with the highest discriminative power, as determined by a random-forest algorithm.[15] Among the 27 individual genes with discriminative power exceeding that of the best clinical variable (age) was MMP11 (probe set 235908_at), which was overexpressed in the treatment-failure group (P=0.03 after adjustment for the false discovery rate). We selected this gene for further immunohistochemical testing, since previous studies have shown that the gene family of matrix metallopeptidases are overexpressed in patients in whom treatment has failed[24] and that MMP11 in particular is expressed by tumor-associated macrophages.[25]

We compared the three data sources for feature selection and settled on a gene-expression profiling model for gene-expression probe sets only, a clinical model based on the International Prognostic Score only, and a combination model including both features. This comparison showed that the AUC value was highest for the gene-expression model, as compared with the clinical and combination models (0.837 vs. 0.625 and 0.821, respectively) (Fig. 1B). The differences in accuracy among the models were most prominent at low false negative rates, with the gene-expression model and the combination model yielding higher true negative rates than the clinical model.

IMMUNOHISTOCHEMICAL ANALYSIS

Our gene-expression study and previous studies by other investigators suggested that a predominance of tumor-infiltrating macrophages, a lack of small B cells, and overexpression of matrix

Table 1. Demographic and Clinical Characteristics of the Two Cohorts of Patients.

Variable	Gene-Expression Profiling (N=130)			Immunohistochemical Analysis (N=166)		
	Treatment Success (N=92)	Treatment Failure (N=38)	P Value	Treatment Success (N=87)	Treatment Failure (N=79)	P Value
Median age (range) — yr	37 (8–80)	46 (12–84)	0.03	33 (16–80)	36 (15–82)	0.21
Male sex — %	51	68	0.08	51	53	0.74
Histologic subtype — %			0.15			0.19
Nodular sclerosis	82	63		89	80	
Mixed cellularity	12	26		6	8	
Lymphocyte-rich	2	3		0	4	
Lymphocyte- depleted	1	5		0	3	
Not classifiable	3	3		6	6	
Stage — %			0.003			0.01
I	14	8		11	3	
II	60	32		53	39	
III	16	37		20	34	
IV	10	24		16	24	
Presence of constitutional symptoms — %	36	50	0.14	40	53	0.10
Tumor size			0.39			0.27
Median (range) — cm†	6 (2–17)	7 (2–26)		6 (0–28)	7 (0–19)	
≥10 cm — %	18	26		33	30	
IPS ≥4 (high risk) — %‡	14	18	0.05	14	20	0.27
Primary treatment — %			1.00			0.32
ABVD chemotherapy with or without radiation	96	95		99	100	
Extended-field radiation alone	4	5		1	0	
Secondary treatment — %						
Autologous stem-cell transplantation	ND	ND		NA	70	
Other therapy with curative intent (CVPP or GDP plus radiation)	ND	ND		NA	8	
Palliative treatment (including single-agent chemotherapy or radiation)	ND	ND		NA	22	

* ABVD denotes doxorubicin, bleomycin, vinblastine, and dacarbazine, CVPP cyclophosphamide, vinblastine, procarbazine, and prednisone, GDP gemcitabine, dexamethasone, and cisplatin, IPS International Prognostic Score, NA not applicable, and ND not done.
† The tumor size was calculated as the longest diameter of the largest involved area.
‡ The IPS ranges from 0 to 7, with higher scores indicating increased risk.

metallopeptidases were correlated with the failure of primary treatment. For these reasons, we selected the markers CD68 (macrophages), CD20 (B cells), and MMP11 for immunohistochemical analysis of a tissue array containing samples from lymph-node biopsies in 166 patients with classic Hodgkin's lymphoma (who were unrelated to the patients in the gene-expression analysis), including 79 patients in whom treatment had failed (Table 1). Of these markers, CD68 and CD20 antibodies are routinely used in the diagnosis of lymphoma. The tissue microarray was also stained for CD30 (a marker for Reed–Sternberg cells) and CD3 (a marker for T cells), but neither the number of CD30+ cells nor the number of CD3+ cells was correlated with outcome (data not shown).

Of these immunohistochemical markers, CD68

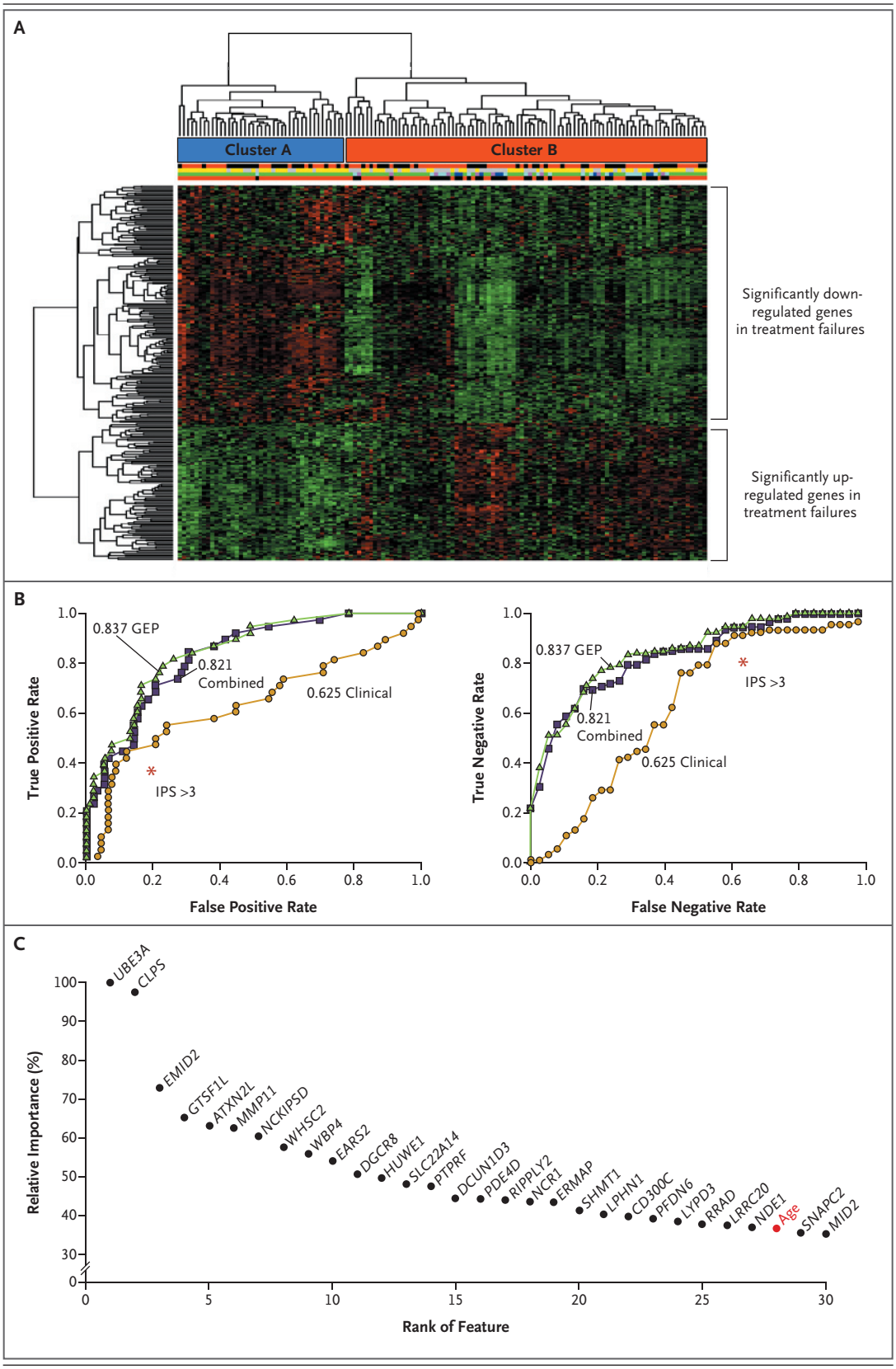


Figure 1 (facing page). Hierarchical Clustering of Gene-Expression Profiles in Hodgkin's Lymphoma, True and False Positive and Negative Rates for Three Models of Outcome Prediction, and the Importance of Individual Genes for Outcome Prediction.

Panel A shows hierarchical clustering of 130 gene-expression profiles for patients with classic Hodgkin's lymphoma. Cluster A has been enriched with primary treatment successes, and Cluster B with both primary treatment successes and failures. Immediately below the cluster bars, the first multicolored bar indicates sex (red for male and black for female), the second bar indicates stage (yellow for limited disease and gray for advanced disease), the third bar indicates the type of treatment failure (green for no treatment failure, purple for refractory, dark blue for early relapse, and light blue for late relapse), and the fourth bar indicates the primary treatment outcome (black for failure and red for success). (For details, see Tables S2 and S3 in the Supplementary Appendix, available with the full text of this article at NEJM.org.) Panel B shows plots of true positive and false positive rates and true negative and false negative rates (receiver-operating-characteristic curves) for three models that were used for feature selection: gene-expression profiling (GEP), a model based on the International Prognostic Score (IPS) for clinical variables, and a model combining these two features. This comparison showed that the value for the area under the curve was highest for the GEP model, as compared with the clinical and combined models (0.837 vs. 0.625 and 0.821, respectively). For comparison with the established IPS, red asterisks indicate an IPS of more than 3, as calculated with the use of IPS thresholds.[4] Panel C shows the relative importance of individual genes for outcome prediction. Relative importance is shown for 30 annotated probe sets (selected with the use of sparse multinomial logistic regression) that were more influential than Ann Arbor staging. Among the 27 individual genes exceeding the importance of the best clinical variable, age (shown in red), was *MMP11*.

stood out because of its significant correlation with primary and secondary treatment outcomes (Fig. 2). Using univariate analysis, we found a significant correlation between the number of CD68+ tumor-infiltrating macrophages and shortened progression-free survival (P=0.03) (Table 2). Patients with a high number of CD68+ cells (an immunohistochemical score of 3) had a median progression-free survival of 2.7 years, whereas during an observation period of 16.4 years, the median survival was not reached in patients with a score of 1 (Fig. 3A). In a multivariate Cox regression model that included factors with respect to the International Prognostic Score and immunohistochemical scores for CD68, CD20, and MMP11, CD68 was not an independent factor for an association with progression-free survival. In contrast, an increased number of CD68+ macrophages correlated with disease-specific survival in both univariate and multivariate analysis (P=0.003 for both comparisons) and outperformed the International Prognostic Score (P=0.03) (Table 2).

The 10-year disease-specific survival rate was significantly lower among patients with a CD68 immunohistochemical score of 3 (59.6%) than among those with a score of 2 (67.4%) or 1 (88.6%) (P=0.003 for all comparisons) (Fig. 3B). The number of tumor-infiltrating macrophages was also correlated with the outcome after secondary treatment. Secondary treatment that was administered with curative intent failed in only 12.5% of patients with a CD68 immunohistochemical score of 1, as compared with failure in 51.7% of those with a score of 2 and in 62.5% of those with a score of 3 (P=0.009 for all comparisons). In particular, there was a significant correlation between the failure of autologous hematopoietic stem-cell transplantation and a CD68 score of more than 1 (P=0.008). A high-risk International Prognostic Score (>3)[4] was not significantly associated with the number of CD68+ macrophages in diagnostic biopsy samples (8.7% for a score of 1, as compared with 18.1% for a score of 2 and 22.9% for a score of 3; P=0.17 for all comparisons). When the analysis was restricted to limited-stage disease, a CD68 score of 1 was associated with a long-term disease-specific survival rate of 100% (P=0.04) (Fig. 3C).

In agreement with the findings of the gene-expression study, MMP11 immunohistochemical staining showed a significant correlation with progression-free survival in both univariate analysis (P=0.008) and multivariate analysis (P=0.009), although not with disease-specific survival (Table 2). Among the cells with positive staining were Reed–Sternberg cells, macrophages, and endothelial cells (data not shown).

Using univariate analysis, we also found that an increased number of CD20+ small B cells (immunohistochemical score, >2) was significantly associated with prolonged progression-free survival (P=0.02) and disease-specific survival (P=0.02) (Table 2). However, the number of CD20+ small B cells was not an independent predictor of survival and was strongly correlated with advanced-stage disease (P=0.002). Neither the number of CD20+ Reed–Sternberg cells nor the presence or

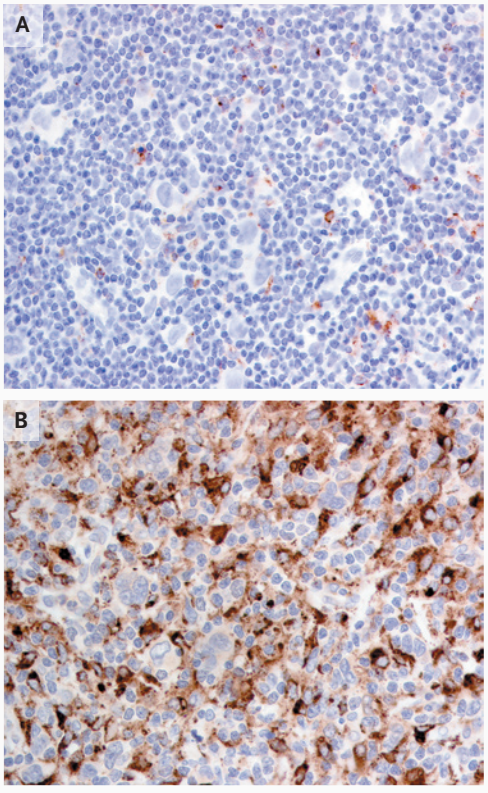


Figure 2. Representative Immunohistochemical Analyses for CD68 in Lymph-Node Biopsy Samples from Two Patients.

Panel A shows a sample obtained from a patient in the treatment-success group, with few CD68+ macrophages, and Panel B shows a sample from a patient in the treatment-failure group, with many CD68+ macrophages. Both patients had the nodular sclerosis subtype of Hodgkin's lymphoma.

absence of primary or secondary lymphoid follicles was associated with the outcome.

DISCUSSION

We found that the overexpression of a macrophage signature in expression-profile studies of diagnostic lymph-node specimens obtained from patients with classic Hodgkin's lymphoma was associated with the failure of primary treatment. Using immunohistochemical analysis, we also found that an increased number of CD68+ cells (a marker of benign macrophages) in the diagnostic sample was associated with a poorer outcome in an independent set of samples from 166 patients. Multivariate analysis revealed that the number of CD68+ cells was also associated with the outcome of secondary treatment, independently of the International Prognostic Score.

Three previous studies that have used expression profiles of the microenvironment in Hodgkin's lymphoma have been reported.[23,24,26] Of these, one identified a gene signature of macrophages but did not show the clinical value of tumor-associated macrophages assessed by means of a common immunohistochemical marker.[23] The association between the number of macrophages and treatment outcome has also been studied in other B-cell cancers.[19,27,28] Differences in survival among patients with various lymphoma subtypes, which are linked to macrophage content, might be explained by the variable presence of macrophages with M1 or M2 differentiation in biopsy samples, indicating distinct biologic features of the tumors.[29] However, in these lymphoma subtypes, including Hodgkin's lymphoma, the functional link between macrophage numbers and the contribution of these cells to the treatment outcome remains unclear.[23,30] Our gene-expression classifier for the outcome of primary treatment outcome revealed *MMP11*, a gene that has been found by other investigators to be expressed in tumor-associated macrophages involved in remodeling of apoptotic lymphatic tissue.[25] Using immunohistochemical analysis, we were able to confirm the correlation between the number of MMP11+ cells and progression-free survival in an independent cohort of patients. However, MMP11 stained many different cell types, including macrophages, and thus did not allow us to identify the particular cells that were responsible for the production of the protein.

Our findings also validate the recent report of a correlation between an increased number of small B cells and a favorable outcome.[26] The recently described correlation between the number of CD20-positive B cells and survival in patients with classic Hodgkin's lymphoma[31] needs to be reassessed in the context of clinical studies showing successful treatment with the addition of rituximab to standard chemotherapy.[32,33]

We report the clinical value of a single marker, CD68, in the identification of tumor-associated

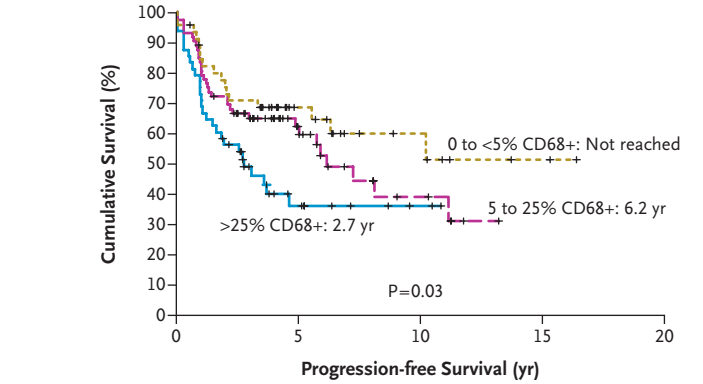
Table 2. Progression-free and Disease-Specific Survival in a Validation Cohort of 166 Patients.*

Variable	Patients with Characteristic	P Value for Progression-free Survival		P Value for Disease-Specific Survival	
		Univariate Analysis	Multivariate Analysis	Univariate Analysis	Multivariate Analysis
	no. (%)				
Demographic data					
Male sex	86 (51.8)	0.90		0.63	
Age >44 yr	54 (32.5)	0.32		0.05	
Immunohistochemical data†					
≥5% CD68+ cells (IHC score, >1)	120 (72.3)	0.03		0.003	0.003
CD20+ cells					
≤10% Background B cells (IHC score, <3)	85 (51.2)	0.02		0.02	
Reed–Sternberg cells	20 (12.0)	0.95		0.59	
Lymphoid follicles	44 (26.5)	0.24		0.34	
≥1% MMP11+ (IHC score, >1)‡	65 (40.6)	0.008	0.009	0.09	
Laboratory data					
Albumin <40 g/liter	68 (41.0)	0.047		0.03	
Hemoglobin <10.5 g/dl	29 (17.5)	0.004		0.11	
White-cell count >15,000/mm^3	23 (13.9)	0.52		0.23	
Lymphocyte count <600/mm^3 or <8%	25 (15.1)	0.13		0.15	
Clinical data					
IPS >3 (high risk)§	28 (16.9)	0.38		0.004	0.03
Advanced-stage disease	125 (75.3)	0.002	0.001	0.05	
Constitutional symptoms	76 (45.8)	0.08		0.48	
Bulky tumor (≥10 cm in diameter)	53 (31.9)	0.82		0.57	

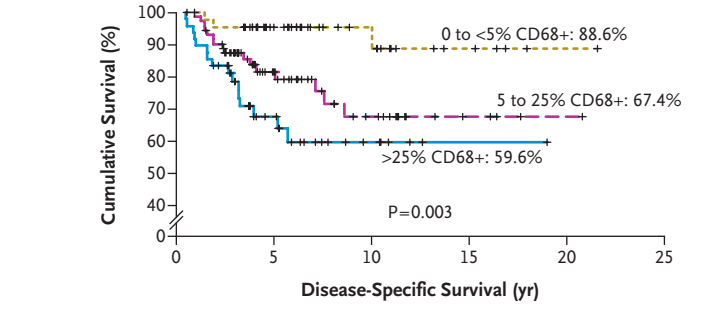
* P values are for the correlation between each factor and survival. Univariate analyses were calculated with the use of a Cox proportional-hazards regression model, and multivariate analyses were performed with a Cox proportional-hazards regression model (forward stepwise likelihood ratio).
† Immunohistochemical (IHC) scores range from 1 to 3 for CD68, from 1 to 4 for CD20, and from 0 to 3 for MMP11, with higher scores indicating a greater proportion of positive cells.
‡ Data regarding immunohistochemical staining were missing for six patients.
§ The International Prognostic Score (IPS) ranges from 0 to 7, with higher scores indicating increased risk.

macrophages by immunohistochemical analysis, an analytic method that can be easily incorporated into a routine diagnostic approach. The use of such markers in combination with well-established clinical risk factors could improve on the predictive value of a single biomarker used alone.

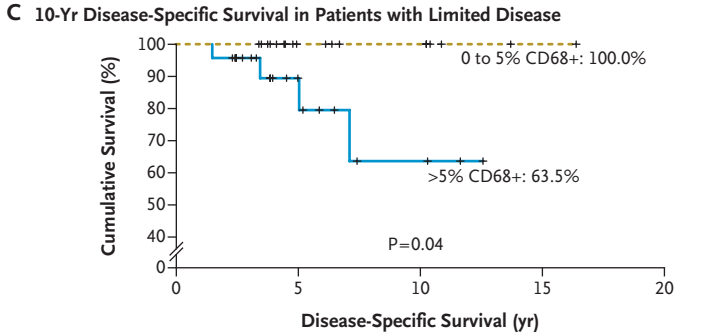
We focused on tumor-associated macrophages because of the strong signal from the gene-expression data and the recently renewed interest in these non-neoplastic cells as major contributors to the biologic features of lymphoma and outcome prediction.[19] The value of assessing the number of tumor-associated macrophages as a biomarker is highlighted by the association between these cells and the outcome after secondary therapy with autologous stem-cell transplantation, a widely used treatment option. Accurate prediction of the outcome after secondary treatments with curative intent would provide better risk stratification for these therapeutic options. Clinical predictors of the outcome after autologous stem-cell transplantation have been of limited value.[34] In addition, our finding that in the tissue-microarray cohort, none of the patients



No. at Risk					
0 to <5% CD68+	46	17	7	2	0
5 to 25% CD68+	72	21	6	0	0
>25% CD68+	48	9	2	0	0



No. at Risk					
0 to <5% CD68+	46	28	13	5	1	0
5 to 25% CD68+	72	33	14	4	1	0
>25% CD68+	48	19	6	1	0	0



No. at Risk					
0 to 5% CD68+	18	9	6	1	0
>5% CD68+	23	10	3	0	0

Figure 3. Survival in a Validation Cohort of 166 Patients, According to the Number of Infiltrating CD68+ Macrophages in Pretreatment Lymph-Node Biopsy Specimens.

The graphs show progression-free survival in all patients (Panel A) and disease-specific survival in all patients (Panel B) and in 41 patients with limited-stage disease (Panel C). According to the immunohistochemical scoring system that was used, a score of 1 indicates less than 5% CD68+ cells, a score of 2 indicates 5 to 25%, and a score of 3 indicates more than 25%. Clinically relevant biomarkers for predicting the outcome of treatment in patients with Hodgkin's disease have not been established. In this study, gene profiling and immunohistochemical analysis were used to find such a marker. A strong association was found between a poor outcome of treatment and an increased number of CD68+ cells in the microenvironment of Reed–Sternberg cells. CD68, a marker of macrophages, outperformed the conventional International Prognostic Score and can be immunohistochemically stained in diagnostic samples of Hodgkin's lymphoma.

with limited-stage disease and a low number of macrophages died is encouraging, since the applicability of the International Prognostic Score is restricted to advanced-stage disease. Thus, the CD68+ macrophage content represents a biomarker with clinical applicability in all stages of classic Hodgkin's lymphoma, both at the time of diagnosis and at the time of relapse.

In summary, our study showed the value of enumerating CD68+ macrophages in diagnostic lymph-node samples for prediction of the outcome after primary treatment and secondary treatment (in particular, autologous stem-cell transplantation). The absence of an increased number of CD68+ cells in patients with limited-stage disease defines a subgroup of patients for whom the rate of long-term disease-specific survival is 100% with the use of available treatments.

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APPENDIX

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