# PROGNOSTIC MOLECULAR FACTORS AND ALGORITHMS IN DIFFUSE LARGE B-CELL LYMPHOMA

Heidi Nyman

Department of Oncology
Helsinki University Central Hospital
University of Helsinki, Finland
and
Molecular and Cancer Biology Programme,
Biomedicum Helsinki
University of Helsinki, Finland

ACADEMIC DISSERTATION

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**Supervised by:**

Docent Sirpa Leppä, MD, PhD
Department of Oncology, Helsinki University Central Hospital
Genome Scale Biology Research Programme, Biomedicum Helsinki
University of Helsinki
Helsinki, Finland

Docent Marja-Liisa Karjalainen-Lindsberg, MD, PhD
Department of Pathology, Helsinki University Central Hospital
University of Helsinki
Helsinki, Finland

**Reviewed by:**

Docent Sirkku Jyrkkiö, MD, PhD
Department of Oncology and Radiotherapy, Turku University Central Hospital
University of Turku
Turku, Finland

Professor Jan Delabie, MD, PhD
Department of Pathology, Rikshospitalet-Radiumhospitalet Medical Centre
University of Oslo
Oslo, Norway

**Opponent:**

Docent Harald Holte, MD, PhD
Chairman of Nordic Lymphoma Group
Department of Oncology, Rikshospitalet-Radiumhospitalet Medical Centre
University of Oslo
Oslo, Norway

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## 1. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred in the text by Roman numerals I-IV:

**I.** Nyman H, Adde M, Karjalainen-Lindsberg ML, Taskinen M, Berglund M, Amini RM, Blomqvist C, Enblad G, Leppä S.
Prognostic impact of immunohistochemically defined germinal center phenotype in diffuse large B-cell lymphoma patients treated with immunochemotherapy.
Blood 2007;109(11);4930-4935.

**II.** Nyman H, Jantunen E, Juvonen E, Elonen E, Böhm J, Kosma VM, Enblad G, Karjalainen-Lindsberg ML, Leppä S.
Impact of germinal center and non-germinal center phenotypes on overall and failure-free survival after high-dose chemotherapy and auto-SCT in primary diffuse large B-cell lymphoma.
Bone Marrow Transplantation 2008;42(2):93-98.

**III.** Nyman H, Jerkeman M, Karjalainen-Lindsberg ML, Banham AH, Enblad G, Leppä S.
Bcl-2 but not FOXP1, is an adverse risk factor in immunochemotherapy-treated non-germinal center diffuse large B-cell lymphomas.
European Journal of Haematology 2009;82(5):364-372.

**IV.** Nyman H, Jerkeman M, Karjalainen-Lindsberg ML, Banham AH, Leppä S.
Prognostic impact of activated B-cell focused classification in diffuse large B-cell lymphoma patients treated with R-CHOP.
Modern Pathology 2009;22(8):1094-1101.

Publication I is included in the doctoral thesis of Magdalena Adde, Uppsala, Sweden.

## 2. ABBREVATIONS

| | |
|---|---|
| aa-IPI | age-adjusted International Prognostic Index |
| ABC | activated B-cell-like |
| ASCT | autologous stem cell transplantation |
| BCL2/6 | B-cell leukemia/lymphoma 2/6 |
| BEAC | carmustine, etoposide, cytarabine and cyclophosphamide |
| BEAM | carmustine, etoposide, cytarabine and melphalan |
| CCL3 | chemokine ligand 3 |
| CCND2 | cyclin D2 |
| CD | cluster of differentiation |
| cDNA | complementary deoxyribonucleic acid |
| CHOEP | cyclophosphamide, doxorubicin, vincristine, etoposide and prednisone |
| CHOP | cyclophosphamide, doxorubicin, vincristine and prednisone |
| CI | confidence interval |
| CLL | chronic lymphocytic leukaemia |
| CNOP | cyclophosphamide, mitoxantrone, vincristine and prednisone |
| CNS | central nervous system |
| CT | chemotherapy |
| DLBCL | diffuse large B-cell lymphoma |
| DNA | deoxyribonucleic acid |
| EBV | Epstein-Barr virus |
| ECOG | Eastern Cooperative Oncology Group |
| EFS | event-free survival |
| EPOCH | etoposide, prednisone, vincristine, cyclophosphamide and doxorubicin |
| $^{18F}$FDG-PET | positron emission tomography with fluorine-18 fluorodeoxyglocose |
| FFS | failure-free survival |
| FL | follicular lymphoma |
| FN1 | fibronectin 1 |
| FOXP1/3 | forkhead box protein 1/3 |
| GC | germinal centre |
| GCB | germinal centre B-cell-like |
| GCET1 | germinal centre B-cell-expressed transcript 1 |
| GELA | Groupe d'Etude des Lymphomes de l'Adulte |
| GEP | gene expression profiling |
| HDT | high-dose chemotherapy |
| ICAM-1 | intracellular adhesion molecule 1 |
| ICT | immunochemotherapy |
| Ig | immunoglobulin |
| IHC | immunohistochemistry |
| IL-2/4/6/10 | interleukin 2/4/6/10 |
| IPI | International Prognostic Index |
| IRF | interferon regulatory factor |

| | |
|---|---|
| LDH | lactate dehydrogenase |
| LMO2 | LIM domain only 2 |
| MACOP-B | methotrexate, doxorubicin, cyclophosphamide, vincristine, prednisone and bleomycin |
| MALT | mucosa-associated lymphoid tissue |
| MHC | major histocompatibility complex |
| MInT | MabThera International Trial |
| mRNA | messenger ribonucleic acid |
| MUM1/IRF4 | multiple myeloma 1 / interferon regulatory factor 4 |
| NF-κB | nuclear factor kappa B |
| NHL | non-Hodgkin lymphoma |
| NK | natural killer |
| non-GCB | non-germinal centre B-cell-like |
| OS | overall survival |
| PFS | progression-free survival |
| PKC | protein kinase C |
| PMLBCL | primary mediastinal large B-cell lymphoma |
| PRDM1β | positive regulatory domain I β |
| R | rituximab |
| RICOVER-60 | rituximab with CHOP in patients aged over 60 years-trial |
| R-IPI | revised International Prognostic Index |
| RNA | ribonucleic acid |
| TMA | tissue microarray |
| TNF | tumour necrosis factor |
| VACOP | etoposide, doxorubicin, cyclophosphamide, vincristine and prednisone |
| VACOP-B | etoposide, doxorubicin, cyclophosphamide, vincristine, prednisone and bleomycin |
| VEGF | vascular endothelial growth factor |
| WHO | World Health Organization |

## 3. ABSTRACT

Diffuse large B-cell lymphoma (DLBCL) is a cancer originating from the lymphatic tissue. It is the most common of the non-Hodgkin lymphomas. As DLBCL is characterized by heterogeneous clinical and biological features, its prognosis varies.

To date, the International Prognostic Index (IPI) has been the strongest predictor of outcome for DLBCL patients and the strongest determinant of a given therapy. However, IPI risk stratification is based on clinical features, and no biological characters of the disease are taken into account. Although knowledge of DLBCL biology has increased, many aspects remain unclear. Gene expression profiling studies have identified two major cell-of-origin phenotypes in DLBCL with different prognoses; the favourable germinal centre B-cell-like (GCB) and the unfavourable activated B-cell-like (ABC) subgroups. By using immunohistochemistry, identification of the GCB and ABC phenotypes has been translated into a clinically applicable approach, defining the subgroups as GCB and non-GCB, respectively. However, results of the prognostic impact of the immunohistochemically defined GCB and non-GCB distinction are controversial, and a molecular classification of DLBCL is not readily applicable in daily clinical practice. Furthermore, since the addition of the CD20 antibody rituximab to chemotherapy has been established as the standard treatment of DLBCL, all molecular markers need to be evaluated in the post-rituximab era of lymphoma therapies.

In this study, we aimed to evaluate the predictive value of immunohistochemically defined cell-of-origin classification in DLBCL patients. The GCB and non-GCB phenotypes were defined according to the Hans algorithm based on the expression of CD10, BCL6 and MUM1/IRF4 among 90 immunochemotherapy- and 104 chemotherapy-treated DLBCL patients. In the chemotherapy group, we observed a significant difference in survival between GCB and non-GCB patients, who had a good and a poor prognosis, respectively. However, in the rituximab group, no prognostic value of the GCB phenotype was observed. Likewise, among 29 high-risk de novo DLBCL patients receiving high-dose chemotherapy and autologous stem cell transplantation, the survival of non-GCB patients was improved, but no difference in outcome was seen between GCB and non-GCB subgroups. In contrast, the predictive value of the classification remained in the control group, which consisted of 34 high-risk DLBCL patients treated with conventional chemotherapy.

Similarly, the results of the individual predictive molecular markers transcription factor FOXP1 and anti-apoptotic protein BCL2 have been inconsistent and should be assessed in

immunochemotherapy-treated DLBCL patients. The markers were evaluated in a cohort of 117 patients treated with rituximab and antracylin-based chemotherapy. Positive FOXP1 expression was observed in 19% of patients, whereas 67% of patients were BCL2-positive. FOXP1 expression could not distinguish between patients, with favourable and those with poor outcomes. In contrast, BCL2-negative DLBCL patients had significantly superior survival relative to BCL2-positive patients. The prognostic impact of BCL2 was primarily observed in the non-GCB subgroup.

Since the results suggested that the Hans algorithm based on the expression of CD10, BCL6 and MUM1/IRF4 was not applicable in immunochemotherapy-treated DLBCL patients, we aimed to further focus on algorithms based on ABC markers. The germinal centre marker BCL6 was omitted from the algorithm because of reported weaknesses in the reproducibility of the staining. We examined the modified activated B-cell-like algorithm based on MUM1/IRF4 and FOXP1 expressions, as well as a previously reported Muris algorithm based on the expression of BCL2, CD10 and MUM1/IRF4 among 88 DLBCL patients uniformly treated with immunochemotherapy. Both algorithms distinguished the unfavourable ABC-like subgroup with a significantly inferior failure-free survival relative to the GCB-like DLBCL patients.

Taken together, our results indicate that the immunohistochemically defined cell-of-origin classification in DLBCL has a prognostic impact in the immunochemotherapy era, when the identifying algorithms are based on ABC-associated markers. We also propose that BCL2 negativity is predictive of a favourable outcome. Further investigational efforts are, however, warranted to identify the molecular features of DLBCL that could enable individualized cancer therapy in routine patient care.

# 4. INTRODUCTION

The incidence of cancer is increasing worldwide. In 2007, about 26 000 people received a cancer diagnosis in Finland (Finnish Cancer Registry 2008). Of these, approximately 1000 (4%) were non-Hodgkin lymphomas (NHLs).

Diffuse large B-cell lymphoma (DLBCL) is the largest NHL entity. The clinical course of the disease is aggressive and patients are often symptomatic. An immediate initiation of treatment is required. The combination of the CD20 antibody rituximab with multi-agent chemotherapy has become the standard of care for de novo DLBCL patients in the 2000s. The addition of rituximab to chemotherapy has improved survival relative to conventional chemotherapy, and currently more than half of the patients can be cured (Coiffier et al. 2002).

The International Prognostic Index (IPI) is a standard approach to estimate prognosis in DLBCL (International Non-Hodgkin´s Lymphoma Prognostic Factor Project 1993). Five clinical parameters, including age, disease distribution, performance status, lactate dehydrogenase (LDH) level and involvement of extranodal sites, distinguish between cases with good and poor outcomes. Although the survival of DLBCL patients has improved, some patients experience relapse or have refractory disease and ultimately die from their lymphoma. In addition to the clinical features of the disease, the heterogeneous biology of DLBCL most likely influences the outcome.

Gene expression profiling (GEP) studies have identified prognostic biological markers and phenotypes in DLBCL (Alizadeh et al. 2000, Rosenwald et al. 2002). The translation of the markers immunohistochemically is a clinically applicable approach with many expectations. This study has focused on evaluating immunohistochemically defined molecular markers and phenotypes in DLBCL patients treated with immunochemotherapy as well as with high-dose chemotherapy (HDT) and autologous stem cell transplantation (ASCT).

# 5. REVIEW OF THE LITERATURE

## 5.1 General aspects of non-Hodgkin lymphomas and diffuse large B-cell lymphoma (DLBCL)

NHLs are heterogeneous malignancies derived from lymphatic tissue. The World Health Organization (WHO) has classified the lymphoid neoplasms based on the Revised European American Lymphoma Classification of Lymphoid Neoplasms (REAL) (Swerdlow et al. 2008). The classification determines the lymphomas according to morphology, immunophenotype and genetic and clinical features. Three major categories of malignancies have been defined: B-cell NHLs, T-cell and NK-cell NHLs, and Hodgkin lymphomas.

The incidence of NHLs has increased in the last decades. In Finland, 1050 new cases were diagnosed in 2007, accounting for about 4% of all malignancies (www.cancerregistry.fi). The NHLs were the sixth most frequent type of cancer in men and in women (incidence: men 12.0/100 000 and women 9.4/100 000). Similarly, in Sweden during the same time period the incidence of NHLs was 16.3/100 000 for men and 12.8/100 000 for women and 1329 new lymphoma diagnoses were reported (www.socialstyrelsen.se).

### 5.1.1 B-cell differentiation and neoplasms

In general, naïve B-cells in the central lymphoid system mature through transformation and proliferation into antibody-producing plasma cells and memory B-cells. Malignancies may develop during this process, and consequently, B-cell neoplasms tend to imitate different stages of normal B-cell differentiation (Figure 1) (Swerdlow et al. 2008).

In the primary phase of differentiation, the precursor B-cell undergoes immunoglobulin gene arrangement and either develops into a naïve B-cell in the peripheral lymphoid tissue or undergoes apoptosis. The naïve B-cell may enter the primary follicle, forming a germinal centre (GC), wherein it matures to a CD10- and BCL6-expressing centroblast. Centroblasts mature further into centrocytes, which later also re-express BCL2 (reviewed in MacLennan 1994). Additionally, the naïve B-cell may directly develop into a plasma cell. Within the germinal centre, the downregulation of BCL6, partly by the action of the IRF4/MUM1 protein (Saito et al. 2007b), is essential for the differentiation of lymphocytes to plasma cells or memory B-cells. On the other hand, upon antigen stimulation, the lymphocytes may undergo apoptosis.

According to the phases of B-cell differentiation, the precursor B-cell neoplasm includes B lymphoblastic leukaemia/lymphoma, whereas the pre-GC neoplasm comprises mantle cell lymphoma. Tumours of the GC B-cells are follicular lymphoma (FL), Burkitt lymphoma, Hodgkin lymphoma and DLBCL. In the last stage of B-cell differentiation, post-GC neoplasms develop, including marginal zone and mucosa-associated lymphoid tissue (MALT) lymphomas, lymphoplasmacytic lymphoma, chronic lymphocytic leukaemia (CLL), plasma cell myeloma and some cases of DLBCL.

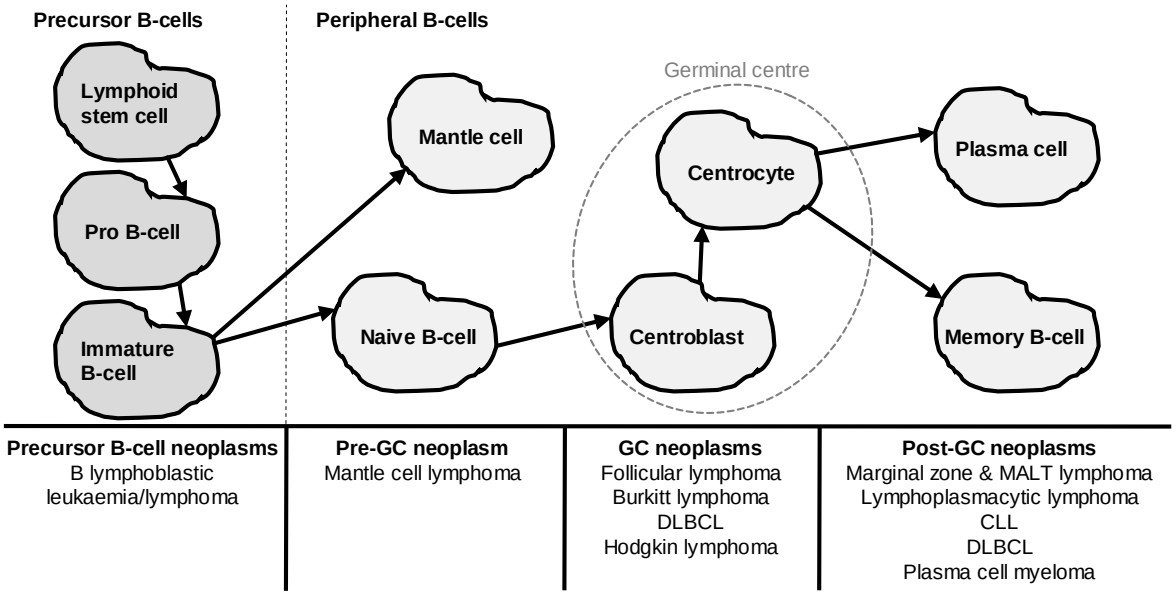


**Figure 1.** Scheme of B-cell differentiation and corresponding B-cell neoplasms. Modified from Swerdlow et al. 2008.

### 5.1.2 Mature B-cell neoplasms

Mature B-cell neoplasms include about 30 different entities, forming the largest category (85%) of all NHLs. The DLBCL is the most common type of NHL, accounting for about 35% of all NHLs, followed by FL (25-30%), MALT (5-10%) and mantle cell lymphoma (5%). The remaining one-third of NHLs contain minor B-cell and T-cell entities (Swerdlow et al. 2008). B-cell lymphomas are identified by a combination of morphological features and distinctive immunophenotypes.

The median age for all mature B-cell neoplasms is about 65 years, and the risk of developing lymphomas increases with age. A slight male predominance is observed.

Acknowledged risk factors for lymphomas are abnormalities of the immune system, including primary and secondary immunodeficiencies. Immunodeficiency may be due to human immunodeficiency virus, transplantation-related immunosuppressive treatment or autoimmune disease (Kassan et al. 1978, Nalesnik et al. 1988, Beral et al. 1991). Infectious agents and chronic infections are also associated with NHLs, e.g. the relation between Epstein-Barr virus (EBV) and endemic Burkitt lymphoma (Prevot et al. 1992).

Based on the clinical behaviour of NHLs, lymphomas can be divided into aggressive and indolent types (Hiddemann et al. 1996). Aggressive lymphomas have a rapid clinical course. The patients are symptomatic and appropriate treatment should be initiated immediately after diagnosis. Survival with aggressive lymphomas is shorter than with indolent entities, although about two of three DLBCL patients can be cured. The indolent types of NHLs, including FL, have a reasonably good prognosis, but advanced stages of the disease are, despite treatment, considered incurable.

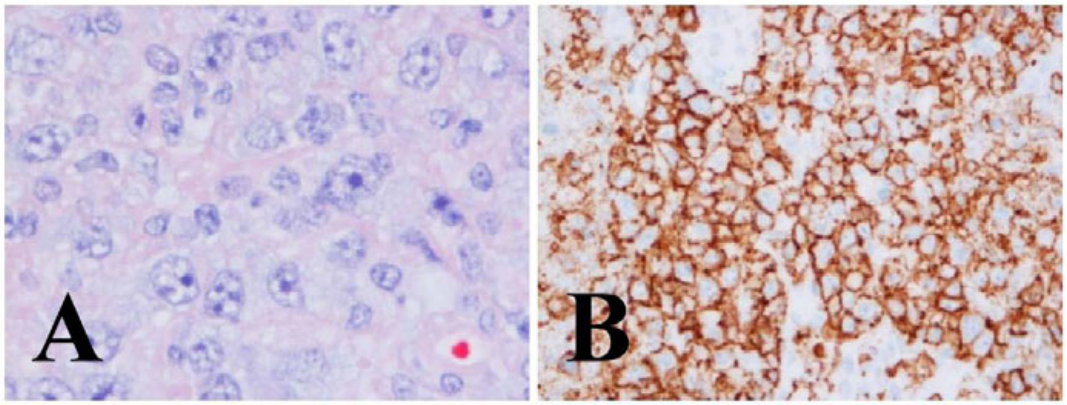


**Figure 2.** Diffuse large B-cell lymphoma lymph node. 1) Large centroblasts growing in a diffuse pattern (haematoxylin and eosin (H&E) stained). 2) Lymphoma B-cells have a high expression of CD20.

### 5.1.3 Classification and pathogenesis of DLBCL

The characteristic morphology of DLBCL is a diffuse proliferation pattern of large lymphocytes that have transformed the nodal structure of the lymph node (Figure 2A). The nuclear size of the lymphoid cell is about twice the size of a normal lymphocyte. The majority of DLBCLs arise from the normal antigen-driven B-cell in the germinal centre of the peripheral lymphoid organ (reviewed in Kuppers et al. 1999). According to the morphological differences and the immunophenotypic profile of DLBCL, the WHO

classification separates the lymphoma into several distinctive entities (Table 1). Three common morphological variants of DLBCL have been identified: centroblastic, immunoblastic and anaplastic. The molecular and immunohistochemical subgroups will be presented in detail in the forthcoming sections. Identification of the subtypes is not only based on morphological features but also on specific clinical presentations of the entities, like the primary DLBCL of the central nervous system (CNS) and the EBV-positive DLBCL of the elderly patients. Furthermore, those heterogeneous cases that are unclassified according to the criteria are acknowledged as a subgroup of DLBCL not otherwise specified (NOS).

**Table 1.** Diffuse large B-cell lymphoma: variants, subgroups and subtypes according to the WHO lymphoma classification 2008 (Swerdlow et al. 2008).

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

- Common morphologic variants
  - Centroblastic
  - Immunoblastic
  - Anaplastic
- Rare morphologic variants
- Molecular subgroups
  - Germinal centre B-cell-like (GCB)
  - Activated B-cell-like (ABC)
- Immunohistochemical subgroups
  - CD5-positive DLBCL
  - Germinal centre B-cell-like (GCB)
  - Non-germinal centre B-cell-like (non-GCB)

**Diffuse large B-cell lymphoma subtypes**

- T-cell/histiocyte-rich large B-cell lymphoma
- Primary DLBCL of the CNS
- Primary cutaneous DLBCL, leg type
- EBV-positive DLBCL of the elderly

**Other lymphomas of large B-cells**

- Primary mediastinal (thymic) large B-cell lymphoma
- Intravascular large B-cell lymphoma
- DLBCL associated with chronic inflammation
- Lymphomatoid granulomatosis
- ALK-positive LBCL
- Plasmablastic lymphoma
- Large B-cell lymphoma arising in HHV-8-associated multicentric Castelman disease
- Primary effusion lymphoma

**Borderline cases**

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

The DLBCL cells frequently express the pan-B-cell markers CD19, CD20, CD22 and CD79a, and in 50-75% of cases also immunoglobulin (Loddenkemper et al. 2004) (Table 2, Figure 2B). In about 10% of the de novo DLBCL, CD5 is expressed on neoplastic cells (Tagawa et al. 2005), although it is distinguished from mantle cell lymphomas by the lack of cyclin D1 expression. The frequency of CD10, BCL6 and IRF4/MUM1 expression varies. The GC markers CD10 and BCL6 are found in 30-60% and 60-90% of cases, respectively. The IRF4/MUM1 marker indicative for the post-GCB phenotype is expressed in 35-65% of cases (Colomo et al. 2003, Berglund et al. 2005, Muris et al. 2006, Amen et al. 2007). A high FOXP1 expression is also demonstrated in post-GCB DLBCLs (Barrans et al. 2004). In contrast to normal B-cells, co-expression of both GCB and post-GCB markers may be observed in lymphoid cells (Falini et al. 2000). Ki-67 expression reflecting the proliferation grade of the DLBCL cells is usually high, in some cases up to 90% (Miller et al. 1994).

DLBCLs have heterogeneous chromosomal abnormalities (Table 2) (Offit et al. 1995, Muramatsu et al. 1997, Chaganti et al. 1998, Kramer et al. 1998). The most common are t(14;18)(q32;q21), involving the *BCL2* gene, and rearrangement of *BCL6* at region 3q27, each manifesting in about one-third of DLBCLs. The *MYC* oncogene present on region 8q24 is rearranged in about 10% of cases. In addition, rearrangements of immunoglobulin chains and somatic hypermutations targeting multiple genetic loci may contribute to the oncogenesis of DLBCL (Pasqualucci et al. 2001).

**Table 2.** Characteristic immunophenotypes and genetics of DLBCL.

| Characteristic | Result |
|---|---|
| **Immunophenotype** | CD19+ |
| | CD20+ |
| | CD22+ |
| | CD79a+ |
| **Cytogenetics** | t(14;18)(q32;q21) |
| | t(3;14)(q27;q32) |
| | t(8;14)(q24;q32) |

## 5.2 Prognostic clinical factors in DLBCL

DLBCL is characterized as a rapidly proliferating lymphoma usually accompanied by lymphadenopathy. However, extranodal presentation may confuse the clinical picture.

The Ann Arbor staging, based on the spreading of the disease to adjacent lymph nodes, was originally developed for patients with Hodgkin's lymphoma (Lister et al. 1989). The classification, currently also applied to NHLs, is less accurate in DLBCL due to the early dissemination and involvement of extranodal sites. However, to manage the disease, evaluation of the extension of the lymphoma is essential. In approximately 50% of patients, the lymphoma is localized to a single lymph node region (stage I) or to multiple lymph node regions restricted to one side of the diaphragm (stage II). Patients with a more widespread stage III disease have affected lymph node regions on both sides of the diaphragm. In cases of involvement of one or more extranodal sites, the lymphoma is considered to be stage IV. Up to 40% of DLBCL patients will have at least involvement of one extranodal site, and the stage is then designated with an E (Harris et al. 1994). Any site may be involved, among others, bone, bone marrow, testis, nasopharynx and gastrointestinal tract. About one-third of patients will present with B-symptoms, including weight loss, fever or night sweats, at the time of diagnosis. If the patient has B-symptoms, the stage of the disease is indicated with a B, otherwise with an A. Due to the rapid progression of the lymphoma, bulk tumours with a diameter over 10 cm are demonstrated in about 30% of patients, and the stage is then designated with an X. Involvement of extranodal sites and several affected lymph node regions have been observed to be negative predictors of survival (Hoskins et al. 1991, Nicolaides et al. 1998, Yang et al. 2009). Moreover, a large tumour mass is associated with poor prognosis (Pfreundschuh et al. 2008a).

The increased use of positron emission tomography with fluorine-18 fluorodeoxyglocose ($^{18F}$FDG-PET) in clinical practice and particularly in staging and response assessment of lymphomas has led to the revision of the response criteria of lymphomas (Cheson et al. 2007). A $^{18F}$FDG-PET-positive lymphoma delineates the extent of the disease, whereas a negative $^{18F}$FDG-PET defines the metabolic remission of the lymphoma. In addition to the role in response evaluation, $^{18F}$FDG-PET has a prognostic value in DLBCL patients. A positive $^{18F}$FDG-PET after chemotherapy treatment or before HDT and ASCT was reported to predict a short progression-free survival (PFS) (Spaepen et al. 2001 & 2003). However, the role of interim $^{18F}$FDG-PET during treatment remains unclear (reviewed in Mikhaeel 2009 and Terasawa et al. 2009).

### 5.2.1 International Prognostic Index

To develop a model for outcome prediction, the International Non-Hodgkin Lymphoma Prognostic Factor Project (1993) retrospectively analysed clinical data on about 3000 aggressive lymphoma patients treated with chemotherapy and defined the following five clinical factors: age over 60 years, stage III or IV disease, elevated serum LDH level, more than one involved extranodal site and an Eastern Cooperative Oncology Group (ECOG) performance status of 2 or 3. The division of patients according to IPI scoring identified risk groups with different survival rates. The 5-year overall survival (OS) observed for the low-risk (none or one factor), low intermediate-risk (two factors), high intermediated-risk (three factors) and high-risk (four or five factors) groups was 73%, 51%, 43% and 26%, respectively (Table 3). The prognostic value of IPI has been confirmed in DLBCL patients (Nicolaides et al. 1998, Wilder et al. 2002). Currently, in clinical practice, IPI risk stratification is the only available tool to estimate risk of relapse and death at diagnosis.

As comorbidity of elderly patients may determine the choice of treatment, an age-adjusted IPI (aa-IPI) was also validated in the IPI Project (International Non-Hodgkin´s Lymphoma Prognostic Factor Project 1993). The aa-IPI risk stratification is based on stage, serum LDH and performance status. Patients with an aa-IPI score of 0 have a favourable outcome, whereas patients with a score of 1, and 2 or 3 have an intermediate and poor prognosis, respectively.

IPI risk stratification was incorporated into clinical practice before immunochemotherapy became a treatment of choice in DLBCL. Sehn et al. (2007) reported a retrospective analysis of the prognostic value of IPI in the post-rituximab era of lymphoma therapies. In a cohort of 365 R-CHOP-treated DLBCL patients, the predictive value of IPI remained, but instead of the originally described four groups, the authors revised the IPI classification (R-IPI) by redistributing the patients into three separate groups of very good (score 0), good (score 1-2) or poor (score 3-5) outcome. Accordingly, a significant difference in survival between the groups was observed, with a four-year PFS of 53%, 80% and 94%, respectively. The corresponding OS rates were 55%, 79%, and 94% (Table 3).

In case of relapsed DLBCLs, the secondary IPI and aa-IPI determined before the second-line HDT combined with ASCT can identify patients with favourable or unfavourable prognosis (Hamlin et al. 2003, Jabbour et al. 2005, Lerner et al. 2007).

**Table 3.** Predicted survival (%) of DLBCL patients according to IPI and R-IPI risk stratifications.

| | No. of patients (%) | 5-yr OS after CT[1] (%) | 4-yr OS after ICT[2] (%) | 4-yr PFS after ICT[2] (%) |
|---|---|---|---|---|
| **Standard IPI risk** | | | | |
| low (0-1) | 28 | 73 | 82 | 85 |
| low-intermediate (2) | 27 | 51 | 81 | 80 |
| high-intermediate (3) | 21 | 43 | 49 | 57 |
| high (4-5) | 24 | 26 | 59 | 51 |
| **R-IPI prognosis** | | | | |
| very good (0) | 10 | NA | 94 | 94 |
| good (1-2) | 45 | NA | 79 | 80 |
| poor (3-5) | 45 | NA | 55 | 53 |

NA, not available.
[1] International Non-Hodgkin´s Lymphoma Prognostic Factor Project (1993); [2] Sehn et al. (2007).

## 5.3 Treatment of de novo DLBCL

A considerable proportion of DLBCL may be cured, with the outcome depending on patient and tumour characteristics. When antracyclin-containing multidrug chemotherapy regimens were standard therapy from the 1970s to the 1990s, approximately half of the patients were cured. Since studies comparing different multi-agent chemotherapies could not demonstrate difference in outcomes between treatments, CHOP was accepted as a first-line regimen for DLBCL patients (Gordon et al. 1992, Cooper et al. 1994, Fisher et al. 1994). Earlier, radiotherapy alone was considered a treatment option, but currently it is administered after chemotherapy to patients with limited or bulky diseases.

Since 2000, treatment results of DLBCL have improved significantly with increased frequency or intensity of the chemotherapy regimen. Pfreundschuh et al. (2004a & 2004b) studied the impact of dose-intensification on the outcome of both young (<60 years) and elderly DLBCL patients. They compared CHOP administered every two or three weeks, with or without etoposide (CHOP-14, CHOP-21, CHOEP-14 and CHOEP-21). In the group of young patients, an improved OS was observed for those receiving the regimen in two-week cycles compared with three-week cycles. The addition of etoposide significantly enhanced the event-free survival (EFS); however, no improvement in OS was detected (Pfreundschuh et al. 2004b). In the trial with elderly patients, the CHOP-14 arm had superior survival to the CHOP-21 arm, but the outcome was not further improved by the addition of etoposide, due to increased toxicity (Pfreundschuh et al. 2004a).

### 5.3.1 Immunochemotherapy

The improved treatment achieved in DLBCL during recent years is primarily the result of combining rituximab with chemotherapy. Rituximab is a recombinant humanized monoclonal antibody targeting the CD20 antigen. CD20 is not present in B-lymphoid stem cells, but is expressed through the maturation of B-cells from pre-B-stage to antibody-secreting plasma cells. The majority (90%) of B-cell neoplasms express CD20. The mechanisms of action of rituximab in vitro include antibody-dependent cell-mediated cytotoxicity, complement-mediated lysis and direct induction of apoptosis (reviewed in Smith 2003).

Initially, rituximab was incorporated in the treatment of indolent lymphomas. However, subsequent to positive results in phase II studies (Coiffier et al. 1998, Vose et al. 2001), several large randomized phase III studies (Table 4) comparing immunochemotherapy with chemotherapy in different subsets of DLBCL patients have been conducted and were further evaluated in a recent meta-analysis (Gao et al. 2009). The positive results in the phase 3 studies place immunochemotherapy into a new standard of care.

The French Lymphoma Group GELA was the first to report results of a significant advantage of R-CHOP treatment relative to CHOP for elderly untreated DLBCL patients (Coiffier et al. 2002). The two-year OS observed for patients treated with eight cycles of R-CHOP-21 was 70%, in comparison with 57% for patients receiving CHOP-21 eight times. The corresponding five-year OS rates were 58% and 45% (Feugier et al. 2005). Similarly, the German Lymphoma Group analysed the benefit of rituximab for young (<60 years) DLBCL patients in the MInT trial (Pfreundschuh et al. 2006). The outcome of the patients with favourable or intermediate-risk disease (aa-IPI 0-1) after R-CHOP-like treatment was superior to that of similar regimens without rituximab. The estimated three-year EFS was 79% after immunochemotherapy compared with 59% after chemotherapy, and the difference in OS remained significant. The ECOG study group randomized elderly patients to receive either CHOP or R-CHOP, followed by rituximab maintenance or no maintenance for patients responding to the induction treatment (Habermann et al. 2006). A benefit of rituximab on PFS was demonstrated, when it was administered either as a part of the induction or maintenance treatment, but no advantage was reported if rituximab was used in both settings. For OS, no difference was observed between the arms, probably because the patients with CHOP received rituximab later on in the maintenance treatment.

**Table 4.** Major randomized studies comparing chemotherapy with rituximab combined with chemotherapy in de novo DLBCL patients.

| DLBCL study | Patient characteristics | EFS or FFS | OS |
|---|---|---|---|
| **GELA**[1] | 399, elderly >60 yrs | 5-yr EFS p=0.00002 | 5-yr OS, p=0.0073 |
| R-CHOP-21x8 | N=202 | 47% | 58% |
| CHOP-21x8 | N=197 | 29% | 45% |
| **MInT**[2] | 824, young <60 yrs | 3-yr EFS, p<0.0001 | 3-yr OS, p<0.001 |
| R-CHOP-likex6 | N=413 | 79% | 93% |
| CHOP-likex6 | N=410 | 59% | 84% |
| **ECOG**[3] | 632, elderly >60 yrs | 3-yr FFS, p=0.003 | 3-yr OS, p=0.05 |
| R-CHOP-21x6-8 | N=318 | 52% | 67% |
| CHOP-21x6-8 | N=314 | 39% | 57% |
| | ---------- | 2-yr FFS, p<0.001 | |
| R-CHOP | N=93 | 77% | NA |
| R-CHOP+mRx4 | N=80 | 79% | NA |
| CHOP+mRx4 | N=94 | 74% | NA |
| CHOP | N=85 | 45% | NA |
| **RICOVER-60**[4] | 1222, elderly >60 yrs | 3-yr EFS, p<0.001 | 3-yr OS, p=0.003 |
| CHOP-14x6 | N=307 | 47% | 68% |
| CHOP-14x8 | N=305 | 53% | 66% |
| R-CHOP-14x6 | N=306 | 67% | 78% |
| R-CHOP-14x8 | N=304 | 63% | 73% |

mR, maintenance rituximab, NA, not available.
[1]Feugier et al. (2005), [2]Pfreundschuh et al. (2006), [3]Habermann et al. (2006),
[4]Pfreundschuh et al. (2008b).

Furthermore, intensification of immunochemotherapy was studied in a randomized RICOVER-60 trial, where six or eight cycles of bi-weekly CHOP-14 with or without rituximab were administered to elderly patients with aggressive B-cell lymphoma (Pfreundschuh et al. 2008b). The results showed that both six and eight cycles of R-CHOP-14 significantly improved survival over CHOP. In addition, the outcome of the patients was better after six cycles than after eight cycles of R-CHOP14, probably because of more toxicity related to the extended treatment protocol. Moreover, in a population-

based retrospective study, the survival of DLBCL patients in the pre- and post-rituximab eras was analysed, and addition of rituximab to chemotherapy was reported to significantly improve survival (Sehn et al. 2005). Similarly, in our experience, the outcome of DLBCL patients at the Department of Oncology in Helsinki University Hospital has improved with immunochemotherapy, as the 3.5-year OS was 72% after R-CHOP and 49% after CHOP treatment (reviewed in Leppä et al. 2009).

### 5.3.2 High-dose therapy (HDT) and autologous stem cell transplantation (ASCT)

The aim of HDT is to improve survival by intensifying the cytotoxic effect of the treatment. As a supportive action, ASCT is performed to reduce potentially fatal side-effects caused by depletion of bone marrow stem cells following treatment. BEAC or BEAM are the most widely used chemotherapy regimens in lymphoma patients.

Randomized trials have yielded conflicting results on the impact of upfront HDT and ASCT in aggressive NHL patients. According to a meta-analysis, low-risk patients have no evidence of improved survival after HDT compared with conventional chemotherapy, and data on the benefit of HDT and ASCT for high-risk patients are controversial (Strehl et al. 2003, Greb et al. 2007, reviewed in Greb et al. 2008). Previously, though, before rituximab was available, combined HDT and ASCT was considered a treatment option for young high-risk DLBCL patients in Finland, as less than half of the patients were cured with conventional chemotherapy (Shipp et al. 1999, Greb at al. 2007). In the immunochemotherapy era, the role of HDT and ASCT as a first-line therapy is unclear, since the treatment-related mortality is more extensive than with conventional chemotherapy.

### 5.3.3 Treatment of relapsed DLBCL

In young patients with an aggressive chemosensitive lymphoma relapse, the use of HDT and ASCT is an attempt to achieve a cure. The addition of rituximab to the induction chemotherapy in the salvage setting prior to HDT and ASCT decreases the risk for secondary relapse (Kewalramani et al. 2004, Vellenga et al. 2008). However, for those DLBCL patients who do not achieve complete remission or who suffer an early relapse after immunochemotherapy, the prognosis is predominantly poor (Martin et al. 2008) and the following treatments are challenging.

## 5.4 Prognostic biomarkers in DLBCL

### 5.4.1 Gene expression profiling

Clinical and morphological heterogeneity of DLBCL suggests that biologically important subtypes, which may help us to tailor the therapy, ought to be defined. The development of DNA microarrays has provided an opportunity to identify gene expression signatures of the tumours that define both novel molecular entities and molecular predictors for survival. A DNA microarray consists of thousands of spots of previously identified genes. The array measures the differences in mRNA levels between the lymphoma samples and the results are usually presented in a heat map, where overexpressed or downregulated genes are shown in red and green, respectively. The profiling in DLBCL has identified several signatures that are both associated with unfavourable outcome after treatment and emphasize the similarities between tumour cells and normal B-lymphocytes (reviewed in Abramson & Shipp 2005). The comparison of studies identifying DLBCL gene signatures is somewhat difficult, as the identified genes are mainly non-overlapping, and methodological and material differences exist. Discrepancies may, for instance, derive from the measured genes, the microarray techniques (oligonucleotide or cDNA arrays), the computational approaches or the patient samples (frozen or paraffin-embedded tissues). In some cases, though, the constructed molecular predictor gene expression signatures have been studied in both preliminary and validation groups (Rosenwald et al. 2002). Unfortunately, the challenging microarray technique and the lack of available frozen material diminish the value of the method in daily clinical practice.

#### 5.4.1.1 Cell of origin

The early GEP studies have demonstrated that DLBCL can be divided into two molecular entities differing in gene expression profiles. The phenotypes share similarities to normal B-lymphocytes, but have shown different clinical outcomes (Alizadeh et al. 2000). The GCB subgroup, accounting for about 50% of cases, had the expression profile of normal GC B-cells. By contrast, the other activated B-cell-like (ABC) subgroup lacked the expression of GC genes and resembled activated B-cells. Initially, a type-3 group was also defined, comprising the unclassified cases. According to the GEP classification, the observed prognosis differed for the two major subtypes after antracyclin-based chemotherapy. GCB patients showed a more favourable outcome, with a five-year OS of 76% compared with 16% for ABC patients. Distinction of the GCB and ABC signatures and predictive impact on survival were independent of any clinical features of the disease

and were further confirmed in other retrospective series with chemotherapy-treated patients (Rosenwald et al. 2002, Shipp et al. 2002, Wright et al. 2003, Monti et al. 2005). One study observed no significant difference in outcome, although the phenotypes were apparent in the profiling (Poulsen et al. 2005).

Molecular profiling can differentiate GCB and ABC DLBCLs according to intracellular pathway activities and oncogenic mechanisms (Rosenwald et al. 2002, Bea et al. 2005, Tagawa et al. 2005, Iqbal et al. 2006, Gleissner et al. 2008). The t(14;18)-deregulating *BCL2* (45%) and the c-*REL* locus amplification on chromosome 2p (16%) occur predominantly, if not exclusively, in the GCB phenotype (Huang et al. 2002, Rosenwald et al. 2002). By contrast, the ABC DLBCLs are characterized by transcriptional overexpression of *BCL2*, chromosomal gains or amplifications in 3q and 18q21-q22 and losses in 6q21-q22 (Bea et al. 2005). Most importantly, a constitutive activation of the anti-apoptotic NF-κB signalling pathway appears in ABC but not in GCB DLBCLs (Davis et al. 2001, Rosenwald et al. 2002, Lam et al. 2005). Additionally, the GCB phenotype has ongoing Ig mutation, while none is observed in the ABC subgroup.

Extranodal lymphomas also have variations in their gene expression signatures. The ABC subtype is commonly seen in primary CNS lymphomas (Camilleri-Broet et al. 2006, Lin et al. 2006) and testicular DLBCL (Booman et al. 2006). By contrast, gastric and intestinal DLBCL cases (Mitchell et al. 2008) are usually GCB subtypes. In addition to the GCB and ABC phenotypes, GEP has also identified the primary mediastinal large B-cell lymphoma (PMLBCL) as an uncommon subtype of DLBCL (Rosenwald et al. 2003, Savage et al. 2003). Currently, however, the PMLBCL is considered a separate entity in the WHO classification (Swerdlow et al. 2008).

#### 5.4.1.2 Additional gene expression-based models in the pre-rituximab era

Several attempts have been made to identify gene expression profiles of DLBCL. The original cell-of-origin signature presented by Alizadeh et al. (2000) included 375 genes. However, the revised signature models identifying GCB and ABC subtypes involved a smaller amount of 100 (Rosenwald et al. 2002) or 27 genes (Wright et al. 2003). In the separate models, disagreement emerged for the unclassified type 3 tumours. Since DLBCL may include up to 40% type 3 tumours, a risk for misclassification is possible (Wright et al. 2003, reviewed in Abramson & Shipp 2005, Monti et al. 2005).

Gene expression signatures other than the GCB and ABC phenotypes may also have an impact on the clinical course of the DLBCL. Several groups have proposed predictor

models based on the expression of 6-17 genes (Rosenwald et al. 2002, Shipp et al. 2002, Lossos et al. 2004). These are largely non-overlapping with the other signatures, with the exception of *BCL6* and fibronectin genes.

The Leukemia and Lymphoma Molecular Profiling Project defined on the basis of hierarchical clustering the "lymph node survival signature" in chemotherapy-treated DLBCL patients (Rosenwald et al. 2002). By selecting highly variable expression of 17 genes, four characteristic groups could be identified: a GC B-cell, proliferating cells, reactive stromal and immune cells in the lymph node, MHC class II complex. Of these, the group with a proliferation signature was associated with poor outcome. By contrast, the outcome was favourable for the three remaining groups representing host responses. Another study used an oligonucleotide microarray technique and identified 13 different predictive genes that contributed to B-cell receptor signalling, regulation of cell cycle and apoptosis and serine/threonin phosphorylation in DLBCL patients with either cured or refractory disease (Shipp et al. 2002). Furthermore, Lossos et al. (2004) evaluated previously defined prognostic genes by applying a quantitative real-time polymerase chain reaction approach. A six-gene model for risk stratification was identified, which included the following genes as the strongest predictors: *LMO2* and *BCL6* involved in germinal centre formation, *CCND2* in cell cycle progression, *BCL2* in apoptosis, *FN1* in cytoskeletal organization and *CCL3* in inflammation. The expression of *BCL2*, *CCND2* and *CCL3* occurring in the ABC signature was correlated with a shorter survival. On the other hand, the GCB phenotype genes *LMO2* and *BCL6* and the lymph-node signature gene *FN1* were correlated with prolonged survival. The impact on survival was independent of the IPI. The expression of MHC class II molecules was correlated with superior survival also independently of the GCB and ABC subtypes (Rosenwald et al. 2002, Rimsza et al. 2004, Rimsza et al. 2006).

The resistance of lymphoma cells to apoptosis may also be clarified with GEP, as subtypes with high expression of anti-apoptotic and cytotoxic effector genes have a poor outcome after chemotherapy (Muris et al. 2007). In addition, optional subtypes of DLBCL were identified by genes involved in the tumour microenvironment and host inflammatory response (Monti et al. 2005). These non-predictive subtypes expressed increased levels of genes associated with oxidative phosphorylation (BCL2 family), B-cell receptor/proliferation and host response (inflammatory cells).

#### 5.4.1.3 Stromal signature

The cell-of-origin classification (Jais et al. 2008, Lenz et al. 2008), and the predictive six-gene model (Malumbres et al. 2008) were reported to retain their predictive value also in DLBCL patients treated with immunochemotherapy. The Leukemia and Lymphoma Molecular Profiling Project reported a novel optimal survival model containing three signatures obtained by diversifying the "lymph node gene signature" (Lenz et al. 2008). In addition to the GCB signature, the model recognized a favourable stromal-1 signature and an unfavourable stromal-2 signature, both of which were characterized by non-malignant cells of the tissue. The stromal-1 group was representative of the extracellular matrix, collagen synthesis and connective-tissue growth factor. On the other hand, genes reflecting tumour angiogenesis and blood vessel density were represented by the stromal-2 signature.

#### 5.4.1.4 NF-κB signalling pathway in DLBCL

The ABC DLBCL expresses several NF-κB target genes and has a constitutively active NF-κB pathway (Davis et al. 2001), which may support lymphocyte proliferation and survival. The NF-κB comprises a family of five members: RelA (p65), RelB, c-Rel, NF-κB1 (p50/105) and NF-κB2 (p52/p100), that control genes associated with the activation and survival of B-cells (reviewed in Li & Verma 2002). The inducible transcription factor is bound as an inactive form to the inhibitor of NF-κB in the cytoplasm. The activation and phosphorylation of NF-κB occurs through the classical-canonical pathway by inflammatory stimuli or the alternative pathway triggered by tumour necrosis factor (TNF)-family members (reviewed in Bonizzi & Karin 2004). In the main classical pathway, the activation is controlled by the IκB kinases that phosphorylate the inhibitor proteins. This process leads to the release of the NF-κB members, which translocate to the nucleus and activate gene transcription. In DLBCL, the activation of NF-κB is mediated by the classical pathway and phosphorylation of p65, which correlates with favourable prognosis (Espinosa et al. 2008). In the ABC subtype, IκB kinases are constitutively active (Davis et al. 2001). The activation of NF-κB in DLBCL is, however, complex, as it is based on genetic lesions of several genes (Compagno et al. 2009).

### 5.4.2 Immunohistochemical models as predictors of DLBCL

Immunohistochemistry (IHC) is a part of the routine diagnostic procedure in most malignancies and of major importance in lymphomas. A predictive protein expression or algorithm based on a limited number of markers is an option to define molecular characteristics of DLBCL and outcome of patients.

#### 5.4.2.1 Cell of origin defined by the Hans algorithm

The GEP-based identification of molecular subgroups has been translated to protein level by using IHC. The GCB and non-GCB subgroups were initially defined according to expression of CD10 and BCL6 (Barrans et al. 2002) or a combination of the previous markers together with MUM1/IRF4 and CD138 (Colomo et al. 2003). However, the results were inconsistent, and none of the IHC findings correlated with gene expression data. Subsequently, an algorithm defining the subgroups was developed.

Hans et al. (2004) created tissue microarray (TMA) blocks from 142 cases that had also been categorized into GCB and ABC subgroups by GEP. The series included 75 GCB, 41 ABC and 26 type 3 cases. The cases were subclassified to either GCB or non-GCB subgroups according to CD10, BCL6 and MUM1/IRF4 expression (Figure 3A). If the case was CD10-positive, it belonged to the GCB subgroup, while both CD10- and BCL6-negative cases belonged to the non-GCB subgroup. If the case was CD10-negative and BCL6-positive, the MUM1/IRF4 expression defined the subgroup; MUM1/IRF4-negative cases were of GCB subtype and MUM/IRF4-positive cases of non-GCB subtype. According to the Hans algorithm, 42% of cases were considered to be GCB and 58% non-GCB. The outcome for the TMA-based classification was observed to be similar to the cDNA-microarray based survival results, as the five-year OS for GCB patients was 76%, as opposed to 34% for patients with the non-GCB phenotype. By comparing the cDNA microarray and TMA classifications, 30 patients were considered to be misclassified. The reported sensitivity for TMA was 71% for the GCB and 88% for the non-GCB subgroups. In survival analysis, the outcome of the misclassified patients correlated best with the results of the TMA definition, as the TMA GCB or microarray ABC patients had a favourable survival. Similarly, the TMA non-GCB and microarray GCB patients had a poor outcome. The predictive value of the phenotype was independent of the IPI risk stratification. The authors concluded that the TMA classification might be more predictive than the microarray classification.

Several studies have subsequently confirmed that the different prognostic subgroups of DLBCL can be identified by analysing a limited number of markers in the Hans algorithm (Berglund et al. 2005, Muris et al. 2006, Sjö et al. 2007, Lee et al. 2008, Seki et al. 2009). However, other studies have shown no difference in the outcome between GCB and non-GCB patients (Colomo et al. 2003, De Paepe et al. 2005, Moskowitz et al. 2005, Amen et al. 2007, Dupuis et al. 2007, Peh et al. 2008, Ilic et al. 2009, Laszlo et al. 2009). Furthermore, separate immunophenotypes in the GCB subgroup have recently been reported to have different survivals. For instance, GCB cases that are CD10- and BCL6-positive have a significantly shorter survival than CD10-negative, BCL6-positive or MUM1/IRF4-negative GCB patients (Anderson et al. 2009). All of these retrospective studies were performed on cohorts treated with CHOP-like chemotherapy. Thus, since the addition of rituximab to chemotherapy was accepted as standard treatment, it was essential to evaluate the prognostic relevance of the cell-of-origin classification in immunochemotherapy-treated DLBCL patients.

#### 5.4.2.2 Other algorithms defining cell of origin

To further define the molecular features of DLBCL immunohistochemically, Muris et al. (2006) developed an algorithm that was based on not only expression of GCB and non-GCB proteins but also anti-apoptotic BCL2 protein. Two different algorithms were presented in the series of chemotherapy-treated DLBCL patients. Firstly, the cases were defined as GCB or ABC-like phenotypes by omitting BCL6 from the algorithm and defining the subtypes according to the expression of CD10 and MUM1/IRF4. If the case was CD10-positive, or CD10- and MUM1/IRF4-negative, it was considered to represent the GCB phenotype. An ABC-like case had negative CD10 and positive MUM1/IRF4 expression. The CD10- and MUM1/IRF4-based algorithm resulted in more ABC-like DLBCL cases than the Hans algorithm, but the predictive power on the outcome was similar for both algorithms. As BCL2 and CD10 were observed to be independent prognostic markers, the algorithm by Muris was further modified to optimize the stratification of the DLCBL into favourable (group 1) and unfavourable (group 2) subgroups (Figure 3B). Group 2 consisted of BCL2-positive, CD10-negative and MUM1/IRF4-positive cases, whereas the other phenotypes belonged to group 1. The favourable and unfavourable subgroups demonstrated a significant difference in survival. The prognostic impact of the algorithm was independent of the IPI risk stratification. Sjö et al. (2007) confirmed the prognostic value of the group 1 and 2 division, but reported that the Hans algorithm had a better prognostic value than the Muris algorithm.

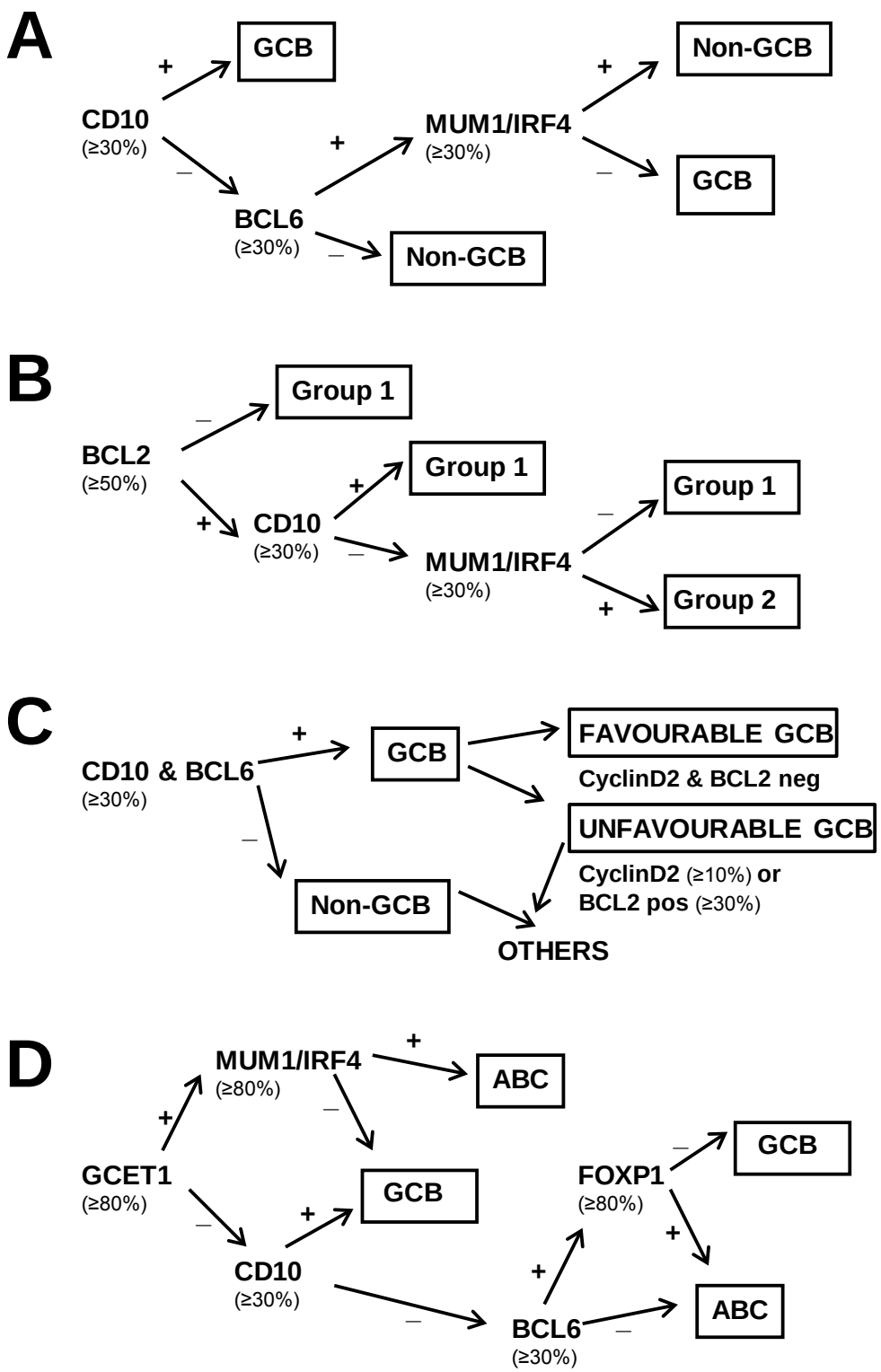


**Figure 3.** Molecular classifications of DLBCL by immunohistochemically defined algorithms. A) Algorithm by Hans et al. (2004). B) Algorithm by Muris et al. (2006). C) Algorithm by Amen et al. (2007). D) Algorithm by Choi et al. (2009).

An additional attempt to improve the classification of DLBCL was based on the algorithm consisting of CD10, BCL6, cyclin D2 and BCL2 stainings (Amen et al. 2007). The cases positive for either CD10 or BCL6 represented the GCB phenotype, whereas CD10- and BCL6-negative cases belonged to the non-GCB phenotype. The GCB subgroup was further divided into cases with favourable (BCL2- and cyclin D2-negative expression) or unfavourable (either BCL2- or cyclin D2-positive) outcome (Figure 3C). The survival analysis demonstrated a significantly improved survival for the favourable GCB patients compared with the others group (non-GCB and poor GCB cases), whereas no prognostic impact was observed for the Hans algorithm.

A novel immunohistochemically defined algorithm classifying DLBCL was validated in both chemotherapy- and immunochemotherapy-treated patients (Choi et al. 2009). By using the five markers GCET1, CD10, BCL6, MUM1/IRF4 and FOXP1, the cases were separated into GCB and ABC-like subgroups. GCET1 is a marker limited to GC-derived lymphomas (Montes-Moreno et al. 2008). The GCB subgroup consisted of the following phenotypes: CGET+/MUM1/IRF4-, GCET1-/CD10+ and GCET1-/CD10-/BCL6+/ FOXP1-, whereas all others were considered to belong to the ABC subtype (Figure 3D). The reported three-year OS was 87% for GCB patients compared with 44% for patients with the ABC-like subtype. The new algorithm was reported to have a 93% concordance with the GEP classification.

### 5.4.3 Individual prognostic markers

The GEP has highlighted the heterogeneity of DLBCLs. Many new proteins have been identified as important prognostic factors in determining outcome of patients (Table 5). To date, the biological markers have improved our knowledge of the DLBCL biology, but much confusion remains (reviewed in Gascoyne 2004, Abramson & Shipp 2005 and Lossos & Morgensztern 2006).

#### 5.4.3.1 BCL6

The transcriptional repressor, BCL6, mediates survival, proliferation and differentiation of B-cells by multifunctionally regulating germinal centre development and T-helper 2-mediated antigen responses (Ye et al. 1997). The proto-oncogene *BCL6* is involved in the chromosomal translocation affecting band 3q27 (Kerckaert et al. 1993, Chang et al. 1996, Seyfert et al. 1996). The *BCL6* gene is normally strongly expressed in GC B- and T-lymphocytes and NHLs originating from GC lymphocytes (Cattoretti et al. 1995).

The prevalence of *BCL6* translocations and rearrangements is reported to be higher in the ABC subgroup than in the GCB subgroup (Iqbal et al. 2007, Shustik et al. 2010). Generally, the presence of *BCL6* gene rearrangements or mutations has no effect on the outcome of DLBCL patients (Bastard et al. 1994, Pescarmona et al. 1997, Kramer et al. 1998, Capello et al. 2000, Jerkeman et al. 2002, Shustik et al. 2010). However, according to some reports, patients with *BCL6* rearrangement have also had a superior (Offit et al. 1994) or inferior (Tibiletti et al. 2009) survival in response to chemotherapy.

High BCL6 mRNA level or BCL6 expression in lymphoma cells has been associated with a significantly improved survival (Lossos et al. 2001, Rosenwald et al. 2002, Hans et al. 2004, Lossos et al. 2004, Iqbal et al. 2007, Sjö et al. 2007, Malumbres et al. 2008, Rimsza et al. 2008, Uccella et al. 2008, Seki et al. 2009), whereas another study reported that high BCL6 expression correlates with poor outcome (Peh et al. 2008). Furthermore, Winter et al. (2006) noted that the addition of R to CHOP interfered with the prognostic value of BCL6. A recent study, however, observed that BCL6 positivity was associated with superior survival after immunochemotherapy (Seki et al. 2009).

#### 5.4.3.2 MUM1/IRF4

MUM1/IRF4 is a lymphocyte-specific member of the IRF family of transcription factors essential for the function of mature B-cells and cytotoxic T-lymphocytes (Mittrucker et al. 1997). The MUM1/IRF4 protein is associated with the terminal phases of B-cell differentiation (Saito et al. 2007b). It also functions as a transcription repressor in early B-cell development (Acquaviva et al. 2008). In addition to plasma cells, the protein is strongly expressed in activated T-cells and a subset of GC B-cells (Falini et al. 2000).

As MUM1/IRF4 is associated with the ABC subtype, the predictive value of the protein has often been evaluated as part of the cell-of-origin algorithms. When the MUM1/IRF4 protein was individually evaluated, positive expression was associated with unfavourable survival in DLBCL patients treated with chemotherapy, and in some cases with HDT supported by ASCT (Hans et al. 2004, Muris et al. 2006, van Imhoff et al. 2006, Amen et al. 2007, Veelken et al. 2007). By contrast, others have reported no prognostic value of MUM1/IRF4 positivity (Berglund et al. 2005, Sjö et al. 2007, Costa et al. 2008, Hallack Neto et al. 2009a).

#### 5.4.3.3 FOXP1

The Forkhead box protein family consists of four transcription factors. The FOXP1 is expressed in different cell types, but its function is largely unknown. B-lymphocytes express FOXP1 at several stages of differentiation (Banham et al. 2001), to a lesser extent in resting B-cells than in activated B-cells (reviewed in Shaffer et al. 2002). The *FOXP1* gene has been reported to be upregulated in the unfavourable ABC subtype (reviewed in Shaffer et al. 2002, Brown et al. 2008, Lenz et al. 2008).

FOXP1 expression was initially associated with poor prognosis (Barrans et al. 2004, Banham et al. 2005), although additional studies with chemotherapy-treated DLBCL patients reported no prognostic impact of FOXP1 on survival (Hans et al. 2004, Amen et al. 2007). High FOXP1 expression has been observed in MALT lymphomas (Streubel et al. 2005) and may be related to transformation to DLBCL (Sagaert et al. 2006).

#### 5.4.3.4 Other biomarkers related to B-cell differentiation and signalling

CD10 is a membrane surface metalloproteinase expressed in lymphoid cells in the follicle centres and is thought to be expressed during the first stages of immunoglobulin heavy chain rearrangements (McIntosh et al. 1999). Although CD10 is lost during the maturation of pro-B-cells to naïve B-cells, it reappears on the cell surface during antigen-dependent germinal centre maturation and may therefore serve as a GCB marker. The predictive value of CD10 for outcome is controversial. An improved prognosis has been reported in patients with high CD10 expression (Ohshima et al. 2001, Hans et al. 2004, Berglund et al. 2005, Muris et al. 2006, Sjö et al. 2007), whereas others have observed no difference in survival between CD10-positive and -negative DLBCL patients (Lossos et al. 2001, Colomo et al. 2003, Fabiani et al. 2004, Amen et al. 2007).

The CD5 antigen is expressed in about 10% of DLBCL patients (Taniguchi et al. 1998), although it is mainly a marker for CLL and mantle-cell lymphomas. Relative to CD5-negative DLBCL patients, cases with high CD5 expression have been reported to have a more widespread disease, involvement of extranodal sites and poorer prognosis after chemotherapy (Yamaguchi et al. 1999 & 2002, Linderoth et al. 2003, Suguro et al. 2006). However, the observation was not confirmed in another patient cohort (Katzenberger et al. 2003). In the immunochemotherapy era, the results have also been controversial, as the prognostic impact of CD5 on survival was significant in patients treated with R-CHOP (Ennishi et al. 2008), while others reported that rituximab improved survival only in CD5-negative cases (Hyo et al. 2009).

**Table 5.** Prognostic molecular markers in DLCBL.

| Biomarker | Proposed outcome | Mechanism | References |
|---|---|---|---|
| **BCL6** | Favourable | Transcription factor/repressor | Hans et al. 2004, Lossos et al. 2004, Iqbal et al. 2007 |
| **MUM1/IRF4** | Unfavourable | Transcription factor | Hans et al. 2004, Muris et al. 2006, Amen et al. 2007 |
| **FOXP1** | Unfavourable | Transcription factor | Barrans et al. 2004, Banham et al. 2005 |
| **CD10** | Favourable | Neutral endopeptidase | Hans et al. 2004, Berglund et al. 2005, Muris et al. 2006 |
| **CD5** | Unfavourable | B-cell differentiation | Yamaguchi et al. 2002, Linderoth et al. 2003, Suguro et al. 2006 |
| **HGAL** | Favourable | GC phenotype | Natkunam et al. 2005 |
| **PKC-β** | Unfavourable | B-cell signaling | Shipp et al. 2002, Hans et al. 2005, Chaiwatanatorn et al. 2009 |
| **LMO2** | Favourable | Transcription factor | Lossos et al. 2004, Natkunam et al. 2008 |
| **BCL2** | Unfavourable | Anti-apoptosis | Lossos et al. 2004, Berglund et al. 2005, Amen et al. 2007 |
| ***p53* mutation** | Unfavourable | Cell cycle regulation | Piris et al. 1994, Ichikawa et al. 1997, Young et al. 2007 |
| **Cyclin D2** | Unfavourable | Cell cycle regulation | Hans et al. 2004 & 2005, Amen et al. 2007 |
| **Mast cells** | Favourable | Microenvironment/host response | Hedström et al. 2007 |
| **FOXP3** | Favourable | Microenvironment/host response | Le Na-Ri et al. 2008 |
| **VEGF** | Unfavourable | Regulator of angiogenesis | Ganjoo et al. 2008, Gratzinger et al. 2008, Jørgensen et al. 2009 |

The role of the specifically IL-4 induced human germinal centre-associated lymphoma *(HGAL)* gene is unknown (reviewed in Lossos & Levy 2003). As the HGAL protein is mainly expressed in the cytoplasm of GC lymphocytes and GCB-derived lymphomas, it may play a role in identifying the GCB subtype (Natkunam et al. 2005). A high HGAL mRNA level was associated with significantly longer survival than a low HGAL expression (Natkunam et al. 2005).

The protein kinase Cβ is a member of the PKC family (α, βI, βII and γ), which plays a role in the activation and survival of B-cells, principally through the activation of the NF-κB pathway (Saijo et al. 2002). PKCβII protein levels have been reported to be higher in non-GCB tumour cells than in GCB cells (Fridberg et al. 2007). According to gene expression studies, the *PKCβ* had prognostic value (Shipp et al. 2002). Likewise, the expression of PKCβ (Hans et al. 2005, Li et al. 2007) and PKCβII (Espinosa et al. 2006, Schaffel et al. 2007) proteins is associated with poor outcome in chemotherapy-treated DLBCL patients. Moreover, high expression of PKCβII is correlated with adverse outcome after immunochemotherapy (Chaiwatanatorn et al. 2009, Riihijärvi et al. 2010).

The *LMO2* is a transcription factor included in the predictive six-gene model (Lossos et al. 2004, Malumbres et al. 2008). The LMO2 protein is expressed in normal GC B-cells and partially in GCB-derived lymphomas (Natkunam et al. 2007, Copie-Bergman et al. 2009). LMO2 protein expression has been shown to have prognostic value, as a high level of LMO2 correlates with favourable survival in both chemotherapy- and immunochemotherapy-treated DLBCL patients (Natkunam et al. 2008). Recently, though, the expression of LMO2 protein was not associated with outcome in immunochemotherapy-treated patients (Copie-Bergman et al. 2009).

Additionally, in DLBCL, improved survival has been linked to expression of CD19 (Kimura et al. 2007), CD21 (Otsuka et al. 2004, Miyazaki et al. 2008), CD40 (Linderoth et al. 2003 & 2007b) and CD23 (Linderoth et al. 2003). The outcome was poor in DLBCL patients with positive CD43 (Mitrovic et al. 2009) and CD138 (Oh & Park 2006) expression. Reduced CD20 expression was also associated with inferior outcome (Johnson et al. 2009). The immunoreactivity of CD44 was associated with favourable survival (Drillenburg et al. 1999, Tzankov et al. 2003) and ICAM-1 with unfavourable outcome (Terol et al. 1998).

#### 5.4.3.5 BCL2 and markers related to apoptosis

The family of BCL2 proteins contains both anti-apoptotic and pro-apoptotic members. *BCL2* is a proto-oncogene promoting B-cell survival by inhibiting apoptosis. BCL2 overexpression is observed in about 50% of DLBCLs. Of these, only 15% have the t(14;18), indicating that other mechanisms regulate the expression (Gascoyne et al. 1997). The *BCL2* is deregulated in all molecular subtypes (Alizadeh et al. 2000). In the GCB subtype, *BCL2* expression is associated with t(14;18), while in the ABC subtype higher BCL2 levels are observed as a consequence of amplification of the *BCL2* gene or NF-κB activation (Rosenwald et al. 2002, Iqbal et al. 2004, Kusumoto et al. 2005). Furthermore,

the GCB lymphomas show high expression levels of pro-apoptotic proteins Bax, Bak and Bid, and low expression of the anti-apoptotic BCL-xL protein (Bai et al. 2004).

The anti-apoptotic role of BCL2 (Hockenbery et al. 1990, Monni et al. 1997, Kramer et al. 1998) suggests an advantage for the survival of lymphoma cells. However, the prognostic value of BCL2 expression remains to be clarified. Several studies in chemotherapy-treated DLBCL patients have shown an adverse impact of BCL2 expression on survival (Hermine et al. 1996, Gascoyne et al. 1997, Barrans et al. 2002, Colomo et al. 2003, Lossos et al. 2004, Berglund et al. 2005, Amen et al. 2007, Veelken et al. 2007, Rimsza et al. 2008, Seki et al. 2009), whereas others have observed no predictive value for BCL2 (Piris et al. 1994, Wilson et al. 2007, Winter et al. 2008). Furthermore, expression of the BCL2 protein and *BCL2* gene aberrations were reported to predict poor outcome only in the ABC subgroup (Iqbal et al. 2006, Obermann et al. 2009).

Caspases are involved in the apotosis signalling pathway, and survivin is an inhibitor protein of apoptosis. Expression of caspase 3 and 8 was observed to correlate with favourable outcome, while caspase 9 was associated with shorter survival of DLBCL patients (Muris et al. 2005). In addition, survivin expression was related to poor outcome (Adida et al. 2000, Watanuki-Miyauchi et al. 2005).

#### 5.4.3.6 p53 and other cell cycle regulators

Cell cycle regulators, like *p53,* are important in regulating the balance between cell proliferation and death. In normal conditions, the p53 protein arrests the cell cycle in response to DNA damage. Mutations in the *p53* gene disturb its regulatory function and lead to uncontrolled cell growth (reviewed in Levine et al. 2006). The incidence of *p53* mutations is lower in haematological malignancies than in other solid tumours (reviewed in Preudhomme & Fenaux 1997 and Peller & Rotter 2003). Several studies have demonstrated that *p53* mutations are associated with poor prognosis (Ichikawa et al. 1997, Moller et al. 1999, Leroy et al. 2002, Kerbauy et al. 2004, Young et al. 2007 & 2008), especially in the GCB subset of patients (Young et al. 2008, Zainuddin et al. 2009). In a few studies, however, no association between *p53* mutations and outcome was found (Osada et al. 1999, Barrans et al. 2002). Results of the impact of p53 protein expression are also inconsistent, as high expression was either associated with shorter survival (Piris et al. 1994, Stewart et al. 2009) or had no prognostic value (Kramer et al. 1996, Jerkeman et al. 2004). The p53+/p16-/p14- and p53+/p21- phenotypes were more frequently observed in the GCB subtype (Paik et al. 2005, Visco et al. 2006), where they were associated with unfavourable outcome. Furthermore, expression of p63 (p53 homologous)

was demonstrated to predict both a poor (Fukushima et al. 2006) and a good (Hallack Neto et al. 2009b) prognosis, as well as to correlate with high proliferation of DLBCL cells (Hedvat et al. 2005).

Cyclins are cell cycle-regulating proteins, often deregulated in malignant cells. In DLBCL, expression of cyclin B1 (Obermann et al. 2005), cyclin D2 (Hans et al. 2004 & 2005, Amen et al. 2007), cyclin D3 (Filipits et al. 2002) and cyclin E (Tzankov et al. 2006) has been associated with poor prognosis.

The nuclear antigen Ki-67 is present in all cycling cells, and the proportion of Ki-67 in positive cells reflects the proliferation rate of the tumour. In DLBCL, the reported prognostic value of Ki-67 is controversial. High Ki-67 expression was demonstrated to be associated with poor prognosis (Grogan et al. 1988, Miller et al. 1994, Sanchez et al. 1998, Broyde et al. 2009), although other studies have reported a favourable outcome for patients with positive Ki-67 expression (Wilson et al. 1997, Jerkeman et al. 2004, Hasselblom et al. 2008b).

#### 5.4.3.7 Microenvironment

The tumour microenvironment plays a crucial role in cancer development, and, for instance, in FL, the immune cells may be more important than tumour cells in predicting survival (Dave et al. 2004, Farinha et al. 2005, Taskinen et al. 2007). In DLBCL, GEP identified a subset of DLBCLs characterized by host inflammatory response (Monti et al. 2005). Genes associated with the tumour microenvironment have been described to be overexpressed in DLBCL patients with continuous remission (Linderoth et al. 2008). At the tissue level, large amounts of lymphoma-infiltrating T-lymphocytes (Ansell et al. 2001) and mast cells (Hedström et al. 2007) were associated with superior outcome in DLBCL patients. However, unlike in FL, the amount of CD68-positive macrophages in the tumour was not observed to be associated with outcome (Hasselblom et al. 2008a). Interestingly, high tumour-associated macrophage content has been noted in DLBCL patients who are cured after chemotherapy (Linderoth et al. 2008).

The transcription factor FOXP3 has been identified as an important regulator of CD4+CD25+ regulatory T-cells and is accepted as their marker (Hori et al. 2003, reviewed in Li et al. 2006). Regulatory T-cells play a role in immunological tolerance and may contribute to tumour progression (reviewed in Beyer & Schultze 2006). An increased number of immunohistochemically defined FOXP3 positive regulatory T-cells was shown to have a favourable prognostic impact on the outcome of DLBCL patients (Lee et al.

2008), although high expression of these cells was also associated with a poor prognosis in high-risk patients (Saez et al. 2009).

Angiogenesis is essential in tumourgenesis and tumour growth (reviewed in Hanahan & Folkman 1996). Aggressive lymphomas have an intensified stromal haemangiogenesis (Ruan et al. 2006), and therefore, pro- and anti-angiogenic growth factors may serve as predictive factors. High expression of VEGF and VEGF receptors correlated with an adverse outcome in DLBCL patients treated with chemotherapy (Ganjoo et al. 2008, Gratzinger et al. 2008, Jørgensen et al. 2009) and immunochemotherapy (Gratzinger et al. 2010).

#### 5.4.3.8 Soluble serum markers

The more studied prognostic markers are the ones evaluated in lymphoma cells and the microenvironment of tissue samples. However, predictive soluble serum markers have also been found in both chemotherapy- and immunochemotherapy-treated DLBCL patients. Examples including elevated serum levels of beta-2 microglobulin, LDH (Suki et al. 1995), adhesion molecule CD44 (Ristamaki et al. 1997), ICAM-1 (Terol et al. 2003), IL-2 receptor (Ennishi et al. 2009, Morito et al. 2009), the TNF-receptor family member Fas (Hara et al. 2009) and L-kynurenine (Yoshikawa el al. 2009) have predicted poor outcome. Soluble markers evaluated in serum samples have practical applicability; however, these markers are not lymphoma-specific and can therefore also reflect other conditions of the patient.

# 6. AIMS OF THE STUDY

The major aim of this thesis was to determine the role of immunohistochemically defined molecular predictors related to cell of origin in DLBCL patients treated with immunochemotherapy.

Specific aims were as follows:

1) To determine the prognostic impact of GCB and non-GCB phenotypes according to the Hans algorithm in DLBCL patients treated with a combination of rituximab and chemotherapy (I).

2) To investigate the predictive value of the cell-of-origin classification in DLBCL patients treated with HDT and ASCT (II).

3) To define the prognostic impact of the ABC-associated markers BCL2 and FOXP1 in immunochemotherapy-treated DLBLCs (III).

4) To develop a novel algorithm and test its applicability in predicting outcome in DLBCL patients treated with immunochemotherapy (IV).

# 7. PATIENTS AND METHODS

## 7.1 Patients and treatments

Patients were selected on the basis of availability of clinical information and histological material. In Studies I-IV, the series consisted partly of the same patient cases. All patient data were collected retrospectively. The following clinical characteristics of each patient were obtained from medical records: age at diagnosis, sex, performance status, Ann Arbor stage at presentation, extranodal involvement, serum LDH level and therapy regimen. IPI, aa-IPI and R-IPI were determined. For survival analysis, the dates for diagnosis, treatment, relapse, last follow-up, and death of any cause were recorded. The endpoints were defined according to the Cheson criteria (Cheson et al. 1999). None of the patients were immunodeficient. All cases were diagnosed by a haematopathologist as CD20-positive DLBCLs, according to the WHO classification (Jaffe et al. 2001). The protocols and samplings were approved by the local Institutional Review Boards, the Ethics Committee at the Department of Surgery at the Hospital District of Helsinki and Uusimaa and the Finnish National Authority for Medicolegal Affairs.

**Study I**

The series consisted of 194 de novo DLBCL patients diagnosed and treated at University Hospitals in Helsinki, Finland, and Uppsala, Sweden. The patients were treated between 1994 and 2004, a time period before rituximab was adapted to standard treatment and a period of immunochemotherapy treatment. Ninety patients received immunochemotherapy, i.e. rituximab combined with a CHOP-like regimen, whereas 104 chemotherapy-treated patients served as a control group. CHOP was administered to about 58% of the cases in both treatment arms, whereas the remaining regimens consisted of CHOEP, VACOP, MACOP-B, EPOCH and CNOP. The study included 107 males and 87 females. The median age of the patients in the rituximab group was 60.9 (range 23-82) years and in the control group 62.0 (range 25-83) years. The median follow-up for immunochemotherapy-treated patients was 27 months and for chemotherapy-treated patients 52 months.

**Study II**

From 1994 to 2002, we collected data on 29 young high-risk DLBCL patients receiving first-line treatment with HDT and ASCT at the Departments of Oncology and Haematology in Helsinki and Kuopio, Finland. The control group consisted of 34 young high-risk chemotherapy-treated patients from Uppsala, Sweden. The young high-risk

patients were aged under 64 years, with an Ann Arbor stage III or IV disease and an aa-IPI≥1. The median follow-up for the ASCT group was 8 years and for the control group 4 years. As an induction treatment, 5-7 cycles of chemotherapy was typically administered. The chemotherapy included CHOP and CHOP-like regimens, and as a consolidation treatment BEAC or BEAM was administered. None of the patients received rituximab.

**Study III**

This cohort consisted of 117 DLBCL patients diagnosed and treated with immunochemotherapy during 2002-2006 at the Departments of Oncology in Helsinki, Finland, and in Uppsala and Lund, Sweden. Of the patients, 76% were treated with an R-CHOP regimen and the rest received R-CHOEP. The median age was 63.3 (range 18-84) years, with 61% of the patients being over 60 years. The median follow-up was 29 months. At the time of data analysis, 28 patients had relapsed and 19 subsequently died.

**Study IV**

In this series, we collected 88 uniformly immunochemotherapy-treated de novo DLBCL patients. The only accepted therapy regimen was R-CHOP. Patients were diagnosed and treated at the Departments of Oncology in Helsinki, Finland, and Lund, Sweden, during 2002-2006. The median follow-up at the time of data analysis was 37 months. The median age of the 42 male and 46 female patients was 66.6 (range 18-84) years.

## 7.2 Immunohistochemistry

Diagnostic paraffin blocks were collected from the patients from Studies I-IV at the time of diagnosis. Only whole-tissue sections were used in the analysis. Paraffin sections of 4-µm thickness were mounted on Vectabond-coated slides and kept at +37°C for 24 h and then stored at 4°C.

**CD10, BCL6, MUM1/IRF4 and BCL2 (I-IV)**

The immunohistochemical stainings were performed according to the following procedure. The sections were kept at 56°C for 30 min. Deparaffinization was carried out by using the sections in xylene, which were rehydrated through a series from graded alcohol to water. For antigen retrieval, sections were treated in a pressure cooker for 2 min in 0.01M sodium citrate buffer (pH 6.0), washed with phosphate-buffered saline (PBS) and incubated in hydrogen peroxidase. After rewashing with PBS, Vectastain ABC mouse kit reagents (Vector Laboratories, USA) were used according to the manufacturer's instructions. Primary monoclonal mouse antibodies CD10, BCL6, MUM1/IRF4 and

BCL2 were diluted 1:200, 1:20, 1:100 and 1:100, respectively, in 0.3% bovine serum albumin (BSA) PBS. After antibody administration at room temperature (RT), sections were incubated overnight at 4°C. During the following day, IHC was completed using the Vectastain ABC kit. The immunoreactions were visualized with 3-amino-9-ethylcarbazole (AEC) or 3,3´-diaminobenzidine (DAB) at RT for 10 min. After washing the slides with PBS and distilled water, the sections were counterstained with Mayer haematoxylin and mounted for microscopy examination.

**FOXP1 (III, IV)**

In the immunohistochemical staining procedure of FOXP1, deparaffinization and rehydration were performed as described above. The sections were then cooked in a microwave oven in trishydroxymethylaminomethane/ethylenediaminetetraacetic acid (Tris/EDTA, pH 9.0) for 3 x 4 min. For immunohistochemical detection, the Dako Cytomation Envision System-HRP DAB kit (Dako Cytomation, Denmark) was used according to the manufacturer's instructions. The FOXP1 (JC12) mouse antibody was diluted 1:80 in PBS and 10% human serum and incubated on the slides for 30 min at RT. After completing the Envision Kit procedure, Mayer haematoxylin was applied for counterstaining, followed by mounting.

A histological section of normal lymph node was used as a positive control for all specific stainings. For the negative control, no primary antibody was applied on the section in the procedure.

**Table 6.** Antibodies used in immunohistochemical stainings.

| Antigen | Antibody | Dilution | Manufacturer |
|---|---|---|---|
| **CD10** | monoclonal mouse | 1:200 | Novocastra Laboratories, United Kingdom |
| **BCL6** | monoclonal mouse | 1:20 | Dako Cytomation, Denmark |
| **MUM1/IRF4** | monoclonal mouse | 1:100 | Dako Cytomation, Denmark |
| **BCL2** | monoclonal mouse | 1:100 | Dako Cytomation, Denmark |
| **FOXP1** | monoclonal mouse | 1:80 | Gift from AH Banham, United Kingdom |

## 7.3 Evaluation of immunoreactivity

The evaluation of immunoreactivity was supervised by an experienced haematopathologist (M-LK-L, Helsinki). The sections were analysed by one or two independent investigators. The immunostainings were evaluated with a Leica DM LB bright-field microscope (Leica Microsystems GmbH) at 40x magnification. Only nuclear staining for BCL6, MUM1/IRF4 and FOXP1, nuclear and cytoplasma staining for BCL2 and membrane staining for CD10 were evaluated in lymphoma cells (Figure 4). No follicular areas were evaluated. According to previous studies (Hans et al. 2004, Berglund et al. 2005), the sections were scored positive for CD10, BCL6 and MUM1/IRF4 if 30%, and for BCL2 if 50% or more of lymphoma cells were positive. The cut-off for FOXP1 expression was deemed positive if 30% (Hans et al. 2004, Banham et al. 2005, Amen et al. 2007), and strongly positive if 100% of lymphoma cells showed positive immunoreactivity (Barrans et al. 2004). In Studies III and IV, the strongly positive 100% cut-off level was used for FOXP1 staining, as the distribution of the positive and negative cases was the most comparable with the previous study (Barrans et al. 2004).

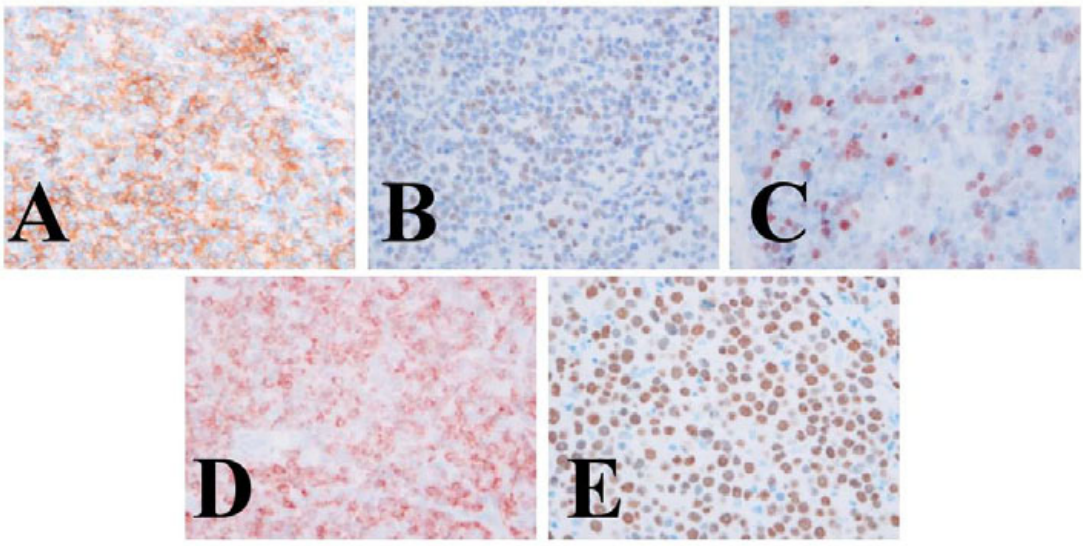


**Figure 4.** Positive expression in immunohistochemical stainings for A) CD10, B) BCL6, C) MUM1/IRF4, D) BCL2 and E) FOXP1.

## 7.4 Immunohistochemical classifications of cell of origin

Three different classifications identifying the cell of origin were used: the Hans algorithm (Studies I-IV), the Muris algorithm (Study IV) and the modified ABC-like algorithm (Study IV).

According to the Hans algorithm (Figure 3A), cases were assigned to the GCB phenotype if CD10 or both CD10 and BCL6 were positive. If both CD10 and BCL6 were negative, the case was assigned to the non-GCB subgroup. In cases where CD10 was negative and BCL6 positive, MUM1/IRF4 expression determined the subgroup; MUM1/IRF4-positive cases were considered to belong to the non-GCB subgroup and MUM1/IRF4-negative cases to the GCB subgroup.

According to the Muris classification in Figure 3B, the cases were assigned to group 1 if BCL2 was negative or if both BCL2 and CD10 were positive. In cases where BCL2 was positive and CD10 negative, MUM1/IRF4 determined the subgroup; MUM1/IRF4-negative cases were considered to belong to group 1 and MUM1/IRF4-positive cases to group 2.

Using the modified ABC-like classification, cases with positive MUM1/IRF4 or FOXP1 expression were considered to belong to the ABC-like subgroup, whereas the rest of cases were assigned as others (Figure 5).

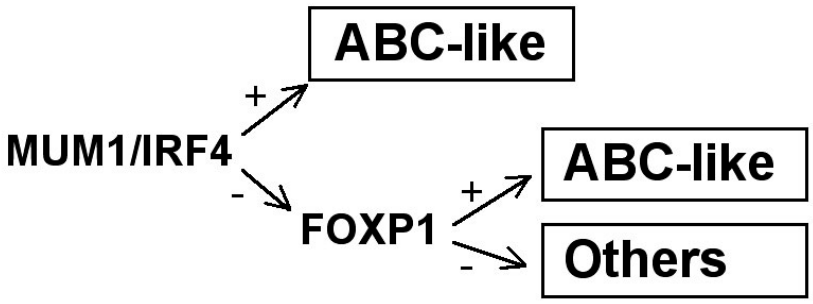


**Figure 5.** Modified activated B-cell-like algorithm.

## 7.5 Statistical methods

Statistical analyses were carried out using Statistical software package SPSS version 11.0 for Macintosh and version 14.0 for Windows (SPSS, Chicago, IL, USA).

To analyse the difference in frequencies of patient characteristics and predictive factors, Chi-square test was used. Reproducibility of immunohistochemical stainings was evaluated by Kappa value. Survival rates were estimated by the Kaplan-Meier method, and the differences were compared by the log rank test. Cox univariate and multivariate analyses were carried out to evaluate the prognostic impact of clinical and molecular factors on survival, acknowledging also the relative risk and 95% confidence intervals (CI). All p-values were 2-tailed, and a probability value of less than 0.05 was considered significant. OS was estimated as the time from diagnosis to death of any cause or to the date of the last follow-up. Failure-free survival (FFS) was determined as the time from date of diagnosis to lymphoma relapse or death.

# 8. RESULTS

## 8.1 Expression of germinal centre B-cell-like (GCB)- and activated B-cell-like (ABC)-associated markers

The expression of the GCB- and ABC-associated markers is presented in Table 7. The samples were CD10-, BCL6-, MUM1/IRF4-, BCL2- and FOXP1-positive in approximately 36%, 58%, 51%, 67% and 19% of cases, respectively. The cut-off percentages used in the survival analysis were the same in each study.

**Table 7.** Expression of cell-of-origin-associated proteins in DLBCL samples.

| | STUDY I | STUDY II | STUDY III | STUDY IV |
|---|---|---|---|---|
| **CD10, cut-off 30%** | | | | |
| Positive | 41% | 34% | 35% | 33% |
| Negative | 59% | 66% | 65% | 67% |
| **BCL6, cut-off 30%** | | | | |
| Positive | 54% | 62% | 59% | 55% |
| Negative | 46% | 38% | 41% | 45% |
| **MUM1/IRF4, cut-off 30%** | | | | |
| Positive | 49% | 54% | 51% | 49% |
| Negative | 51% | 46% | 49% | 51% |
| **BCL2, cut-off 50%** | | | | |
| Positive | NA | NA | 67% | 66% |
| Negative | | | 33% | 34% |
| **FOXP1, cut-off 100%** | | | | |
| Positive | NA | NA | 19% | 18% |
| Negative | | | 81% | 82% |

## 8.2 Prognostic impact of Hans algorithm-based cell-of-origin classification on survival (I, II)

In these series, the immunohistochemically defined GCB and non-GCB phenotypes were determined for all patients based on the expression of CD10, BCL6 and MUM1/IRF4 according to the Hans algorithm.

### 8.2.1 Outcome in immunochemotherapy-treated DLBCL patients (I)

**Clinical characteristics**

Baseline and treatment characteristics of 90 immunochemotherapy-treated patients and 104 controls treated with an antracyclin-based, CHOP-like chemotherapy regimen were compared. The patient and clinical characteristics were well balanced between the groups. According to IPI, 60% of subjects in the rituximab group and 63% in the control group had a low-risk disease, with an Ann Arbor stage of III or IV in 73% and 68% of cases, respectively.

The immunochemotherapy group consisted of 56% GCB and 44% non-GCB patients. The distribution was comparable in the chemotherapy group, as the GCB phenotype was observed in 45% and non-GCB phenotype in 55% of cases (p=0.200). The immunohistochemically defined non-GCB group included more males than females compared with the GCB subgroup. No significant differences in age, Ann Arbor stage and IPI score were observed between phenotypes or treatments.

**Survival analysis**

To analyse the survival of DLBCL patients in the pre- and post-rituximab eras, we compared the outcome of the immunochemotherapy and control groups. At 2 years, a significant difference in FFS and OS emerged between the groups. The FFS was 69% in the rituximab group and 45% in the control group. The corresponding OS rates were 79% and 61%. These survival differences were further observed in supplementary analysis in all age and IPI subgroups. A complete response was achieved after treatment in 70% of the patients in the rituximab group and in 79% of patients in the control group (p=0.250).

According to the IPI, the series consisted of about 60% low-risk and intermediate-risk (IPI 0-2) patients. The low-risk patients showed a significantly better survival than the high-risk (IPI 3-5) patients. In the rituximab group, the 2-year FFS was 79% for low-risk and 56% for high-risk patients (p=0.028), and the corresponding 2-year OS was 88% and 67% (p=0.030). Similar differences in the survival were observed in the control group (FFS 57% vs. 26%, p=0.001, and OS 75% vs. 39%, p<0.001). Cox multivariate analysis confirmed IPI as an independent prognostic factor for FFS and OS in chemotherapy- and immunochemotherapy-treated patients.

Consistent with previous studies (Hans et al. 2004, Berglund et al. 2005), chemotherapy-treated patients with the GCB phenotype had a significantly better survival than those with the non-GCB phenotype (FFS 59% vs. 34%, p=0.001, and OS 73% vs. 51%, p=0.012). Conversely, when the outcome was evaluated in immunochemotherapy-treated patients,

no difference in survival was observed between the molecular subgroups (FFS 72% vs. 67%, p=0.593, and OS 77% vs. 82%, p=0.936). In the rituximab group, the adverse impact of the non-GCB phenotype was eliminated.

### 8.2.2 Outcome in DLBCL patients treated with ASCT-supported HDT (II)

**Clinical characteristics**

Twenty-nine young high aa-IPI patients treated with HDT and ASCT, and 34 chemotherapy-treated control patients showed no difference in median age (ASCT 44.4 years and controls 52.5 years) or gender (ASCT: male 52%, female 48%, and controls: male 53%, female 47%). The ASCT group consisted of 93% high-risk patients according to the aa-IPI, whereas in the control group only 59% of patients had a high-risk disease (p=0.003). However, no significant difference was observed for Ann Arbor stage in the groups. Distribution of the immunophenotypes was equal. In the ASCT group, 45% of the patients belonged to the GCB and 55% to the non-GCB phenotype. In the control group, the corresponding division was 56% and 44%.

**Survival analysis**

The 2-year FFS rate was 64% in the ASCT group and 46% in the control group (p=0.020). In OS, a trend for a superior outcome was observed in patients treated with ASCT relative to the chemotherapy-treated patients (75% vs. 63%, p=0.183). According to the aa-IPI, high-risk patients had a worse outcome than low-risk patients, but due to the small number of low-risk patients the difference in survival remained non-significant.

To evaluate the impact of treatment on the predictive value of immunophenotype, we analysed the survival according to GCB and non-GCB subtypes. In the chemotherapy-treated group, non-GCB cases had a very poor survival relative to GCB patients (FFS 20% vs. 66%, p<0.001, and OS 47% vs. 77%, p=0.004). Conversely, no difference in outcome was observed between GCB and non-GCB patients treated with HDT and ASCT (FFS 67% vs. 62%, p=0.804, and OS 83% vs. 61%, p=0.585). We further compared the outcomes to evaluate the effect of treatment on the predictive value of the phenotype. According to phenotype and treatment, no significant difference in survival was present in GCB patients after ASCT or chemotherapy treatment (FFS 67% vs. 66%, p=0.821, and OS 83% vs. 77% p=0.414). However, in non-GCB patients, the ASCT-treated subgroup had a significantly better outcome than the control group (FFS 62% vs. 20%, p<0.001, and OS 69% vs. 47%, p=0.009). Cox regression analysis confirmed the prognostic value of treatment in non-GCB patients (FFS: RR 0.094, 95% CI 0.031-0.285, p<0.001, and OS: RR 0.225, 95% CI 0.078-0.653, p=0.006).

**Table 8.** Association of IPI and cell-of-origin phenotype on survival.

| **DLBCL patients treated with ICT (I)** | **N** | **1-yr FFS (%)** | **2-yr FFS (%)** | **P FFS** | **1-yr OS (%)** | **2-yr OS (%)** | **P OS** |
|---|---|---|---|---|---|---|---|
| **All** | 90 | 82 | 69 | | 89 | 79 | |
| **IPI** | | | | 0.028 | | | 0.030 |
| low 0-2 | 54 | 87 | 79 | | 94 | 88 | |
| high 3-5 | 36 | 75 | 56 | | 80 | 67 | |
| **Hans algorithm** | | | | 0.593 | | | 0.936 |
| GCB | 50 | 80 | 72 | | 88 | 77 | |
| non-GCB | 40 | 85 | 67 | | 90 | 82 | |
| **DLBCL patients treated with CT (I)** | **N** | **1-yr FFS (%)** | **2-yr FFS (%)** | **P FFS** | **1-yr OS (%)** | **2-yr OS (%)** | **P OS** |
| **All** | 104 | 61 | 45 | | 80 | 61 | |
| **IPI** (missing 1) | | | | 0.001 | | | <0.001 |
| low 0-2 | 65 | 75 | 57 | | 92 | 75 | |
| high 3-5 | 38 | 38 | 26 | | 63 | 39 | |
| **Hans algorithm** | | | | 0.001 | | | 0.012 |
| GCB | 47 | 78 | 59 | | 85 | 73 | |
| non-GCB | 57 | 46 | 34 | | 76 | 51 | |
| **DLBCL patients treated with HDT and ASCT (II)** | **N** | **1-yr FFS (%)** | **2-yr FFS (%)** | **P FFS** | **1-yr OS (%)** | **2-yr OS (%)** | **P OS** |
| **All** | 29 | 86 | 64 | | 93 | 75 | |
| **Hans algorithm** | | | | 0.804 | | | 0.585 |
| GCB | 13 | 92 | 67 | | 100 | 83 | |
| non-GCB | 16 | 81 | 62 | | 88 | 61 | |
| **DLBCL patients treated with CT (II)** | **N** | **1-yr FFS (%)** | **2-yr FFS (%)** | **P FFS** | **1-yr OS (%)** | **2-yr OS (%)** | **P OS** |
| **All** | 34 | 56 | 46 | | 82 | 63 | |
| **Hans algorithm** | | | | <0.001 | | | 0.004 |
| GCB | 19 | 79 | 66 | | 90 | 77 | |
| non-GCB | 15 | 27 | 20 | | 73 | 47 | |

## 8.3 Markers associated with ABC phenotype (III)

**Clinical characteristics**

In Study III, a group of 117 R-CHO(E)P treated de novo DLBCL patients was analysed. Approximately two-thirds of the patients had disseminated stage III or IV disease, and 39% had high IPI (3-5) risk scores.

A high BCL2 immunoreactivity was observed in 78 lymphomas (67%), whereas 39 cases were negative. The expression of BCL2 was associated with the non-GCB phenotype (p=0.011). No relationship was observed between BCL2 and FOXP1 expression.

The expression of FOXP1 was scored positive if 100% of the lymphoma cells had a uniform strong nuclear expression. Twenty-two (19%) of the lymphomas stained strongly for FOXP1, whereas the remaining 95 cases were negative. Distribution of FOXP1 immunoreactivity was comparable with a previous study (Barrans et al. 2004). We also evaluated the cut-off level of 30% for FOXP1-positive lymphoma cells (positive 39 (33%), negative 78 (67%)), observing no difference in outcome relative to the 100% cut-off level. A significant relationship emerged between FOXP1 and the non-GCB phenotype (p<0.001).

**Survival analysis**

The estimated 3-year FFS and OS rates were 71% and 80% for all patients, respectively. When the survival was analysed according to IPI risk stratification, low-risk patients had an improved outcome relative to high-risk patients (FFS 86% vs. 49%, p=0.001 (Figure 6A), and OS 89% vs. 69%, p=0.027). The division of the patients by cell-of-origin distinction according to the Hans algorithm showed no difference in outcome between GCB and non-GCB cases (FFS 76% vs. 67%, p=0.157, and OS 82% vs. 79%, p=0.636).

The results of this study further showed that BCL2 had prognostic value in this patient cohort. According to the Kaplan-Meier analysis, a worse outcome was observed after R-CHO(E)P treatment for BCL2-positive than for BCL2-negative patients. The 3-year FFS was 86% for BCL2-negative patients and 61% for BCL2-positive patients (p=0.001) (Figure 6B). A significantly difference in OS was also observed (negative 86% vs. positive 76%, p=0.034).

The predictive impact for BCL2 on outcome was further analysed separately in the GCB and non-GCB subgroups. For patients with the non-GCB phenotype, a significant impact

of BCL2 on 3-year FFS (negative 100% vs. positive 58%, p=0.011) and a trend for a difference in OS (negative 100% vs. positive 74%, p=0.082) were observed. By contrast, in GCB patients, BCL2 expression had no significant prognostic impact on survival (FFS, negative 85% vs. positive 68%, p=0.076, and OS, negative 85% vs. positive 78%, p=0.225).

The prognostic role of BCL2 was further confirmed in Cox analyses. According to univariate analyses, both IPI and BCL2 had a significant prognostic impact. In multivariate analyses, BCL2 remained an independent prognostic factor for FFS, but not for OS (FFS: RR 4.965, 95% CI 1.091-22.601, p=0.038, and OS: RR 3.592, 95% CI 0.820-15.736, p=0.090).

FOXP1 expression did not predict outcome in immunochemotherapy-treated patients. The 3-year FFS was 65% for FOXP1-positive and 72% for FOXP1-negative patients (p=0.476) (Figure 6C). The corresponding OS rates were 72% and 81% (p=0.898).

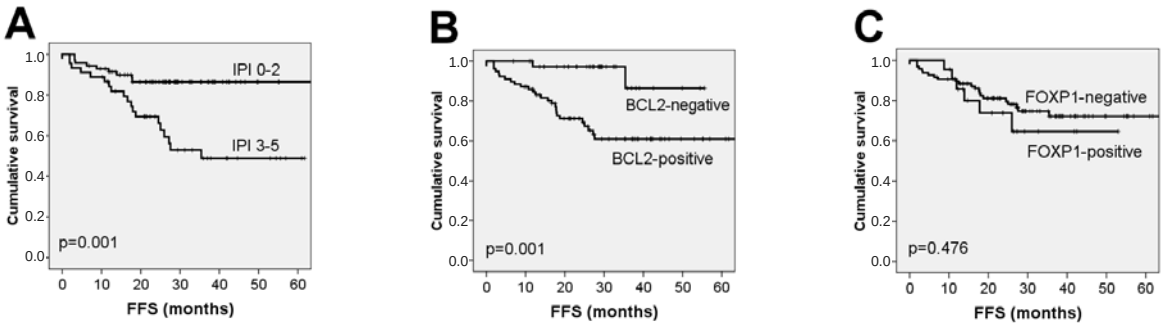


**Figure 6.** Failure-free survival according to A) IPI, B) BCL2 expression and C) FOXP1 expression.

## 8.4 Prognostic impact of modified cell-of-origin algorithms (IV)

**Clinical characteristics**

Both IPI and R-IPI were retrospectively determined for the 88 R-CHOP-treated patients. Sixty-two percent had a low-risk disease according to IPI (IPI 0-2), whereas the remaining patients had a high-risk disease (IPI 3-5). According to the R-IPI risk stratification, 7%, 55% and 38% of patients had very good, good and poor prognosis, respectively. Of the patients, 49% had involvement of one or more extranodal sites at the time of diagnosis, and 53% had advanced stage III or IV disease.

**Survival analysis**

For all patients, the 3-year FFS was 72% and the OS 77%. According to the IPI and R-IPI risk stratifications, a significant difference in survival rates was present. The FFS was 84% for IPI low-risk and 53% for IPI high-risk patients (p=0.002). The difference in OS was similar (IPI low-risk 85% vs. IPI high-risk 63%, p=0.012). Concordantly, the R-IPI distinguished three risk groups with different outcomes, FFS 100%, 82% and 53% for very good, good and poor risk groups, respectively. The corresponding OS rates observed in the R-IPI risk groups were 100%, 83% and 63%. No difference in outcome emerged between patients with nodal and those with extranodal involvement.

### 8.4.1 Modified ABC-like classification and other classifications

In the patient cohort, MUM1/IRF4 expression was observed in 43 patients and FOXP1 expression in 16 patients. According to the modified ABC-like classification, 46 cases were assigned to the ABC-like subgroup and the remaining 42 cases to the subgroup of “others”. The classification correlated significantly with CD10 ($p<0.001$) and BCL6 expression ($p=0.001$), and Hans ($p<0.001$) and Muris algorithms ($p<0.001$). The rate of misclassification in relation to the Hans algorithm was 14% and in relation to the Muris algorithm 16%. The measure of agreement between the modified ABC-like and Hans classifications was 0.727.

Clinical characteristics were well balanced between the subgroups. The ABC-like subgroup consisted of more elderly patients and cases with nodal restricted disease.

According to Kaplan-Meier analysis, the ABC-like subgroup had a significantly inferior FFS rate (63% vs. 82%, p=0.048) (Figure 7A). For patients with the ABC-like phenotype, a trend for an adverse OS was also observed (69% vs. 85%, p=0.110).

According to the Muris algorithm, group 2 consisted of 32 patients with the post-GCB immunophenotype. The rest of the patients (64%) belonged to group 1. Patients in group 2 showed an adverse outcome relative to patients in group 1. The FFS was 59% in group 2, compared with 81% in group 1 (p=0.041) (Figure 7B), and the corresponding OS was 67% and 82% (p=0.159).

When the cell of origin was determined using the Hans algorithm, 48% of cases belonged to the GCB subgroup and the remainder had the non-GCB phenotype. The differences in clinical outcomes between the GCB and non-GCB patients were non-significant (FFS 75 % vs. 70%, p=0.631 (Figure 7C), and OS 77% vs. 76%, p=0.894).

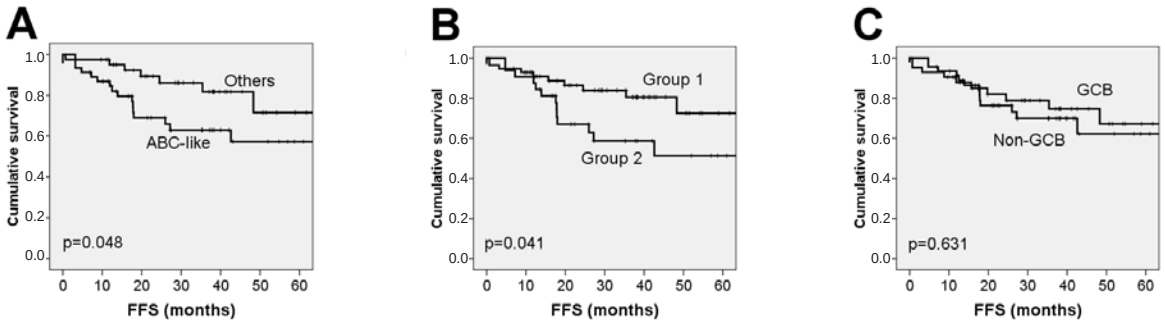


**Figure 7.** Failure-free survival according to A) modified ABC-like algorithm, B) Muris algorithm and C) Hans algorithm.

**Table 9.** Association of IPI, BCL2, FOXP1 and cell-of-origin phenotype on survival.

| **DLBCL patients treated with R-CHO(E)P (III)** | **N** | **1-yr FFS (%)** | **3-yr FFS (%)** | **P FFS** | **1-yr OS (%)** | **3-yr OS (%)** | **P OS** |
|---|---|---|---|---|---|---|---|
| **All** | 117 | 89 | 71 | | 93 | 80 | |
| **IPI** (missing 1) | | | | 0.001 | | | 0.027 |
| low 0-2 | 71 | 92 | 86 | | 94 | 89 | |
| high 3-5 | 45 | 84 | 49 | | 91 | 69 | |
| **Hans algorithm** | | | | 0.157 | | | 0.636 |
| GCB | 55 | 91 | 76 | | 91 | 82 | |
| non-GCB | 62 | 87 | 67 | | 95 | 79 | |
| **BCL2 expression** | | | | 0.001 | | | 0.034 |
| positive | 78 | 85 | 61 | | 91 | 76 | |
| negative | 39 | 97 | 86 | | 97 | 86 | |
| **FOXP1 expression** | | | | 0.476 | | | 0.898 |
| positive | 22 | 86 | 65 | | 96 | 72 | |
| negative | 95 | 89 | 72 | | 94 | 81 | |
| **DLBCL patients treated with R-CHOP (IV)** | **N** | **1-yr FFS (%)** | **3-yr FFS (%)** | **P FFS** | **1-yr OS (%)** | **3-yr OS (%)** | **P OS** |
| **All** | 88 | 90 | 72 | | 93 | 77 | |
| **IPI** (missing 1) | | | | 0.002 | | | 0.012 |
| low 0-2 | 54 | 92 | 84 | | 94 | 85 | |
| high 3-5 | 33 | 85 | 53 | | 91 | 63 | |
| **Hans algorithm** | | | | 0.631 | | | 0.894 |
| GCB | 42 | 89 | 75 | | 90 | 77 | |
| non-GCB | 46 | 91 | 70 | | 96 | 76 | |
| **Muris algorithm** | | | | 0.041 | | | 0.159 |
| group 1 | 56 | 91 | 81 | | 93 | 82 | |
| group 2 (post-GCB) | 32 | 88 | 59 | | 94 | 67 | |
| **Modified ABC-like algorithm** | | | | 0.048 | | | 0.110 |
| others | 42 | 95 | 82 | | 95 | 85 | |
| ABC-like | 46 | 85 | 63 | | 91 | 69 | |

# 9. DISCUSSION

## 9.1 Immunohistochemically defined cell-of-origin distinction in DLBCL (I, II)

The addition of rituximab to conventional chemotherapy has significantly improved the outcome of DLBCL patients during the last decade. Currently, the IPI risk stratification is the only prognostic tool in use in daily clinical practice. However, our knowledge of the biology of DLBCL has significantly improved from the findings attained in GEP studies. As the cell of origin identified by either the GEP (Alizadeh et al. 2000) or IHC (Hans et al. 2004) methodology was observed to be prognostic for patients treated with chemotherapy, we decided to identify the prognostic role of the GCB and non-GCB phenotypes in immunochemotherapy-treated patients. When this study commenced, no analyses of cell-of-origin distinction in rituximab-treated patients had been published. In Study I, we observed that according to the Hans algorithm the IHC-defined GCB patients had improved survival relative to non-GCB patients treated with chemotherapy. This finding was consistent with previous studies (Hans et al. 2004, Berglund et al. 2005). By contrast, the prognostic value of the cell-of-origin distinction disappeared in patients treated with a combination of rituximab and chemotherapy. In both treatment groups, only the IPI risk stratification could distinguish between patients with good and poor prognosis.

In the studies subsequently published in the immunochemotherapy era, the results on the prognostic impact of GCB and non-GCB phenotype have been controversial. Consistent with our findings, several studies have not observed any difference in survival between GCB and non-GCB patients treated with immunochemotherapy (Saito et al. 2007a, Ennishi et al. 2008, Uccella et al. 2008, Wilson et al. 2008, Copie-Bergman et al. 2009, Ilic et al. 2009, Kyllönen et al. 2009, Seki et al. 2009, Song et al. 2009, Xia et al. 2010). The immunochemotherapy regimen has consisted of rituximab in combination with CHOP-like chemotherapy. However, Fu et al. (2008) reported that in a series of both EPOCH- and R-EPOCH-treated DLBCL patients, OS was significantly better for the group of GCB patients than in patients with the non-GCB phenotype. With the EFS, a significant difference was observed in chemotherapy-treated patients, whereas only a trend for a difference was seen in immunochemotherapy-treated patients. In addition, in an immunochemotherapy-treated Japanese cohort, superior survival was observed for GCB patients relative to non-GCB patients (Morito et al. 2009).

In Study II, we evaluated the clinical impact of immunophenotyping in DLBCL patients treated with HDT supported with ASCT and noted that the survival was similar for the GCB and non-GCB subgroups. Other subsequent studies (Veelken et al. 2007, Costa et al. 2008, Hallack Neto et al. 2009a) have also reported that GCB and non-GCB DLBCL patients had a similar survival after HDT and ASCT, and that the outcome was independent of cell-of-origin distinction. On the other hand, the trials of the Dutch Hemato-Oncology Association (HOVON) for poor-risk DLBCL patients showed that the GCB phenotype had a significant positive impact on survival relative to the non-GCB phenotype (van Imhoff et al. 2006). However, rituximab was not included in the treatment regimens in these studies.

Our results in Studies I and II revealed that the survival of all DLBCL patients, but particularly non-GCB patients, improved in response to modification of conventional chemotherapy. Those lymphomas that relapsed after intensified treatments were most refractory to salvage therapies, and the patients died rapidly due to lymphoma. The mechanism by which non-GCB patients benefit more from intensified regimens than GCB patients is unknown, but is likely to be associated with biological features of the disease.

The biological characteristics related to aggressive disease in ABC-type DLBCLs include defective apoptosis signalling caused by overexpression of the anti-apoptotic *BCL2*, high expression of NF-κB and constitutive activation of Iκ kinase (Davis et al. 2001, Rosenwald et al. 2002, Iqbal et al. 2004, Bea et al. 2005, Lam et al. 2005). By contrast, the *BCL6* gene is principally expressed in GCB DLBCLs (Alizadeh et al. 2000, Rosenwald et al. 2002, Wright et al. 2003).

NF-κB is an important mediator of resistance to apoptosis. Lymphoma cells with constitutively active NF-κB have been observed to be resistant to chemotherapeutic agents, and inhibition of NF-κB to increase the sensitivity of lymphoma cells to therapy in vitro (Wang et al. 1999). The induced cell cycle arrest and apoptosis by inhibition of NF-κB are mainly seen in cell lines representing ABC-type lymphomas, suggesting that this particular mechanism could contribute to the poor outcome of these lymphomas (Davis et al. 2001). Rituximab, on the other hand, may control the NF-κB pathway and play a role in the sensitization of lymphomas to chemotherapeutic agents. Rituximab has been observed to inhibit expression of the components of the NF-κB pathway, consequently decreasing the activity of the pathway in chemoresistant DLBCL cell lines (Jazirehi et al. 2005). This may provide a survival benefit for the non-GCB subgroup. As rituximab decreases the activity of the NF-κB pathway, the expression of the NF-κB target gene *PRDM1β*, a regulator of the differentiation of mature B lymphocytes to plasma cells, is

also reduced (Liu et al. 2007). Liu et al. (2007) observed that expression of *PRDM1β* correlated with poor outcome in chemotherapy-treated non-GCB patients. However, the addition of rituximab eliminated the survival difference, further supporting the more beneficial effect of rituximab in non-GCB patients. The NF-κB pathway was also noted to induce the expression of cytokines IL-6 and IL-10 through the signal transducer and activator of transcription (STAT) 3 protein signalling in ABC DLBCLs (Lam et al. 2008), although the consequences of this action remain unclear. Furthermore, in studies performed with DLBCL and other B-cell lymphoma cell lines, rituximab has been described to inhibit the constitutively active phosphatidylinositol-3-kinase/AKT (PI3K/AKT) (Smith et al. 2005, Uddin et al. 2006, Suzuki et al. 2007), mitogen-activated protein kinase kinase 1/2 (MAPK) (Vega et al. 2004) and extracellular signal-regulated kinase 1/2 (ERK) pathways (Jazirehi et al. 2004), but the impact of these pathways on the outcome of DLBCL patients still needs to be clarified.

The association of GCB phenotype with a more favourable outcome may be a consequence of the expression of BCL6 since BCL6 represses the expression of the *PRDM1* gene (Shaffer et al. 2000, Tunyaplin et al. 2004), transcription of *NFκB1* (Li et al. 2005) and activity of NFκB (Perez-Rosado et al. 2008). The GCB DLBCLs also show prominent levels of pro-apoptotic molecules Bax, Bak and Bid, possibly contributing to the good response to therapy (Bai et al. 2004). IL-4 target genes have been observed to be differentially expressed in the GCB and non-GCB subgroups (Lu et al. 2005), and IL-4 can sensitize the GCB cells to chemotherapy and rituximab (Sarosiek et al. 2009).

It is, however, important to note that the identified biological characteristics of GCB and non-GCB lymphomas have not yet clarified the controversial findings obtained in studies of the prognostic impact of immunohistochemically defined cell-of-origin distinction for survival. These clinical and methodological factors that may influence the results will be discussed further in the following section.

## 9.2 Markers and algorithms associated with the ABC phenotype (III, IV)

Since the immunohistochemical findings of the prognostic impact of the cell of origin for survival were conflicting, we decided to evaluate other markers and alternative algorithms, predominantly associated with the ABC phenotype. We focused on the expression of MUM1/IRF4, FOXP1 and BCL2 in immunochemotherapy-treated de novo DLBCL patients. Although the studies were retrospective, we aimed to minimize treatment-related biases by including only patients who had received uniform therapy.

Before the initiation of our study, the prognostic impact of FOXP1 had not been evaluated in immunochemotherapy-treated DLBCL patients. Moreover, the results concerning the predictive value of BCL2 expression had been controversial. In Study III, the expression of FOXP1 and BCL2 proteins was found to be associated with the non-GCB phenotype. This finding is consistent with previous studies on chemotherapy-treated patients (Barrans et al. 2004, Hans et al. 2004, van Imhoff et al. 2006). In survival analysis, no difference was observed in the outcome between FOXP1-positive and -negative patients. The number of FOXP1-positive cases was relatively small (19%), possibly restricting the survival analyses. By contrast, BCL2-negative patients had a significantly better outcome than BCL2-positive patients, both among all patients and especially in the non-GCB subgroup. Consistent with our findings, BCL2 negativity at the mRNA and protein levels has been reported to be a favourable feature in immunochemotherapy-treated patients (Malumbres et al. 2008, Winter et al. 2008). Similarly, when the BCL2 expression was evaluated separately in the GCB and non-GCB subgroups, the BCL2 expression was predictive in GCB patients treated with immunochemotherapy, and patients with the BCL2-negative GCB phenotype had the most favourable prognosis (Song et al. 2009). In other studies, addition of rituximab to chemotherapy resulted in a loss of the negative prognostic impact of BCL2 on survival (Mounier et al. 2003, reviewed in Shivakumar & Armitage 2006, Wilson et al. 2007, Fu et a. 2008, Wilson et al. 2008, Seki et al. 2009, Song et al. 2009).

BCL2 is a regulator of apoptosis and its overexpression has been observed to result in chemotherapy resistance of lymphoma cells both in vitro and in vivo (Hermine et al. 1996, Kramer et al. 1998, Barrans et al. 2002). Rituximab, on the other hand, induces apoptosis and sensitizes lymphoma cells to chemotherapeutic agents by downregulating BCL2 and BCL-xL expression (Alas & Bonavida 2001, Jazirehi et al. 2003). The results of studies in cell culture indicate that rituximab could eradicate the negative impact of BCL2 expression on survival. Additionally, rituximab was observed to inhibit IL10 (Alas & Bonavida 2001, Alas et al. 2001), which functions as a regulator of BCL2 (Voorzanger et al. 1996, Weber-Nordt et al. 1996), and to consequently interfere with the expression of BCL2. However, interactions between BCL2 and IL10 gene polymorphisms have recently been described to cause R-CHOP resistance in DLBCLs (Park et al. 2009). Furthermore, in lymphomas with *BCL2* and *MYC* translocations, patients negative for BCL2 expression have been reported to have a significantly superior survival relative to BCL2-positive patients (Johnson et al. 2009). Thus, multiple details of the lymphoma genome complicate the interpretation of findings.

In Study IV, we focused on ABC-associated markers. We evaluated MUM1/IRF4 and FOXP1 positivities and developed a modified ABC-like classification. As a result, we were able to distinguish the ABC-like subgroup of DLBCL patients with poor survival in response to immunochemotherapy. Similarly, according to the Muris classification based on the expression of BCL2, CD10 and MUM1/IRF4, the group of BCL2-positive post-GCB patients had an inferior survival relative to the other patients. Although a correlation existed in the phenotypes identified by the modified ABC-like classification and the Hans algorithm, no survival difference was observed between GCB and non-GCB patients when the Hans algorithm was used. The difference in the prognostic impact between the separate algorithms may be associated with BCL6 immunostaining. In the Hans algorithm, BCL6 has an effect on the classification of the phenotype in about 70% of CD10-negative DLBCLs. There is a risk for misclassification of these DLBCLs, as a poor reproducibility of BCL6 staining and interpretation of immunoreactivity have been found (de Jong et al. 2009).

Consistent with our findings in the immunochemotherapy era, Sjö et al. (2007) had previously confirmed the prognostic value of the Muris algorithm in chemotherapy-treated DLBCL patients. Similarly, the value of the modified ABC-like classification needs to be further tested in other patient cohorts and prospective studies. Nevertheless, confirmatory results of the negative impact of FOXP1 and MUM1/IRF4 expression on survival were recently reported. An immunofluorescence in situ (FISH) index, defined by two of the three ABC markers (positivity for FOXP1, MUM1/IRF4 or *BCL6* breakpoint), was observed to be associated with an unfavourable outcome in immunochemotherapy-treated DLBCL patients (Copie-Bergman et al. 2009).

The benefit of the proposed algorithms in DLBCL is their ability to define the subgroups based on only two to five markers. However, the algorithms are dependent on the prognostic effect of each marker as well as its hierarchical position in the algorithm. For example, if a staining is unreliable and difficult to score, the algorithm may not classify the subgroups correctly. Furthermore, diverse routine practices in different centres may influence the results obtained with algorithms.

## 9.3 Clinical and methodological variables that determine the predictive value of molecular markers in DLBCL (IV)

GEP-based measurement of mRNA levels in lymphomas identified molecular characteristics and cell-of-origin classification of DLBCL. However, the microarray

technique is currently not available for routine clinical use due to lack of standardized commercially accessible tests. The requirement for fresh or frozen tissue samples also limits the methodology. Although RNA has been extracted from paraffin blocks, this methodology is rarely feasible. Immunohistochemical class prediction and determination of prognostic markers, in turn, would be more practical. To date, unfortunately, the reported IHC-defined results have been inconsistent. A number of clinical and methodological factors are likely to account for the variable results. Clinical factors include patient and treatment-related characteristics. Methodological factors are related to the immunohistochemical method and include variations in techniques, scoring criteria and inter-and intraobserver variability.

### 9.3.1 Clinical variables

Selection of patient series varies in different studies. The cohorts are either retrospectively or prospectively collected. The specific age groups, distribution of gender, populations, and possible inclusion of relapsed patients in the studies may also create biases. Furthermore, survival of the patients may be affected by the involvement of nodal or extranodal regions, as those with local lymphomas have a more favourable outcome than patients with a disseminated disease. The chemotherapy regimens used in different countries are globally observed to be non-uniform, mostly due to historical routines. Also, during the time period when rituximab treatment was initiated and the immunochemotherapy era started, there were several treatment options. Likewise, the OS may be influenced by the effective second-line treatment (rituximab, HDT and ASCT, or palliative treatment) in patients who relapse after first-line treatment.

Currently, potential new treatment approaches are under investigation in lymphomas. Novel therapies, including biological drugs, may have an effect on the prognostic impact of all of the factors studied thus far in DLBCL. When new treatments are applied in therapy routines, the previously recognized prognostic markers require reassessment. As well with new therapies, other previously unidentified biological factors of DLBCL may play an important role in prognosis.

Potential future treatments in DLBCL include radioimmunotherapy, which has already become an option in FL and mantle cell lymphoma. 90Y-ibritumumabtiuxetan targets radiation to B-lymphoma cells by CD20 guidance. The response rates observed for 90Y in phase I/II trials are approximately 50% for DLBCL patients (Witzig et al. 1999, Gordon et al. 2004). Other phase II/III studies have been performed to evaluate the use of radioimmunotherapy in elderly refractory DLBCL patients who are not suitable candidates

for HDT and ASCT (Morschhauser et al. 2007), as well as following CHOP as a consolidation therapy in previously untreated elderly patients (Zinzani et al. 2008), with promising results. Furthermore, the second generation of anti-CD20 antibodies (veltuzumab, ofatumumab), with structural and functional differences to rituximab, has been created to improve the efficacy of treatments. Results in phase I/II studies of veltuzumab in relapsed and refractory B-cell NHLs show good tolerability of the drug and promising objective responses (Morschhauser et al. 2009), which will be further evaluated in ongoing clinical trials. Biologically designed therapies with indications in other cancer entities are also under investigation in DLBCL. Results are expected from ongoing phase 3 trials, after initial results on the VEGF-A antibody bevacizumab (Stopeck et al. 2009), the PKCβ inhibitor enzastaurin (Robertson et al. 2007) and the mammalian target of the rapamycin (mTOR) inhibitor everolimus (Yee et al. 2006).

### 9.3.2 Methodological variables

Incorporation of IHC-defined predictive markers into clinical practice is not yet appropriate, partly due to the lack of validation of the methodology. Variation in laboratory techniques is related to differences in the use of TMA or whole-tissue sections, fixation of slides, staining protocols, and use of different antibodies. The TMA technique allows simultaneous staining and analysis of a large number of tumours, but in some cases cores can be missing. Debatable is also the representativeness of a TMA sample compared with a whole-tissue section, as the architecture of the lymphoma may be indistinctive in the TMA cores. According to a report by Hedvat et al. (2002), the TMA was comparable with whole sections in IHC analysis of several commonly used markers of NHL such as CD10, CD20 and BCL2. However, determination of the GCB phenotype from TMA sections was unreliable due to difficulties in the scoring of BCL6 on TMAs relative to whole-tissue sections (Linderoth et al. 2007a). Scoring of the data also diverges, as different cut-off levels are used. The cut-off levels are often determined according to previous data or, for example, as the median positive value of the staining. For instance, the cut-off level of the markers in the Hans algorithm was generally determined as more than 30% positivity of the lymphoma cells (Hans et al. 2004, Berglund et al. 2005, Amen et al. 2007), but in other studies variations in the cut-off level - between 10% and 50% - have been used (Muris et al. 2006, Veelken et al. 2007). The positivity of a staining is usually determined by one or two pathologists. The result is therefore dependent on the knowledge of the pathologist, leading to a risk for bias. This problem could probably be eradicated by the use of rational statistical methods (Tzankov et al. 2009).

In 2003, the Lunenburg Lymphoma Biomarker Consortium was established as an international collaboration in order to unify IHC methods in biomarker research. Thus far, the group has focused on standardization of measurements and validation of the prognostic relevance of biomarkers in DLBCL (de Jong et al. 2007 & 2009). They have especially concentrated on technical and intra-observer variations in scoring interpretation. The study included eight experienced laboratories that used diverse protocols and antibodies in the stainings of eight proteins on identical TMAs. Although the same antibody clone was used, staining intensities were noted to differ significantly, and technical artefacts were also observed. After eliminating the discrepancies in the stainings, the scorings were evaluated by nine pathologists. The reproducibility was excellent or very good for several markers, i.e. CD10, CD20, MUM1/IRF4, CD5 and BCL2. However, poor reproducibility was noted for Ki-67 and BCL6. Initially, the agreement observed for the classification of GCB and non-GCB was 57%, but after optimizing the stainings the agreement improved to 77% (de Jong et al. 2009). The IHC for subclassification of DLBCL was reported to be feasible. The intra- and interobserver variation for BCL2 has also been evaluated and determined to be acceptable in assessments of lymphoma (Borlot et al. 2008).

A goal of future studies will be to analyse both RNA and tissue samples by collecting both frozen and fresh, and paraffin-embedded materials from all patients. In research and in clinical practice, the immunohistochemical technique needs to be optimized and the scoring criteria strictly followed. Later, by combining several markers in different samples of a patient and by using diverse techniques and methods (i.e. tissue and serum samples, IHC and in situ hybridization), the reliability and value of predictive markers should improve. The molecular properties of DLBCL will likely tailor treatment decisions in the future, as demonstrated by the proteasome inhibitor bortezomib, which enhances the activity of chemotherapy in ABC, but not GCB, DLBCLs (Dunleavy et al. 2009).

# 10. CONCLUSIONS

Diversity in its clinical presentation, morphology and immunophenotype indicate that DLBCL is a heterogeneous disease. GEP studies have contributed to improved knowledge of the molecular features of DLBCL. In addition to tumour-specific divergences, crosstalk between tumour cells and the microenvironment might also be important. Currently, GEP is not accessible in routine clinical use. However, identification of the molecular phenotypes and markers has been translated to an applicable approach by using IHC.

In these studies, we evaluated the prognostic impact of IHC-defined molecular markers and algorithms in DLBCL patients treated with immunochemotherapy or HDT and ASCT. The main conclusions were as follows:

1. The prognostic impact of the Hans algorithm cell-of-origin classification, based on the expression of CD10, BCL6 and MUM1/IRF4, is eliminated in DLBCL patients treated with immunochemotherapy. Correspondingly, the GCB and non-GCB high-risk patients treated with HDT and ASCT have a similar survival.

2. In immunochemotherapy-treated DLBCL patients, BCL2 negativity is associated with superior survival. By contrast, FOXP1 expression does not have a prognostic impact on survival.

3. Modified algorithms primarily focusing on ABC-associated markers identify the ABC-like subgroup of DLBCL patients with a poor prognosis after immunochemotherapy.

Although our results suggest that the proposed modified ABC-like classification and the expression of BCL2 may identify DLBCL patients with an unfavourable outcome, these findings need to be re-evaluated in prospective studies. Knowledge about the biology of DLBCL in combination with the clinical features of the disease will hopefully define reliable prediction tools and provide tailored treatments for future daily clinical practice.

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Heidi Nyman

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