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# ABBREVIATIONS

aaIPI	Age-adjusted International Prognostic Index
ABC	Activated B-cell like
ASCT	Autologous stem cell transplantation
Bcl-2	B-cell lymphoma gene-2
Bcl-6	B-cell lymphoma gene-6
CD	Cluster of differentiation
CHOP	Cyclophosphamide, doxorubicin, vincristine, prednisone
CHOEP	CHOP plus etoposide
CI	Confidence interval
CNOP	Cyclophosphamide, mitoxantrone, vincristine, prednisone
CR	Complete remission
CRu	Complete remission, uncertain
CT	Computed tomography
CTL	Cytotoxic T lymphocyte
ECOG	Eastern Cooperative Oncology Group
ESR	Sedimentation rate
FOXP3	Forkhead box protein 3
GCB	Germinal centre B-cell like
HR	Hazard ratio
IFN	Interferon
IPI	International Prognostic Index
LDH	Lactate dehydrogenase
MHC	Major histocompatibility complex
MUM-1	Multiple myeloma oncogene-1
NF- $\kappa$ B	Nuclear factor-kappa B
NK	Natural killer
OS	Overall survival
p-AKT	Phosphorylated AKT
PFS	Progression-free survival
PR	Partial remission
RT	Radiotherapy
RT-PCR	Reversed transcriptase-polymerase chain reaction
Th	T helper
TIA-1	T cell intracytoplasmic antigen-1
TILs	Tumour infiltrating lymphocytes
TNF	Tumour necrosis factor
T <sub>regs</sub>	Regulatory T cells
VACOP-B	Etoposide, doxorubicin, cyclophosphamide, vincristine, bleomycin, prednisone

# INTRODUCTION

## Lymphoma classifications

Malignant lymphomas are tumours originated from cells in the lymphatic system (lymph nodes, spleen, and other lymphatic tissues). While Hodgkin's disease has been a well-defined disease for more than 150 years, definitions and delimitations of the other lymphoma subtypes, the non-Hodgkin lymphomas (NHL), have been less clear and several diagnostic classification systems have been used for the last forty years. Before 1966 the NHL were divided into reticulum cell sarcoma, lymphosarcoma and giant follicle lymphoma. The Rappaport classification 1966 [1] recognised several entities based on growth pattern (diffuse vs nodular) and cytomorphology (histiocytic, lymphocytic, mixed). In the 1970s, the Lukes-Collins [2] and Kiel classifications [3] incorporated knowledge about immunology (T vs B phenotypes) and lymphocyte physiology, but none of these or other classifications reached world-wide acceptance. The Working Formulation (WF) 1982 [4] was an attempt to find a common platform between co-existing classifications but still described the entities based on cytomorphology (the term "large cell" replaced "histiocyte"), growth pattern (diffuse vs follicular) and three different "grades", based on survival curves, without taking T vs B phenotypes into account. The term "diffuse large B-cell lymphoma" (DLBCL) was not established until 1994 when the Revised European-American Classification of Lymphoid Neoplasms (REAL) [5] was introduced. REAL incorporated all available morphologic, immunologic, genetic and clinical data for each diagnosis and this system became generally accepted and subsequently constituted the basis for the current WHO-classification, established 2001 [6] and updated 2008. DLBCL corresponds to diffuse centroblastic, B-immunoblastic and anaplastic large B-cell lymphoma in the Kiel classification. It is not possible to exactly delineate DLBCL from the WF scheme, but diffuse large cell (group G), large cell immunoblastic (group H) and diffuse mixed small and large cell (group F) lymphoma encompass DLBCL [6].

## Definition & characteristics

DLBCL is a heterogeneous disease, morphologically, clinically and biologically. In tissue sections, the tumour is characterized by a proliferation of blastic B-cells with a diffuse growth pattern. In immunohistochemical staining, the cells almost invariably express the B-cell markers CD20 and CD79a and expression of the proliferation marker Ki-67 is varying but relatively high [7]. Infiltration of lymphocytes and histiocytes, as also sclerosis, can be present to a variable degree. Extranodal involvement, i.e. outside lymph nodes, spleen, thymus and Waldeyer's Ring, is common. Special subtypes occur, e.g. primary DLBCL of

the CNS, mediastinal large B-cell lymphoma, Tcell/histiocyte-rich large B-cell lymphoma, primary cutaneous DLBCL, primary effusion lymphoma and intravascular large B-cell lymphoma. DLBCL is the most common lymphoma entity and has been reported to account for 33% of all NHL in one non-population based study [8] and 40% and 33%, respectively, in two population-based registries [9, 10], but chronic lymphocytic leukaemia was not included in these latter registries, indicating that the true DLBCL proportion is lower. Most cases are *de novo* DLBCL, without a previously known lymphoma, but a composite picture of DLBCL and follicular lymphoma can occur. DLBCL can also develop from a previously known low grade lymphoma, i.e. transformed lymphoma. DLBCL belongs to the “aggressive” lymphomas, a loosely defined clinical term denoting lymphomas with rapid clinical course if not treated but possible to cure [11]. The term approximately corresponds to the “intermediate grade” (i.e. groups D-G) and “high grade” group H in WF.

## Treatment

Before the introduction of combination chemotherapy in the mid 1960s, practically no patients were cured except for a proportion of stage I-II [12, 13] patients treated with RT [14]. The chemotherapy combinations MOPP (nitrogen mustard, vincristine, procarbazine, prednisone), C-(M)OPP (nitrogen mustard replaced by cyclophosphamide) [15] and combined vincristine, cyclophosphamide, cytarabine and methotrexate [16] were the first reported regimens with curative potential for patients with stage III-IV diffuse histiocytic lymphoma. After introduction of doxorubicin [17, 18], the CHOP regimen [19] given every third week (CHOP-21) became standard therapy, with a reported long-term cure rate of 32% for stage II-IV diffuse histiocytic lymphoma [20]. In the 1980s, multidrug regimens (e.g. mBACOD, ProMACECytaBOM, MACOP-B) were introduced with favourable outcome data in phase II studies, but randomized studies on aggressive lymphomas failed to demonstrate a survival benefit for the new regimens [21, 22]. Thus, CHOP or CHOP-like regimens remained as standard therapy for stage II-IV disease, resulting in a long-term OS of approximately 40-45%. In recent years, however, shortening of the cycle intervals to two weeks (CHOP-14) [23] and especially combining the anti-CD20 antibody rituximab (R) with CHOP-21 [24-27] or R-CHOP-14 [28], has resulted in improved OS, in the range of 9-13%, and R-CHOP has become standard therapy for stage II-IV DLBCL.

For patients with early stage disease (stage I - localized stage II), RT alone was initially used, but since addition of chemotherapy was shown to be beneficial [29, 30], short chemotherapy combined with involved field RT or chemotherapy alone became routine. The chemoradiotherapy alternative has produced excellent results (long-term OS exceeding 90%) for patients with non-bulky, stage I disease without other risk factors [31, 32], but in recent years the benefit of RT has been questioned, especially after the introduction of rituximab.

Consolidating ASCT has not been proven to be beneficial as part of first line therapy [33] but is indicated for first relapse patients responding to second line chemotherapy [34].

## **Prognostic factors**

In a given therapy era a search for prognostic factors at diagnosis are valuable in the risk estimation of the patient, can facilitate comparisons between studies and identify candidates for alternative therapies and regarding biological markers, bring insight into tumour biology and therapy resistance.

## **Clinical factors**

IPI, the prevailing clinical score system, is based on a large (2031 patients), multi-institutional study on aggressive lymphomas, treated with different doxorubicin-containing regimens in phase II and III studies between 1982 and 1987. Age >60 years, stage III+IV, bad performance status (ECOG 2-4), increased serum LDH level, >1 extranodal site, B symptoms and bulky disease had all shown prognostic influence in previous studies and so they did in this study, in univariate analyses, as also low serum albumin level and involvement of spleen, bone marrow, liver, lung and CNS did. All the variables, except for serum albumin, were chosen for multivariate Cox regression analysis; high age, bad performance status, increased serum LDH level, stage III+IV and >1 extranodal sites persisted as independent predictors for inferior OS. Four prognostic groups were identified depending on the number of risk factors present, low risk (0-1 factors), low-intermediate (2), high-intermediate (3) and high risk (4-5) group; estimated OS at 5 years was 73, 51, 43 and 26%, respectively. For patients  $\leq$ 60 years of age, the aaIPI model was constructed by LDH level, stage and performance status; low risk (0 factors), low-intermediate (1), high-intermediate (2) and high risk group (3). Estimated OS at 5 years was 83, 69, 46 and 32%, respectively. The aaIPI model was also applicable for patients older than 60 years [35].

## **Biological factors**

### **Cytogenetics**

The most common chromosome abnormalities involve the bcl-6 gene on chromosome 3, considered to be crucial in DLBCL pathogenesis [36, 37]. T(3;14) and other 3q27 translocations, from which the bcl-6 gene originally was cloned [38], occur in approximately 15-25% of the DLBCL cases but studies on the prognostic value have shown conflicting results [39-41]. Mutations of the bcl-6 gene are more frequent, 50-60% of DLBCL cases [42, 43], and have been associated with a favourable prognosis [43, 44]. T(14;18) translocation involving the bcl-2 gene on chromosome 18 is seen in 15-20% of the DLBCL cases but has not shown to be prognostic [41, 45]. Myc 8q24 translocations, t(8;14) and variants, occur in 7-10% [41, 46, 47] and has been attributed worse

prognosis, but it has been unclear to what extent a concomitant t(14;18) translocation or p53 dysregulation has contributed. However, in a recent study, myc translocation was associated with worse PFS and OS independent of t(14;18) [48]. Also, amplification of the 8q24 gene can occur [49] and in one study, overexpressed myc mRNA was seen in 30% (15/48) of the DLBCL cases, largely independent of a myc translocation, and was associated with worse outcome [50]. Additionally, a large number of other chromosomal aberrations have been described in DLBCL [49, 51, 52].

### **Gene expression profiling**

Gene expression profiling with DNA microarray techniques has led to essential new knowledge about the molecular background of DLBCL [53-57]. In the “stage of B-cell differentiation” or “cell-of-origin” concept, two subtypes of DLBCL were defined depending on their mRNA expression pattern, the “germinal centre B-cell like” (GCB), with overexpression of typical germinal centre-associated genes, e.g. CD10, bcl-6, LMO2, and the “activated B-cell like” (ABC) subtype with an expression pattern like in vitro stimulated blood B-lymphocytes, e.g. c-myc, cyclin D2, bcl-2, c-FLIP, MUM-1 (IRF-4) and CD 44. The GCB subtype had significantly better OS than the ABC subtype [53]. Another study showed that the transcription factor NF-κB was constitutively activated in the ABC subtype, which could explain the strong expression of cyclin D2, bcl-2, c-FLIP and MUM-1 [58]. The cell-of-origin concept was subsequently confirmed in a larger study, where also an unspecific subtype (Type 3) was identified. The GCB subtype had significantly better OS than the other two; estimated OS at 5 years was 60% and 35-39%, respectively. Additionally, different gene signatures were identified, the unfavourable “proliferation signature” and the favourable “germinal centre B-cell signature”, “lymph node signature” (genes associated with extracellular matrix, connective tissue, macrophages and NK cells) and “MHC class II signature” [55]. In order to find especially important prognostic genes, the mRNA expression of 36 genes was investigated with RT-PCR on 66 patients; bcl-6, LMO2 and fibronectin-1 were the most favourable, while bcl-2, cyclin D2 and SCYA3 were the most unfavourable [59]. Gene expression profiling studies have also shown that mediastinal large B-cell lymphoma has a profile similar to Hodgkin’s disease [56, 60].

Since gene expression profiling and RT-PCR methods are complicated and not generally available in clinical practice, it is important to find relevant prognostic biomarkers on the protein level, using routine immunohistochemical methods. So, in the forthcoming chapters, studies on the protein level are reviewed.

### **Cell-of-origin concept**

CD10 is a membrane metalloproteinase expressed in a variety of tissues, but in secondary lymph organs restricted to germinal centre cells [61]. In DLBCL,

CD10 overexpression occurs in approximately one third of the patients [62-64] and has in univariate analyses been attributed improved OS [62-65] or no impact on OS [66, 67].

Bcl-6 is a transcriptional repressor, almost exclusively expressed in germinal centre B-cells [68], which promotes proliferation via inhibition of p27 and BLIMP1 (resulting in increased myc activity), blocks differentiation of the germinal centre B-cells via inhibition of BLIMP1 and attenuates inflammation [69]. In immunohistochemical staining, overexpressed bcl-6 is seen in the majority of DLBCL cases, usually ranging from 55 to 80% [39, 62, 64, 66, 67] and has been associated with better OS [62, 64, 70] or with no impact on OS [65-67].

The transcription factor MUM-1 is normally expressed in plasma cells and activated T-cells but is also considered to be expressed in late (bcl-6-) intra-germinal centre B-cells. In DLBCL, MUM-1 is overexpressed in approximately 40-60% of the cases and has, as a single factor, been associated with inferior OS in some [64, 65] but not in other [62, 66, 71] studies.

By incorporating MUM-1 with CD10 and bcl-6 into a model which correlated to the gene expression profiling data [53, 55], two types of DLBCL were identified, the GCB and non-GCB; the latter corresponded to the ABC type and Type 3 [64]. The GCB group had a significantly better OS (estimated at 5 years, 76% vs 34%), also evident after adjustment for a two-group IPI model. The cut-off value 30% was used for all three markers and the GCB type was defined as CD10+ alone (regardless of the other two) or CD10-/bcl-6+/MUM-1- while CD10-/bcl-6- (regardless of MUM-1) or CD10-/bcl-6+/MUM-1+ was considered as non-GCB. Using the same algorithm, the GCB phenotype has been shown to predict a favourable prognosis, independent of IPI, in one [62] but not in another [41] study.

## **Proliferation and cell cycle**

In general terms, a disturbed balance between proliferation and apoptosis is a hallmark for development of all malignant diseases [72]. The master controller of apoptosis and cell cycle is the tumour suppressor gene p53, which in case of “stress” or DNA damage induces cell cycle arrest and repair, or apoptosis. Point mutation of p53 is present in 10-20% of the DLBCL cases and has been associated with worse outcome [73-75]. Mutation leads to a defective protein, detectable by immunohistochemistry, but p53 protein expression has not shown to add prognostic information [76-78]. But, the p53+/p21- combination has been associated with inferior outcome [75, 79].

Cyclins are proteins controlling the activity of their corresponding cyclin dependent kinases (CDK) in specific phases of the cell cycle; cyclin D (D1-D3) in phase G1, and thereafter cyclin E, A and B. The CDK inhibitor p27 arrests the cell cycle in the “restriction point” (G1-S) and is considered to be an important tumour suppressor [69], but studies on its prognostic role have not shown

consistent results [66, 80]. But, consistent with previous gene expression profiling studies, overexpressed cyclin D2 protein has been shown to predict inferior OS [71]. Also cyclin D3 overexpression has been associated with worse outcome in one [81] but not in another larger [71] study, and overexpressed cyclin E has been described as an adverse factor in one study [82]. The prognostic impact of cyclin A is unclear; in one study cyclin A did not predict survival [83], but that study was rather small and interpretation was hampered by incomplete clinical data. Moreover, the prognostic impact of the most established proliferation marker Ki-67 has been unclear. The proportion of Ki-67+ cells in tumour specimens has been the standard measure of cell proliferation, i.e. “proliferation index”, since Ki-67 is expressed in proliferating cells throughout the cell cycle (G1, S, G2, M) [84] but is not detectable with immunohistochemical methods in resting cells (G0) [85]. The prevailing notion has attributed high Ki-67 expression worse prognosis, mainly based on rather small studies on aggressive lymphomas [86, 87] but subsequent larger DLBCL studies have shown conflicting results [71, 77, 78].

## **Apoptosis**

Apoptosis is a strictly controlled cell death program, characterized by cell shrinkage, chromatin condensation and DNA fragmentation with sustained cellular integrity. The formed “apoptotic bodies” are then degraded, e.g. by macrophages [88]. In general, dysregulation of the apoptotic machinery is considered to be involved in both tumour development and chemotherapy resistance. Normal apoptosis comprises activation of the “stress-induced” (intrinsic), the death-receptor (extrinsic) or the perforin/granzyme B induced programs. Chemotherapy seems to act mainly by inducing apoptosis through the “stress-induced” program [89], where pro- and anti-apoptotic members of the bcl-2 family are essential regulators. After being activated, Bax and Bak initiate the apoptotic process by opening pores in the mitochondrial outer membrane, leading to release of substances e.g. cytochrome c, with subsequent activation of caspase 9, which in turn activates the effector caspases 3, 6 and 7. The anti-apoptotic bcl-2 and bcl-xL proteins, as also MCL1 and others, are potent inhibitors of Bax and Bak. Pro-apoptotic “BH3-only” members, like Bad, inactivate bcl-2 and bcl-xL or, like Bid, directly activate Bax and Bak [89]. IAPs (inhibitors of apoptosis), e.g. XIAP and survivin, are important inhibitors on the caspase level. The “extrinsic” program is activated by stimulation of the “death receptors” FAS, TRAIL-R and TNF-R, resulting in activation of caspase 8, which directly activates the effector caspases 3 (6 and 7) or activates the intrinsic program via truncation (i.e. activation) of Bid. An important inhibitor of caspase 8 is c-FLIP.

p53 regulates apoptosis by direct transcriptional activation of the bcl-2 family members and the extrinsic death receptors. Additionally, the bcl-2 family is modulated by posttranslational modifications (e.g. phosphorylation) by protein



kinases, e.g. AKT (protein kinase B). AKT plays a central role in the phosphatidylinositol 3-kinase (PI3K)/AKT pathway mediating pro-survival signals in response to growth factor or cytokine stimulation.

### ***Dysregulated apoptosis***

Bcl-2 protein is overexpressed in a majority (50-75%) of DLBCL patients and bcl-2 positivity has been associated with inferior OS in several [62, 66, 76, 77, 90] but not in all [64, 71, 78] pre-rituximab studies. Moreover, overexpression of XIAP [91] has been associated with inferior survival, as also has been shown for survivin in one of two studies [71, 92], while studies on Bax protein have shown diverging results [67, 93, 94]. Notably, in one study overexpression of c-FLIP predicted better outcome [91].

Galectin-3 is a beta-galactoside-binding lectin with anti-apoptotic properties, normally expressed in a variety of tissues [95]. It can also be aberrantly overexpressed in DLBCL and has been associated with resistance to Fas-induced apoptosis [96]. However, no published studies have addressed the prognostic role of galectin-3 expression in DLBCL.

### ***Relation between proliferation, apoptosis and cell-of-origin***

The original gene expression profiling studies found a correlation between non-GCB (“ABC”) and bcl-2<sup>+</sup> expression [53], subsequently supported by an immunohistochemical study [64]. The gene expression profiling studies also showed that the unfavourable ABC type was overrepresented by genes in the “proliferation signature” [53, 55]. On the other hand, an immunohistochemical correlation study found a positive correlation between the expression of proliferation markers (Ki-67, cyclin A) and germinal centre proteins (CD10 and bcl-6) [97] but also between the proliferation markers and pro-apoptotic proteins (Bax, Bak, Bad, Bid) [98]. However, that study did not include clinical or survival data. Previously, supportive for a correlation between proliferation and apoptosis has been a small study on relapsed aggressive lymphomas, in which low Ki-67 expression correlated to bcl-2<sup>+</sup> [99], but no other studies have investigated such possible correlations and their prognostic relevance.

### ***Rituximab and apoptosis***

Rituximab has been described to act indirectly by inducing complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) [100] but also by inducing apoptosis [101]. Rituximab seems to be able to directly activate the intrinsic apoptotic program [102] but also to sensitize cells to chemotherapy-induced apoptosis by downregulating the protein kinase activity in intracellular anti-apoptotic pathways (p38MAPK/NF-κB/Bcl-2, Src/Raf-1/ERK1/2/BclxL, NF-κB/BclxL and PI3K/AKT/Bad/BclxL) and by sensitizing Fas-mediated apoptosis [103]. Moreover, rituximab-resistant DLBCL cell lines have shown i) hyperactivation of the p38MAPK/Bcl-2, ERK1/2/BclxL

and NF- $\kappa$ B/Bcl<sub>x</sub>L pathways with overexpression of bcl-2 and bcl-xL proteins [103], ii) decreased expression of Bax and Bak [104] or iii) hyperactivation of the PI3K/AKT pathway with overexpressed MCL1 protein [102]. Moreover, AKT kinase has been found to be abnormally activated in DLBCL [105, 106], and some data support that AKT-mediated defective apoptosis could have prognostic relevance in DLBCL [106, 107]. But, very little is known about the prognostic role of proteins involved in these pathways among patients treated with rituximab and chemotherapy [102], except for bcl-2 [108-110].

### **Cell surface and other markers**

The T-cell marker CD5 is expressed in 7-9% of *de novo* DLBCL cases and has been associated with inferior survival [67, 111, 112]. But, better outcome has been reported in single studies regarding CD40+ [67], CD21S+ [113] and LMO2+ [114], the latter consistent with gene expression profiling data [53, 59]. Expression of the adhesion receptor molecule CD44 has been associated with lymphoma dissemination [115] and worse outcome in DLBCL [116-118], but ICAM-1 (intercellular adhesion molecule-1) expression, only studied in small patient series, has been attributed a favourable prognosis [119, 120] or no prognostic influence [117]. Consistent with gene expression profiling data [55, 121], high MHC class II protein expression on tumour cells has been associated with favourable prognosis [122, 123]. However, in a recent gene expression profiling study on R-CHOP treated patients, MHC class II signature was not prognostic [124]. MHC class I mRNA expression has been associated with better outcome [119] but on the protein level no prognostic impact has been reported [125]. Overexpressed PKC-beta, a protein kinase involved in B-cell signaling, has been found to be an adverse prognostic factor on the mRNA [57] and protein level [71]. Overexpression of the transcription factor FOXP1 has also been associated with worse outcome in some [126, 127] but not in other [64, 71, 110] studies.

### **Tumour microenvironment**

#### ***Tumour infiltrating lymphocytes (TILs)***

The immune system is considered to be involved in the control of cancer development, i.e. tumour immune surveillance [128]. This system includes an innate immune response, in which NK cells participate, and an adaptive immune response with induction of tumour specific CD4+ Th cells and activation of CD8+ CTLs. The CTLs then recognise MHC class I/antigen expressing target cells and induce apoptosis, which occurs in at least three ways; the perforin/granzyme program, the Fas ligand-Fas induced program, and indirectly by CTL-produced IFN- $\gamma$  and TNF- $\alpha$  [129]. In various cancer studies, large numbers of TILs have predicted improved survival [130-132]. In contrast, a large amount of CTLs has been associated with worse outcome in various lymphoma entities, such as Hodgkin's disease [133-135], anaplastic large T-cell

lymphoma [136] and unspecified peripheral T cell lymphoma [137]. In DLBCL, the prognostic role of TILs has been unclear. Gene expression profiling data attributed patients with high expression of genes in the “lymph node signature” a better outcome [55], but that study did not elucidate the role of TILs more specifically, and only a few and rather small flow cytometric and immunohistochemical studies with conflicting results have been published [121, 125, 138-141].

T<sub>regs</sub>, a small subset of Th cells typically defined as CD4+CD25+ cells, play an important role in the immune system by suppressing self-reactive T cells and thereby inhibiting autoimmunity, but have also been described to hamper anti-tumour responses and thereby promoting tumour growth [142]. Since CD25 also can be expressed on non-regulatory, activated CD4+ T cells, other markers have been proposed to better define Tregs; the transcription factor FOXP3 and possibly LAG-3 (lymphocyte activation gene-3) seem to be the most specific thus far [143, 144]. In carcinoma studies, a large number of FOXP3+ T<sub>regs</sub> has been associated with worse outcome [145-147]. In contrast, FOXP3+ has been shown to predict better prognosis in follicular lymphoma [148, 149], Hodgkin’s disease [133], cutaneous T-cell lymphoma [150] and extranodal NK/T-cell lymphoma [151], but no previous studies have addressed the prognostic significance of T<sub>regs</sub> in DLBCL.

### ***Other markers***

As mentioned, gene expression profiling studies have found an association between high expression of genes expressed in reactive stromal cells (e.g. fibronectin, and macrophages) and favourable outcome [55, 119, 124] while overexpression of genes related to endothelial cells and angiogenesis, e.g. VEGF, predicted worse survival [57, 124]. However, immunohistochemical studies have not been able to confirm a favourable impact of increased macrophage (CD68+) infiltration [152] or an unfavourable influence of increased VEGF expression [153-155]. However, one study has attributed increased mast cell infiltration a better outcome [156].

## **Population-based studies**

Randomised clinical trials are indeed the most adequate to evaluate new treatment options, but such trials comprise selected cohorts of patients and a substantial number of patients are most likely excluded for reasons such as high age, co-morbidity, bad performance status, physician bias or patient refusal. A population-based study on a cohort representative of a defined population offers advantages, especially external validity, i.e. the applicability of the results to the general population, and also allowing estimations of prevalence and distributions of risk factors [157].

In DLBCL, previous Dutch and Danish population-based studies showed that at least one third of the population did not receive recommended therapy with curative intent [9, 10]. But, most patients in these studies were diagnosed in the 1980s and early 1990s. In contrast, in a retrospective population-based study on 292 DLBCL patients treated 1999-2002, practically all (94%) were started on therapy with curative intent [158]. However, the median age seemed to be rather low (63 years) compared to the other population-based series, 67 and 65 years, respectively [9, 10]. Moreover, none of these studies showed data on how many of the patients who really accomplished planned therapy.

The IPI study [35] was large but emanated from patients treated in the 1980s, and it is reasonable to believe that the patient cohort was heavily selected (median age was 56 years). Additionally, that study did not only comprise DLBCL patients. In DLBCL, IPI has been shown to be predictive in non-population-based studies but has only been validated in a few population-based series, namely in the Dutch series of 376 patients treated in the 1980s [9] and in subgroups of the large Danish cohort [10].

# AIMS OF THE STUDY

The objectives of the present study were to:

- retrospectively evaluate treatment, outcome and clinical prognostic factors (including IPI) in a population-based cohort of patients,
- investigate the prognostic role of tumour-infiltrating lymphocytes in relation to clinical factors,
- investigate the prognostic role of proliferation markers in the context of anti-apoptotic proteins and GCB/non-GCB phenotypes, as also in relation to clinical factors,
- investigate the prognostic role of anti- and pro-apoptotic proteins in relation to clinical factors, in patients treated with rituximab and chemotherapy.



# PATIENTS AND METHODS

## Statement of official approval

All studies (Paper I-IV) were approved by The Regional Ethics Review Board, Göteborg.

## Patients

### Paper I

All adult patients ( $\geq 18$  years of age) with *de novo* DLBCL diagnosed between January 1995 and December 2000 were identified from the population-based Regional Lymphoma Registry of the Western Sweden Health Care Region. In order to find all DLBCL patients, cases registered as DLBCL, unspecified high grade B-cell lymphoma, follicular large cell/follicular lymphoma grade 3, B-lymphoblastic lymphoma, Burkitt lymphoma and unspecified NHL were selected for a first review (n=701). Tumour specimens were collected, centrally reviewed and reclassified according to the WHO classification [6]. Out of these primarily selected patients, and after exclusion of patients with previously known indolent lymphomas, the diagnosis of DLBCL was established in 535 individuals. All subtypes of DLBCL were included in the study. Clinical information was collected from the original case books at the different hospitals and centrally reviewed. Two main therapy groups were retrospectively identified, depending on therapy intention, i) a “curative intent” group and ii) a “palliative” group. Potentially curative therapy for stage II-IV patients mainly consisted of CHOP or CHOP-like regimens, with at least six cycles in responding patients, while most stage I patients received chemoradiotherapy with at least three cycles of CHOP followed by involved field RT. Chemoradiotherapy was also considered as an appropriate option for patients with non-bulky, localized stage II disease. In the regional guidelines 1995, RT alone was an option for patients with small stage I tumours and normal LDH, but these patients (n=6) were not included in the curative intent group in this study. For a small number of patients, the therapeutic intention was retrospectively difficult to determine, but those who were properly staged and started on one, full-dose cycle of chemotherapy were assigned to the curative intent group. Response assessment was retrospectively performed according to the International Workshop Criteria [159]. Consolidating RT was considered for patients in CRu or PR (and CR after initial bulky disease, the year 2000) and high risk patients in first CR were offered ASCT (the year 2000). Responding relapse patients younger than 65 years were offered ASCT.

## **Paper II and III**

From the population-based cohort of 376 patients treated with curative intent in Paper I, all patients were included if the following criteria were fulfilled: i) the patients had not primary CNS lymphoma or post-transplant lymphoproliferative disorder (or small cell bone marrow infiltration, Paper III), ii) the patients had completed a potentially curative treatment (i.e. patients who discontinued therapy due to reasons other than poor treatment response, such as toxicity or concomitant disease, were not included), and iii) adequate amount and quality of paraffin-embedded biopsy material was available for additional immunostaining and successful immunostaining was performed. Out of 316 (Paper II) and 301 (Paper III) patients who fulfilled the clinical inclusion criteria, 195 (Paper II) and 199 (Paper III) patients were included after successful immunostaining.

## **Paper IV**

All adult patients with *de novo* DLBCL diagnosed in the Western Sweden Health Care Region during the years 2005 and 2006 and in a part of the region (Göteborg and Borås) during 2007, were identified from the Swedish Cancer Registry. All DLBCL patients fulfilling the following criteria were included: i) the patients had not primary CNS lymphoma or post-transplant lymphoproliferative disorder, ii) the patients had been started on rituximab and chemotherapy with curative intent, iii) the patients had completed such therapy, i.e. patients who discontinued therapy due to toxicity or concomitant disease were not included; all responding patients received 6-8 cycles of R-CHOP or R-CHOEP or, if non-bulky localized disease, three cycles of R-CHOP followed by involved field RT (30-40 Gy), and iv) adequate amount and quality of paraffin-embedded biopsy material was available for additional immunostaining and successful immunostaining was performed. Clinical information was obtained from the patient case books. One-hundred and forty patients fulfilled the first two clinical criteria, 121 patients completed therapy and 106 patients were included after successful immunostaining for all the markers. Recommended therapy was determined according to aaIPI; R-CHOP-21 for patients with aaIPI=0-1 (or short R-CHOP-21 plus involved field RT if non-bulky, localized disease), R-CHOP-14 and R-CHOEP-14 for aaIPI=2-3 patients older and younger than 60 years, respectively. Some of the patients attaining at least PR underwent consolidating RT, and responding relapse patients younger than 65 years were offered ASCT.

## **Immunohistochemistry**

### **Paper II – IV**

One paraffin-embedded block containing representative material of DLBCL was chosen for production of serial whole-tissue sections with a thickness of 4



microns. After routine deparaffinisation, rehydration and a microwave antigen retrieval step (750 W, 8 minutes; 350 W, 15 minutes), immunostaining was performed to demonstrate CD3, TIA-1, perforin and FOXP3 (Paper II), Ki-67, cyclin A, galectin-3 (Paper III), bcl-xL, p-AKT (AKT, phospho T308), MCL1, Bax, Bak, (Paper IV), bcl-2, CD10, bcl-6 and MUM-1 (Paper III and IV), using the Dako Envision Detection System (horseradish peroxidase/3,3'-diaminobenzidine+) in a TechMate Horizon Autostainer (Paper II and III) and TechMate500Plus Autostainer (Paper IV). Mayer's haematoxylin staining was used as nuclear counterstaining. Tonsillar tissue served as positive controls for all markers except for galectin-3 (prostate). On the different slides of each tumour, isolated square formed specimen areas of approximately 0.25 - 1 cm<sup>2</sup> were marked out, considered to be representative for the tumour section with diffusely distributed blast cells and evenly distributed Ki-67+ cells. Enumeration of CD3+, TIA-1+, perforin+ (Paper II), Ki-67, cyclin A (Paper III) and bcl-xL (Paper IV) was performed on four computer saved images from different fields in the selected area; a total area of 0.11 mm<sup>2</sup> was manually counted for each case. For CD3, TIA-1, perforin and bcl-xL, the number of positive cell profiles/mm<sup>2</sup> specimen area was calculated, while for Ki-67 and cyclin A, the number of positive cell profiles out of all cell profiles was counted and registered as percentages. The number of FOXP3+ and p-AKT+ cells was counted in four different full microscopic fields of vision within a total area of 0.68 mm<sup>2</sup> (FOXP3, Paper III) and 0.95 mm<sup>2</sup> (p-AKT, Paper IV) and expressed as the number of positive cells/mm<sup>2</sup> specimen area. For CD10, bcl-6 and MUM-1, the proportion of positive cells was estimated and cases with at least 30% positive cells were considered positive, according to Hans et al and their algorithm for defining GCB vs non-GCB phenotype was used (see page 11). Similarly, bcl-2 positivity was estimated, with the cut-off value 30% in Paper III, 30% and 50% in Paper IV. Regarding galectin-3 (Paper III), a qualitative estimation was made; in most cases the vast majority of cells were clear-cut positive (n=40) or negative (n=141), but 18 cases showed focal or weak positivity and those were also considered as positive in further analyses. The MCL1 staining (Paper IV) was highly positive in most patients; thus, estimation was performed according to <50, 50-80 and >80% positive fractions. In the Bax and Bak staining (Paper IV) practically all tumour cells stained positive in every patient, and therefore a discriminating estimation was not possible. For detection of cytotoxic cells in Paper II we chose TIA-1, which codes for an membrane protein in cytotoxic granules, mainly in CTLs (regardless of their activation state) and NK cells, and perforin, a pore-forming protein found in cytoplasmic granules of activated CTLs and NK cells. In order to further characterize the TIA-1+ cell population, immunostaining was performed in six cases with CD56 and a double-staining with TIA-1 and CD8.

## Statistics

PFS was measured from date of diagnosis to date of disease progression, death related to lymphoma treatment (only applicable for the cohort in Paper I and the curative intent group, n=140, in Paper IV), relapse or latest follow-up. Death unrelated to lymphoma or its treatment was censored at the time of death. OS was measured from date of diagnosis to date of death of any cause, or latest follow-up. The Chi-square and Mann-Whitney U-tests were used when comparing categories against categorical and continuous data, respectively. Survival was estimated by the Kaplan-Meier method and the log-rank test was used when comparing two groups. A p-value <0.05 was considered statistically significant. The Cox proportional hazard regression model was used for uni- and multivariate analyses. For the continuous immunohistochemical markers, each one was tested as a continuous variable in Cox regression and only if a significant impact was found, categorization was performed; the value that separated the patient material into two groups with the most significantly different outcome was chosen as cut-off value (Paper II), or categorization was done according to the quartile distribution (Paper III and IV). The follow-up time for all patients was estimated by the reversed Kaplan-Meier method [160].

# RESULTS

## Paper I

### Clinical characteristics, treatment and response

Five hundred and thirty-five patients were identified, corresponding to 23,7% of all lymphomas in the region, or 26% of all NHL, or ~34% of the NHL if chronic lymphocytic leukaemia cases were excluded (Centre of Oncology, Sahlgrenska University Hospital). Treatment with a curative intent was started in 376 patients, i.e. 70% of all patients. The median age for all patients was 73 (range 19-99) years and for the curative intent group 68 (range 19-87) years. Patient characteristics are summarized in Table I.

Table I. Characteristics at presentation

	All patients (n=535)	Curative intent group (n=376)
	%	%
Sex, female / male	48 / 52	45 / 55
Age		
≤ 60 y	27	36
61-70	17	22
71-80	34	35
> 80	22	7
Stage		
I	18	20
II	27	34
III	11	12
IV	34	34
Extranodal involvement	59	56
Extranodal > 1 site	12	11
Bone marrow involved	13	14
Serum LDH increased	43	46
Performance status > 1	29	21
Spleen involved	12	13
Waldeyer involved	13	14
Bulky disease	24	24
B symptom	45	45
Hb < 115 g/L	31	29
ESR ≥ 30 mm	27	28
Albumin < 35 g/L	30	28
IPI / aalPI		
L	34 / 27	43 / 33
LI	16 / 20	20 / 26
HI	17 / 21	20 / 24
H	16 / 13	12 / 11
missing	17 / 19	5 / 6

Missing data for each variable, see Table II.

Involvement, other extranodal sites, % of all patients (% of curative intent group): Gastrointestinal 11 (10); liver 6 (4); skin 6 (5); skeleton 6 (6); lung 5 (4); CNS 4 (5); other 24 (23); missing data in 5-8 % of all patients, 0 % in curative intent group.

Curative intent group: Chemotherapy alone was given to 301 patients, while 75 patients received chemoradiotherapy; CHOP (n=254), other doxorubicin-containing regimens (n=44), CNOP (n=63) and other regimens (n=15). Forty-five patients, i.e. 12% of the entire group, did not complete the therapy, because of deaths (n=13; cardiopulmonary or infectious complications), non-fatal toxicity (n=19) and other reasons (n=13). Consequently, 331 patients completed the therapy, corresponding to 62% of the entire cohort. For the group older than 70 years, only 43% accomplished therapy. Out of all 376 patients, the complete remission rate (CR and CRu) was 61% and 14% attained PR. No significant difference in complete remission rate was seen between patients older and younger than 68 years (p=0.86). For the small, selected subset of patients older than 80 years who received treatment with curative intent (27/115), 12 (44%) achieved a CR or CRu.

Palliative group: This group comprised 157 patients, 53% females and 47% males, with median age 81 (range 30-99) years. Thirty-two percent of the female vs 27% of the male patients belonged to this group (p=0.15). No significant sex difference was found regarding median age but 58% of the females vs 41% of the males (p=0.03) had bad performance status (ECOG >1). The reasons for omitting curative therapy were high age and concomitant disease (27%), high age (18%), concomitant disease (17%), poor performance status (17%), other (9%) or not specified (12%).

## **Survival**

At a median follow-up of 74 months, the median OS for all patients was 22 (95% CI 16-32) months and PFS 11 (95% CI 8-16) months; the estimated OS and PFS at five years were 37% and 36%, respectively (Figure 1a). In the curative intent group, the median OS was 55 (95% CI 42-71) months and PFS 40 (95% CI 19-69) months; the estimated OS and PFS at five years were 48% and 46%, respectively (Figure 1b). The median OS for the palliative group was 3 (95% CI 2-5) months, range 0-122 months and estimated 5-year OS 9%.

## **Risk factors**

Curative intent group: In addition to well-known risk factors, also male sex had a negative impact on OS (Table II) and PFS (Figure 2). However, bulky disease was not a significant risk factor, either for PFS (p=0.17) or OS (Table II), and neither did age >68 vs ≤68 years affect PFS (p=0.14). IPI and aaIPI strongly predicted OS, but the survival difference was small between the two intermediate risk groups and was statistically significant only in the aaIPI model (p=0.047) (Figure 3). In Cox regression analysis, the negative impact of male sex on PFS and OS was independent of IPI and aaIPI (Table III; IPI model data not shown). Adjustment for doxorubicin- vs mitoxantrone-containing chemotherapy did not affect the results; only minimal changes in the numerical values were noticed (data not shown). The complete remission rate

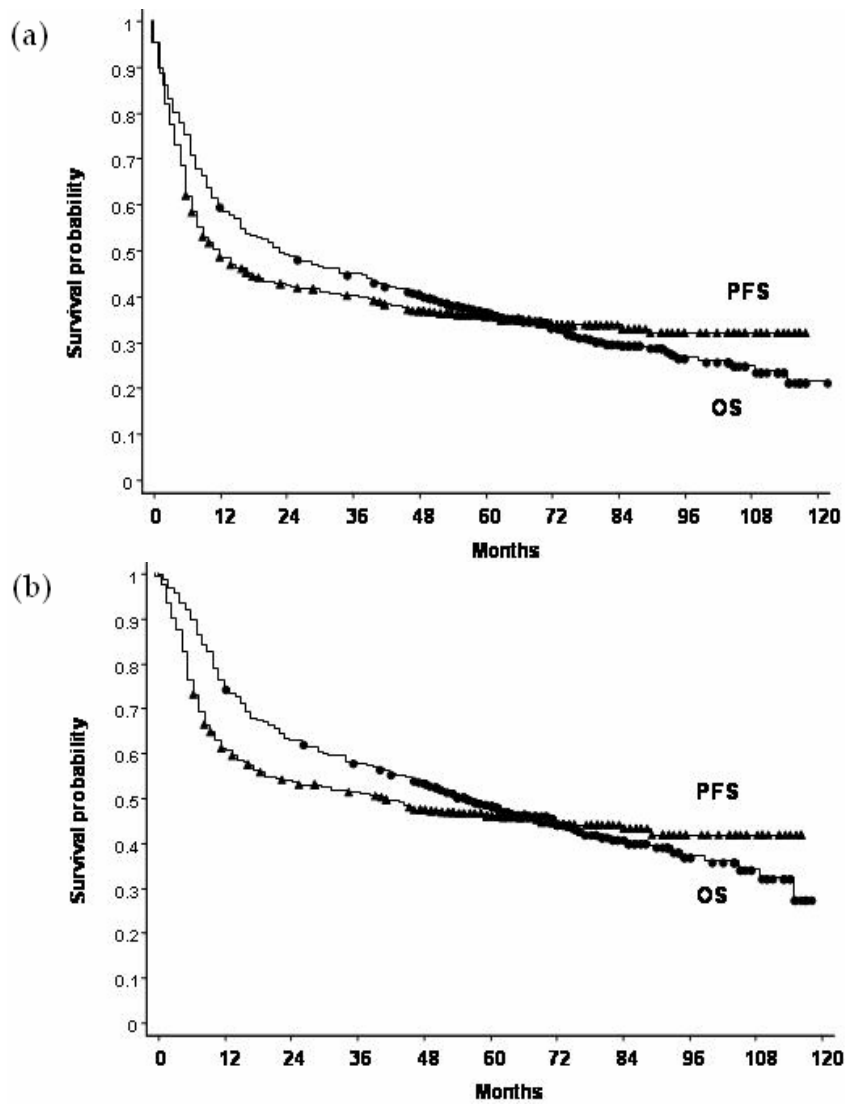


Figure 1. Overall (OS) and progression-free (PFS) survival for all patients (a) and patients in the curative intent group (b).

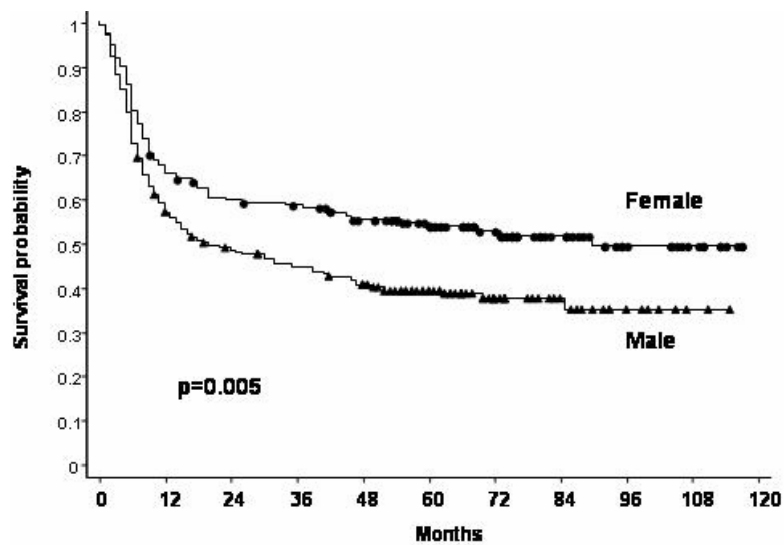


Figure 2. Progression-free survival in the curative intent group; female vs male.

Table II. Prognostic factors for overall survival; univariate analysis

Characteristic	Curative intent group (n=376)			All patients (n=535)		
	HR (95 % CI)	p-value	Missing %	HR (95 % CI)	p-value	Missing %
Sex, male vs female	1.5 (1.1-2.0)	0.004	0	1.1 (0.9-1.4)	0.31	0
Age >60 vs ≤ 60	1.6 (1.2-2.1)	0.002	0	2.1 (1.7-2.8)	< 0.0001	0
Stage III-IV vs I-II	2.5 (1.9-3.3)	< 0.0001	0	2.8 (2.2-3.5)	< 0.0001	10
Extranodal sites > 1 vs ≤ 1	1.5 (1.0-2.2)	0.04	1	1.9 (1.4-2.5)	< 0.0001	11
LDH elevated vs normal	2.0 (1.5-2.6)	< 0.0001	5	2.1 (1.7-2.7)	< 0.0001	13
Perf status >1 vs ≤ 1	2.4 (1.8-3.2)	< 0.0001	1	3.1 (2.5-3.9)	< 0.0001	1
Extranodal no vs yes	1.8 (1.4-2.4)	< 0.0001	0	2.1 (1.7-2.6)	< 0.0001	5
Bone marrow, yes vs no	2.0 (1.4-2.8)	< 0.0001	2	2.1 (1.6-2.8)	< 0.0001	15
Gastrointestinal, yes vs no	0.8 (0.5-1.3)	0.47	0	1.0 (0.7-1.4)	0.91	7
Liver, yes vs no	4.7 (2.7-8.2)	< 0.0001	0	4.7 (3.2-6.8)	< 0.0001	7
Skin, yes vs no	1.9 (1.1-3.4)	0.02	0	1.9 (1.3-2.8)	0.001	5
Skeleton, yes vs no	0.8 (0.5-1.5)	0.54	0	0.9 (0.6-1.5)	0.76	7
Lung, yes vs no	2.3 (1.3-4.2)	0.004	0	2.4 (1.6-3.7)	< 0.0001	7
CNS, yes vs no	2.0 (1.2-3.4)	0.009	0	1.9 (1.2-3.0)	0.005	6
Spleen, yes vs no	2.3 (1.6-3.3)	< 0.0001	0	2.0 (1.5-2.7)	< 0.0001	7
Bulky, yes vs no	1.0 (0.7-1.4)	0.97	1	1.2 (0.9-1.5)	0.17	8
Waldeyer, yes vs no	1.4 (0.9-2.1)	0.12	0	1.2 (0.9-1.6)	0.30	7
B symptoms, yes vs no	1.6 (1.2-2.1)	0.001	0	1.7 (1.4-2.1)	< 0.0001	3
ESR >30 vs ≤ 30	1.5 (1.0-2.0)	0.03	32	1.6 (1.2-2.1)	0.0002	38
Albumin <35 vs ≥ 35	1.7 (1.3-2.3)	0.0004	16	2.0 (1.5-2.5)	< 0.0001	22
Hb <115 vs ≥ 115	1.5 (1.1-2.0)	0.009	1	1.6 (1.3-2.0)	< 0.0001	7

Missing %, proportion of patients with missing data regarding the specified factor.

Table III. Prognostic factors in curative intent group; multivariate analysis

	Overall survival		Progression-free survival	
	HR (95 % CI)	p-value	HR (95 % CI)	p-value
<b>A. Individual IPI factors &amp; significant, other factors</b>				
Stage III-IV vs I-II	1.9 (1.3-2.6)	0.0002	2.4 (1.7-3.4)	< 0.0001
Performance status >1 vs ≤ 1	1.9 (1.3-2.6)	0.0002	1.7 (1.2-2.4)	0.002
Age > 60 vs ≤ 60	1.6 (1.2-2.2)	0.0044	1.0 (0.7-1.4)	0.99
LDH increased vs normal	1.5 (1.1-2.0)	0.019	1.4 (0.99-1.9)	0.053
Extranodal sites > 1 vs ≤ 1	0.7 (0.4-1.1)	0.11	0.7 (0.4-1.1)	0.09
Sex: Male vs female	1.7 (1.3-2.4)	0.0002	1.8 (1.3-2.4)	0.0002
Liver yes vs no	3.5 (1.9-6.5)	0.0001	3.4 (1.8-6.4)	0.0002
Lung yes vs no	2.9 (1.5-5.4)	0.0011	2.4 (1.3-4.6)	0.006
CNS, yes vs no	2.9 (1.6-5.3)	0.0004	2.4 (1.3-4.4)	0.006
<b>B. aalPI model &amp; significant, other factors</b>				
aalPI model		< 0.0001		< 0.0001
0 risk factor	1.0 reference		1.0 reference	
1 risk factor	1.9 (1.3-2.9)	0.0008	2.2 (1.5-3.4)	0.0002
2 risk factors	2.7 (1.8-4.0)	< 0.0001	3.3 (2.2-5.0)	< 0.0001
3 risk factors	5.7 (3.6-9.1)	< 0.0001	5.6 (3.5-9.1)	< 0.0001
Sex: Male vs female	1.7 (1.3-2.3)	0.0003	1.8 (1.4-2.5)	< 0.0001
Liver yes vs no	2.8 (1.6-5.2)	0.0007	3.1 (1.7-5.6)	0.0004
Lung yes vs no	2.6 (1.4-4.7)	0.003	2.5 (1.3-4.5)	0.005
CNS, yes vs no	2.5 (1.4-4.5)	0.002	2.2 (1.2-4.0)	0.009

did not differ between males and females (60 vs 61%,  $p=0.78$ ), but more men relapsed (46 vs 25%,  $p=0.001$ ). No statistically significant difference in clinical characteristics or treatment modalities was demonstrated between males and females.

All patients: Individual risk factors are presented in Table II; age predicted survival but sex did not. Yet, in the group with complete IPI data (444 patients), all statistically significant multivariate findings from the curative intent group were confirmed.

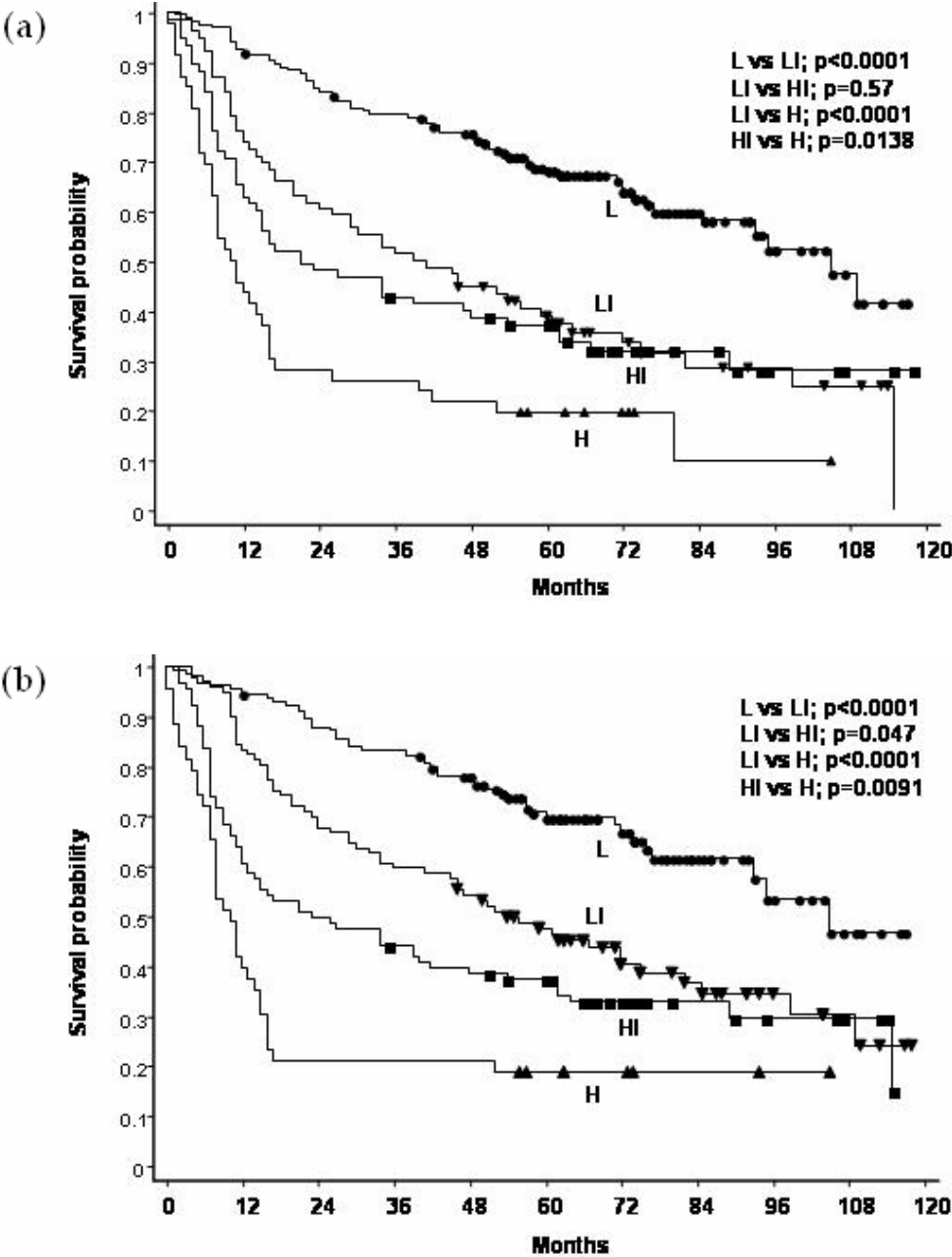


Figure 3. Overall survival in the curative intent group, IPI (a) and aaIPI (b); low (L), low-intermediate (LI), high-intermediate (HI) and high (H) risk groups.

## Paper II - III

### Patient characteristics

One hundred and ninety-five (Paper II) and 199 (Paper III) patients were included. Table IV shows the clinical characteristics. The median follow-up in paper II and III was 74 and 75 months, respectively. In paper II, estimated 5-year PFS was 55% and OS 61%, and in paper III 54% and 60%, respectively.

Table IV. Characteristics of patients with diffuse large B-cell lymphoma in Paper I - IV

	Paper I (n=376)	Paper II (n=195)	Paper III (n=199)	Paper IV (n=106)
	%	%	%	%
Median age	68 y	66 y	66 y	64 y
Sex, female / male	45 / 55	48 / 52	47 / 53	45 / 55
Age > 60 years	64	62	61	62
Stage I	20	21	20	11
II	34	38	41	35
III	12	14	15	28
IV	34	27	24	26
Bulky disease, yes	24	26	24	28
B-symptom, yes	45	42	42	39
Extranodal disease, yes	56	46	44	41
Extranodal sites > 1	11	8	7	12
Bone marrow involved	14	11	9	7
Performance status (ECOG) > 1	21	16	15	23
Serum LDH increased	46	49	50	63
IPI				
low (0-1 factors)	43	47	47	30
low-intermediate (2)	20	22	22	37
high-intermediate (3)	20	18	18	16
high (4-5)	12	10	9	17
unknown	5	3	4	0
Type of treatment				
chemotherapy	80	77	77	97
chemoradiotherapy	20	23	23	3
Type of chemotherapy				
doxorubicin-containing	79	84	83	100
mitoxantrone-containing	17	16	17	0
other	4	0	0	0
Rituximab	0	0	0	100

Missing values in Paper I, see Table II.



## Immunohistochemical findings

Paper II: The median numbers of CD3+, TIA-1+, perforin+ and FOXP3+ cells were 723 (range 0-6946), 536 (0-5714) and 241 (0-4170) and 67 (range 0-1770) cells/mm<sup>2</sup> tumour area. Figure 4 (A-D) shows the typical pattern of high and low expression of TIA-1 and FOXP3. The TIA-1/CD8 and CD56 staining confirmed a CTL origin in most of the TIA-1+ cells. Fever was associated with a larger number of TIA-1+ ( $p=0.02$ ), perforin+ ( $p=0.002$ ) and CD3+ cells ( $p=0.03$ ), but not FOXP3+ cells ( $p=0.57$ ).

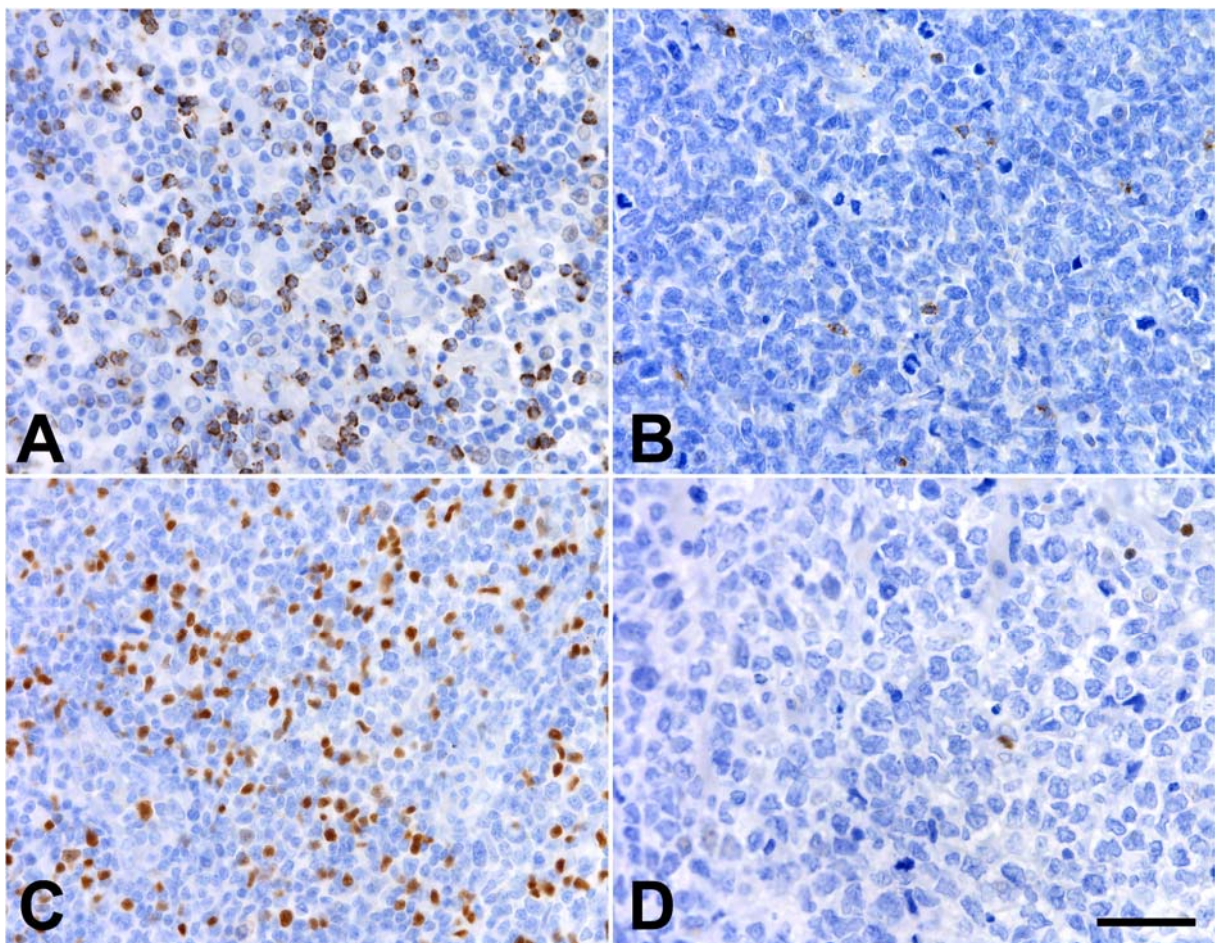


Figure 4. Immunohistochemical detection of (A) high TIA-1 expression, (B) low TIA-1 expression, (C) high FOXP3 expression, and (D) low FOXP3 expression. Bar denotes 50  $\mu$ m.

Paper III: The proportion of Ki-67+ cells varied between 14 and 95% (mean 61%, median 62%) and cyclin A+ cells ranged between 1 and 58% (mean 21%, median 20%). A GCB phenotype was found in 46% of the patients (CD10+ 33%, bcl-6+ 62%, MUM-1+ 60%); bcl-2 was positive in 75% and galectin-3 in 29% of the patients.

## Prognostic value of the immunohistochemical markers

### TILs (Paper II)

The number of TIA-1+ cells (continuous variable) correlated to PFS ( $p=0.03$ ) and OS ( $p=0.02$ ), in favour of a low number. After categorization, the group with low TIA-1 expression ( $\leq 260$  cells/mm<sup>2</sup>) had better outcome than the other group; estimated PFS at 5 years was 67% vs 50% ( $p=0.03$ ) and OS was 73% vs 57% ( $p=0.03$ ) (Figure 5).

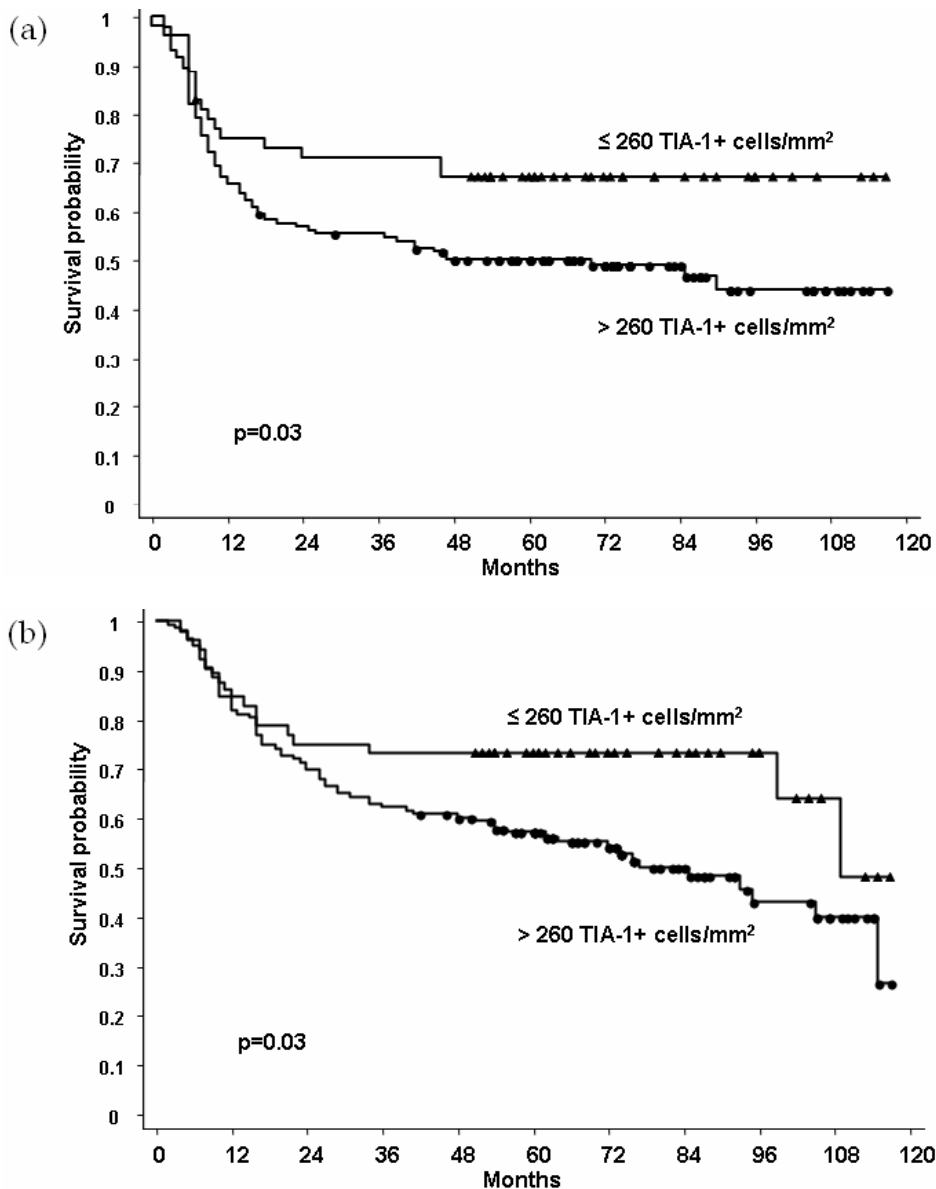


Figure 5. Progression-free (a) and overall (b) survival according to low and high expression of TIA-1.

The clinical characteristics for the two TIA-1 groups were well balanced except for sex and doxorubicin- vs mitoxantrone-containing treatment (Table V); after adjustment for these variables and the IPI model in Cox regression, low TIA-1+

group still predicted better PFS (HR 0.75, 95% CI 0.31-0.99; p=0.05) but not OS (p=0.21). Similar results were obtained when only looking at the group treated with doxorubicin-containing chemotherapy; the low TIA-1+ group still had a better PFS (p=0.02) after adjustment for IPI and sex. Consistently, high number of perforin+ cells (continuous variable) correlated with worse PFS (p=0.01) and OS (p=0.02), but after categorization (<280 vs  $\geq$ 280 cells/mm<sup>2</sup>), the difference in PFS and OS did not reach statistical significance, p=0.08 and p=0.10, respectively. Neither FOXP3 nor CD3 expression predicted survival.

Table V. Characteristics of 195 patients with small and large number of TIA-1+ cells

	TIA-1+ $\leq$ 260 cells/mm <sup>2</sup>	TIA-1+ $>$ 260 cells/mm <sup>2</sup>	p-value
	(n=52) %	(n=143) %	
Median age	67 y	65 y	0.53
Sex, female / male	62 / 38	43 / 57	0.02
Stage I+II / III+IV	58 / 42	59 / 41	0.83
Bulky disease ( $\geq$ 10 cm)	31	25	0.40
Extranodal disease	52	43	0.29
Extranodal sites $>$ 1	8	8	
Bone marrow involvement	8	13	0.32
Performance status (ECOG) $>$ 1	12	18	0.29
Serum LDH elevated *	46	52	0.48
B-symptoms	42	42	
IPI			
low risk (0-1)	50	47	0.81
low-intermediate risk (2)	21	23	0.63
high-intermediate risk (3)	19	19	
high risk (4-5)	8	11	0.60
unknown	8	2	0.08
Type of first line treatment			0.39
chemotherapy	73	79	
chemoradiotherapy	27	21	
Type of chemotherapy			0.01
doxorubicin-containing	94	80	
mitoxantrone-containing	6	20	
Consolidating radiotherapy	21 #	21 #	
Consolidating ASCT	6 #	3 #	0.40

\* Missing LDH values in 8 % and 2 % of the patients in the low and high TIA-1 group, respectively. # Percent of responding patients.

### Proliferation markers & GCB/non-GCB & anti-apoptotic proteins (Paper III)

Cox regression with Ki-67 as a continuous variable indicated worse PFS (p=0.028) with lower Ki-67 percentages, but not worse OS (p=0.19). After categorization into three groups according to the 25th and 75th percentiles, the lowest quartile (Ki-67 <49%) group had worse outcome than the other two (Figure 6 a-b), also after adjustment for clinical factors (Table VI).

When dividing the material into two groups, no survival difference was demonstrated between Ki-67 >73% and ≤73% (data not shown), while the group with Ki-67 <49% had worse outcome than the Ki-67 ≥49% group (PFS at 5 years, 40 vs 59%, p=0.007; OS 49 vs 64%, p=0.026, respectively). Serum LDH level was significantly higher in the Ki-67 <49% than Ki-67 ≥49% group (p=0.05), but no other characteristics differed significantly (data not shown). After adjustment for the aaPI model, sex, age and doxorubicin-containing therapy, Ki-67 <49% persisted as a predictor for inferior PFS (HR 1.9, p=0.005) and OS (HR 1.8, p=0.02). The cyclin A expression had no impact on PFS or OS (data not shown).

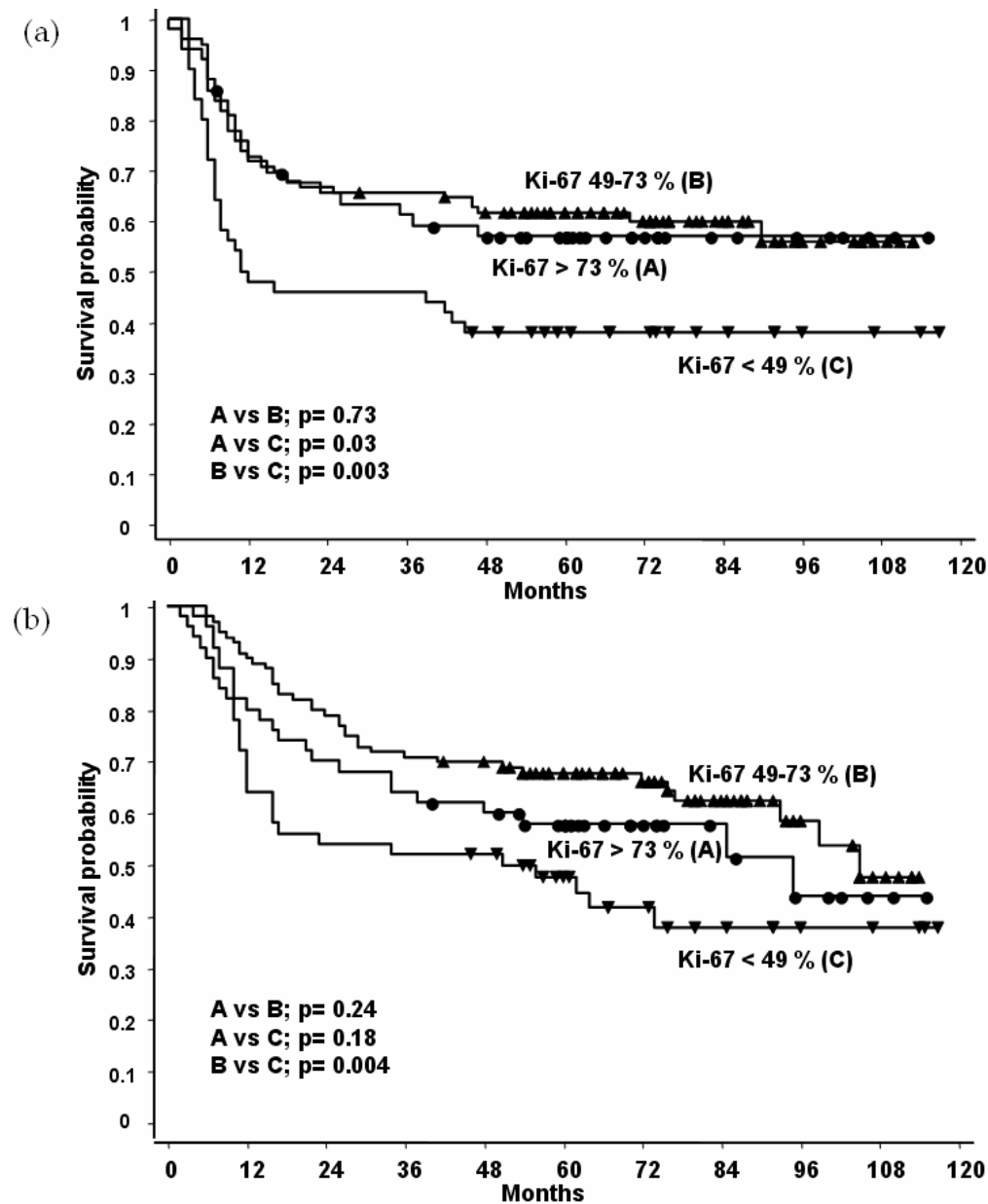


Figure 6. Progression-free (a) and overall (b) survival according to the Ki-67 expression.

Table VI. Ki-67 expression and clinical factors; multivariate analysis

	Progression-free survival		Overall survival	
	HR (95 % CI)	p-value	HR (95 % CI)	p-value
Ki-67 > 73 %	1.0 reference		1.0 reference	
Ki-67 49-73 %	1.3 (0.7-2.3)	0.38	0.9 (0.5-1.6)	0.69
Ki-67 < 49 % *	2.3 (1.3-4.3)	0.007	1.6 (0.9-3.0)	0.11
Sex, male vs female	2.3 (1.5-3.7)	0.0004	2.4 (1.5-3.9)	0.0003
aaIPI model		<0.0001		0.0003
0 risk factor	1.0 reference		1.0 reference	
1 risk factor	2.6 (1.4-4.8)	0.003	2.0 (1.1-3.7)	0.02
2 risk factors	3.8 (2.0-7.0)	<0.0001	2.6 (1.5-4.8)	0.001
3 risk factors	6.4 (3.0-14.0)	<0.0001	4.9 (2.3-10.7)	<0.0001
Doxorubicin yes vs no	0.6 (0.3-1.0)	0.05	0.5 (0.3-0.9)	0.02
Age > 60 vs ≤ 60	0.8 (0.5-1.3)	0.42	1.2 (0.7-2.0)	0.45

\* Ki-67 < 49 % vs 49-73 %: HR 1.8, p=0.02 (OS), HR 1.8, p=0.02 (PFS).

Bcl-2 predicted survival; PFS at 5 years was 47% vs 76% (p=0.001) and OS 55% vs 74% (p=0.014) for the bcl-2+ vs bcl-2- group, respectively. Non-GCB vs GCB phenotype had major impact on survival; at 5 years, estimated PFS was 43% vs 71% (p=0.0001) and OS 49% vs 72% (p=0.0002), respectively. After adjustment for aaIPI model, age, sex and doxorubicin vs mitoxantrone therapy, non-GCB phenotype independently predicted worse PFS and OS (HR 2.4, p=0.001 for both). Similarly, bcl-2+ was as an independent predictor for PFS (HR 2.7, p=0.006) but only as a trend for OS (HR 1.8, p=0.06) after adjustment for the clinical factors.

Moreover, low Ki-67 (<49% vs ≥49%) predicted worse PFS (HR 1.9, p=0.007) and OS (HR 1.7, p=0.025), independent of non-GCB/GCB (PFS: HR 2.5, p=0.0004; OS: HR 2.3, p=0.0008) and clinical factors. When Ki-67 instead was combined with bcl-2, similar results were obtained except that the impact of bcl-2+ expression not reached statistical significance regarding OS (HR 1.7, p=0.09). Galectin-3 expression had no impact on PFS (p=0.99) or OS (p=0.96).

## Paper IV

### Patients, therapy, survival and clinical factors

Clinical characteristics for the 106 DLBCL patients are summarised in Table IV. The median age was 64 (range 27-84) years. Three patients received three cycles of R-CHOP-21 and involved field RT. Fifty-eight patients received R-CHOP-21, 33 patients R-CHOP-14 and 12 patients R-CHOEP-14. The median follow-up was 30 months. Estimated 3-year PFS was 72% and OS 83%. For comparison, estimated 3-year PFS was 68% and OS 75% for all 140 patients who were started on rituximab and chemotherapy with curative intent. The traditional IPI model was not valid in the study series of 106 patients (the two

low risk curves superimposed, as also the two high risk curves did) but IPI 0-2 vs 3-5 factors distinguished two groups with different PFS (estimated at 3 years 83% vs 53%, respectively,  $p=0.004$ ) and OS (87% vs 72%,  $p=0.04$ ). Also male sex predicted worse PFS ( $p=0.04$ ), but not OS ( $p=0.74$ ). Moreover, IPI and sex independently affected PFS (IPI=3-5: HR=3.0,  $p=0.005$ ; male sex: HR=2.6,  $p=0.03$ ). Also in the “intention-to-treat” material ( $n=140$ ), IPI=3-5 and male sex independently predicted worse PFS (HR=3.0,  $p=0.0006$  and HR=2.9,  $p=0.004$ , respectively) and OS (HR=3.5,  $p=0.0008$  and HR=2.1,  $p=0.06$ , respectively).

## **Immunohistochemical findings**

The median numbers of p-AKT+ and bcl-xL+ cells were 69 (15-334) and 805 (range 55-3773) cells/mm<sup>2</sup> tumour area, respectively. Bcl-2 was positive in 81% (cut-off 30%) and 69% (cut-off 50%) of the cases. Regarding MCL1, only 2% of the patients had <50% positive cells, 10% had 50-80% and 88% had >80% positive cells. The expression of p-AKT did not correlate to MCL1, bcl-xL or bcl-2 expression (data not shown). The GCB phenotype was expressed in 58% of the patients (CD10+ 38%, bcl-6+ 84%, MUM-1+ 50%). The non-GCB group was overrepresented by bcl-2+ patients (cut-off 50%,  $p=0.011$ ; 30%,  $p=0.007$ ) but the p-AKT expression was not higher in the non-GCB group than in the GCB group ( $p=0.29$ ).

## **Prognostic value of the biomarkers**

An increasing number of p-AKT+ cells/mm<sup>2</sup> (continuous variable) showed a weak trend for worse PFS ( $p=0.14$ ) and OS ( $p=0.12$ ). However, more women and patients older than 60 years had higher p-AKT expression (data not shown); when adjusting for sex and IPI, the p-AKT expression significantly predicted PFS ( $p=0.02$ ) and as a trend OS ( $p=0.06$ ). Categorization into three groups according to the 25th and 75th percentiles showed that patients in the highest quartile (>108 positive cells/mm<sup>2</sup>,  $n=27$ ) had worse outcome than the <25th and 25-75th groups, while no significant survival difference was detected between the two latter groups (data not shown). When comparing the highest quartile group with the remainder, the high p-AKT+ group had worse PFS (HR=2.7, 95% CI, 1.1-6.3,  $p=0.02$ ) and OS (HR=3.6, CI 1.3-9.9,  $p=0.01$ ), independent of IPI (PFS: HR=3.1, CI 1.4-6.6,  $p=0.004$ ; OS: HR=2.9, CI 1.1-7.5,  $p=0.03$ ) and sex (PFS: HR=3.4, CI 1.4-8.5,  $p=0.008$ ; OS: HR=1.8, CI 0.7-5.2,  $p=0.25$ ). Table VII presents the clinical characteristics and Figure 8 (A-B) a typical immunohistochemical pattern for high vs low p-AKT expression.

Also Bcl-2 overexpression predicted worse outcome, with larger survival difference with cut-off 50% than 30% (data not shown). For further analyses, the cut-off value of 50% was used. Estimated PFS at 3 years was 64% and 90% for bcl-2+ and bcl-2- ( $p=0.005$ ), respectively; corresponding figures for OS were 78% and 92% ( $p=0.05$ ). After adjustment for IPI and sex, bcl-2+ still predicted PFS (HR=3.9, 95% CI= 1.1-13.4,  $p=0.03$ ) but not OS (HR=3.7,  $p=0.21$ ).

Furthermore, bcl-2+ and high p-AKT expression independently predicted outcome, also after adjustment for sex and IPI (bcl-2+: PFS, HR=3.9, p=0.03; OS, HR=3.6, p=0.10; High p-AKT: PFS, HR=2.6, p=0.02; OS, HR=3.2, p=0.02). Neither MCL1 nor bcl-xL expression had any impact on survival (data not shown). Since virtually all tumour cells stained positive for Bax and Bak, no prognostic information was obtained from these markers.

GCB vs non-GCB phenotype had no impact on OS (HR=1.5, p=0.38) but the non-GCB group showed a trend for worse PFS in univariate analysis (HR=2.1, p=0.06) but this trend disappeared (p=0.39) when the clinical factors were taken into account. On the other hand, bcl-2+ remained predictive for worse PFS (HR=4.6, p=0.02) after adjustment for GCB/non-GCB phenotype.

Table VII. Characteristics of 106 patients with high vs low p-AKT expression

	p-AKT+ highest quartile	p-AKT+ remainder	p-value
	(n=27) %	(n=79) %	
Median age	69 y	63 y	0.13
Sex, female / male	70 / 30	37 / 63	0.002
Age > 60 years	81	56	0.01
Age ≥ 75 years	15	23	0.36
Stage III+IV	52	54	0.82
Bulky disease (≥ 10 cm)	26	29	0.75
Extranodal disease	41	41	0.98
Extranodal sites > 1	11	13	0.83
Performance status >1	11	27	0.08
Increased serum LDH	78	58	0.06
IPI			
0-2 factors	67	67	0.97
3-5 factors	33	33	

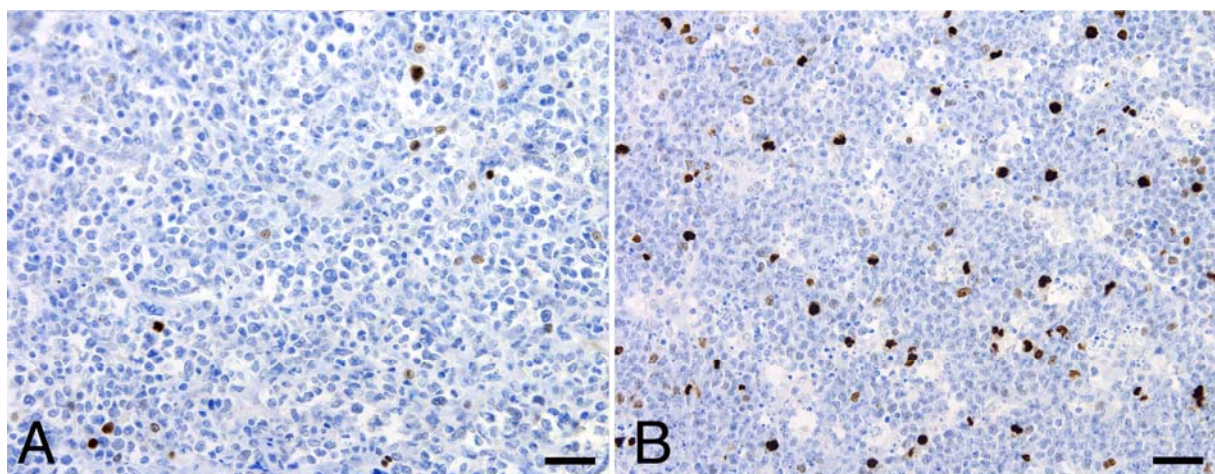


Figure 8. Immunohistochemical detection of (A) low p-AKT expression and (B) high p-AKT expression. Bar denotes 50 µm.





# DISCUSSION

## Patient selection and clinical factors

In Paper I, we presented a large population-based cohort of DLBCL patients with detailed data on characteristics, treatment and outcome. We retrospectively categorized therapy intention, which deserves some comments. Certainly one cannot rule out the possibility of some bias in such procedure, but on the other hand and more importantly, we also analysed how many patients who really accomplished therapy. One potential bias, however, could be that some patients originally registered as unspecified NHL were reclassified as DLBCL and thereby included in the study (n=17), but in fact, 11 of those received appropriate DLBCL treatment and were therefore included in the curative intent group. Nevertheless, in this cohort of 535 patients, 70% received treatment with a curative intent, with a median age of 68 years. But 12% of those discontinued the treatment due to toxicity, i.e. 62% of all 535 patients actually received a potentially curable therapy. Moreover, in the subgroup older than 70 years, merely 43% completed the treatment. So, we conclude that therapy is still difficult to accomplish in a considerable number of patients, mostly elderly, in a general DLBCL population. Reasonably, this fact is also valid in the rituximab era, since DLBCL patients must manage to receive at least 6 cycles of CHOP. Moreover, the IPI and aaIPI models were confirmed to be strong predictors for OS and PFS. Surprisingly, we also found that male sex had a negative impact on PFS and OS in the curative intent group, independent of IPI. The response rate did not differ between males and females but more men relapsed and died due to lymphoma. We cannot explain this discrepancy; no statistically significant differences in baseline characteristics or given therapy between females and males were detected, but despite that, a clinically relevant difference cannot be totally excluded. In the palliative group, prognostic information was lacking to a great extent, but more women had poor performance status, which could raise the suspicion that females with favourable performance status were overrepresented in the curative intent group, but they were not. In the literature, sex was not found to predict survival in the first large prognostic index study on aggressive lymphoma [161] or in the much larger IPI study [35]. However, two-thirds of the patients were males in both studies and the median age was only 50 and 56 years, respectively. Additionally, not only DLBCL patients were included in these studies. In population-based DLBCL studies, sex perspective has been rather sparsely discussed. To our knowledge, the prognostic impact of sex was not reported from the Dutch material [9] or from the entire Danish registry material [162]. However, some other data support the finding that males could have worse outcome; in a study on 220 aggressive lymphoma patients older than 60 years [163], male sex was

recognized as one of the factors predicting higher disease-specific mortality and all-cause mortality. Furthermore, in a community based study on diffuse aggressive lymphomas, male sex predicted shorter event-free survival independent of stage, LDH and performance status [164].

The patient material in Paper IV (rituximab and chemotherapy) was somewhat more selected than in Paper II and III, since not all patients treated with curative intent in the region received rituximab in the beginning of the study period. Nevertheless, we found that a two-group IPI model predicted survival, and ones again, male sex predicted worse outcome. Analysis of further, separate clinical factors was not performed due to the rather small sample size in the study. Also other studies have shown that IPI has prognostic impact in R-CHOP treated patients, as a two-group model similar to the one in our study [109, 110, 165] or as a three-group model in larger studies [166, 167]. The finding that male sex predicted worse outcome must be interpreted with caution, but it seems important that the sex perspective be taken into account in future studies as a possible prognostic factor.

## **TILs**

In Paper II, which represents the largest immunohistochemical study on TILs in DLBCL (195 patients), a small number of tumour-infiltrating TIA-1+ CTLs cells predicted better PFS, independent of clinical factors. Furthermore, high expression of perforin tended to be associated with worse outcome. Previously, only a few and rather small immunohistochemical studies have addressed the prognostic influence of CTLs and other TILs in DLBCL. In an early study on 82 patients, a small number of Leu-2+ (CD8+) cells predicted short relapse-free survival, but not OS [140]; however, interpretation is hampered by the lack of adjustment for clinical factors. Moreover, in a very small immunohistochemical study (22 patients), as part of a large gene expression profiling study, cases with the lowest MHC class II expression (unfavourable prognosis) had lower CD8+ counts than cases with the higher MHC class II expression [121]. In contradiction, in a study on 70 patients, less than 15% activated CTLs predicted longer PFS and OS, independent of clinical factors [125]. In addition, a gene expression profiling study (46 patients) showed that intense “cellular cytotoxic immune response” pattern predicted worse survival and was associated with high granzyme B+ expression in immunostaining [141]. In conclusion, even if the TIA-1 finding in our study has to be interpreted with some caution, it seems to strengthen a concept that a small amount of CTLs at diagnosis is indicative of better outcome. Indeed, similar findings have also been presented for some other lymphoma subtypes [133-137]. Since recruitment and activation of tumour-specific CTLs are considered to be important for effective tumour control, one could expect that large amount of CTLs predicted better prognosis. But, tumour cells are known to use several mechanisms to escape an anti-tumour immune response, e.g. downregulation of MHC class I and II molecules, loss of co-

stimulatory molecules or secretion of immunosuppressive cytokines. Also, even if tumour-specific cytotoxic cells are present, their function could be compromised by direct tumour-induced inhibition, e.g. by reduction of lytic granule exocytosis [168], defective T cell receptor signaling [169] or upregulation of inhibitory NK cell receptors [170]. Moreover, it has been demonstrated that lymphoma TILs could possess similar functional defects as T cells in inflammatory lymph nodes [171], suggesting that tumour cells induce a state of chronic inflammation in their vicinity, e.g. by the release of cytokines, leading to compromised function of recruited cytotoxic cells in an antigen-independent manner. Indeed, elevated plasma levels of inflammatory or immunosuppressive cytokines such as interleukin (IL)-6, TNF- $\alpha$  and IL-10 have previously been reported as negative prognostic factors in DLBCL patients [172-174]. In our study, the small subset of patients with fever had significantly more TIA-1+ and perforin+ cells, indicating an association with an inflammatory state at least for these patients. Also, besides being pyrogenic, TNF- $\alpha$  induces migration of cytotoxic cells into sites of inflammation, which could lead to a recruitment of tumour-antigen unspecific cytotoxic cells into the tumour area. Altogether, it seems that an accumulation of cytotoxic cells not necessarily leads to effective killing of tumour cells.

One possible mechanism for inhibition of CTL activity could be the presence of tumour-infiltrating T<sub>regs</sub>, but FOXP3 expression did not predict survival in the present study. When considering results from the carcinoma studies, one would expect that large number of FOXP3+ cells predicted worse outcome. Indeed, in vitro studies on human non-Hodgkin B cells have shown that intratumoural CD4+CD25+ cells can inhibit the proliferation of activated anti-tumour CTLs, resulting in decreased lysis of the tumour cells [175]. However, unlike the carcinoma studies, FOXP3+ has been associated with better prognosis in follicular lymphoma, Hodgkin's disease and some T-cell lymphomas [133, 148-151]. The reasons for the opposite findings are not easily explained, but the action of T<sub>regs</sub> in lymphoma could be more complex than just inhibition of the immune response. Since it has been shown that FOXP3+ or CD4+CD25+ cells directly can suppress and even kill normal B cells [176, 177], it has been hypothesised that T<sub>regs</sub> also could inhibit tumour B-cells [148]. On the other hand, follicular lymphoma has a generally indolent course, which is in strong contrast to DLBCL. The finding in our study, in which the number of FOXP3+ cells neither did correlate with disease extent at diagnosis nor prognosis, could possibly indicate that T<sub>regs</sub> play a less important role in DLBCL than in other lymphoma entities. However, although FOXP3 has been considered to be the most specific marker so far, the delimitations and properties of human T<sub>regs</sub> seem to be very complex [144, 178, 179]. Recently, two immunohistochemical studies on FOXP3 and DLBCL have been published; in one study (96 patients), a large amount of FOXP3+ cells predicted better OS, significant also after adjustment for IPI [180], but concern regarding the methodology may be raised; the cut-off

value was determined by an unknown ROC curve, the FOXP3 calculation was done with a special software, the median follow up time was very short (16 months) and both R-CHOP and CHOP patients were included. In another study (125 patients), FOXP3+ was remarkably associated with better “disease-specific” survival ( $p=0.05$ ) in the GCB subgroup but worse survival in the non-GCB subgroup ( $p=0.06$ ), in univariate analyses [181], but these findings became non-significant after adjustment for age and stage, and no therapy data was presented. Taken together, we conclude that it is not shown that the number of  $T_{regs}$  predicts survival in DLBCL.

## **Proliferation marker Ki-67**

In paper III (199 patients), low Ki-67 expression (<49%, the lowest quartile) predicted inferior survival, also after adjustment for clinical factors, while patients with high Ki-67 expression (>73%, i.e. the highest quartile) did not do worse than the remainder. The prognostic role of Ki-67 protein expression has been unclear. A number of factors could explain the diverging results, e.g. sample size and patient selection or differences in immunohistochemical methodology and statistics. The proliferation index (growth fraction) was originally defined as the proportion of Ki-67+ cells out of all nucleated cells, analysed in areas characteristic for the tumour [182]. It was also suggested that in cases with variable staining, e.g. due to lymphocyte infiltration, quantification was performed in areas with the greatest Ki-67 staining [7]. These authors found that centroblastic and B-immunoblastic lymphoma patients with low Ki-67 expression had worse outcome [183]. In contrast, in a study on 105 patients with diffuse large cell lymphoma (DLBCL,  $n=83$ ), Ki-67 >60% predicted inferior OS, independent of stage and B symptoms [86, 184], and in another study on 60 patients with aggressive lymphoma (diffuse large cell type,  $n=41$ ), Ki-67 >80% (11 patients) similarly predicted worse OS [87]. In these studies, quantification was done in areas with the highest expression, and Ki-67+ cells out of all large cells [86] or out of all cells [87] were counted. In addition, in one study on 140 DLBCL patients, Ki-67 >20% ( $n=77$ ) was associated with shorter OS, independent of IPI [78]. But, that study used special software for cell counting and the Ki-67 percentages were remarkably low (range 3-56%). In contrast, but consistent with our findings, a prospective study on 185 DLBCL patients showed that patients with Ki-67 <60% ( $n=116$ ) had worse PFS and OS than those with Ki-67 60-90% [77]. In that study, Ki-67+ cells out of all tumour cells were counted in random areas. However, other studies on 114 [185], 128 [66] and 200 [71] DLBCL patients have not found Ki-67 to be predictive for survival. In the latter study, tissue microarray technique was used, the Ki-67+ fraction out of all tumour cells was estimated (not counted) and results from the core with the highest expression was presented. In our study, quantification was done in representative areas and Ki-67+ cells out of all nucleated cell profiles

were counted. We did so for practical reasons and this procedure was also considered to reflect clinical practice where a quick estimation of the proliferation index is performed. Certainly, it is not possible from available data to compare the methods, but the median Ki-67+ percentage in our study (62%) was in accordance with other studies (55-65%) [182, 186]. So, to summarize the large DLBCL studies, our study (Paper III) and another one [77] found a negative prognostic impact of low Ki-67 expression, one study found the opposite [78], but with difficulty to interpret the software counted data, while no impact of Ki-67 expression was demonstrated in another large study [71]. Taken together, our results strengthen the concept that low Ki-67 expression could be an adverse prognostic factor in DLBCL, possibly reflecting less chance for tumours with many non-proliferating cells to respond to chemotherapy [99, 187]. Indeed, the group with low Ki-67 tended to have fewer complete responders ( $p=0.06$ ) and had more relapses ( $p=0.04$ ) than patients with higher Ki-67 expression. One possibility would be that bcl-2 overexpression explained the worse outcome in the low Ki-67 group, according to a possible relation between bcl-2 and proliferation [188, 189] and indeed we found a correlation between bcl-2+ (and galectin-3+) and low Ki-67+. But, the negative prognostic effect of low Ki-67 expression persisted after adjustment for bcl-2, a finding that also has been described in a previous study [77].

From a practical clinical point of view, perhaps a more important finding was that high Ki-67 expression ( $>73\%$ ) not predicted worse outcome, even though some caution is justified due to the limited number of patients in the upper range ( $n=50$ ). In addition, no conclusions can be drawn regarding the possible prognostic role of extremely high Ki-67 values, since only five patients had Ki-67  $>90\%$ . On the other hand, in a study comparing Ki-67 estimates between laboratories, there was low reproducibility regarding the possibility to separate Ki-67 76-95% vs  $>95\%$  groups, suggesting that they should be grouped together [190].

## Cell-of-origin

In Paper III it was shown that the GCB phenotype, according to the algorithm by Hans et al [64], strongly predicted a favourable outcome independent of IPI among patients treated with chemotherapy, which is consistent with another study [62]. In the R-CHOP era, gene expression profiling data have shown that GCB still predicts better outcome [124]. But, immunohistochemical studies using the previously mentioned algorithm, [110, 165, 191] and Paper IV, have not demonstrated a benefit for GCB phenotype patients, even if such a difference was seen in univariate analysis in one study [191]. However, these four studies were smaller (90-131 patients) than the gene expression profiling study, but also a recently published study on 279 patients failed to demonstrate a better outcome for the GCB group [167]. Therefore, another algorithm using CD10, bcl-6, MUM-1, GCET1 and FOXP1, with better concordance with gene

expression profiling data than the former algorithm, has recently been proposed [192].

## Apoptosis

In Paper III, the negative prognostic impact of bcl-2 overexpression was confirmed, significant also after adjustment for clinical factors. To summarize studies from the pre-rituximab era, bcl-2+ overexpression has been associated with worse survival, independent of IPI, in several studies [62, 66, 76, 77, 167]. But, there are also studies (138 to 200 patients) in which bcl-2 has not predicted survival [64, 71, 78]. Paper IV comprised 106 patients treated with rituximab and chemotherapy. We found that bcl-2+ predicted worse PFS, independent of clinical factors, but no significant difference was seen regarding OS, probably due to a rather short follow up. Our findings partly contrast to previous immunohistochemical studies on R-CHOP treated patients; in three studies on 140, 131 and 279 patients, respectively, bcl-2 did not predict survival [108, 167, 191]. On the other hand, in the prospective US Intergroup study comparing CHOP with R-CHOP +/- R maintenance, bcl-2+ had negative impact on failure-free survival among R-CHOP treated patients (n=107) in multivariate analysis [109]. Furthermore, bcl-2+ predicted worse survival in a recent study on 117 patients [110]. All studies referred to used 50% as cut-off value. So, even if rituximab seems to overcome at least some of the bcl-2 induced chemotherapy resistance [193], our study strengthens the notion that bcl-2+ still predicts less chance for sustained remission, possibly reflecting rituximab resistance [102, 103].

We also found in Paper IV that high p-AKT protein expression predicted inferior survival after adjustment for sex and IPI. Interestingly, high p-AKT and bcl-2+ expression independently predicted worse survival, indicating different ways of resistance. The serine-threonine kinase AKT plays a central role in the PI3K/AKT pathway in which PI3K phosphorylates membrane bound phosphatidylinositol-diphosphate (PIP2) to generate phosphatidylinositol-trisphosphate (PIP3), permitting phosphorylation (activation) of AKT on the Thr308 and Ser473 residues in the presence of PDK1 and mTORC2, respectively. P-AKT then mediates a range of pro-survival signals for anti-apoptosis, proliferation, cell growth (via mTORC1) and angiogenesis [194]. In our study there was no correlation between p-AKT and MCL1 or between p-AKT and bcl-xL expression. Neither did bcl-xL+ or MCL1+ predict survival with the methodology used in our study. This could possibly indicate that the negative prognostic impact of p-AKT+ related to other mechanisms than inhibition of apoptosis. On the other hand, p-AKT not only inhibits apoptosis via Bad/Bcl-xL and GSK-3 $\beta$ /MCL1 but also via downregulation of the transcription factors FOXO and p53 (via activation or MDM2), upregulation of NF- $\kappa$ B activity and direct inhibition of pro-caspase 9 [194]. In DLBCL some clinical and in vitro data support the association between overexpressed AKT,

dysregulated apoptosis and worse prognosis [106, 107] but no studies, to our knowledge, have addressed the prognostic influence of p-AKT protein expression at diagnosis in patients treated with immunochemotherapy. So, even if the p-AKT finding in the present study has to be interpreted with some caution, and needs to be confirmed in other studies, high p-AKT expression seems to predict worse outcome. Thus, inhibition of the activated PI3K/AKT pathway could possibly be of clinical interest in this subgroup of DLBCL patients. Indeed, AKT inhibitors such as perifosine [195] and the novel compound GSK690693 [196] have been shown to induce apoptosis in lymphatic leukaemia cell lines, and in a recent in vitro study on DLBCL cells a synergistic effect was seen by combining rapamycin and a histone deacetylase inhibitor, which inhibited AKT via mTORC2 and induced apoptosis [197].

# CONCLUSIONS

In a large population-based cohort of DLBCL patients, it was shown that

- a considerable percentage of patients did not accomplish potentially curable treatment,
- there was no significant difference in the complete remission rate or PFS between patients younger and older than 68 years, among those treated with curative intent,
- IPI and aaIPI strongly predicted PFS and OS
- male sex predicted inferior PFS and OS, independent of IPI.

From the population-based material, it was shown that

- a small number of tumour-infiltrating TIA-1+ CTLs was associated with better outcome, suggesting that immunohistochemical analysis of the number of CTLs at diagnosis can provide additional prognostic information,
- the amount of tumour-infiltrating FOXP3+ T<sub>regs</sub> did not predict survival, possibly indicating that such cells are of less clinical importance in DLBCL.
- low rather than high Ki-67 expression could have prognostic relevance in DLBCL, independent of bcl-2 and GCB/non-GCB phenotype.

In R-CHOP treated patients it was found that

- high p-AKT expression predicted inferior survival, independent of bcl-2 and clinical factors, suggesting that immunohistochemical analysis of p-AKT at diagnosis provides additional prognostic information, a finding that needs to be confirmed in future studies,
- bcl-2 overexpression could have prognostic relevance also in the era of immunochemotherapy
- IPI was still predictive for survival
- male sex predicted worse outcome, a finding that needs to be confirmed in larger cohorts of patients treated with immunochemotherapy.



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