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*Anna Kaisa Pasanen*

# A TRANSLATIONAL STUDY ON THE ROLES OF REDOX MOLECULES, CELL CYCLE REGULATORS AND CHEMOKINE RECEPTORS AS PROGNOSTIC FACTORS IN DIFFUSE LARGE B-CELL LYMPHOMA

*UNIVERSITY OF OULU GRADUATE SCHOOL;*
*UNIVERSITY OF OULU,*
*FACULTY OF MEDICINE,*
*INSTITUTE OF CLINICAL MEDICINE,*
*DEPARTMENT OF ONCOLOGY AND RADIOTHERAPY;*
*OULU UNIVERSITY HOSPITAL*

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**Pasanen, Anna Kaisa, A translational study on the roles of redox molecules, cell cycle regulators and chemokine receptors as prognostic factors in diffuse large B-cell lymphoma.**

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## *Abstract*

Lymphomas are a group of more than 70 different malignancies arising from lymphoid tissues and diffuse large B-cell lymphoma (DLBCL) is the most common subtype of lymphoma. More than 70% of DLBCL patients can be cured with modern therapy, but some patients still die of the disease. The recognition of patients with adverse prognosis, justifying deviation from standard treatment and risking severe side effects, is problematic. The aim of this study was to identify potential biological factors for the prediction of poor treatment response and central nervous system (CNS) relapse in DLBCL patients.

The study included 263 lymphoma patients. 205 patients had a DLBCL, and 37 of these represented primary CNS lymphoma (PCNSL). Immunohistochemistry was used to determine the expression of oxidative stress markers 8-hydroxydeoxyguanosine (8-OHdG) and nitrotyrosine, as well as antioxidant enzymes manganese superoxide dismutase (MnSOD), thioredoxin (Trx) and gamma cysteine ligase (GCL) from samples representing reactive lymphoid tissue and B-cell derived lymphomas. From DLBCL samples staining was also conducted for cell cycle regulating proteins p16, p53, p21 and p27 and chemokine receptors CXCR4, CXCR5 and CCR7. Immunoelectron microscopy (IEM) for CXCR4 and CXCR5, and their ligands CXCL12 and CXCL13 was performed on additional samples from reactive lymphoid tissue, nodal DLBCL, secondary CNS lymphoma and PCNSL.

Factors associated with adverse prognosis included expression of nitrotyrosine, Trx and GCL. A prognostic score reflecting the degree of cell cycle dysregulation within each patient's tumour identified 3 distinct prognostic groups among DLBCL patients. High cytoplasmic CXCR5 expression was associated with CNS involvement, whereas nuclear CXCR4 expression correlated with nodal disease.

These results demonstrate the considerable biological heterogeneity seen within DLBCL, but further research is needed to confirm them. High antioxidant activity and the accumulation of damage to cell cycle regulating pathways separated patient groups with a poor prognosis that might benefit from new types of treatment. Chemokine receptor expression seems to play a role in the CNS tropism of DLBCL, and, if confirmed, could in the future contribute to more effective targeting of CNS prophylactic therapies.

*Keywords:* antioxidants, biological markers, cell cycle, central nervous system, chemokine receptors, diffuse large B-cell lymphoma, immunoelectron microscopy, immunohistochemistry, oxidative stress, prognosis, survival

**Pasanen, Anna Kaisa, Translationaalinen tutkimus redox-molekyylien, solusyklin säätelijöiden ja kemokiinireseptorien rooleista ennusteellisina tekijöinä diffuusissa suurisoluisessa B-solulymfoomassa.**

Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta, Kliinisen lääketieteen laitos, Syöpätaudit ja sädehoito; Oulun yliopistollinen sairaala

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## *Tiivistelmä*

Lymfoomat ovat yli 70 erilaisen maligniteetin muodostama ryhmä imukudoksen syöpiä, ja diffuusi suurisoluinen B-solulymfooma (engl. DLBCL) on yleisin lymfoomatyyppi. Yli 70 prosenttia DLBCL-potilaista pystytään parantamaan nykyaikaisilla hoidoilla, mutta osa potilaista kuolee edelleen tautiin. Nämä potilaat tarvitsisivat tehokkaampia hoitoja vakavien haittavaikutusten riskistä huolimatta, mutta huonon ennusteen potilaiden tunnistaminen etukäteen on vaikeaa. Tutkimuksen tavoitteena oli löytää biologisia tekijöitä DLBCL-potilaiden hoitovasteen ja taudin keskushermostossa (engl. CNS) uusiutumisen ennustamiseen.

Aineisto sisältää 263 lymfoomapotilasta. 205 potilaalla on DLBCL, ja 37:llä näistä primaari aivolymfooma (PCNSL). Immunohistokemiallisilla värjäyksillä määritettiin oksidatiivisen stressin markkereiden 8-hydroksideoksiguanosiinin (8-OHdG) ja nitrotyrosiinin, sekä antioksidanttientsyymien mangaanisuperoksidi-dismutaasin (MnSOD), tioredoksiinin (Trx) ja gammakysteiiniligaasin (GCL) ilmentyminen reaktiivista imukudosta sekä B-soluperäisiä lymfoomia edustavissa näytteissä. DLBCL-näytteistä määritettiin lisäksi solusykliä säätelevien proteiinien p16, p53, p21 ja p27 sekä kemokiinireseptorien CXCR4, CXCR5 ja CCR7 ilmentyminen. Lisäksi reaktiivista imukudosta, imusolmuke-DLBCL:aa, sekundaarista CNS-lymfoomaa ja PCNSL:aa edustavista näytteistä määritettiin immunoelektronimikroskooppisesti reseptorien CXCR4 ja CXCR5 sekä ligandien CXCL12 ja CXCL13 ilmentyminen.

Tulosten mukaan voimakas nitrotyrosiini-, Trx- ja GCL-positiivisuus ovat yhteydessä huonoon ennusteeseen. Solusyklin säätelyhäiriön vaikeusastetta kuvaava ennusteellinen pisteytys jaotteli DLBCL-potilaat kolmeen ennusteelliseen ryhmään. Runsas sytoplasminen CXCR5-positiivisuus oli yhteydessä CNS-tautiin, kun taas tumapositiivisuus CXCR4:lle korreloi imusolmuketautiin.

Tutkimustulokset kuvaavat DLBCL:n merkittävää biologista heterogeenisyyttä, mutta tulosten varmistamiseksi tarvitaan lisää tutkimuksia. Korkea antioksidanttiaktiivisuus ja solusyklin säätelyhäiriöiden kasautuminen erottivat huonoennusteisia potilasryhmiä, jotka voisivat hyötyä uudenlaisista hoidoista. Kemokiinireseptorien ilmentyminen vaikuttaisi olevan yhteydessä DLBCL:n CNS-hakuisuuteen, ja tulosten varmistuessa ekspressioprofiilien analysointia voitaisiin tulevaisuudessa hyödyntää ennaltaehkäisevien hoitojen tehokkaammassa kohdentamisessa.

*Asiasanat:* antioksidantit, biologiset merkkiaineet, diffuusi suurisoluinen B-solulymfooma, ennuste, immunoelektronimikroskopia, immunohistokemia, kemokiinireseptorit, keskushermosto, oksidatiivinen stressi, selviytyminen, solunjakautuminen

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Oulu, August 2013 Anna Kaisa Pasanen

# Abbreviations

| | |
|---|---|
| 8-OHdG | 8-hydroxydeoxyguanosine |
| ABC-DLBCL | activated B-cell-like diffuse large B-cell lymphoma |
| ABVD | doxorubicin, bleomycin, vinblastine and dacarbazine |
| AIDS | acquired immunodeficiency syndrome |
| ATT | alternating triple therapy |
| BBB | blood brain barrier |
| BBBD | blood brain barrier disruption |
| BCR | B-cell receptor |
| BEACOPP | cyclophosphamide, doxorubicin, etoposide, procarbazine, prednisone, bleomycin and vincristine |
| BSA | bovine serum albumin |
| CCR | CC chemokine receptor |
| CDK | cyclin-dependent kinase |
| CDKI | cyclin-dependent kinase inhibitor |
| CEOP | cyclophosphamide, epirubicin, vincristine and prednisone |
| CHOEP | cyclophosphamide, doxorubicin, vincristine, etoposide and prednisone |
| CHOP | cyclophosphamide, doxorubicin, vincristine and prednisone |
| CLL | chronic lymphocytic leukaemia/ small lymphocytic lymphoma |
| CNS | central nervous system |
| CT | computer tomography |
| CXCR | CXC chemokine receptor |
| DBD | DNA-binding domains |
| DHAP | dexamethasone, high-dose cytarabine and cisplatin |
| DLBCL | diffuse large B-cell lymphoma |
| DLBCL, NOS | diffuse large B-cell lymphoma, not otherwise specified |
| DSS | disease-specific survival |
| EATCL | enteropathy-associated T-cell lymphoma |
| EBV | Epstein-Barr virus |
| ESHAP | etoposide, methylprednisone, cytarabine and cisplatine |
| FCM | flow cytometry |
| FL | follicular lymphoma |
| GCB-DLBCL | germinal center B-cell-like diffuse large B-cell lymphoma |
| GCL/ $\gamma$ -GCS | gamma cysteine ligase / gamma-glutamylcysteine synthetase |
| GEP | gene expression profiling |

| | |
|---|---|
| GPCR | G-protein coupled receptor |
| GPX1 | glutathione peroxidase 1 |
| GSH | glutathione |
| Gy | Gray |
| $H_2O_2$ | hydrogen peroxide |
| HAART | highly active antiretroviral treatment |
| HBV | hepatitis B virus |
| HCV | hepatitis C virus |
| HDT/ASCT | high-dose chemotherapy with autologous stem cell transplantation |
| HIV | human immunodeficiency virus |
| HL | Hodgkin lymphoma |
| HTLV-1 | human T-lymphotropic virus type 1 |
| ICE | ifosfamide, etoposide and carboplatin |
| IEM | immunoelectronmicroscopy |
| IFRT | involved-field radiotherapy |
| IPI | International Prognostic Index |
| LD | lactate dehydrogenase |
| MALT | marginal zone lymphoid tissue |
| MCL | mantle cell lymphoma |
| MnSOD | manganese superoxide dismutase |
| MRI | magnetic resonance imaging |
| mRNA | messenger ribonucleic acid |
| NF-κB | nuclear factor kappa-B |
| NHL | non-Hodgkin lymphoma |
| $NO^{\cdot}$ | nitric oxide |
| NOPP | mitoxantrone, vincristine, prednisone and procarbazine |
| NSCLC | non-small cell lung cancer |
| OS | overall survival |
| PBS | phosphate buffered saline |
| PCNSL | primary central nervous system lymphoma |
| PFS | progression-free survival |
| RA | rheumatoid arthritis |
| RFS | relapse-free survival |
| R-IPI | revised International Prognostic Index |
| ROC | receiver operating characteristic |
| ROS | reactive oxygen species |

| | |
|---|---|
| sCNSL | secondary central nervous system lymphoma |
| SLE | systemic lupus erythematosus |
| SS | Sjögren's syndrome |
| TBS | tris-buffered saline |
| Trx | thioredoxin |
| WHO | World Health Organization |

# List of original publications

The thesis is based on the following publications, which are referred to in the text by their Roman numerals:

I Pasanen AK, Kuitunen H, Haapasaari K-M, Karihtala P, Kyllönen H, Soini Y, Turpeenniemi-Hujanen T & Kuittinen O (2012) Expression and prognostic evaluation of oxidative stress markers in an immunohistochemical study of B-cell derived lymphomas. Leuk Lymphoma 53: 624–631.

II Pasanen AK, Haapasaari KM, Jantunen E, Soini Y, Turpeenniemi-Hujanen T, Bloigu R, Lilja L, Kuittinen O & Karihtala P (2012) Oxidative stress and redox state-regulating enzymes have prognostic relevance in diffuse large B-cell lymphoma. Exp Hematol Oncol 1:2.

III Pasanen AK, Haapasaari KM, Peltonen J, Soini Y, Jantunen E, Bloigu R, Turpeenniemi-Hujanen T & Kuittinen O (2013) Cell cycle regulation score predicts relapse-free survival in non-germinal centre diffuse large B-cell lymphoma patients treated by means of immunochemotherapy. Eur J Haematol 91: 29–36.

IV Pasanen AK, Lemma S, Haapasaari K-M, Sippola A, Sormunen R, Soini Y, Jantunen E, Koivunen P, Salokorpi N, Bloigu R, Turpeenniemi-Hujanen T & Kuittinen O (2013) Similar chemokine receptor profiles in primary central nervous system lymphoma and secondary central nervous system involvement of systemic diffuse large B-cell lymphoma – possible biomarkers for patient selection for central nervous system prophylaxis. Manuscript.

# Contents

# 1 Introduction

Lymphomas are a heterogeneous disease group entailing more than 70 different entities (Swerdlow *et al.* 2007). The incidence of lymphomas has been rapidly rising in the last 50 years, and in 2011, 1,319 new lymphomas were diagnosed in Finland (Finnish Cancer Registry, Cancer Statistics (2011) at http://www.cancer.fi/syoparekisteri/, accessed 6/12/2013). The rising incidence makes lymphomas a significant disease group also from a socioeconomic point of view. The most common subtype of lymphoma, covering around 30% of all new cases, is diffuse large B-cell lymphoma (DLBCL). DLBCL is an aggressive disease presenting with considerable biological heterogeneity, and in the 20$^{th}$ century it was associated with mortality rates of up to 70%. In the beginning of the 21$^{st}$ century, however, the introduction of the CD20 monoclonal antibody rituximab into clinical practice constituted a breakthrough in the treatment of DLBCL. Today, more than 70% of DLBCL patients can be cured with immunochemotherapy (Cabanillas 2010).

Despite the remarkable improvement seen in the prognosis of DLBCL patients, some patients still succumb to their disease. Around one third of DLBCL patients present with a refractory or recurring disease. After relapse, only 20% of DLBCL patients can be cured with further chemotherapy, and the aim should, therefore, be effective frontline treatment (Gisselbrecht *et al.* 2010). Another disease group with a dismal prognosis is DLBCL of the central nervous system (CNS). Primary CNS lymphomas (PCNSL), as well as the secondary CNS involvement of an extracranial disease (sCNSL), are rare forms of DLBCL. Although promising results have been reported with new experimental treatments, at the moment CNS lymphoma still constitutes a condition with extremely high mortality rates (Angelov *et al.* 2009).

DLBCL is usually sensitive to both chemotherapy and radiotherapy, and many effective therapy modalities exist. It seems that also CNS relapses could be prevented by intravenous high-dose methotrexate (Holte *et al.* 2013). More aggressive therapy, however, has more severe side effects, and treatment-related mortality is a serious concern. This makes patient selection and the targeting of aggressive treatments paramount. For the moment, no clinically applicable biological markers able to effectively predict patient prognosis, treatment response or disease relapse in DLBCL patients have been identified. Risk assessment is presently based on clinical characteristics, which are no longer able to identify patients with a very poor prognosis. The mechanisms causing drug

resistance in some patient populations are also poorly understood. Better prognostic and predictive markers are, therefore, needed, and the heterogeneous nature of DLBCL indicates a requirement for disease-specific biological markers.

Some potentially significant prognostic markers might be found in molecules reflecting the redox state of tumour tissue. Aetiological and prognostic significance for end products of cellular oxidation as well as antioxidant enzymes has been found in several solid malignancies (Karihtala & Soini 2007). In lymphoid malignancies, very little data exists on the expression and prognostic significance of oxidative stress markers and antioxidant enzymes. These are molecules directly influencing the function of certain chemotherapeutic drugs used to treat DLBCL, and studying their expression might in part aid in explaining the differential drug response in patient subgroups.

Cell cycle regulators are essential in the control of cell proliferation and death. In cancer, this regulation is inherently compromised. The most important pathways regulating cell cycle progression and mitosis in mammals are the Rb, p53 and p27 pathways, and damage in one or more of these pathways occurs in practically all human cancer (Sherr 2000). Prognostic significance has also been shown in both solid and haematological malignancies. In lymphomas, studies in the era of modern immunochemotherapy are, however, scarce. Treatment is the single most important factor determining the significance of prognostic markers, and considering the revolutionary effect rituximab has had on the treatment results of DLBCL, data preceding its emergence cannot be applied to present day.

Data explaining the CNS tropism of DLBCL is very much lacking. Chemokines and their receptors guide the migration and homing of cells into specific tissues, and roles in organ-specific metastases, including brain metastases, of solid tumours have been discovered (Zlotnik *et al.* 2011). Also in lymphomas they seem to affect disease dissemination, and differential chemokine receptor expression has been reported by one study in PCNSL, compared with systemic DLBCL and other nodal lymphomas (Jahnke *et al.* 2005). Their role in the secondary CNS involvement of systemic DLBCL has, however, not been investigated.

The aim of the present study was to identify biological markers with potential for the early identification of therapeutically challenging patient subgroups. By assessing the expression patterns of redox molecules, cell cycle regulators and chemokine receptors, we wanted to gain new insights into the biology of DLBCL as a whole, as well as to identify unique biological features of prognostic

subgroups. This might also reveal new therapeutic implications for patients with distinct phenotypes.

# 2 Review of the literature

## 2.1 Lymphomas

Lymphomas are a heterogeneous group of malignancies arising from lymphoid tissues. The classification of lymphomas has historically been very challenging and many different classifications have been presented, starting with the rough division into only Hodgkin lymphoma (HL) and non-Hodgkin lymphomas (NHL). As biological data has increased, the classification has become more reflective of the biological variation that exists within the disease group, and in the latest classification by the World Health Organization (WHO), lymphomas are separated into up to 76 different subtypes (Swerdlow *et al.* 2008).

Lymphomas can be divided into B-cell and T-/NK-cell derived neoplasms according to the cell line from which they have originated. HL is also considered a separate entity although it has been discovered to be predominantly B-cell derived. (Swerdlow *et al.* 2008.) B-cell derived lymphomas include both aggressive and indolent disease subtypes and are more common than the mostly aggressive T-cell lymphomas. Among the most common B-cell derived lymphomas are diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), HL, chronic lymphocytic leukaemia/small lymphocytic lymphoma (CLL), marginal zone lymphoid tissue (MALT) lymphoma and mantle cell lymphoma (MCL). (The Non-Hodgkin's Lymphoma Classification Project 1997.) DLBCL, which is the most common subtype of lymphoma covering around 30% of all cases, will be discussed in detail later. FL is the most common form of indolent lymphoma, and accounts for approximately 20% of all lymphomas. FL is composed of germinal centre B-cells and typically presents with an at least partially follicular growth pattern in neoplastic tissues (Swerdlow *et al.* 2008). A central cytogenetic aberration, which is seen in up to 80% of FL cases, is the translocation (t14;18)(q32;q21) resulting in overexpression of the antiapoptotic Bcl-2 protein (Horsman *et al.* 1995). Around 10–15% of all lymphomas represent HL, which is considered an aggressive disease entity and is most commonly seen in young adults. This subtype is characterized by a small number of scattered large malignant cells in neoplastic tissues, surrounded by a large colonization of benign inflammatory cells. (Swerdlow *et al.* 2008.) These malignant cells have been named Hodgkin-Reed-Sternberg cells and are predominantly of B-cell origin. CLL and MALT lymphoma are indolent lymphoma subtypes that are slow

to disseminate and both account for approximately 7% of NHLs. Around 3–10% of NHLs represent MCL. This disease entity includes both aggressive and indolent variants, but at the moment most patients cannot be cured (Swerdlow *et al.* 2008). The translocation t(11;14)(q13;q32) leading to overexpression of cell cycle promoting cyclin D1 is seen in practically all MCL cases, and it is considered to be the primary genetic event in the development of MCL (Bosch *et al.* 1994). FL, HL, CLL and MCL typically present as nodal diseases, whereas DLBCL frequently shows additional extranodal involvement and MALT lymphoma mostly involves extranodal mucosal tissues.

### 2.1.1 Epidemiology

In the year 2011, 1,319 new lymphomas were diagnosed in Finland, covering 4.4% of all new cancer cases (Finnish Cancer Registry 2011). 128 of the new lymphoma cases represented HL, and its incidence has remained fairly stationary in the past decades. The number of new NHLs, however, has nearly tripled in the last 30 years, with a mean of 424 yearly cases in the early 1980s compared with the 1,191 new cases diagnosed in 2011. For reasons unknown, NHLs are quickly becoming one of our most prevalent cancer types. In 2011 NHLs were the 7$^{th}$ most common cancer type among men in Finland and the 9$^{th}$ most common cancer type among women (Finnish Cancer Registry 2011).

Lymphomas are slightly more common among men than women, with 731 new lymphomas diagnosed in Finnish men and 588 in women in 2011. Certain age groups also have increased risk of lymphoma. HL has two age-related incidence peaks, being most common among 20- to 40-year-olds and then again in people over 60. The incidence of NHLs starts to increase rapidly in age groups over 60, and the highest incidence rates are found among 60- to 64-year-olds in both men and women (Finnish Cancer Registry 2011). There is a clear geographical variation in the incidence of lymphomas, with higher incidence rates found in developed countries, indicating a possible association with higher socioeconomical status (IARC, Globocan (2008) at http://globocan.iarc.fr/, accessed 3/11/2013).

### 2.1.2 Aetiology

The aetiology of lymphomas remains largely unknown. Although several individual risk factors have been identified, they account for only a small

proportion of all cases. Acquired or congenital immunodeficiency is known to be associated with increased risk of lymphoma. Compared with the general population, patients with acquired immunodeficiency syndrome (AIDS) have more than a 100-fold risk of developing NHL, and most of these are high-grade lymphomas (Goedert 2000, Levine *et al.* 1992). This risk has significantly decreased with the emergence of the modern highly active antiretroviral treatment (HAART), but it is, however, still slightly higher compared with immunocompetent populations (Clifford *et al.* 2005). Immunosuppressive medications after organ transplantation lead to at least a 6-fold increase in NHL risk, and with transplants other than the kidney the risk may be as high as 38-fold (Adami *et al.* 2003). Patients with a congenital immunodeficiency syndrome have a higher than average lifetime risk of cancer, and NHL is the most frequently seen cancer type in this patient group (Filipovich *et al.* 1992).

An association has been found between several autoimmune disorders and NHL. A large meta-analysis published in 2005 reported increased NHL risks associated with Sjögren's syndrome (SS) (18.8-fold risk), systemic lupus erythematosus (SLE) (7.4-fold risk) and rheumatoid arthritis (RA) (3.9-fold risk) (Zintzaras *et al.* 2005). Among RA patients, a higher NHL risk was associated with cytotoxic (5.1-fold risk) and biological drugs (11.5-fold risk) compared with patients receiving conventional antirheumatic treatment (2.5-fold risk). MALT lymphoma is the most common lymphoma subtype seen in SS patients, whereas patients with RA most often present with a DLBCL (Voulgarelis *et al.* 1999, Baecklund *et al.* 2003). Patients with coeliac disease have a 16- to 19-fold risk of NHL (Catassi *et al.* 2002). The association is strongest for enteropathy-associated T-cell lymphoma (EATCL), but other types of B- and T-cell lymphomas are also seen (Catassi *et al.* 2002, Mathus-Vliegen *et al.* 1994).

Certain infective agents are also known to promote lymphomagenesis. Epstein-Barr virus (EBV) has high prevalence worldwide and increases the risk for many lymphomas such as HL, endemic Burkitt's lymphoma, T-cell/NK-cell lymphomas and lymphomas associated with immunodeficiency in e.g. human immunodeficiency virus (HIV) infected patients or after organ transplantation (Hjalgrim & Engels 2008). Human T-lymphotropic virus type 1 (HTLV-1) is endemic in southwestern Japan, the Caribbean and South America, and causes adult T-cell leukaemia/lymphoma in around 2–6% of carriers (Arisawa *et al.* 2000). Hepatitis B virus (HBV) seems to cause a nearly 3-fold increase in NHL risk and patients infected with hepatitis C virus (HCV) display increased incidence rates for DLBCL, MALT lymphoma and lymphoplasmacytic

lymphoma (Ulcickas *et al.* 2007, de Sanjose *et al.* 2008). Also some bacterial infections contribute to the development of lymphoma. Helicobacter pylori infections can cause gastric MALT lymphoma, whereas Campylobacter jejuni seems to be associated with small intestinal NHL and Chlamydia psittaci with ocular adnexal lymphoma (Wotherspoon *et al.* 1991, Lecuit *et al.* 2004, Ferreri *et al.* 2004).

## 2.2 Diffuse large B-cell lymphoma

### *2.2.1 Epidemiology and clinical features*

Approximately one third of all new NHLs represent DLBCL, making it the most common lymphoma subtype in western countries. Like NHLs in general, the incidence of DLBCL has been on the rise in the past few decades. It is mostly seen among the elderly, but can occur at any age.

DLBCL can manifest as a nodal or an extranodal disease, and around 40% of DLBCL cases are restricted to extranodal sites at diagnosis (Harris *et al.* 1994). DLBCL can develop anywhere in the body, but some of the most frequently affected extranodal sites include the gastrointestinal tract, bone marrow, liver, spleen and testis. Clinically it usually presents as a rapidly growing tumour mass affecting lymph nodes or an extralymphatic site. Symptoms, if any, depend highly on the tumour site.

### *2.2.2 Histological diagnosis, classification and biology*

The diagnosis of DLBCL is based on tissue morphology and immunohistochemical stainings performed on tumour tissue samples. In histological samples DLBCL is characterized by a diffuse tissue infiltration by large neoplastic B-cells. Normal tissue architecture has usually been partially or totally compromised by the diffuse malignant growth. The neoplastic B-cells in DLBCL have large nuclei at least twice the size of normal quiescent lymphocytes or a size equal to or exceeding that of a normal macrophage. Additionally, these malignant cells display open, blastic chromatin and basophilic cytoplasm.

DLBCL was originally considered a single disease, but has proven to actually comprise several biologically different entities. In the latest WHO classification, T-cell/histiocyte-rich large B-cell lymphoma, primary DLBCL of the CNS,

primary cutaneous DLBCL (leg type) and EBV-positive DLBCL of the elderly are separated as their own distinct DLBCL subtypes (Swerdlow *et al.* 2008). Other DLBCL cases fall into the category of DLBCL, not otherwise specified (NOS). There is, however, still marked biological heterogeneity in this subgroup. Three common morphological variants – centroblastic, immunoblastic and anaplastic – can be separated. Distinct disease entities can also be identified by molecular and immunohistochemical analysis. Alizadeh *et al.* (2000) have identified three distinct molecular subtypes among DLBCL patients using gene expression profiling (GEP): the germinal center B-cell-like (GCB), the activated B-cell-like (ABC) and type 3 DLBCL. These subtypes arise from different stages of B-cell development. GCB subtype cases present with the gene expression profile of germinal center B-cells, whereas the ABC subtype has the profile of activated peripheral B-cells. Cases that cannot be classified as GCB or ABC subtype are included in the category of type 3 DLBCL. Hans *et al.* (2004) have described a corresponding immunohistochemical subtyping into germinal center (GC-) and non-GC DLBCL by determining the expression of certain germinal center (CD10 and Bcl-6) and postgerminal centre (MUM-1 epitope of IRF4) biomarkers in patient samples. Other potentially more accurate algorithms for this cell of origin phenotyping have also been suggested, but at the moment the Hans algorithm is still the most frequently used (Muris *et al.* 2006, Choi *et al.* 2009, Nyman *et al.* 2009b, Meyer *et al.* 2011). None of these immunohistochemical algorithms are as accurate as GEP, and they are able to correctly identify the subtype in around 80% of cases. Another immunohistochemical subgroup that can be separated is CD5-positive DLBCL. CD5 is an antigen typically expressed by malignant cells in CLL, and around 10% of DLBCLs also express CD5. This phenotype is associated with adverse prognosis (Miyazaki *et al.* 2011).

The pathogenesis of DLBCL has long been unclear, but research done in the past decade has started to gradually elucidate the underlying molecular mechanisms. The molecular subtypes determined by GEP are associated with specific genetic alterations, reflecting their different cells of origin. For example, translocations involving the oncogene *BCL-2* are restricted to the GCB subtype, whereas *BCL-6* translocations are more common in the ABC subtype (Huang *et al.* 2002, Iqbal *et al.* 2007). Furthermore, constitutive activation of the antiapoptotic nuclear factor kappa-B (NF-κB) pathway has been discovered to be a hallmark of the ABC subtype (Davis *et al.* 2001). Two prominent upstream pathways seem to sustain this constitutive NF-κB activation, stemming from chronic active B-cell receptor (BCR) signalling and constitutive MYD88

signalling. (Shaffer *et al.* 2012.) In addition to NF-κB activation, BCR and MYD88 signalling also leads to the activation of several other downstream pathways that promote proliferation and survival. In general, DLBCL displays alterations in genes associated with cell cycle regulation, cell survival and apoptosis, and mutations of the prominent *MYC* oncogene, for example, occur in around 5–10% of DLBCL cases (Sanchez-Beato *et al.* 2012, Savage *et al.* 2009).

### *2.2.3 Treatment*

The traditional chemotherapy regimen used to treat DLBCL since the 1970s includes cyclophosphamide, doxorubicin, vincristine and prednisone (the CHOP regimen). At the beginning of the 21st century, the addition of CD20 antibody rituximab to the CHOP regimen (R-CHOP) revolutionized the treatment results of DLBCL. It was found to significantly improve outcome in all age groups and today R-CHOP is considered the standard treatment for DLBCL, curing around 70% of patients (Coiffier *et al.* 2002, Pfreundschuh *et al.* 2006, Pfreundschuh *et al.* 2008).

In patients under 60 years old, adding etoposide to the CHOP regimen (CHOEP) seems to be more effective compared with CHOP, but no survival benefit was found when including rituximab (Pfreundschuh *et al.* 2004a, Pfreundschuh *et al.* 2006). Dose-density is also under investigation in efforts to optimize treatment. Traditionally CHOP/CHOEP is administered every 21 days (CHOP/CHOEP-21), but a more intense treatment schedule with 14-day intervals (CHOP/CHOEP-14) seemed to be superior in the pre-rituximab era (Pfreundschuh *et al.* 2004a, Pfreundschuh *et al.* 2004b). Adding rituximab to CHOP-14 (R-CHOP-14) has been shown to improve outcome in elderly DLBCL patients versus CHOP-14 (Pfreundschuh *et al.* 2008). Recent studies comparing R-CHOP-14 and R-CHOP-21 have, however, shown that in the rituximab era dose-densification does not seem to be beneficial (Cunningham *et al.* 2013, Delarue *et al.* 2013). The number of treatment cycles administered in aggressive DLBCL varies from 6 to 8, although at least with R-CHOP-14, it seems that no added survival benefit is achieved by using more than 6 cycles (Pfreundschuh *et al.* 2008).

The benefit of radiotherapy as a consolidation after immunochemotherapy is for the moment unclear, but some studies have reported a survival benefit after R-CHOP treatment (Phan *et al.* 2010, Marcheselli *et al.* 2011). After individual case selection, radiotherapy is generally administered to patients with a bulky tumour

(over 5 cm in size) and to patients who have residual tumour mass after chemotherapy. A randomized study on the issue is currently being conducted.

CNS relapse is a fatal complication occurring in around 5% of DLBCL cases, but nearly half of these relapses can be prevented by prophylactic therapy with high-dose methotrexate and cytarabine (Zinzani *et al.* 1999, Bos *et al.* 1998, Holte *et al.* 2013). Based on clinical risk assessment, some institutions combine prophylactic therapy with R-CHOP-type immunochemotherapy in high-risk patients. The optimal therapy regimen for CNS prophylaxis is yet to be identified, but it seems that intrathecal methotrexate alone is insufficient, and intravenous administration of high-dose methotrexate is likely also required (Siegal & Goldschmidt 2012).

Most patients can be cured with R-CHOP treatment, but in around one third of DLBCL patients the disease will recur. High-dose chemotherapy with autologous stem cell transplantation (HDT/ASCT) is an intense treatment modality used to treat relapsed aggressive DLBCL. As frontline treatment in aggressive B-cell lymphoma, no survival benefit is achieved with HDT/ASCT compared with R-CHOP-type treatment (Schmitz *et al.* 2012, Ghielmini *et al.* 2013). Before the rituximab era, HDT/ASCT was efficient as a second-line therapy modality for DLBCL, and almost 50% of disease relapses could be cured with HDT/ASCT (Philip *et al.* 1995). Today, however, with frontline immunochemotherapy, the 3-year event-free survival rate after relapse is only 21% (Gisselbrecht *et al.* 2010). This demonstrates the need for biological markers allowing more efficient patient stratification and identification of patients for whom R-CHOP is inadequate.

### *2.2.4 Clinical prognostic factors*

Prognostic factors are highly dependent on the treatment used, and due to the tremendous effect rituximab has had on the prognosis of DLBCL, all prognostic factors pre-dating rituximab need to be re-assessed. At the moment, patient prognostication and treatment choices are based on clinical prognostic factors instead of disease-specific biological characteristics.

Clinical prognostic factors are useful tools for patient prognostication in clinical practice. Their drawback is, however, that they do not necessarily reflect the underlying biology of the patients' particular disease. This is especially noteworthy when dealing with a disease like DLBCL, which presents with considerable biological heterogeneity. Disease-specific biology is an important

factor determining the responsiveness of a malignancy to a specific therapy modality and should be considered in the choice of therapy.

### *The International Prognostic Index*

The only method of prognostication currently commonly used in clinical practice is the International Prognostic Index (IPI) stratification (IPI project 1993). This is a scoring based on the following clinical factors: age over 60 years, advanced disease stage (stage III-IV), poor WHO performance status (>1), elevated serum lactate dehydrogenase (LDH) level and the existence of more than one extranodal lesion at diagnosis (Table 1). The patients get an IPI score of 0–5 based on the number of adverse prognostic factors they present with. The patients are stratified into four risk groups as follows: low (0–1 points), low/intermediate (2 points), high/intermediate (3 points) and high (4–5 points). Before the rituximab era this stratification separated prognostic groups with a 5-year overall survival (OS) varying from 73% in the low risk group to 26% in the high-risk group (IPI project 1993). The incorporation of rituximab into treatment has improved outcome in all IPI classes, and in R-CHOP treated patients the 3-year OS rates in the low- versus high-risk groups vary from 91% to 59% (Ziepert *et al.* 2010) (Table 2). As even patients in the poor prognostic group have a 3-year OS of over 50%, deviation from standard R-CHOP treatment outside of clinical trials is not justified, and better prognostic factors are needed.

**Table 1. Factors producing one point each in the International Prognostic Index (IPI) classification.**

| Parameter | Poor prognostic factor |
|---|---|
| Age | >60 years |
| Stage | III-IV |
| WHO performance status | >1 |
| Serum lactate dehydrogenase | Elevated level |
| Extranodal lesions at diagnosis | >1 |

**Table 2. Prognostic subgroups according to the IPI classification in CHOP vs. R-CHOP treated patients (IPI Project 1993, Ziepert *et al.* 2010).**

| IPI risk group | IPI points | 5-year overall survival (CHOP) | 3-year overall survival (R-CHOP) |
|---|---|---|---|
| Low | 0-1 | 73% | 91% |
| Low/intermediate | 2 | 51% | 81% |
| High/intermediate | 3 | 43% | 65% |
| High | 4-5 | 26% | 59% |

### *2.2.5 Biological prognostic factors*

The search for efficient biological prognostic factors for DLBCL has been active for years, but so far none have been validated well enough to use in clinical practice. Table 3 summarizes the most significant adverse biological factors in the rituximab era.

#### *The cell of origin phenotypes*

Initially, the GEP-based cell of origin phenotypes described by Alizadeh *et al.* (2000) seemed to be associated with differential prognosis, and patients in the ABC subgroup suffered from a significantly poorer outcome than patients in the GCB subgroup. This survival impact was also reported between the immunohistochemically defined GC and non-GC phenotypes in patients treated without rituximab (Hans *et al.* 2004, Muris *et al.* 2006, Choi *et al.* 2009). In the era of modern immunochemotherapy, conflicting results have been reported on the prognostic value of the immunohistochemical cell of origin phenotyping (Nyman *et al.* 2007, Seki *et al.* 2009, Fu *et al.* 2008, Meyer *et al.* 2011). However, a study using GEP for subtyping has still reported a markedly lower survival rate in the ABC subtype, indicating that the conflicting study results possibly stem from the inaccuracy of immunohistochemical algorithms (Lenz *et al.* 2008a). The biological differences between these molecular subtypes have been recognised, but due to high costs of GEP and the question of reliability associated with immunohistochemical algorithms, this subtyping is so far not used for prognostication in clinical practice.

### *MYC mutations*

*MYC* is an oncogene encoding a transcription factor with many properties relating to cell survival and proliferation. Constitutive Myc protein expression significantly promotes lymphomagenesis (Adams *et al.* 1985). Mutations of the *MYC* gene leading to overexpression are found in approximately 5–10% of DLBCL cases, and they are strong predictors of poor survival also in the rituximab era (Savage *et al.* 2009, Rimsza *et al.* 2008, Barrans *et al.* 2010, Akyurek *et al.* 2012). Savage *et al.* (2009) have reported a 5-year OS rate of only 33% in DLBCL patients with *MYC* rearrangements. Immunohistochemical overexpression of Myc protein is more commonly seen without an underlying mutation, resulting from changes in other regulating mechanisms.

### *BCL-2 mutations*

Bcl-2 is an antiapoptotic factor that is frequently overexpressed in DLBCL due to genetic rearrangements or other regulating mechanism (Merino *et al.* 1994, Mounier *et al.* 2003, Horn *et al.* 2013). t(14;18)(q32;q21) is the most commonly seen translocation leading to Bcl-2 overexpression. It appears in 17–28% of DLBCL cases and is more common in the GC subtype (Weiss *et al.* 1987, Hill *et al.* 1996, Huang *et al.* 2002). In R-CHOP treated patients, the data concerning the prognostic value of Bcl-2 overexpression is contradicting. Some studies have found negative prognostic value mostly in the ABC subtype, whereas other studies report no prognostic effect (Iqbal *et al.* 2006, Nyman *et al.* 2009a, Mounier *et al.* 2003, Wilson *et al.* 2007). Then again, Visco *et al.* (2012) found *BCL-2* rearrangements to predict poor survival in GCB-DLBCL patients but not in the activated B-cell subtype.

### *BCL-6 mutations*

*BCL-6* is a transcriptional repressor implicated in lymphomagenesis, with a central role in B-cell differentiation (Shaffer *et al.* 2000). Rearrangements of the *BCL-6* gene are seen in approximately 30% of DLBCL cases (Akyurek *et al.* 2012, Horn *et al.* 2013). Conflicting results have been reported on the prognostic significance of *BCL-6* rearrangements as well as Bcl-6 protein expression in the rituximab era (Akyurek *et al.* 2012, Horn *et al.* 2013, Copie-Bergman *et al.* 2009, Shustik *et al.* 2010, Winter *et al.* 2006). Akyurek *et al.* (2012) have demonstrated

negative prognostic impact for *BCL-6* rearrangements in univariate analysis, and a similar non-significant trend has also been shown by Shustik *et al*. (2010). In both studies, however, the prognostic effect was lost in multivariate analysis. Then again, Horn *et al.* (2013) have reported poor prognostic value for low Bcl-6 protein expression, whereas *BCL-6* rearrangements had no prognostic effect in their study. According to a prospective study conducted by Winter *et al.* (2006), Bcl-6 protein expression has lost its prognostic value in the rituximab era.

### *Double-hit lymphomas*

Cases with the simultaneous mutation of *MYC* and *BCL-2*, or very rarely *BCL-6*, have been named as "double-hit" lymphomas and are associated with a significantly poorer outcome than average (Aukema *et al.* 2011). This double-mutation occurs in only around 4% of DLBCL cases, but these patients have a very poor median survival rate of 9–17 months in R-CHOP treated patients (Akyurek *et al.* 2012, Green *et al.* 2012, Johnson *et al.* 2009, Johnson *et al.* 2012). Immunohistochemical overexpression of Myc and Bcl-2 proteins with or without an underlying mutation is seen in around 20–30% of DLBCL cases. In the unmutated cases other regulating mechanisms cause the overexpression of Myc and Bcl-2 proteins and these patients also have decreased survival rates, with a 3-year OS of approximately 40% (Green *et al.* 2012, Johnson *et al.* 2012, Hu *et al.* 2013). This double-hit category could be considered a poor prognostic subgroup in need of more efficient treatment than R-CHOP.

### *TP53 mutations*

*TP53* is an essential tumour-suppressing gene with many functions such as mediation of cell cycle arrest and apoptosis (Vousden & Prives 2009). It is mutated in over 50% of all human cancers and 12–23% of DLBCL (Hollstein *et al.* 1994, Zainuddin *et al.* 2009, Young *et al.* 2007, Leroy *et al.* 2002). Several studies have reported negative prognostic impact of *TP53* mutations in the pre-rituximab era, and mutations in the DNA-binding domains (DBD) of *TP53* have seemed especially impairing (Ichikawa *et al.* 1997, Leroy *et al.* 2002, Young *et al.* 2007, Zainuddin *et al.* 2009). The prognostic value of *TP53* mutations – especially in the DBD – seems to persist also in patients treated with R-CHOP (Xu-Monette *et al.* 2012). In a study by Xu-Monette *et al.* (2012), the 5-year OS

of DLBCL patients with wildtype *TP53* has been 65.9% versus 47.8% in patients with a mutated *TP53* gene (p=0.0005).

**Table 3. Some of the most significant biological adverse prognostic factors in DLBCL in the rituximab era.**

| Prognostic factor | Method | Study |
|---|---|---|
| Double-hit lymphoma | FISH[1] | Green *et al.* J Clin Oncol 2012 |
| | | Johnson *et al.* J Clin Oncol 2012 |
| Myc and Bcl-2 overexpression | IHC[2] | Green *et al.* J Clin Oncol 2012 |
| | | Johnson *et al.* J Clin Oncol 2012 |
| | | Hu *et al.* Blood 2013 |
| *MYC* rearrangements | FISH | Savage *et al.* Blood 2009 |
| *MYC* overexpression | GEP | Rimsza *et al.* Blood 2008 |
| ABC[3] subtype | GEP[4] | Lenz *et al.* N Engl J Med 2008 |
| *TP53* mutations in the DBD[5] | Resequencing microarray | Xu-Monette *et al.* Blood 2012 |
| *TP53* deletions | PCR[6] | Jardin *et al.* Blood 2010 |
| *CDKN2A/p16* deletions | PCR | Jardin *et al.* Blood 2010 |

[1]FISH = fluorescence in situ hybridization, [2]IHC = immunohistochemistry, [3]ABC = activated B-cell-like, [4]GEP = gene expression profiling, [5]DBD = DNA-binding domain, [6]PCR = polymerase chain reaction

### *2.2.6 CNS lymphoma*

DLBCL expresses tropism for the CNS, but the pathophysiology behind this phenomenon is unknown. CNS-DLBCL, presenting as either PCNSL or secondary CNS involvement of extracranial DLBCL, is a persisting therapeutic challenge.

#### *Primary CNS lymphoma*

PCNSL is a rare disease that comprises around 1–2% of all non-Hodgkin lymphomas, and 95% of PCNSLs represent DLBCL (Hochberg *et al.* 2007). The incidence of PCNSL, like that of DLBCL, is rising, but the reason for this rise is unknown. Immunodeficient people, e.g. patients with HIV or AIDS, used to have increased risk of PCNSL associated with EBV infection, but modern HIV-treatment seems to have diminished this risk (Sparano *et al.* 1999). Increasing incidence rates have also been reported among immunocompetent populations, especially in the elderly (Villano *et al.* 2011).

The pathophysiology of PCNSL is poorly understood. This is a disease that involves the brain and meninges, and intraocular involvement is also seen at times. PCNSL cells have been found to exhibit strong CNS tropism leading to homing of malignant B-cells into the CNS, possibly implicating an extracranial origin for this disease (Jiang *et al.* 2010). The biology behind this distinct migratory pattern is not known. GEP studies have shown that – compared to systemic DLBCL and normal lymph node tissue – PCNSL has differential expression of pathways relating to extracellular matrix and cell adhesion properties (Tun *et al.* 2008, Sung *et al.* 2011). This would suggest that the interaction between CNS microenvironment and lymphoma cells probably has an integral role in the pathogenesis of PCNSL. Most PCNSL cases represent the non-GC phenotype of DLBCL, although they also show characteristics of the GC phenotype (Raoux *et al.* 2010, Montesinos-Ronger *et al.* 2008). PCNSL typically presents with an angiocentric morphology, with perivascular reactive T-cells surrounded by malignant B-cells. Disease progression leads to a more diffuse infiltration of the brain parenchyma by malignant B-cells.

The traditional treatment for PCNSL has been chemotherapy combined with radiotherapy, but the median OS with this treatment is only 2.5 years (Raoux *et al.* 2010). The blood brain barrier (BBB) constitutes a major problem in the treatment of CNS lymphomas. It prevents the penetration of most substances into the CNS, or only small amounts pass through but therapeutic levels are not reached. The BBB disruption (BBBD) treatment is a new, experimental treatment where the BBB is momentarily disrupted by concentrated mannitol infusion, allowing drugs into the CNS. This treatment seems to be more effective compared with traditional therapy and is even considered potentially curative in a previously incurable disease group (Angelov *et al.* 2009).

### *Secondary CNS lymphoma*

CNS recurrence develops in around 5% of DLBCL cases (Zinzani *et al.* 1999, Bos *et al.* 1998, van Besien *et al.* 1998). Despite the excellent control rates seen in systemic DLBCL with the emergence of R-CHOP therapy, the incidence of CNS relapse has decreased only marginally, if at all (Tai *et al.* 2011, Yamamoto *et al.* 2010). However, the manifestation of CNS relapses has changed and instead of massive systemic disease together with CNS involvement, solitary CNS recurrences present a new challenge. As with PCNSL, little is known about the pathophysiology of sCNSL. DLBCL cases in the double-hit category are known

to have a high risk of CNS relapse, which occurs in 9–50% of these patients (Kridel & Dietrich 2011). Also, sCNSL patients with the non-GC phenotype respond poorly to treatment and have shorter survival rates compared with the GC phenotype (Patil *et al.* 2009).

The treatment results of sCNSL are very poor, and the median OS in these patients is less than 6 months (Jahnke *et al.* 2006). Most sCNSL cases occur within a year from DLBCL diagnosis, which suggests early CNS dissemination of malignant cells (Siegal & Goldschmidt 2012). The BBB provides lymphoma cells protection against chemotherapy, leading to early disease relapse. Considering the poor treatment results of sCNSL, the efforts are for the moment focused on the prevention of disease dissemination into the CNS. Prophylactic treatment with intravenous high-dose methotrexate and cytarabine seems to be effective (Tilly *et al.* 2003, Holte *et al.* 2013). A study by the Nordic Lymphoma Group, using a 3-year follow-up time, showed that with the administration of high-dose methotrexate and high-dose Ara-C (cytarabine) after 6 cycles of R-CHOEP-14, around 50% of the anticipated CNS relapses could be prevented (Holte *et al.* 2013).

Due to high toxicity, the prophylactic treatment is only justified in patients with high risk of CNS relapse. At the moment, risk assessment is based on certain clinical features, including age over 60 years, elevated serum LDH levels, advanced stage, high IPI score and extranodal involvement, especially of tissues such as testis or breast, as well as paranasal or paraspinal tumour location (Siegal & Goldschmidt 2012). For now, these clinical features provide the best-known method for risk stratification. Testicular involvement is a strong independent predictor of CNS relapse in DLBCL, and up to 34% of these patients will develop a CNS recurrence within 10 years of diagnosis (Zucca *et al.* 2003). Apart from patients with testicular involvement, only about 20% of the high-risk patients identified by these clinical features would actually develop a CNS relapse and benefit from the prophylactic treatment, underlining the need for more efficient predictive biological factors (Boehme *et al.* 2007). The role of flow cytometry (FCM) in the prediction of CNS relapse is under active investigation at the moment. Compared to conventional cytology, FCM is a more sensitive method for screening cerebrospinal fluid for neoplastic cells, and it seems that FCM+ patients have an increased risk of developing CNS recurrence even if cytology remains negative, making these patients candidates for prophylactic treatment (Quijano *et al.* 2009, Benevolo *et al.* 2012). The definitive role of FCM in clinical practice is, however, yet to be established.

## 2.3 Oxidative stress

Reactive oxygen species (ROS) are small highly reactive oxygen containing particles constitutively produced by cells as a result of aerobic respiration. ROS have both endogenous (mitochondria, cytochrome P450 metabolism, inflammatory cells etc.) and exogenous (e.g. radiation and smoking) sources (Nathan & Cunningham-Bussel 2013, Valavanidis *et al.* 2009). If not eliminated by antioxidant enzymes, ROS rapidly oxidize cellular structures causing damage to the DNA, lipids, proteins and practically any adjacent structures. ROS have important physiological functions, e.g. acting as transcription factors and participating in the immune defence. When ROS production exceeds its elimination, however, a state of oxidative stress occurs. Oxidative stress leads to accumulating damage in cellular macromolecules, and is associated with the pathogenesis of several different diseases such as asthma, atherosclerosis and cancer (Karihtala & Soini 2007).

### *2.3.1 Oxidative stress and carcinogenesis*

Oxidative stress and ROS promote carcinogenesis in multiple ways (Figure 1). Tumours typically exhibit chronic inflammation and hypoxia deriving from accelerated growth and insufficient circulation. This leads to increased ROS production and creates a selection pressure towards resistance to oxidative stress and apoptosis, as well as accelerating angiogenesis and proliferation (Hussain *et al.* 2003). Furthermore, in addition to causing direct DNA damage and genetic instability, ROS also activate transcription factors and proto-oncogenes such as *C-FOS*, *C-JUN* and *C-MYC* (Jaruga *et al.* 1994, Toyokuni *et al.* 1995). By enhancing protease enzyme activity oxidative stress promotes invasion and metastasising properties (Toyokuni *et al.* 1995). Persistent oxidative stress in tumour tissue also leads to the activation of counteracting systems, most importantly increased antioxidant enzyme production (Landriscina *et* al. 2009). The cytotoxic effects of doxorubicin, etoposide, cytarabine and glucocorticoids, drugs that are traditionally used to treat lymphomas, are mediated through ROS and oxidative stress (Kamio *et al.* 2003, Tome *et al.* 2011). Therefore, tumour tissue with upregulated antioxidative processes is likely to be less sensitive towards these anticancer drugs.

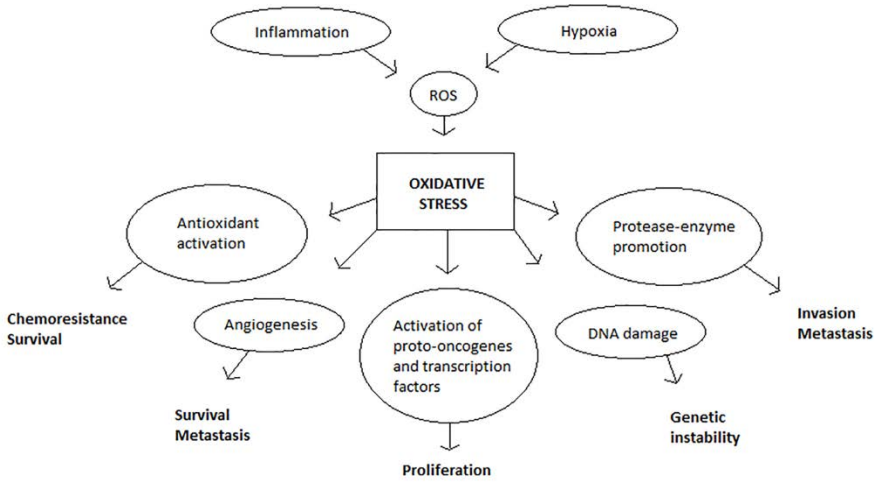


**Fig. 1. Oxidative stress deriving from excessive ROS production promotes carcinogenesis in multiple ways.**

### *2.3.2 Markers and evaluation*

Evaluating the redox state of tumour tissue is problematic and many sources of error exist. Complicating factors include, for example, the very short lifespan of ROS and the presence of other redox state regulating molecules, physiological sources of ROS in tissues, artificial growth environment in cell cultures; in addition, the process of taking a biopsy can itself result in oxidative contamination of the tissue sample.

Immunohistochemical staining for specific end products of cellular oxidation and antioxidant enzymes is one of the most commonly used methods for redox state evaluation, enabling the localization of oxidative and antioxidative activity. The most frequently used markers of oxidative stress include 8-hydroxydeoxyguanosine (8-OHdG) and nitrotyrosine (Valavanidis *et al.* 2009, Karihtala & Soini 2007). 8-OHdG is the stable end product of oxidative damage to the DNA, whereas nitrotyrosine reflects damage caused by nitric oxide ($NO^{\cdot}$) and its derivatives such as the potent free radical peroxynitrite.

Antioxidant enzymes act as a first-line defence against free radicals and are also important reflectors of the redox state. Manganese superoxide dismutase

(MnSOD) is one of the most important antioxidant enzymes in mammals (Holley *et al.* 2012). It resides in the mitochondria where ROS are mostly produced as a side product of the mitochondrial ATP synthesis. Another major family of antioxidants are the nuclear thioredoxin (Trx) enzymes. In addition to regulating cell redox state, Trx serves as a growth factor, inhibits apoptosis and activates transcription factors such as NF-κB (Powis & Montfort 2001, Hirota *et al.* 1999). Glutathione (GSH) is an antioxidant enzyme with many physiological functions, and, in addition to redox signalling, it participates in basic cellular phenomena such as protein synthesis, DNA synthesis and repair, and proliferation (Ballatori *et al.* 2009). The production of GSH is controlled by another antioxidant enzyme, gamma cysteine ligase or gamma-glutamylcysteine synthetase (GCL/γ-GCS), which is another molecule commonly used to measure redox activity in tumour tissues.

### *2.3.3 Oxidative stress and cancer prognosis*

Markers of oxidative stress, as well as redox state regulating enzymes, have been shown to have prognostic effect in many solid malignancies. High expression levels of 8-OHdG have been connected with poor prognosis in at least colorectal carcinoma, ovarian cancer and malignant melanoma (Sheridan *et al.* 2009, Karihtala *et al.* 2009, Murtas *et al.* 2010). In breast cancer patients, it seems that low levels of 8-OHdG predict poor survival, whereas high levels of nitrotyrosine are associated with a large primary tumour (Sova *et al.* 2010, Karihtala *et al.* 2004). Poor prognostic effect for nitrotyrosine has also been reported in metastatic melanoma and oesophageal carcinoma (Ekmekcioglu *et al.* 2000, Kato *et al.* 2001). Increased expression of antioxidants Trx and MnSOD has been associated with poor prognosis and metastatic disease in gastrointestinal malignancies (Kinnula *et al.* 2004, Raffel *et al.* 2003). Furthermore, resistance to a variety of chemotherapeutic agents has been reported in connection with Trx and GCL expression (Yokomizo *et al.* 1995, Iwao-Koizumi *et al.* 2005, Bailey *et al.* 1992, Soini *et al.* 2001).

### *2.3.4 Oxidative stress and lymphoma*

Increased ROS production and oxidative stress is known to cause apoptosis in lymphocytes via the mitochondrial pathway (Wilkinson *et al.* 2012). ROS cause down-regulation of Bcl-2 related antiapoptotic proteins, enabling mitochondrial

outer membrane permeabilization and the release of cytochrome c and other apoptotic factors into the cytosol, initiating the degradation of cellular components. Alterations in this mitochondrial apoptosis pathway, mainly the increased expression of mitochondrial antioxidant enzymes, have been shown to lead to resistance to oxidative stress. Tome *et al.* (2012) have demonstrated in a lymphoma tissue culture under oxidative stress that overexpression of the antioxidant enzyme catalase in mitochondria alters the activity of several redox related pathways and increases chemoresistance to doxorubicin and dexamethasone, as well as to drugs that do not have directly ROS-mediated effects, such as vincristine and cyclophosphamide.

GSH is a major component in the intracellular ROS defence. The synthesis of GSH is controlled by the antioxidant enzyme GCL, whereas its ROS-eliminating function is mediated by another member of the GSH-family, the downstream effector glutathione peroxidase 1 (GPX1). GPX1 eliminates hydrogen peroxide by reducing it and subsequently oxidizing GSH. In a study by Andreadis *et al.* (2007) the expression of GPX1 was associated with early treatment failure and poor disease-specific survival in DLBCL patients. The adverse prognostic effect was more pronounced in the ABC-subtype. Preceding Andreadis *et al.* (2007), only one other study had been published examining the role of redox-state regulating enzymes in lymphomas. Tome *et al.* (2005) examined the expression of multiple antioxidant enzymes, including MnSOD, GPXs and Trx, in DLBCL tissue, and developed a redox signature score based on the expression of these molecules. In the score, they divided the studied genes into two groups, with group A including enzymes from e.g. the SOD and GSH families, whereas the Trx enzymes were included in group B. They then subtracted the sum of mRNA expression values in group B from the sum value of group A. The study concluded that the decreased expression of antioxidant enzymes together with increased function of the Trx system is associated with poor overall survival in DLBCL. Andreadis *et al.* (2007) also tested the redox signature score in their material, but found no significance. On the contrary, they concluded that the GPX1 component of the score alone appeared to carry some significance, but the association was with increased expression and poor survival. These two studies with contradicting results represent the only data available on the prognostic effect of redox state reflecting molecules in lymphomas.

## 2.4 Cell cycle regulators

### *2.4.1 Cell cycle*

The process of cell proliferation, *i.e.,* the cell cycle, consists of four phases that are under strict regulation. DNA is replicated in the synthesis phase (S phase), and in the mitosis phase (M phase) the cell divides into two identical daughter cells. S and M phases are separated by two necessary gap phases, G1 and G2, during which the cell prepares for mitosis. Cells can also exit the cell cycle and enter a quiescent stage named G0.

Cyclins are molecules that drive the cell cycle by forming active holoenzymes with their catalytic partners, cyclin-dependent kinases (CDKs). CDK production in cells is constitutive, whereas cyclins accumulate periodically and control cell cycle transitions. G1/S transition is a critical restriction point in cell division (Figure 2). (Lundberg & Weinberg 1999, Sherr 2000.) The G1/S transition is promoted by D-type cyclins (cyclin D1, D2 and D3) interacting with CDKs 4 and 6, as well as cyclin E with catalytic partner CDK2. After peaking at the G1/S transition, cyclin E is degraded and replaced by cyclin A. Cyclin A drives the S and G2 phases supporting DNA synthesis and preparation for mitosis. Cyclins B1 and B2 are produced in the M phase and control the completion of mitosis. While cyclins and CDKs promote the cell cycle, CDK-inhibitors (CDKIs) function as cell cycle arresting agents. (Sherr 2000.) Two classes of CDKIs have been identified: the INK4 and Cip/Kip families. The INK4 CDKIs include proteins $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$, and inhibit the cyclin D-dependent kinases CDK4 and 6. CDKIs in the CIP/KIP family inhibit CDK2 and include proteins $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$.

D-type cyclins are unstable molecules and their synthesis and activity depend on constant mitogenic stimuli. These outside growth signals are, therefore, necessary for cell cycle progression through the G1 phase and transition into the S phase. The function of cyclin E-CDK2 complexes – enforced by cyclin D dependent kinases – is mitogen-independent and after progression through the G1/S restriction point cell cycle completion becomes independent of outside mitogenic stimuli. (Lundberg & Weinberg 1999.) The G1/S restriction point is regulated by retinoblastoma (Rb), p53 and p27 tumour suppressor pathways (Figure 2). (Sherr 2000.)

### *G1/S regulating pathways*

Retinoblastoma protein (pRb) is a powerful antiproliferative agent that prevents cell division by repressing the transcription of genes necessary for DNA synthesis. (Cobrinik 2005.) The CDKI $p16^{INK4a}$ stabilizes pRb, whereas the activation of cyclin D-dependent kinases (CDK 4 and 6) leads to phosphorylation of pRb and enables cell cycle progression. E2F transcription factors are growth-promoting molecules, inhibited by pRb, that also induce the synthesis of cyclin E. The accumulation of cyclin D and subsequent inactivation of pRb, therefore, promotes cyclin E production which further reinforces the G1/S transition and makes cell cycle progression mitogen-independent. Together the proteins p16, cyclin D – CDK4 and 6 and pRb form the Rb pathway.

p53 protein is one of the most prominent tumour suppressors in mammals, with a wide variety of functions relating to different cellular processes. Importantly, as a response to cellular stress signals, such as DNA damage, hypoxia or oncogene activation, it can either cause cell cycle arrest or induce apoptosis. (Bálint & Vousden 2001.) It also induces the production of the CDKI $p21^{Cip1}$, which in turn inhibits cell cycle progression. MDM2 is a negative p53 regulator that functions both by blocking the transcription of the *TP53* gene and by promoting p53 degradation. $p14^{ARF}$ – encoded by the same gene as $p16^{INK4a}$ but with differential sequencing – stabilizes p53 by inhibiting the function of MDM2. The proteins p14, MDM2, p53 and p21 make up the p53 pathway.

Another important tumour suppressor is the CDKI $p27^{Kip1}$, which is abundantly expressed in resting cells and causes rapid cell cycle arrest when induced. It is inhibited by cyclin E-CDK2 complexes, and together they form the p27 pathway. (Lee & Kim 2009.) Interestingly, p27 is also required for the formation of active cyclin D-CDK 4/6 complexes. When bound by these enzyme complexes, p27 is in an inactive form and unable to prevent cell cycle progression. On the contrary, it enables the function of cyclin D-dependent kinases, subsequently promoting the function of cyclin E-CDK2 complexes, enforcing p27 degradation and further promoting the G1/S transition.

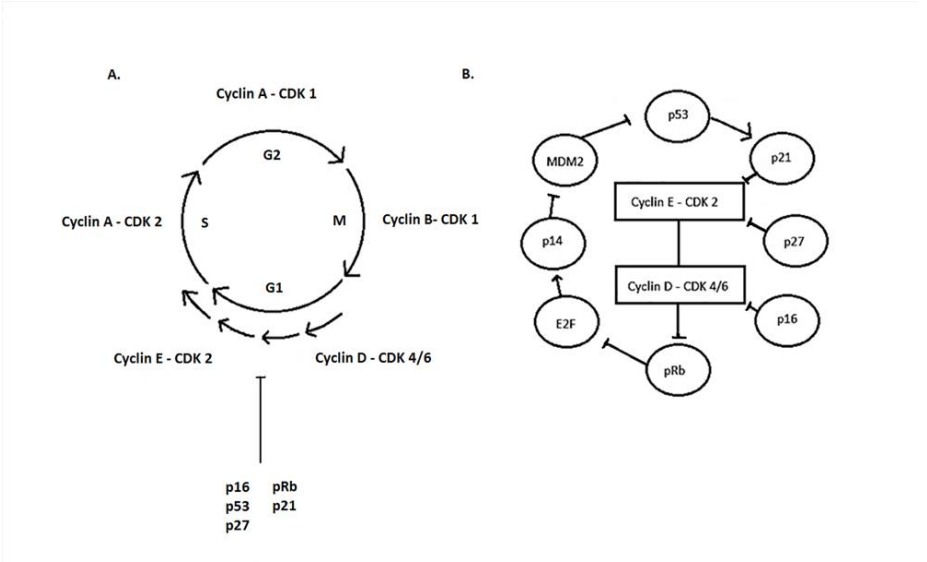


**Fig. 2. Diagrams depicting the different phases of the cell cycle together with the regulators of the G1/S transition. (A) The G1/S transition is promoted by cyclin D-CDK 4/6 and cyclin E-CDK2 complexes, whereas proteins p16, pRb, p53, p21 and p27 inhibit cell cycle progression. (B) p16, p21 and p27 inhibit the function of cyclin-CDK complexes, whereas pRb inhibits E2F-transcription factors necessary for DNA synthesis in the S phase. p53 can cause cell cycle arrest or induce apoptosis in several ways, e.g. through the induction of p21. MDM2 is a p53 inhibitor, whereas p14 is a p53 stabilizing factor.**

### *2.4.2 Cell cycle regulation and carcinogenesis*

The imbalance between cell proliferation and death is a key phenomenon in the development of malignancies, and regulation of the G1/S transition is frequently compromised in human cancers. Alterations in the G1/S regulating pathways have been found to hold both aetiological and prognostic value in several malignancies. Alterations in the Rb pathway occur frequently in human malignancies, and negative prognostic impact has been shown in e.g. laryngeal carcinoma, gastrointestinal and haematological malignancies, as well as in ovarian cancer (Scambia *et al.* 2006). In non-small cell lung cancer (NSCLC) the immunohistochemical phenotype Rb-/p53+ was associated with adverse prognosis (Xu *et al.* 1996). *TP53* is mutated in most human cancers and the

presence of these mutations seems to predict poor survival in at least breast cancer, head and neck squamous cell carcinoma and haematological malignancies (Robles & Harris 2010). Expression and prognostic significance of p27 expression has also been extensively studied in different cancer types. Most studies report adverse prognostic effect for low p27 expression in at least NSCLC, head and neck cancer, colorectal carcinoma, prostate cancer, breast cancer and ovarian cancer (Chu *et al.* 2008).

### *2.4.3 Cell cycle regulation and lymphoma*

Cell cycle deregulation has an integral role in the development and prognosis of lymphomas as well. Inactivation of the *CDKN2A* gene by homozygous deletion, point mutations or promoter hypermethylation leads to loss of p16 protein expression, and is mostly found in aggressive and transformed lymphoma, but rarely in low-grade diseases (Pinyol *et al.* 1998). *TP53* mutations are found in approximately 12.5% of lymphoid malignancies, and in addition to DLBCL they correlate with aggressive disease and poor survival also in other lymphomas, such as CLL, FL, MCL and adult T-cell leukaemia/lymphoma (Newcomb *et al.* 1995, Cheung *et al.* 2009). Overexpression of cyclin D1 is a defining characteristic in MCL, deriving from the translocation t(11;14) which is found in essentially all MCL cases (Bosch *et al.* 1994).

#### *DLBCL*

Concomitant alterations in any two of the Rb, p53 and p27 regulatory pathways have been reported to occur in approximately 50% of DLBCL cases, whereas the simultaneous alteration of all three pathways seems to occur in around one third of patients (Bai *et al.* 2007). The simultaneous defect of multiple regulatory pathways was found to hold negative prognostic value in the pre-rituximab era. The immunohistochemical phenotype p16-p14-p53+ was associated with adverse outcome in GC-DLBCL patients (Paik *et al.* 2005). In another study, patients with a combination of mutated *TP53*, inactive *CDKN2A* and overexpression of p27 presented with extremely poor overall survival (Sánchez-Beato *et al.* 2001). Members from single tumour suppressor pathways were also found prognostic. The immunohistochemical phenotype p53+p21- seems to correlate to some extent with a mutated *TP53* and was associated with poor survival in GC-DLBCL (Visco *et al.* 2006). The overexpression of p27 – possibly reflecting the accumulation of

an inactive protein – has also been associated with poor survival in DLBCL patients in the pre-rituximab era (Sáez *et al.* 1999).

The parameters predicting poor survival in R-CHOP treated patients include deletions of *CDKN2A* and *TP53*, *TP53* mutations located in the DBD, as well as the immunohistochemically assessed p21 positivity and low p27 expression (Jardin *et al.* 2010, Xu-Monette *et al.* 2012, Winter *et al.* 2010, Seki *et al.* 2010). The *CDKN2A* gene encodes both $p16^{INK4a}$ and $p14^{ARF}$, connecting the Rb and p53 pathways and explaining the relatively high frequency of concomitant alterations in these pathways. Jardin *et al.* (2010) showed that the deletions of *CDKN2A* and *TP53* genes were independent predictors of unfavourable outcome also in the rituximab era, and the simultaneous deletion of both these genes led to early disease-related death in all affected patients in this study. As also shown by others, *CDKN2A* deletions were found to occur predominantly in the ABC phenotype of DLBCL (Jardin *et al.* 2010, Lenz *et al.* 2008b).

## 2.5 Chemokines and their receptors

### *2.5.1 Physiological roles*

Chemokines are small chemotactic cytokines that have many physiological functions. (Zlotnik *et al.* 2011.) There are two types of chemokines, homeostatic and inflammatory. Homeostatic chemokines are constitutively produced by tissues and they participate in functions such as organogenesis, angiogenesis and cell proliferation. They also guide the physiological migration of lymphocytes and stem cells. The production of inflammatory chemokines is induced as a response to inflammatory stress signals, and they are responsible for the recruitment of lymphocytes to the site of inflammation.

Currently, 48 chemokines and 19 chemokine receptors have been identified. (Zlotnik *et al.* 2006, Zlotnik *et al.* 2011.) Chemokines and their receptors can be divided into four families: alpha- (CXC-), beta- (CC-), gamma- (C-) and delta- (CX3C-) chemokines/receptors. Chemokines induce a response only in cells expressing their respective receptors and it is, therefore, the chemokine receptor profile of cells that determines their migratory patterns. Chemokine receptors belong to the group of 7 transmembrane domain G-protein coupled receptors (GPCRs). Most chemokine receptors can bind more than one ligand and most ligands have more than one receptor. Ligand binding causes a conformational

change in the receptor, triggering an intracellular signalling cascade leading to a cellular response, such as chemotaxis. After binding, the ligand-receptor complex is internalized – preventing prolonged activity – and either transported into lysosomes for degradation or recycled back to the cell membrane (Marchese *et al.* 2008). The CXC chemokine receptor 4 (CXCR4), for example, has been found to mostly undergo degradation after ligand binding, and only a small proportion of receptors is recycled back to the cell membrane (Tarasova *et al.* 1998). The CC chemokine receptor 7 (CCR7) has two ligands, CCL19 and CCL21, and where exposure to CCL19 causes rapid receptor internalization, the same does not occur as response to CCL21 exposure (López-Giral *et al.* 2004). This might be due to the need for cells to remain CCL19 responsive to enable migration to T-cell zones in lymph nodes.

The most essential molecules guiding lymphocyte homing are CXCR4 and its ligand CXCL12, CXCR5 and ligand CXCL13 and CCR7 with its two ligands CCL19 and CCL21 (Zlotnik *et al.* 2011). These are homeostatic chemokines and receptors that have many important physiological roles. The CXCR4/CXCL12 axis is essential for normal development of the CNS, as well as haematopoiesis and development of cardiovascular organs. Experiments done in genetically altered mice show that the deficiency of either of these molecules leads to fatal anomalies in vascular development, haematopoiesis and cardiogenesis, leading to early death in utero (Tachibana *et al.* 1998). CXCL12 is produced by organs such as the lungs, liver, kidney, skeletal muscle, bone marrow and the brain (Figure 3) (Kucia *et al.* 2005). Small amounts are also produced by lymph nodes. During haematopoiesis, the CXCR4/CXCL12 interaction keeps early B-cells in their niche in the bone marrow. As B-cells mature, they start expressing CXCR5 and CCR7 that enable the dislodgement of mature B-cells from the bone marrow and into the circulation (Honczarenko *et al.* 2006). The chemokine ligands for CXCR5 and CCR7 are mostly produced by secondary lymphoid organs, causing the mature circulating B-cells to migrate into lymph nodes. CXCR5 is essential for the recruitment and entering of B-cells into lymph nodes, whereas CCR7 has a similar role with T-cells. Synchronized interactions between these chemokine receptors facilitate the normal development and organization of secondary lymphoid tissues during organogenesis (Ohl *et al.* 2003).

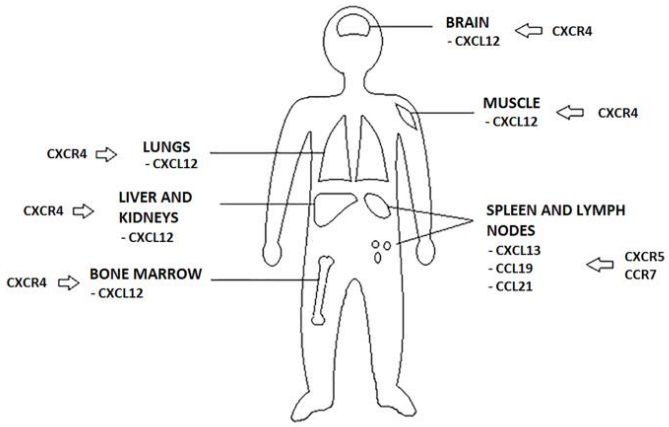


**Fig. 3. A mannequin presenting sources of lymphocyte-attracting chemokines in the human body. CXCL12 is widely produced in different extranodal tissues, whereas the production of CXCL13, CCL19 and CCL21 is mostly focused in secondary lymphoid tissues.**

### *2.5.2 Roles in carcinogenesis*

Chemokines and their receptors have been shown to promote carcinogenesis in several ways. (Lazennec & Richmond 2010.) Chemokines can function as growth factors promoting cell survival and proliferation, as well as increasing angiogenesis. By promoting inflammatory conditions they provide favourable circumstances for transformation of cells. They also increase invasion by affecting cellular adhesion properties and components of the matrix as well as induce cell migration, contributing to increased metastasizing capacity of malignancies.

#### *Solid malignancies*

The role of the CXCR4/CXCL12 axis is the most established in solid malignancies. Tumours often develop hypoxic conditions due to rapid growth and insufficient circulation. Hypoxia induces the production of CXCR4 in cells, promoting angiogenesis and cell motility (Oh *et al*. 2012). This leads to increased vasculature and prevention of hypoxic cell death within the tumour, as well as

increased motility and metastasizing capacity of malignant cells. Nuclear expression of CXCR4 has been connected with poor prognosis and lymph node metastases in hepatocellular carcinoma, colorectal cancer and non-small cell lung cancer (Xiang *et al.* 2009, Wang *et al.* 2010, Na *et al.* 2008). Overexpression of CXCR4 seems to contribute to the development of brain-metastasis in NSCLC and colorectal cancer, and possibly also in breast cancer by way of increasing vascular permeability and promoting BBB penetration of malignant cells (Chen *et al.* 2011, Mongan *et al.* 2009, Lee *et al.* 2004). Nuclear localization of CXCR5 has been connected with a high Gleason score and aggressive disease in prostate cancer, whereas overexpression of CCR7 correlates with lymph node metastases in NSCLC (Singh *et al.* 2009, Takanami 2003).

### *Lymphomas*

The expression of CXCR4, CXCR5 and CCR7 also contributes to the development and dissemination of lymphomas. CLL, MCL and FL are subtypes of non-Hodgkin lymphoma that usually present with widely spread nodal disease. These subtypes have also been found to express high levels of the chemokine receptors CXCR4, CXCR5 and CCR7 (López-Giral *et al.* 2004, Kurtova *et al.* 2009). Furthermore, Rehm *et al.* (2009) have found that mediastinal large B-cell lymphoma cells express only low levels of CXCR5 and CCR7, which might hinder nodal dissemination in this subtype of DLBCL. In lymphomas, only one study has been published dealing with the microlocalization of chemokine receptors. Jahnke *et al.* (2005) have investigated the expression of chemokine receptors CXCR4, CXCR5 and CCR7 in patients with PCNSL and peripheral lymphoma. They found that in PCNSL the chemokine receptor expression localized in the nucleus and the cytoplasm, but not on the cell membrane, which might explain the absence of systemic dissemination in this lymphoma type. Membrane expression was seen in peripheral lymphomas. Considering these findings, as well as findings in solid malignancies, chemokines and their receptors constitute an attractive target for investigation in attempts to further elucidate the obscure pathophysiology of CNS lymphoma and neurotropism.

# 3 Aims of the present study

Despite the ground-breaking effect rituximab has had on the prognosis of DLBCL, some patients still die of their lymphoma. This mostly results from the development of a systemic chemoresistant disease relapse, or a recurrence in the CNS, where the BBB blocks out anticancer drugs creating a safe haven for malignant lymphocytes. These patients could potentially benefit from individually tailored frontline therapies. However, the identification of patients with poor prognosis, justifying deviation from traditional R-CHOP and risking severe side effects, constitutes a persisting challenge.

Oxidative stress markers, as well as redox state regulating molecules, have been found to hold prognostic value in a variety of solid tumours, and some conflicting data from the pre-rituximab era exists also in lymphomas. Components from cell cycle regulating pathways were proven prognostic pre-rituximab, but little is known of their role in patients treated with modern immunochemotherapy. Chemokine receptors determine the migratory paths of cells, and their expression guides organ-specific metastases in solid tumours. Possible roles have also emerged in CNS lymphomas.
The specific aims of the present study were:

1. To evaluate the expression and biological significance of oxidative stress markers and antioxidant enzymes in a broad spectrum of B-cell derived lymphomas.
2. To evaluate the prognostic significance of oxidative stress markers and antioxidant enzymes in DLBCL patients treated with modern immunochemotherapy.
3. To evaluate the prognostic role of cell cycle regulating molecules in DLBCL patients treated with modern immunochemotherapy.
4. To evaluate the expression of lymphocyte guiding chemokine receptors in patients with systemic or CNS-DLBCL, and make a preliminary assessment of their potential as future biomarkers for patient selection for CNS prophylactic therapies.

# 4 Materials and methods

## 4.1 Patient population

The material consisted of 263 lymphoma patients. 205 patients had a DLBCL, and 37 of these represented PCNSL. Detailed subtype distribution is presented in Table 4. Studies I and IV also included reactive lymph node samples from 7 healthy patients as controls. Detailed patient information was collected retrospectively from DLBCL, HL and FL patients, using hospital records at Oulu and Kuopio University Hospitals. From the other patients, only diagnostic information was obtained. All available R-CHOP treated DLBCL patients were included in the studies. With the other subtypes, a suitable number of consecutive patients were selected from hospital records.

**Table 4. Distribution of the lymphoma subtypes and reactive lymph node samples included in the studies.**

| Lymphoma subtype | Number of patients |
|---|---|
| DLBCL | 205 |
| PCNSL | 37 |
| FL | 18 |
| HL | 19 |
| CLL | 7 |
| MCL | 7 |
| MALT lymphoma | 7 |
| Reactive lymph node | 7 |

### *4.1.1 Diagnostic work-up and treatment*

The lymphomas included in the series were diagnosed and treatments initiated between the years 1990–2011 in the University Hospitals of Oulu and Kuopio. Diagnoses were based on histopathological examination and immunohistochemical studies of tissue samples taken from lymph nodes or extralymphatic tumour sites. Diagnostic work-up included patient history, physical examination, blood chemistry, histopathological examination of tumour tissue samples and a bone marrow aspiration and biopsy, as well as imaging with either whole body computer tomography (CT) or a thoracic X-ray and abdominal ultrasound. From patients with neurological symptoms, including all PCNSL

patients, also a sample of cerebrospinal fluid was collected, and magnetic resonance imaging (MRI) was conducted on the whole brain area.

Treatment details were collected from DLBCL, HL and FL patients. DLBCL patients were treated with CHOP-type regimens, including CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone), CHOEP (CHOP + etoposide) and CEOP (cyclophosphamide, epirubicin, vincristine and prednisone), with or without rituximab. Chemotherapy was administered at intervals of 14 or 21 days. If possible considering side effects and tumour localization, involved-field radiotherapy (IFRT) up to 40 Gy (2.0 Gy per fraction, 5 fractions per week) was given to the site of initial tumour mass of DLBCL patients with a bulky tumour of more than 5 cm in diameter or residual tumour mass after chemotherapy. CNS prophylaxis with high-dose systemic and i.t. methotrexate was given to patients at high risk of CNS relapse. Factors indicating high risk included having an IPI score of 3 or higher, primary involvement of testis, breast or base of skull and paraspinal tumour location.

17 out of 19 patients with HL received ABVD treatment (doxorubicin, bleomycin, vinblastine and dacarbazine). One elderly patient with stage I disease received only radiotherapy as primary treatment, and one patient was treated with BEACOPP (cyclophosphamide, doxorubicin, etoposide, procarbazine, prednisone, bleomycin and vincristine). 15 out of 18 FL patients received CHOP-type chemotherapy, with or without rituximab. Two patients were primarily treated with single chlorambucil, and one patient received alternating triple therapy (ATT), including CHOP, ESHAP (etoposide, methylprednisone, cytarabine and cisplatine) and NOPP (mitoxantrone, vincristine, prednisone and procarbazine) regimens. Some patients also received IFRT.

## 4.2 Immunohistochemistry

Tumour tissue samples were collected from patients at the time of diagnosis, then fixed in formalin and embedded in paraffin. 3-µm sections were cut from paraffin blocks and placed on SuperFrostPlus glass slides (Menzel-Gläser, Braunschweig, Germany). Slides were incubated in +37 °C for 4 hours (studies I-III) or overnight (study IV), then deparaffinated in a clearing agent Histo-Clear (National Diagnostics, Atlanta, GA, USA) or xylene (p53 staining) and rehydrated in descending ethanol series. Antigen retrieval was done in the microwave oven using Tris EDTA, pH 9, in the p53, p16 and CCR7 stainings, and a sodium citrate buffer, pH 6, when staining with the other antibodies. Endogenous peroxide

activity was blocked with incubation in a 3% $H_2O_2$ solution. Immunostaining continued according to manufacturers' instructions using the primary antibodies and staining methods presented in Table 5. Incubation with primary antibodies was carried out in a humidity chamber at room temperature for 1 hour (p53 staining in study III and all stainings in study IV) or at +4 °C overnight (studies I-II, and study III excluding p53). Between the different stages of the staining procedure, slides where washed with phosphate buffered saline (PBS) when using the Vectastain Elite ABC kit and Tris-buffered saline (TBS) with the other kits. Staining was concluded by counterstaining with haematoxylin, dehydration and mounting with Histomount (National Diagnostics, New Jersey, USA). Previously known positive and negative control samples were included in each series. In negative controls, the primary antibody was replaced by PBS or TBS, depending on the detection system used.

**Table 5. Antibodies and immunohistochemical staining methods included in the studies.**

| Study | Detected molecule | Primary antibody | Dilution | Source of antibody | Immunostaining method |
|---|---|---|---|---|---|
| I-II | 8-OHdG | MOG020P | 1:50 | JaICA, Nikken SEIL Co., Ltd, Fukuroi, Shizuoka, Japan | Histostain-Plus Bulk Kit, LAB-SA Detection System, Invitrogen Corporation, CA, USA |
| I-II | Nitrotyrosine | 06-284 | 1:2000 | Upstate (Millipore), New York, NY, USA | Novolink Polymer Detection System Kit, Novocastra/Leica Biosystems, NewcastleLtd, Newcastle upon Tyne, UK |
| I-II | Trx | 705 | 1:1000 | American Diagnostica, Inc., Stamford, CT, USA | Vectastain Elite ABC Kit (goat IgG), Vector Laboratories Inc., CA, USA |
| I-II | GCL | sc-22755 | 1:100 | Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA | Novolink Polymer Detection System Kit, Novocastra/Leica Biosystems, NewcastleLtd, Newcastle upon Tyne, UK |
| I-II | MnSOD | S5069 | 1:1000 | Sigma-Aldrich, Inc., St.Louis, MO, USA | Novolink Polymer Detection System Kit, Novocastra/Leica Biosystems, NewcastleLtd, Newcastle upon Tyne, UK |
| III | p16 | MA1074 | 5 µg/ml | Boster Biological Technology Co, Ltd., Wuhan, China | Novolink Polymer Detection System Kit, Novocastra/Leica Biosystems, NewcastleLtd, Newcastle upon Tyne, UK |

| Study | Detected molecule | Primary antibody | Dilution | Source of antibody | Immunostaining method |
|---|---|---|---|---|---|
| III | p21 | MS-230 | 1:200 | NeoMarkers for Lab Vision Corporation, Fremont, CA, USA | Novolink Polymer Detection System Kit, Novocastra/Leica Biosystems, NewcastleLtd, Newcastle upon Tyne, UK |
| III | p27 | RB-9019 | 1:1000 | NeoMarkers for Lab Vision Corporation, Fremont, CA, USA | Novolink Polymer Detection System Kit, Novocastra/Leica Biosystems, NewcastleLtd, Newcastle upon Tyne, UK |
| III | p53 | p53-DO7 | 1:100 | Novocastra/Leica Biosystems Newcastle Ltd., Newcastle-upon-Tyne, UK | Dako REAL™EnVision™ Detection System Peroxidase/DAB, Rabbit/Mouse, Dako Denmark A/S, Glostrup, Denmark |
| III | Ki-67 | NCL-L-Ki67-MM1 | 1:50 | Novocastra/Leica Biosystems Newcastle Ltd., Newcastle-upon-Tyne, UK | EnVision + System-HRP (DAB) kit, Dako North America, Inc., Carpinteria, CA, USA |
| IV | CXCR4 | H00007852-MO5 | 1:150 | Abnova, Taipei, Taiwan | Novolink Polymer detection System Kit, Novocastra/Leica Biosystems Newcastle Ltd., Newcastle-upon-Tyne, UK |
| IV | CXCR5 | MAB190 | 1:2000 | R&D Systems, Minneapolis, MN, USA | Novolink Polymer detection System Kit, Novocastra/Leica Biosystems Newcastle Ltd., Newcastle-upon-Tyne, UK |
| IV | CCR7 | PAB14776 | 1:100 | Abnova, Taipei, Taiwan | Novolink Polymer detection System Kit, Novocastra/Leica Biosystems Newcastle Ltd., Newcastle-upon-Tyne, UK |

| Study | Detected molecule | Primary antibody | Dilution | Source of antibody | Immunostaining method |
|---|---|---|---|---|---|
| II-IV | CD10 | NCL-CD10-270 | 1:100 | Novocastra/Leica Biosystems Newcastle Ltd., Newcastle-upon-Tyne, UK | Dako REAL™EnVision™ Detection System Peroxidase/DAB, Rabbit/Mouse, Dako Denmark A/S, Glostrup, Denmark |
| II-IV | Bcl-6 | anti-human Bcl-6 | 1:50 | DakoCytomation, Glostrup, Denmark | Dako REAL™EnVision™ Detection System Peroxidase/DAB, Rabbit/Mouse, Dako Denmark A/S, Glostrup, Denmark |
| II-IV | MUM-1 | anti-human MUM-1 antibody | 1:100 | DakoCytomation, Glostrup, Denmark | Dako REAL™EnVision™ Detection System Peroxidase/DAB, Rabbit/Mouse, Dako Denmark A/S, Glostrup, Denmark |

## 4.3 Immunoelectronmicroscopy

For immunoelectronmicroscopy (IEM) in study IV tumour tissue samples were first fixed in 4% paraformaldehyde with 2.5% sucrose in 0.1M PBS for 2 hours, then immersed in 2.3M sucrose in PBS at +4 °C and frozen with liquid nitrogen. Leica EM FC7 ultramicrotome was used to cut thin cryosections from samples, and these sections were then incubated in a blocking solution of 0.2% gelatin-PBS followed by 0.1% glycine-PBS. A solution of 1% bovine serum albumin (BSA) and PBS was used to dilute primary antibodies and gold conjugates, and to perform washings between different stages of the staining procedure. In single immunolabelling, 60 minutes of incubation with primary antibodies for CXCR4, CXCR5, CXCL12 and CXCL13 was performed, followed by 30 minutes of incubation in a bridging antibody of a rabbit-anti-mouse IgG (Zymed) with the monoclonal antibodies (CXCR4, CXCR5 and CXCL12). After washings another 30 minutes of incubation in a protein A-gold complex (size 10nm) followed. In double labelling, after incubation with primary antibodies for CXCR4 and CXCR5, Zymed and the protein A-gold complex, free binding sites on protein A were blocked using 1% glutaraldehyde in 0.1M PBS. Samples were then incubated with the second antibody for CXCL12 or CXCL13, followed by another protein A-gold complex (size 5nm) incubation of 30 minutes. With control samples, the labelling procedure was performed without the primary antibodies.

## 4.4 Sample evaluation

### *4.4.1 Immunohistochemistry*

Immunohistochemical stainings were reviewed by two independent observers blinded from the clinical data using a multihead microscope. In study I a comprehensive analysis was made of the immunohistochemical staining patterns for 8-OHdG, nitrotyrosine, Trx, GCL and MnSOD in several types of B-cell derived malignancies, including 18 cases of DLBCL, 18 cases of FL and 19 HL cases, as well as cases with CLL, MCL and MALT lymphoma, 7 of each. 6 reactive lymph node samples were also included in the analysis. Both the intensity of positive immunoreaction as well as percentage of positive cells was evaluated. The analysis was done separately for malignant B-cells and, when stromal positivity occurred, also for the surrounding stromal cells. In the analysis,

cases with positivity in 0–5% of cells were considered negative. Slides were then graded positive at level 1, 2, 3 or 4, when 6–25%, 26–50%, 51–75% or 76–100% of cells were positive, respectively. For statistical analysis, positivity levels 0–3 (0–75% of cells positive) were combined into a low expression group, level 4 cases forming a high expression group. The slides were also graded into levels 1–3 in relation to the intensity of the positive immunoreaction, with 1 standing for light, 2 for moderate and 3 for strong staining intensity.

In study II, with 106 cases of DLBCL, the same evaluation method was used but only staining in malignant B-cells was analysed, and the cases were divided into two groups according to staining intensity. In the 8-OHdG, nitrotyrosine, Trx and MnSOD stainings, cases from negative to moderate staining intensity formed one group and cases with strong staining intensity another. In the GCL staining the grouping was negative to light versus moderate to strong intensity.

In study III the proportion of positive malignant cells for p16, p21, p27 and p53 was analysed in 120 DLBCL samples. A cut-off point of 25%, determined by statistical analysis, was used to assign cases as either positive (25–100% of malignant cells positive) or negative (0–24% of positivity). Due to shortage of available tumour tissue, the staining for p21 could not be analysed in 2, and p27 in 5 cases. Based on literature on the regulation of Rb, p53 and p27 pathways and the immunohistochemical phenotypes reflecting their function, a prognostic score was obtained. In the scoring, cases with dysregulation of 0–1 of these pathways formed a favourable prognostic group, whereas cases where all of these pathways were compromised made up a poor prognostic group. The rest of the cases were assigned to an intermediate prognostic group. In our study, p16 negativity was considered to reflect dysfunction of the Rb pathway and p21 negativity that of the p53 pathway, whereas p27 positivity was considered a marker of p27 pathway dysregulation. In the poor prognostic group the more specific combination of p53 positivity and p21 negativity was used to identify p53 pathway dysfunction, whereas in the favourable prognostic group p21 status alone was considered sufficient. The immunohistochemical phenotypes included in each prognostic group are presented in Table 3 of study III.

In study IV, immunohistochemical stainings for the chemokine receptors CXCR4, CXCR5 and CCR7 were performed in 89 DLBCL samples, including 21 sCNSL samples and 35 brain samples from PCNSL cases. Only staining in malignant B-cells was analysed. First, the localization of positive immunoreaction was determined (nucleus, cytoplasm and/or cell membrane), followed by the

estimation of the proportion of positive malignant cells according to these individual cellular locations. Immunoreaction in each compartment was graded either negative, or positive at levels 1 or 2, when 1–79% or 80–100% of cells, respectively, were positive.

Immunohistochemical stainings for CD10, Bcl-6 and MUM-1 were performed and analysed in studies II-IV to determine the molecular subtype of DLBCL cases. Slides were graded as either positive or negative for each cellular marker using a cut-off point of 30% of malignant cells positive. The staining analysis and subsequent determination of the GC or non-GC phenotype was conducted according to the algorithm developed by Hans *et al.* (2004).

Micrographs demonstrating the immunohistochemical staining patterns were acquired by using the Olympus BX41 microscope (Olympus, Center Valley, PA, USA) and Olympus DP11 digital microscope camera (Olympus, Center Valley, PA, USA). Images were imported into HP Photo & Imaging software (Hewlett-Packard Company, Palo Alto, CA, USA) and, if necessary, image brightness was increased.

### *4.4.2 Immunoelectronmicroscopy*

Samples from 1 reactive lymph node, 1 case of nodal DLBCL, 1 case of sCNSL (brain sample) and 2 PCNSL cases were stained first with a single antibody for CXCR4, CXCR5, CXCL12 and CXCL13. Double immunolabelling for receptor-ligand complexes (CXCR4/CXCL12 and CXCR5/CXCL13) was also conducted in the reactive lymph node, sCNSL and PCNSL samples. The reactive lymph node was only stained for CXCR5/CXCL13 complexes, whereas both receptor-ligand pairs were determined from the CNS samples. Tissue sections were embedded in methylcellulose and examined with the Philips CM100 transmission electron microscope (FEI Company, Eindhoven, The Netherlands). A general assessment of the electron-microscopic expression patterns was first made by a group of 3–5 observers. For a more substantial analysis, 24–26 micrographs were acquired from each patient sample after double immunolabelling, using the Morada CCD camera (Olympus Soft Imaging Solutions GMBH, Munster, Germany), and mean values for receptor/ligand/complex expression on the membrane and in the cytoplasm were calculated. The mean expression densities for these molecules were calculated by measuring the length of cell membrane from each micrograph, adding these together and dividing the sum value of molecules with the membrane length. Similarly, the cytoplasmic molecule

expression was assessed by counting all molecules within 1 $um^2$ of cytoplasm from each micrograph, adding these together and then dividing the sum with the complete area included in the assessment (24–26 $um^2$ of cytoplasm). The expression densities were determined as n/100um of membrane and n/100$um^2$ of cytoplasm.

## 4.5 Statistical analysis

Receiver operating characteristic (ROC) analysis was used to determine the cut-off point for positivity in the immunohistochemical stainings in study III. In all studies, correlations between the expression levels of the different protein markers and clinical parameters were determined using the 2-sided Pearson's chi-square test. Multivariate analysis using the Cox regression model was attempted in study III, but could not be carried out because of the limited size of the patient series. Survival analysis in studies I-III was performed with the parameters disease-specific survival (DSS) and progression-free survival/relapse-free survival (PFS/RFS). DSS was defined as the time elapsed from the date of diagnosis to the date of death from lymphoma, and PFS/RFS was calculated similarly from the date of diagnosis to the relapse date. Patients who died from causes other than lymphoma or dropped out of the follow-up disease-free were censored. The Kaplan-Meier method was used to perform survival analyses and significance was determined with the log-rank test. P-values of less than 0.05 were considered significant. Statistical analyses were performed using the SPSS software (Chicago, IL, USA), versions 17.0–20.0, in the different studies.

## 4.6 Ethical aspects

Good laboratory practice was followed when handling tissue samples in our laboratories. Encoded markings were used in patient samples and identifying information was stored separately in a locked cabinet and room, to protect the patients' privacy. Data analyses were also performed without the patients' identifying information. The studies have not affected patient care or follow-up, and study information is not included in the patients' hospital records. Paraffin-embedded tissue samples used in the studies were collected for diagnostic purposes, and the patients were, therefore, not subjected to additional physical risk. The permission to use paraffin-embedded tissue samples for research

purposes was granted by the Finnish National Supervisory Authority for Welfare and Health (6622/05.01.00.06/2010). The samples used for IEM analysis in study IV were taken in addition to diagnostic tissue samples, and written consent for this was obtained from all patients. The studies were approved by the Ethical Committees of Oulu University Hospital (42/2010, 23.6.2010) and Kuopio University Hospital (98/2009, 22.9.2009).

# 5 Results

## 5.1 Immunohistochemical staining patterns

### *5.1.1 Oxidative stress markers and redox state regulating enzymes*

*Expression in reactive lymphoid tissue*

Samples from 6 reactive lymph nodes were included in the analysis in study I. Light cytoplasmic positivity for 8-OHdG was seen in practically all follicle cells, and one sample also presented with a germinal center blast cell population with strong cytoplasmic positivity. Some cells with a strong immunoreaction were seen in the subcapsular regions, and a few lightly stained lymphocytes appeared in the marginal zones. Strong cytoplasmic positivity was seen in a blast cell population. Two samples had no 8-OHdG expression, and all reactive lymph node samples were negative for nitrotyrosine. Positivity was more substantial for the antioxidant enzymes. Positivity for Trx and GCL was seen in all germinal centre cells. Centrocytes presented with strong nuclear immunoreaction for Trx, whereas marginal zones were negative. Macrophages showed mostly cytoplasmic Trx expression, but some had nuclear positivity as well. GCL and MnSOD were also extensively expressed by macrophages, and MnSOD presented with a granular staining pattern.

*Expression in B-cell derived lymphomas*

Study I was conducted as a pilot study in a small number of patients from various lymphoma subtypes, including 18 DLBCLs, 19 HLs, 18 FLs, 7 CLLs, 7 MCLs and 7 MALT lymphomas, whereas study II had a larger series of 106 DLBCL patients. Immunohistochemical staining patterns are presented in Figure 1 of study I.

A positive immunoreaction for 8-OHdG was seen in both the nucleus and cytoplasm of malignant B-cells, whereas nitrotyrosine presented with a cytoplasmic staining pattern. Positivity for these markers was also seen in stromal cells. In study I we found that the expression of 8-OHdG was more pronounced in DLBCL compared with the more indolent lymphoma subtypes. The expression of nitrotyrosine was again greatest in FL and HL. Most MCL and MALT lymphoma

cases were negative for nitrotyrosine (71% and 67% of cases, respectively). Study II confirmed the extensive expression of oxidative stress markers in DLBCL, and equal expression levels of both 8-OHdG and nitrotyrosine were discovered.

All of the studied antioxidant enzymes presented with a cytoplasmic staining pattern in B-cell derived lymphomas, although in DLBCL, HL and FL samples, Trx was also seen in the nucleus. Macrophages showed strong positivity for Trx. MnSOD had a specific granular staining pattern, mostly of light intensity. In some cases of DLBCL, FL, MCL and MALT lymphoma, an additional subpopulation of lymphoma cells existed, presenting with strong MnSOD intensity. A positive immunoreaction for MnSOD also presented in reactive stromal cells.

In comparison with the oxidation metabolites, the expression of antioxidant enzymes was more substantial in the indolent lymphoma subtypes. Trx expression was generally fairly low, but HL samples presented with extensive positivity, which was uniformly of grade 3 intensity. GCL positivity was found extensively in all studied subtypes. Expression of MnSOD was the highest in MALT lymphoma, followed by MCL, CLL and FL, whereas DLBCL and HL cases had stronger staining intensity. The expression levels and intensities of all studied markers as well as their distribution between the different lymphoma subtypes are presented in Table 3 of study I. Table 4 of study I summarizes correlations between the different markers.

### *5.1.2 Cell cycle regulators*

Samples from 120 DLBCL patients were stained for cell cycle regulating proteins p16, p21, p27 and p53 in study III. All protein markers presented with a nuclear staining pattern (Figure 1 of study III). 33.3% of cases were graded positive for p16, 10.8% for p21, 70.0% for p27 and 50.8% for p53. p16 positivity correlated with positivity for p53 and the immunophenotype p53+p21- ($p=0.01$ and $p=0.013$, respectively).

#### *Associations between cell cycle regulators and redox molecules*

Some associations were seen between expression of p16 and p27 proteins, and the expression levels of oxidative stress markers and antioxidant enzymes. p16 negativity was associated with strong cytoplasmic nitrotyrosine intensity ($p=0.041$), whereas p27 positivity correlated with strong nuclear and cytoplasmic

8-OHdG intensity, as well as moderate to strong cytoplasmic intensity for GCL (p=0.004, p=0.022 and p=0.017, respectively). No associations presented with proteins from the p53 pathway, or with the prognostic score.

### *5.1.3 Chemokine receptors*

Immunohistochemical staining for the chemokine receptors CXCR4, CXCR5 and CCR7 was performed on samples from 35 PCNSL cases, 21 secondary CNS lymphomas and 33 systemic DLBCLs in study IV, and staining patterns are presented in Figure 1 of study IV. Positivity for CXCR4 was seen in the cell cytoplasm and, in 46% of the samples, also in the nucleus. Two samples had membranous CXCR4 expression. Positivity in the CXCR5 immunostaining localised either to the cytoplasm alone, or both to the cytoplasm and the cell membrane. 69% of the samples presented with a combination of cytoplasmic and membranous positivity. CCR7 expression was scarce, and 68% of samples were altogether negative. Nuclear positivity was seen in a small proportion of cells in 29% of the samples, and two samples had also membranous staining. High nuclear CXCR4 expression was associated with low cytoplasmic CXCR5 expression, when using 80% cell positivity as a cut-off point for high expression (p=0.021). No other correlations were found between the chemokine receptors.

## 5.2 Electron-microscopic expression patterns

IEM analysis was performed on samples from 1 reactive lymph node, 1 nodal DLBCL, 1 secondary CNS lymphoma (brain biopsy) and 2 PCNSL cases, after staining with antibodies for chemokine receptors CXCR4 and CXCR5 as well as their ligands CXCL12 and CXCL13. Electron-microscopic expression patterns are presented in Figure 3 of study IV, whereas Figure 4 of study IV presents the expression densities of CXCR5 and CXCL13 in the different diagnosis groups.

### *5.2.1 CXCR4 and CXCL12*

In reactive lymphoid tissue, CXCR4 presented with a nuclear expression pattern, whereas CXCL12 was barely seen at all. The expression of both these molecules was scarce in the nodal DLBCL sample. The few receptors and ligands that were seen were randomly located in the nucleus and cytoplasm, as well as on the membrane of malignant B-cells. Malignant B-cells in the CNS samples expressed

higher numbers of CXCR4 and CXCL12 compared with the lymph node samples, but the expression localised to the cytoplasm and on the membrane, and proper nuclear expression was not seen in PCNSL or sCNSL. CXCL12 was occasionally seen in the nucleus, but this scarce expression could be considered as background label.

### *5.2.2 CXCR5 and CXCL13*

Reactive lymphocytes presented with cytoplasmic and membranous CXCR5 and CXCL13 expression. In the cytoplasm, CXCR5 molecules were mostly located within small vesicles. In the sample representing nodal DLBCL, the expression of these molecules was again low. A few molecules were seen in the cytoplasm, but expression mostly localised to the cell membrane. Compared with the others, the expression of CXCR5 and CXCL13 was significantly higher in the malignant B-cells of the CNS samples, especially in the ones representing PCNSL. In fact, the cytoplasmic expression densities for receptors and complexes were more than twice as high in the PCNSL samples compared with the sCNSL sample, and around 4 to 5 times as high as in the reactive lymph node sample. In the CNS samples, the molecules were mostly localised in the cytoplasm, specifically in the endoplasmic reticulum, but a fair amount also presented on the cell membrane. Even though the expression rates were highest in PCNSL and sCNSL, the relative proportion of CXCR5 receptors binding its ligand CXCL13 were, contrarily, highest in the reactive lymph node sample.

## 5.3 Clinical correlations and prognostic significance

The 3-year RFS and DSS rates for DLBCL patients in our series were 78.0% and 83.4%, respectively (Figure 4). Background parameters predicting poor RFS as well as CNS involvement are presented in Table 6. Table 7 summarizes the discovered correlations between all studied markers and patient prognosis, as well as clinical correlations.

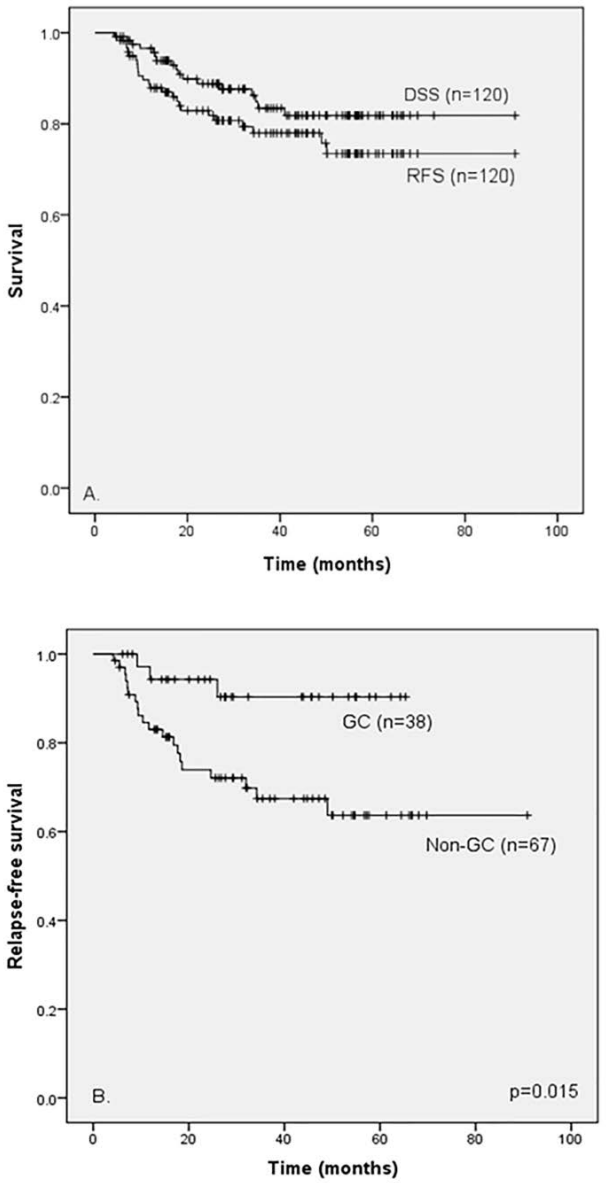


**Fig. 4. Survival curves for DLBCL patients. (A) RFS and DSS rates in the complete patient series. (B) RFS rates for the GC and non-GC phenotypes.**

**Table 6. Background parameters predicting poor RFS as well as CNS involvement.**

| Parameter | Poor prognosis | CNS involvement |
|---|---|---|
| Non-GC phenotype | x | |
| Stage IV | x | x |
| B symptoms | x | x |
| Bcl-6 negativity | x | |
| Elevated LDH | | x |
| >1 extranodal lesion | | x |
| IPI score 3-5 | | x |

### *5.3.1 Prognostic value of oxidative stress markers and antioxidant enzymes*

Preliminary survival analysis was performed in FL and DLBCL patients in study I. Among FL patients, no significant survival correlations were found in relation to expression of oxidative stress markers or antioxidant enzymes. In DLBCL, high positivity (>75% of malignant cells positive) for 8-OHdG was associated with poor PFS (p=0.032), and, furthermore, non-significant trends for poor PFS emerged from high positivity for GCL and low positivity for MnSOD (cut-off 75%) (p=0.093 and p=0.067, respectively).

In study II the staining intensity of malignant B-cells seemed to present with more variation than the proportional extent of positive cells, and intensities were used to group cases for survival analysis. In this study, performed in a larger DLBCL series, the expression of nitrotyrosine, Trx and GCL seemed to divide groups with significant differences in patient survival. Survival curves are presented in Figure 1 of study II. Strong staining intensity for Trx was associated with poor PFS and DSS in DLBCL patients, with 5-year PFS rates of 57.4% in cases with strong Trx positivity, vs. 85.1% in other cases (p=0.046 and p=0.015, respectively). When analysed separately in the GC and non-GC phenotypes, this survival correlation seemed actually to be limited to the non-GC phenotype, where the corresponding 5-year PFS-rates were 31.6% vs. 78.4% (p=0.01). The proportion of positive malignant cells correlated with staining intensity in the GCL staining, and, in line with the trend discovered in study I, moderate to strong staining intensity for GCL was associated with poor prognosis (p=0.049). Another prognostic correlation was found between strong cytoplasmic nitrotyrosine expression and poor PFS (p=0.006).

**Table 7. The discovered associations between marker expression and patient survival, as well as correlations with other parameters.**

| Marker | Prognostic effect | Extranodal involvement | CNS involvement | IPI 3-5 | Stage IV | Age | Bcl-6 |
|---|---|---|---|---|---|---|---|
| 8-OHdG | | ↑ | | ↑ | | | |
| Trx | PFS↓ DSS↓ | | | | | | |
| GCL | PFS↓ | | | | | | |
| Nitrotyrosine | PFS↓ | | | | | | |
| p16 (-) | | | | | | | ↓ |
| p27 (+) | | | | | ↑ | | |
| Cell cycle regulation score | PFS↓ DSS↓ | | | | | | |
| Nuclear CXCR4 | | ↓ | | | | ↓ | |
| Cytoplasmic CXCR5 | | | ↑ | | | | |

### 5.3.2 Cell cycle regulators

#### Clinical correlations of cell cycle regulators

Cell cycle regulators from the Rb and p27 pathways were associated with other prognostic factors. p16 negativity correlated with Bcl-6 negativity (p=0.020), and p27 positivity was associated with stage III-IV disease (p=0.026). Proteins from the p53 pathway did not show clinical correlations individually or when combined. No correlations were found between the prognostic score and clinical parameters or the IPI classification. No association presented between the prognostic score and expression of the proliferation marker Ki-67, either.

#### Cell cycle regulating proteins and survival

The individual cell cycle regulating proteins were not prognostic. However, combinations of two and three markers were able to identify prognostic subgroups in the complete DLBCL patient series, as well as separately among the non-GC phenotype patients. The marker combinations with prognostic value are presented in Table 8.

Survival analysis was also performed using combinations of correlating cell cycle regulating markers and redox molecules. The combination of p16 negativity and high cytoplasmic intensity for nitrotyrosine predicted inferior outcome, with 5-year PFS rates of 42.9% versus 77.8% (p=0.030). No survival correlations presented with the other marker combinations.

#### Prognostic score

Based on the immunohistochemical evaluation of the function of Rb, p53 and p27 pathways, a prognostic score was obtained. Patients with dysfunction of 0–1 of these pathways formed a favourable prognostic group, while patients with disruption of all the pathways were separated as a poor prognostic group (immunohistochemical phenotype p16-p53+p21-p27+). The rest of the patients made up an intermediate prognosis group. Due to missing staining results, 7 patients were excluded from the analysis. Therefore, 113 patients remained in the complete series; 62 out of these patients had the non-GC phenotype.

**Table 8. Marker combinations with poor or favourable prognostic value in the whole DLBCL patient series and among non-GC phenotype patients.**

| Whole DLBCL series | 3-year RFS | p-value | Non-GC DLBCL | 3-year RFS | p-value |
|---|---|---|---|---|---|
| p16-p21- | 69.7% vs. 88.4% | 0.026 | p16-p21- | 45.6% vs.86.7% | 0.004 |
| p21-p27+ | 68.3% vs. 91.9% | 0.029 | p21-p27+ | 48.5% vs. 87.5% | 0.018 |
| p16+p27- | 100% vs. 73.5% | 0.043 | p16+p27- | 100% vs. 59.5% | 0.046 |
| - | - | - | p16-p27+ | 48.9% vs. 78.5% | 0.049 |
| p16-p21-p27+ | 65.3% vs. 85.2% | 0.053 | p16-p21-p27+ | 41.6% vs. 80.4% | 0.007 |
| p16+p21-p27- | 100% vs. 73.1% | 0.048 | - | - | - |
| - | - | - | p16-p53+p27+ | 36.7% vs. 71.3% | 0.029 |
| - | - | - | p16-p53+p21- | 44.4% vs. 73.8% | 0.020 |

The score had significant prognostic value as regards both RFS and DSS of DLBCL patients (see Figure 2 of study III for survival curves). In the complete patient series, the 3-year RFS rates in the favourable, intermediate and poor prognostic groups were 100%, 73.1% and 64.3%, respectively (p=0.034). The corresponding 3-year DSS rates were 100%, 80.5% and 73.1% (p=0.038). The greatest significance was, however, discovered when performing survival analyses separately among the non-GC phenotype patients. In this subgroup, the 3-year RFS rates were 100% in the favourable group, 62.6% in the intermediate group and 24.3% in the poor prognosis group (p=0.003). The 3-year DSS rates for the non-GC phenotype were 100%, 72.3% and 48.6%, respectively (p=0.003).

### *5.3.3 Chemokine receptors and CNS involvement*

Different chemokine receptor expression profiles for systemic and CNS lymphoma emerged in the analysis of immunohistochemical stainings. The expression profiles associated with the different diagnosis groups are presented in Figure 2 of study IV. Systemic DLBCL cases without CNS involvement presented more often with high nuclear CXCR4 expression (>80% malignant cell positivity), and this phenotype was, therefore, associated with a low risk of CNS involvement (p=0.003). These patients also typically presented with a nodal manifestation of DLBCL (p=0.025). On the other hand, high proportion of malignant cells expressing cytoplasmic CXCR5 was a risk factor for CNS disease (p=0.039). Patients with the combination of low nuclear CXCR4 and high cytoplasmic CXCR5 expression were more prone to CNS involvement (p=0.018). When analysing according to cellular localization instead of cell positivity rates, cases with CXCR4 expression in both the cytoplasm and the nucleus of malignant cells had more often an extracranial DLBCL, compared with cases where positivity was restricted to the cytoplasm (p=0.032). This localization pattern was more common in young patients under 60 years of age (p=0.013).

# 6 Discussion

DLBCL is an aggressive malignancy presenting with considerable biological heterogeneity. It is also a disease that typically responds well to a wide variety of treatment modalities, and effective therapeutic options, such as the standard R-CHOP regimen and the intensified HDT/ASCT used to treat relapsed disease, are available. One third of DLBCL patients present with a refractory disease or develop a relapse later during the disease course. Only 20% of these patients can be cured with salvage high-dose chemotherapy combined with ASCT (Gisselbrecht *et al.* 2010). Rituximab, dexamethasone, high-dose cytarabine and cisplatin (R-DHAP) and rituximab, ifosfamide, etoposide and carboplatin (R-ICE) have equal efficacy as a salvage therapy preceding ASCT (Gisselbrecht *et al.* 2010). However, when separating the GC and non-GC subtypes, it seems that R-DHAP works better among GC-DLBCL patients, whereas non-GC phenotype lymphomas seem to respond better to other therapies, such as bortezomib and lenalidomide (Thieblemont *et al.* 2011, Dunleavy *et al.* 2009, Hernandez-Ilizaliturri *et al.* 2011). This further demonstrates the relevance of biological subtyping in DLBCL, as well as the significance of biological features in treatment response. Aggressive treatments should be targeted at patients for whom R-CHOP is insufficient and who are likely to suffer a disease relapse in the future. The identification of these patients presents a continuing challenge. Furthermore, 5% of DLBCL cases recur in the CNS, but the prophylactic therapy can only be justified in high-risk patients. The clinical characteristics presently used can only predict CNS recurrence with 20% accuracy, which calls for biological predictive markers.

## 6.1 Oxidative stress and treatment response

Oxidative stress is a carcinogenic state in cells, and augmented expression levels as well as prognostic value for cellular oxidation products have been found in several solid malignancies (Karihtala & Soini 2007). In this study, the results implicated a role for oxidative stress also in B-cell derived lymphomas. Increasing expression of oxidation metabolites was observed in a series of samples evolving from reactive lymph nodes to indolent lymphomas and, finally, aggressive lymphomas. Whether the increasing oxidative stress during lymphoma evolution is the reason behind or the consequence of a more aggressive disease is unclear. Aggressive malignancies have higher cell proliferation and

apoptosis/necrosis rates, which also lead to a more hypoxic environment, increased inflammation and increased ROS production. Aggressive diseases with high oxidative stress also tend to have increased antioxidant activity, induced by the accumulating ROS. This activity protects the malignant cells from ROS-induced apoptosis and enables tumour growth. Then again, continuous ROS production has also been shown to sustain activation of the BCR signalling cascade, promoting B-cell activation and proliferation (Wheeler & DeFranco 2012). The present results, as well as results from the pre-rituximab era, indicate that the activity of antioxidant systems, versus the expression of oxidative stress markers, may be more significant as regards the prognosis and therapy response of DLBCL patients. Strong staining intensity for the oxidation marker nitrotyrosine, did, however, also show negative prognostic value in this study.

Drug resistance is a major problem in cancer therapy. In DLBCL, many of the standard chemotherapeutic agents in use mediate apoptosis by increasing cellular levels of ROS and thus driving the cells over the apoptotic threshold. Strong staining intensity for antioxidant enzymes Trx and GCL was associated with adverse prognosis in the present study. In line with these findings, increased expression levels of Trx as well as members of the GSH family, including GCL, have been shown to be associated with decreased sensitivity to doxorubicin, etoposide and cyclophosphamide (Yokomizo *et al.* 1995, Tsai-Turton *et al.* 2007). In a study conducted in malignant bladder and prostate cell lines, decreasing Trx expression in malignant cells was found to increase their sensitivity to several superoxide producing chemotherapeutic agents, including doxorubicin and etoposide, as well as UV-radiation (Yokomizo *et al.* 1995). Sensitivity to the tubulin-targeting agent vincristine was not affected. Consistent with this data, in an experiment conducted in our laboratory, Trx knockout has been found to increase the sensitivity of DLBCL cells to doxorubicin (unpublished data). Cyclophosphamide acts by reducing intracellular GSH levels and inducing apoptosis. High expression of GSH seems to decrease sensitivity to cyclophosphamide, and combining another GSH-depleting agent buthionine sulfoximine with treatment with cyclophosphamide has produced better response rates in ovarial carcinoma cells with high GSH expression than either of these agents alone (Tsai-Turton *et al.* 2007). A similar finding has been reported by Asano *et al.* (2009) in leukemia cells with high GCL expression. These cells, initially doxorubicin resistant, were shown to be able to restore their doxorubicin sensitivity after exposure to indomethacine, an inhibitor of GCL promoter

activity. These studies suggest that the addition of Trx- or GSH-/GCL-depleting agents into therapy regimens could provide better response rates in DLBCL patients with chemoresistant diseases and high antioxidant activity.

There are two studies executed pre-rituximab on the effect of oxidative stress and redox state regulating enzymes on DLBCL prognosis, and both reported prognostic roles for increased expression of certain antioxidant enzymes. In line with the findings of the present study, Tome *et al.* (2005) suggested that high Trx expression, combined with low expression of catalase, GPX1 and MnSOD, would be associated with adverse prognosis. Andreadis *et al.* (2007), in turn, found that GPX1 expression could be the only significant factor influencing DLBCL prognosis, and that high levels of GPX1 could predict poor survival. GPX1 is a downstream effector of GSH, whereas GCL is the rate-limiting enzyme controlling the production of GSH. In the present study, strong intensity for GCL was a poor prognostic factor, and, presuming a linear correlation between the expression rates of GCL and GPX1, these findings are consistent with the findings of Andreadis *et al*. (2007).

The mitochondrial antioxidant MnSOD functions by reducing superoxide anions into hydrogen peroxide ($H_2O_2$) (Halliwell *et al.* 1999). $H_2O_2$, although less reactive than superoxide anions, is still an oxidant and needs to be further reduced by other antioxidants, such as the GSH or Trx systems. If these systems are inactive, accumulation of $H_2O_2$ and oxidative stress follows. MnSOD can, therefore, act as either an anti-apoptotic or a pro-apoptotic molecule, depending on the function of other antioxidant systems. The association and dependence on other antioxidant systems could explain why no survival correlations emerged in this study in relation to MnSOD expression, whereas both Trx and GCL were prognostic.

A study regarding the expression of redox molecules in FL has also been conducted in our research group, and in that study none of these redox markers proved prognostically significant (unpublished data). This might be due to the central role of the mitochondrial apoptosis pathway in cellular oxidative stress response (Wilkinson *et al.* 2012). Normally, oxidative stress leads to down-regulation of Bcl-2, enabling apoptosis. In FL, however, the translocation t(14;18) leads to constitutive transcription of the translocated *BCL-2* gene. Therefore, Bcl-2 effect is not eliminated and apoptosis via the mitochondrial pathway is prevented. This might explain the lack of prognostic value in this disease group. The t(14;18) translocation is also seen in around 30% of DLBCL cases with the GC phenotype, reflecting the germinal centre origin shared by both FL and GC-

DLBCL (Iqbal *et al.* 2004). In our study, the prognostic effect of Trx was not seen in GC-DLBCL patients, and it could be speculated whether this might be due to the presence of a *BCL-2* translocation in a proportion of these patients, preventing the apoptotic effect of ROS inducing drugs.

## 6.2 Cell cycle regulation and prognosis

The regulation of cell proliferation and death is inherently compromised in malignant diseases, and damage occurs particularly often in the mechanisms controlling the G1/S transition of the cell cycle, *i.e.,* in the regulatory Rb, p53 and p27 pathways. By analysing the expression of immunohistochemical markers reflecting the function of these pathways a prognostic score was obtained, where the favourable group had damage in 0–1 of these pathways while in the poor prognostic group all 3 of these pathways were compromised. The remaining patients formed a group with an intermediate prognosis. The score was most significant among non-GC DLBCL patients, where the 3-year relapse rate of patients varied from 0 in the favourable group to almost 80% in the poor prognosis group. The accumulation of cellular damage caused by chemotherapy normally leads to either cell cycle arrest and damage repair, or, if damage is extensive, to apoptosis. In GC-DLBCL, even though the cell cycle arresting mechanisms are damaged, the cell can still be programmed for cell death to prevent the proliferation of a damaged cell clone. In non-GC DLBCL, however, apoptosis is prevented by the constant anti-apoptotic activity mediated by NF-κB, giving malignant cells significant survival and proliferation advantage (Davis *et al.* 2001). This phenomenon might also explain why the prognostic effect of the cell cycle regulation score was limited to the non-GC subtype of DLBCL.

The G1/S restriction point in the tumours of the poorest prognostic group has significantly weakened, allowing constant cell cycle progression. Overexpression of cyclin D1 causes a similar regulatory dysfunction in MCL, continuously driving G1/S transition and cell cycle progression (Bosch *et al.* 1994). The incorporation of cytarabine into standard R-CHOP-type therapy has been found to significantly improve prognosis in MCL, and considering the common site of dysfunction, it is possible that the patients in our poor prognosis group might also benefit from cytarabine treatment (Geisler *et al.* 2008). Cytarabine acts in the S phase of the cell cycle by interfering with DNA synthesis and preventing mitosis (Grant 1996). It is most efficient in rapidly proliferating cells, and thus it seems

plausible that it might work in the poorest prognostic group where both cell cycle arresting and apoptotic mechanisms are compromised. Another interesting candidate for investigation would be the mTOR inhibitor temsirolimus, which, among other things, controls the transcription of cyclin D1 (Yuan *et al.* 2009). Further research is warranted to determine the optimal therapy schemes for patients with severe cell cycle dysregulation.

### 6.3 Chemokine receptor expression and CNS tropism

Chemokines and their receptors are responsible for guiding the physiological migration and homing of cells in the body. The major chemokine receptors guiding the homing of lymphocytes are CXCR4, CXCR5 and CCR7. As a developmental remnant, CXCR4 and its ligand CXCL12 are physiologically produced in the CNS, as well as other extranodal organs including the bone marrow. (Zlotnik *et al.* 2011.) Ligands for CXCR5 and CCR7, on the other hand, are mostly produced by lymph nodes, and together these molecules guide the normal organization of secondary lymphoid tissue. Little is known about the mechanisms underlying CNS tropism in DLBCL, but the cytoplasmic localization of these chemokine receptors has previously been shown in PCNSL (Jahnke *et al.* 2005). No studies have been conducted dealing with the expression of these molecules in secondary CNS lymphomas.

In PCNSL, Jahnke *et al.* (2005) have previously reported the presence of cytoplasmic and nuclear expression of CXCR4, CXCR5 and CCR7, whereas extracranial B-cell lymphomas presented with membranous expression as well. We also found that the high cytoplasmic expression of CXCR5 correlated with CNS disease. However, it was the nuclear localization of CXCR4 that associated with nodal disease in our series, and a high proportion of malignant cells expressing nuclear CXCR4 acted as a CNS protective factor. Nuclear CXCR4 expression was also seen in reactive lymph nodes in the IEM analysis, supporting a hypothesis of a physiological expression pattern in lymph nodes and nodal DLBCL. The specific function of nuclear CXCR4 expression is currently unclear, but this expression pattern has also been connected with lymph node metastasis in several solid malignancies, for example hepatocellular carcinoma, colorectal cancer and NSCLC (Xiang *et al.* 2009, Wang *et al.* 2010, Na *et al.* 2008). No nuclear CXCR4 expression was seen in the samples representing CNS lymphoma.

In this study, high cytoplasmic CXCR5 expression correlated with CNS involvement. Both immunohistochemistry and IEM revealed significant

overexpression of CXCR5 in PCNSL samples, compared with the others. PCNSL was followed by sCNSL, and the expression was lowest in the reactive lymph node samples. Contrarily, the proportion of CXCR5 binding its ligand CXCL13 was highest in the reactive lymph node and lowest in the CNS samples, demonstrating more efficient receptor function in healthy tissue. Together with the fact that most of the cytoplasmic CXCR5 molecules seen in PCNSL samples localized into the endoplasmic reticulum, this might indicate defective folding of CXCR5 in CNS lymphomas and subsequent accumulation of an unfolded or misfolded protein.

New information on the biology of PCNSL has emerged in recent years. Jiang *et al.* (2010) showed in a murine model that PCNSL cells exhibit strong and highly selective tropism for the CNS, indicating a possible extracranial origin for this disease, followed by homing to the CNS. If the present hypothesis is correct and the malignant cells in PCNSL have flawed CXCR5 folding mechanisms, these cells may be unresponsive to the CXCL13 produced by lymph nodes and instead respond to the CXCL12 excreted in the CNS, and end up homing there. In line with this, the IEM samples representing PCNSL and sCNSL in our series had more substantial cytoplasmic and membranous CXCR4 expression compared with nodal DLBCL and reactive lymph node samples. Furthermore, NF-κB is known to increase CXCR4 production, indicating that the constitutively active NF-κB signalling seen in non-GC DLBCL would make this subtype more prone to migrate to the CNS in the absence of functioning CXCR5 (Helbig *et al.* 2003). Most PCNSL cases have been shown to represent non-GC DLBCL, and 85% of the PCNSL cases also in this study had the non-GC phenotype. In these series, non-GC phenotype was, however, not a risk factor for secondary CNS lymphoma. Despite the similarities seen between these two disease entities, this would indicate the existence of some underlying differences.

## 6.4 Future prospects

In the present study, it was evaluated whether redox molecules, cell cycle regulators and chemokine receptors could serve as possible future factors in identifying DLBCL patients with a poor prognosis. Ultimately the goal would be to optimise DLBCL treatment through individualisation and tailoring of therapy according to the patients' disease-specific biology. Discovering markers able to identify patients at risk of poor treatment response or disease relapse would

enable better targeting of suitable first-line treatment leading to higher cure rates. On the other hand, lighter therapies with less side effects and shorter hospitalization periods could be administered to patients with a favourable prognosis. These patients would also be eligible for shorter and less intense follow-up protocols. Efficient patient stratification and individual treatment tailoring would thus lead to lower mortality and higher cost-effectiveness.

Poor prognostic subgroups were found in patients with strong expression of the antioxidant enzymes Trx and GCL. Antioxidant expression is associated with drug resistance and poor treatment response, suggesting that these patients might benefit from the incorporation of Trx or GCL depleting agents into therapeutic regimens. Furthermore, chemotherapeutic agents functioning through ROS-independent mechanisms, such as methotrexate, might lead to better response rates in these patients.

The prognostic value of strong Trx expression was especially pronounced in the non-GC phenotype patients, where constitutive NF-κB signalling further prevents induction of apoptosis. The cell cycle regulation score obtained in this study was also more efficient for patient stratification and prediction of disease relapse in the non-GC phenotype. In addition to the constant survival signal provided by NF-κB, patients in the poor prognosis group have severe dysfunction of cell cycle arresting systems in their tumours. These patients might benefit from treatment with agents restricting the overly active NF-κB signalling, such as bortezomib or lenalidomide. Also, treatment with agents directly preventing cell cycle progression might prove beneficial in the poor prognosis patient group. Potential candidates for further research might include temsirolimus, a transcriptional inhibitor of cyclin D1, and cytarabine, a molecule that interferes with DNA synthesis in the S phase.

Another interesting discovery made in this study was the similar chemokine receptor profiles seen in samples from PCNSL and sCNSL. This indicates certain biological similarities between these disease entities, compared to systemic disease and reactive lymphoid tissue. It seems that cytoplasmic overexpression of CXCR5 may be associated with CNS tropism in DLBCL, whereas nuclear CXCR4 expression seems to correlate with a low risk of CNS disease. Taken together the present data suggests that, in the future, chemokine receptor expression might have potential in the prediction of CNS relapse in DLBCL patients. Further studies clarifying the specific roles of these molecules are warranted.

In this study, interesting prognostic correlations emerged from the expression of antioxidant enzymes and cell cycle regulators in DLBCL, and possible roles for chemokine receptors in the prediction of CNS relapse were also discovered. These studies were, however, conducted retrospectively in limited patient populations, and further research is required to verify these findings. Experiments in lymphoma cell cultures and animal models could provide further insights into the roles of antioxidant enzymes and cell cycle regulators in predicting treatment response and determining optimal therapy. Overrepresentation of CNS lymphoma in study IV prevents the calculation of positive and negative predictive values of chemokine receptor expression, and larger prospective studies are needed to definitively determine its predictive significance. If confirmed, however, these results might facilitate more effective targeting of CNS prophylactic therapies among DLBCL patients in the future.

# 7 Conclusions

In the present study, the expression of several biological factors was evaluated in B-cell derived lymphomas. The prognostic value of redox molecules and cell cycle regulators was investigated in DLBCL patients treated uniformly with modern immunochemotherapy. Also, the expression of chemokine receptors in tissue samples from reactive lymph nodes, systemic DLBCL, sCNSL and PCNSL was evaluated, and distinct expression profiles were identified for nodal disease versus CNS lymphoma. The immunohistochemically determined non-GC phenotype had adverse survival impact compared with GC phenotype patients, and very poor prognostic groups were identified among the non-GC DLBCL patients. These patients might benefit from therapy other than the traditional R-CHOP. For example, patients with overexpression of antioxidant enzymes might respond better to antioxidant-depleting or ROS-independent chemotherapeutic agents, and in patients with cell cycle dysregulation, agents directly affecting cell cycle progression might be beneficial.

The specific conclusions drawn from this study are:

1. The amount of oxidative stress seems to increase when comparing reactive lymph nodes to indolent lymphomas and, finally, to aggressive lymphomas, implicating an increasing oxidative stress during lymphomagenesis.
2. Strong staining intensity for nitrotyrosine, Trx and GCL predicts poor PFS in DLBCL. The increased activity of antioxidant systems might contribute to the development of chemoresistance in these patients. A very poor prognostic group was identified among non-GC DLBCL patients with strong positivity for Trx.
3. The accumulation of damage in different cell cycle regulating pathways seems to progressively worsen DLBCL patients' prognosis. A cell cycle regulation score was developed, and it was able to effectively stratify non-GC DLBCL patients into distinct prognostic groups.
4. Distinct chemokine receptor profiles were identified for systemic DLBCL and CNS lymphoma. High nuclear CXCR4 expression was associated with nodal manifestation of DLBCL and a low risk of CNS disease, whereas high cytoplasmic CXCR5 positivity correlated with CNS involvement. According to the IEM results, reactive lymphoid tissue had a nuclear CXCR4 expression pattern, differing from the cytoplasmic expression seen in the CNS samples. Compared with reactive lymphoid tissue, CXCR5 was significantly

overexpressed in PCNSL and to a slightly lesser extent in sCNSL. The proportion of CXCR5 binding ligand CXCL13 was, however, higher in the reactive lymph node compared with the CNS samples, reflecting more efficient receptor function. It seems that the chemokine receptors CXCR4 and CXCR5 might have potential as future biomarkers used in selecting patients for CNS prophylactic therapies.

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# Original publications

I Pasanen AK, Kuitunen H, Haapasaari K-M, Karihtala P, Kyllönen H, Soini Y, Turpeenniemi-Hujanen T & Kuittinen O (2012) Expression and prognostic evaluation of oxidative stress markers in an immunohistochemical study of B-cell derived lymphomas. Leuk Lymphoma 53: 624–631.

II Pasanen AK, Haapasaari KM, Jantunen E, Soini Y, Turpeenniemi-Hujanen T, Bloigu R, Lilja L, Kuittinen O & Karihtala P (2012) Oxidative stress and redox state-regulating enzymes have prognostic relevance in diffuse large B-cell lymphoma. Exp Hematol Oncol 1:2.

III Pasanen AK, Haapasaari KM, Peltonen J, Soini Y, Jantunen E, Bloigu R, Turpeenniemi-Hujanen T & Kuittinen O (2013) Cell cycle regulation score predicts relapse-free survival in non-germinal centre diffuse large B-cell lymphoma patients treated by means of immunochemotherapy. Eur J Haematol 91: 29–36.

IV Pasanen AK, Lemma S, Haapasaari K-M, Sippola A, Sormunen R, Soini Y, Jantunen E, Koivunen P, Salokorpi N, Bloigu R, Turpeenniemi-Hujanen T & Kuittinen O (2013) Similar chemokine receptor profiles in primary central nervous system lymphoma and secondary central nervous system involvement of systemic diffuse large B-cell lymphoma – possible biomarkers for patient selection for central nervous system prophylaxis. Manuscript.



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