Esa Kari

CLINICAL IMPACT OF ANTIOXIDANT ENZYMES PRX6 AND TRX AND THEIR REGULATORS NRF1 AND NRF2 IN DIFFUSE LARGE B-CELL LYMPHOMA
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Kari, Esa, Clinical impact of antioxidant enzymes Prx6 and Trx and their regulators Nrf1 and Nrf2 in diffuse Large B-cell lymphoma.

University of Oulu Graduate School; University of Oulu, Faculty of Medicine; Medical Research Center Oulu; Oulu University Hospital

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Abstract

Diffuse large B-cell lymphoma (DLBCL) is the single most common lymphoid malignancy in Western world. Excess oxidative stress and antioxidative enzyme expression have previously been associated with adverse disease presentation and poor prognosis in DLBCL. The present study evaluated the clinical relevance of two antioxidant enzymes—peroxiredoxin 6 (Prx6) and thioredoxin-1 (Trx)—, one oxidative stress marker—8-hydroxydeoxyguanosine (8-OHdG)—, and four antioxidative, response-related transcription factors nuclear factor erythroid 2-related factor 1 (Nrf1) and factor 2 (Nrf2), Kelch ECH associating protein 1 (Keap1) and BTB (broad complex (BR-C), tramtrack (ttk), bric-a-brac/poxvirus (bab)) domain and Cap'n'collar (CNC) homolog 1 (Bach1) in DLBCL patients and cell culture (Trx).

The pre-treatment blood serum levels of Prx6 were found to be significantly lower among DLBCL patients compared to healthy controls, and these levels were observed to rise to the controls’ levels after treatments. Low Prx6 levels were associated with neutropenic infections; thus, Prx6 might be a potential marker for selecting patients to receive white blood cell growth factors.

All tissue assessed transcription factors, excluding Bach1, had prognostic implications among high-risk DLBCL patients. Low nuclear expression of Nrf1, high cytoplasmic expression of Nrf1, high nuclear expression of Nrf2 and low cytoplasmic expression of Keap1 were related to poor overall survival. Nuclear Nrf2 and Bach1 expressions were associated with adverse clinical features in DLBCL.

Lymphoma cell’s cytoplasmic Trx expression was associated with superior survival with DLBCL patients who received etoposide-containing, high-dose chemotherapy or R-CHOEP. In the cell culture model, knockdown of Trx resulted in sensitization of lymphoma cells to doxorubicin and decreased the response to etoposide. Trx appears to be predict the response to etoposide (R-CHOEP).

Keywords: 8-hydroxydeoxyguanosine (8-OHdG), BTB domain and CNC homolog 1 (Bach1), diffuse large b-cell lymphoma, Kelch ECH associating protein 1, nuclear factor erythroid 2-related factor 1 &2, peroxiredoxin 6, thioredoxin-1
Kari, Esa, Antioksidatiivististen entsyyminen Prx6 ja Trx ja niiden säätelijöiden Nrf1 ja Nrf2 kliininen vaikutus diffuusissa suurten B-solujen lymfoomassa.

Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta; Medical Research Center Oulu; Oulun yliopistolinnan sairaala

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Tiivistelmä

Diffuusi suurten B-solujen lymfooma (DLBCL) on yleisin yksittäinen imusolmukesyöpä länsimaissa. Runsas oksidatiivinen stressi ja antioksidatiivistisen entsyyminen ilmentymenä on aiemmin yhdistetty huonoon taudintekoon ja ennusteeseen DLBCL:ssä. Tässä tutkimuksessa arvioitiin kahden antioksidatiivistisen entsyymin peroksiredoksiini 6 (Prx6) ja tioredoksiini-1 (Trx), yhden oksidatiivisen stressin markkerin 8-hydroksideoksyguanosiinin (8-OHdG) sekä neljän antioksidatiivistä vasteesta liittyvän transkriptiotekijän tumafaktori 2- liittyvää faktori 1 (Nrf1) ja faktori 2 (Nrf2), Kelch ECH assosioituva proteiini 1 (Keap1) ja BTB (broad complex (BR-C), tramtrack (ttk), bric-a-brac/poxvirus (bab)) domeeni ja Cap'n'collar (CNC) homologi 1 (Bach1) kliinistä merkitystä DLBCL-potilaille sekä soluviljellyssä (Trx).


Kaikilla kudoksesta arvioiduilla transkriptiotekijöillä, pois lukien Bach1, oli ennusteellista merkitystä korkean riskin DLBCL-potilaille. Matala Nrf1-tumaekspresio, korkea Nrf1-sytoplasmakspresio, korkea Nrf2-tumaekspresio ja matala Keap1-sytoplasmakspresio liittyivät huonoon kokonaiselinaikaan. Nrf2- ja Bach1-tumaekspressiot assosioituivat huonompaan taudinkuvaan DLBCL:ssä.

Lymfoomasolujen Trx-sytoplasmakspresio liittyi parempaan ennusteeseen DLBCL-potilailta, jotka saivat hoidoksi etoposidia sisältävän annos hoidolle tai R-CHOEP-kurin. Soluviljellämissa Trx:n tuotannon kumoaminen aiheutti lymfoomasolujen herkistymistä doktorusisiin ja resistenssi etoposidille. Trx vaikuttaa ennustavan vastetta etoposidille (R-CHOEP-hoidolle).

Asiasonat: 8-hydroksideoksyguanosiini (8-OHdG), BTB (BR-C), diffuusi suurten B-solujen lymfooma, Kelch ECH assosioituva proteiini 1 (Keap1), peroksiredoksiini 6, tioredoksiini-1, ttk ja bab domeeni ja CNC homologi 1 (Bach1), tumafaktorit 2-liittyvää faktori 1 (Nrf1) ja faktori 2 (Nrf2)
To define is to limit.
-Oscar Wilde
Acknowledgements

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Oulu, August 2019

Esa Kari
Abbreviations

8-OHdG 8-hydroxy-2-deoxyguanosine
ABC activated B-cell
AOE antioxidant enzyme
ASCT autologous stem-cell transplantation
Bach1 BTB (BR-C, ttk and bab) domain and CNC homolog 1
BBBD blood brain barrier disruption
BCL-2 B-cell lymphoma-2 protein
BCL-6 B-cell lymphoma-6 protein
BCR B-cell receptor
BEAC carmustine, etoposide, cytarabine, and cyclophosphamide
BEAM carmustine, etoposide, cytarabine, and melphalan
BL Burkitt lymphoma
BM bone marrow
CD cluster of differentiation
CLL chronic lymphocytic leukaemia
COO cell of origin
CR complete response
CT computer tomography
DLBCL diffuse large B-cell lymphoma
DSS disease-specific survival
EBV Epstein-Barr virus
EBVMCU EBV-positive mucocutaneous ulcer
ELISA enzyme-linked immunosorbent assay
FACS fluorescence-activated cell sorting
FBS fetal bovine serum
FL follicular lymphoma
GC germinal centre
GCB germinal centre B-cell
GCL glutamate–cysteine ligase
GPX glutathione peroxidase
GSH glutathione
H_{2}O_{2} hydrogen peroxide
HDT high-dose therapy
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HGBL high-grade B-cell lymphoma
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>HHV8</td>
<td>human herpes virus 8</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HL</td>
<td>Hodgkin’s lymphoma</td>
</tr>
<tr>
<td>i.e.</td>
<td>id est</td>
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<tr>
<td>IG</td>
<td>immunoglobulin</td>
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<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
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<tr>
<td>IPI</td>
<td>International Prognostic Index</td>
</tr>
<tr>
<td>Keap1</td>
<td>Kelch ECH associating protein 1</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>LN</td>
<td>lymph node</td>
</tr>
<tr>
<td>NHL</td>
<td>non-Hodgkin’s lymphoma</td>
</tr>
<tr>
<td>NKT cell</td>
<td>natural killer T cell</td>
</tr>
<tr>
<td>NOS</td>
<td>not otherwise specified</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Nuclear factor erythroid 2 related factor</td>
</tr>
<tr>
<td>Maf</td>
<td>musculoaponeurotic fibrosarcoma</td>
</tr>
<tr>
<td>MALT</td>
<td>mucosa-associated lymphoid tissue</td>
</tr>
<tr>
<td>MCL</td>
<td>mantle cell lymphoma</td>
</tr>
<tr>
<td>MnSOD</td>
<td>manganese superoxide dismutase</td>
</tr>
<tr>
<td>mTOR</td>
<td>mechanistic target of rapamycin</td>
</tr>
<tr>
<td>MUM-1</td>
<td>multiple myeloma oncogene-1</td>
</tr>
<tr>
<td>MYC</td>
<td>Myelocytomatosis gene</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-kappa B</td>
</tr>
<tr>
<td>OR</td>
<td>odds ratio</td>
</tr>
<tr>
<td>OS</td>
<td>overall survival</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>oxidative phosphorylation</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCDLBCL</td>
<td>primary cutaneous diffuse large B-cell lymphoma</td>
</tr>
<tr>
<td>PCNSL</td>
<td>primary central nervous system (diffuse large B-cell) lymphoma</td>
</tr>
<tr>
<td>PFS</td>
<td>progression-free survival</td>
</tr>
<tr>
<td>PMLBCL</td>
<td>primary mediastinal large B-cell lymphoma</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulphonyl fluoride</td>
</tr>
<tr>
<td>PR</td>
<td>partial response</td>
</tr>
<tr>
<td>Prx</td>
<td>peroxiredoxin</td>
</tr>
<tr>
<td>R-CHOP</td>
<td>rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone</td>
</tr>
</tbody>
</table>
R-CHOEP  rituximab, cyclophosphamide, doxorubicin, vincristine, etoposide, and prednisolone
R-DHAP  rituximab, dexamethasone, high-dose cytarabine, and cisplatin
R-GDP  rituximab, gemcitabine, cisplatin, and dexamethasone
R-GEMOX  rituximab, gemcitabine, and oxaliplatin
R-MINE  rituximab, mesna, ifosfamide, mitoxantrone, and etoposide
RFS  relapse-free survival
ROC  receiver operating characteristic
ROS  reactive oxygen species
siRNA  small interfering ribonucleic acid
SLL  small lymphocytic lymphoma
SOD  superoxide dismutase
THRLBCL  T-cell/histiocyte-rich large B-cell lymphoma
Trx  thioredoxin
WHO  World Health Organisation
Original publications

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


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1 Introduction

Diffuse large B-cell lymphoma (DLBCL) is a heterogenous group of malignancies with a potentially fatal outcome. DLBCL’s prognosis has improved drastically with the introduction of the first, monoclonal antibody, rituximab to therapy repertoire in 1997. DLBCL’s current challenges lie in patient risk stratification, selection of right treatment modalities and treatment of chemorefractory and relapsed disease.

DLBCL as a disease entity has changed over the years along with the new, updated WHO classifications. The latest 2017 revision divided EBV+ DLBCL in the elderly into EBV+ mucocutaneous ulcer; EBV+ DLBCL not otherwise specified (NOS), while double- and triple-hit lymphomas are now categorised as a separate entity among high grade B-cell lymphoma (HGBL) group. WHO currently still recognizes 7 distinct DLBCL entities in which DLBCL NOS covers the bulk of the cases. Information from DLBCL as a disease and further subdivisions are expected to increase in the future.

Oxidative stress and antioxidant enzymes (AOE) are important in many physiological processes. Conversely, aberrant redox balance has been discovered in many cancers, including DLBCL. AOE s have been associated with pathogenesis, aggressive behaviour and resistance to therapy. This area has seen an increased number of publications during the recent years. The goal of this work was to further explore the clinical implications of AOE s and their upstream regulators based on previous findings.
2 Review of the literature

2.1 Lymphomas

Lymphomas are hematological malignancies that arise from lymphatic cells. Their progenitor is a lymphocyte, which can be a B-cell, T-cell or Natural killer cell (NKT-cell) in any state of differentiation. The most typical manifestation occurs in lymph nodes, but extralymphatic diseases are also seen frequently. The B-cell-derived lymphomas are the most prevalent group of lymphoma comprising > 90% of cases, especially in the western world (Armitage & Weisenburger, 1998; Armitage, 1997). Lymphomas are divided into Hodgkin’s lymphoma (HL) and non-Hodgkin’s lymphoma (NHL) for historical reasons. HL comprises 10% of all lymphomas (Shankland, Armitage, & Hancock, 2012).

2.1.1 Lymphoma classification

Current lymphoma classification is based on the revised version of WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues from 2017. The most common NHL is diffuse large B-cell lymphoma (DLBCL), followed by follicular lymphoma (FL); together they cover > 60% of lymphomas (Figure 1).

HL is divided into nodular lymphocyte predominant HL and classic HL, which has four distinct histological classes. T-cell lymphomas have various subtypes. Based on the International Classification of Diseases for Oncology (ICD-O), the Revised WHO IV recognizes 93 different lymphomas, 12 precursor lymphoid neoplasms, 2 immunodeficiency-associated lympho-proliferative disorders and 11 histiocytic and dendritic cell neoplasms (Swerdlow et al., 2017).

2.1.2 Aetiology and epidemiology

A 2018 worldwide estimate was that there were 509,590 (2.8% of all cancers) new patients with NHL and 79,990 (0.4%) new patients with HL. In Finland these numbers were 1321 (4.0%) and 162 (0.5%), respectively. Lymphoma is the 7th most common malignancy globally and the 6th most common malignancy in Finland (Globocan). The incidence of lymphomas has been on the rise during the past few years (Morton et al., 2014).
A common lymphoid progenitor develops from a pluripotent haematopoietic stem cell in the bone marrow (BM). It further differentiates into prolymphocyte, a lymphoblast. The development of B- and T-cells disseminates after this stage. B-cells migrate into the lymph nodes, and T-cells migrate into the thymus to undergo further differentiation. The lifespan of B-cells and T-cells differs, as the B-cells’ main function is to produce immunoglobulins (IG) as a part of the adaptive immune system. B-cells undergo antigen selection in the lymph nodes. Cells unable to encounter their antigen undergo apoptosis. Cells that receive growth stimulus through their B-cell receptor enter the germinal centre (GC) where they become centroblasts. They undergo somatic hypermutation and heavy chain class switching, developing then into centrocytes. Cells that exit the GC differentiate into IG-producing plasma cells or memory B-cells. B-cells interact with antigen-presenting cells and T-cells and express various stage-dependent surface proteins during this intricate process (Swerdlow et al., 2017). Neoplasms can arise at multiple, different stages (Figure 2).
The exact causes of lymphoma are unknown. Cancer is often related to ageing, as carcinogenesis requires time and exposure to carcinogens, and it benefits from immunosenescence (Hoffe & Balducci, 2012; Mancuso et al., 2018). The known risk factors for lymphomagenesis include immunodeficiency (de Miranda, Björkman, & Pan-Hammarström, 2011), autoimmune disease (Baecklund, Smedby, Sutton, Askling, & Rosenquist, 2014), immunosuppression (Van Leeuwen et al., 2009), chronic viral infection (HIV, EBV, HTLV-1, HHV8, and hepatitis C) (Bigoni et al., 1996; Chadburn, Abdul-Nabi, Teruya, & Lo, 2013; Ito, Kusunoki, Mochida, Yamaguchi, & Mizuochi, 2011; Roschewski & Wilson, 2012; Vicario et al., 2018) and chronic bacterial infection (Helicobacter pylori, Borrelia burgdorferi, Chlamydia psittaci, Chlamydia pneumonia, Chlamydia trachomatis, Campylobacter jejuni, Achromobacter xylosidans) (Adam et al., 2014; Chanudet et al., 2007; Lecuit et al., 2004; Morgner, Bayerdörffer, Neubauer, & Stolte, 2000; Schöllkopf et al., 2008). Exposure to some environmental toxic agents, radiation and earlier therapies with chemotherapeutics may play a role.

Notable differences exist in disease epidemiology. Precursor lymphoid neoplasms occur predominantly in children. Of mature B-cell neoplasms, some cases of DLBCL and Burkitt lymphoma (BL) are seen in children (Swerdlow et al., 2017). Otherwise, the median patient age is 60 – 70 years, excluding primary mediastinal large B-cell lymphoma (PMLBCL) with a median age of 35 years (Martelli, Ferreri, Di Rocco, Ansuinelli, & Johnson, 2017). Apart from FL and PMLBCL, lymphoma is somewhat more common in males (Swerdlow et al., 2017). FL and DLBCL are relatively more common in western countries, BL in equatorial Africa and T-cell and NTK cell lymphomas in Asia. Genetics and race can significantly affect the relative disease presentation (Swerdlow et al., 2017).
Fig. 2. B-cell differentiation in relation to major B-cell neoplasms. B-ALL/LBL, B-lymphoblastic leukaemia / lymphoma; MCL, mantle cell lymphoma; FL, follicular lymphoma; BL, Burkitt lymphoma; DLBCL, diffuse large B-cell lymphoma; HL, Hodgkin's lymphoma; MZL, marginal zone lymphoma; MALT, mucosa associated lymphoid tissue; LPL, lymphoplasmacytic lymphoma; CLL/SLL, chronic lymphocytic leukaemia / small lymphocytic lymphoma; PCM, plasma cell myeloma (Swerdlow et al., 2017).

2.2 Diffuse large B-cell lymphoma

DLBCL is the single, most-common lymphoma in adults. It is characterised as a neoplasm of medium-to-large lymphoid B-cells with a nuclear size equal to or larger than those of macrophages or more than twice the size of those of normal lymphocytes, with a diffuse growth pattern (Swerdlow et al., 2017). DLBCL is subdivided into several groups, but no generally accepted criteria exist for
subclassification for the bulk of cases. This group is collectively referred to as DLBCL, not otherwise specified (NOS) (Martelli et al., 2013).

2.2.1 Aetiology and epidemiology

DLBCL accounts for 25 – 40% of NHL and can occur at any age. It is slightly more common in males. Median patient age is > 60 years (Anderson, Armitage, & Weisenberger, 1998; Armitage, 1997). DLBCL, NOS, commonly represents de novo disease, but a secondary transformation from more indolent types of lymphoma (FL, CLL/SLL, MZL, HL) is possible. Immunodeficiency and EBV infection predispose people to DLBCL (Dojcinov et al., 2011; Mueller, 1999). Exposure to pesticides, fertilizers and some medical drugs has also been suggested as an aetiological factor (Martelli et al., 2013; Weisenberger, 1985).

2.2.2 Biology and subtypes

In addition to DLBCL, NOS, T-cell/histiocyte-rich large B-cell lymphoma (THRLBCL), primary central nervous system (diffuse large B-cell) lymphoma (PCNSL), primary cutaneous diffuse large B-cell lymphoma (PCDLBCL), EBV-positive diffuse large B-cell lymphoma, NOS, EBV-positive mucocutaneous ulcer (EBVMCU), and diffuse large B-cell lymphoma associated with chronic inflammation are held as distinct disease entities under the DLBCL-spectrum.

DLBCL can manifest as a nodal or extranodal disease (Anderson et al., 1998; Armitage, 1997). Common extranodal sites include the gastrointestinal tract, bone, testes, spleen, Waldeyer ring, salivary glands, thyroid, liver, kidneys, and adrenal glands (Swerdlow et al., 2017). BM involvement may be concordant (large lymphoid cells in the marrow) or discordant (low-grade lymphoid cells in the marrow) (Brudno et al., 2016). Lymph-node histology typically represents diffusely proliferating, large lymphoid cells that have replaced normal lymph node architecture. Perinodal infiltration may be present. DLBCL, NOS, has three common morphological subtypes: centroblastic, immunoblastic, and anaplastic variant. Morphology currently has an unclear clinical significance (Engelhard et al., 1997; Ott et al., 2010). DLBCL, NOS, can be divided into germinal centre B-cell (GCB) and activated B-cell (ABC) subtypes with gene expression profiling; however, 10-15% remain unclassified. GCB type is of GC origin and ABC type of post-GC origin (Alizadeh et al., 2000; Wright et al., 2003). These subtypes differ by genetic profile, treatment response and prognosis (Table 1). The Hans’ algorithm
can be used to distinguish GCB from non-GCB type (Figure 3) when gene expression profiling is unavailable (Hans et al., 2004). Non-GCB type DLBCL is more often associated with a CNS recurrence (Ollila & Olszewski, 2018).

![Fig. 3. Hans’ algorithm. Immunohistochemical staining proportion ≥ 30% is considered positive. GCB, germinal centre B-cell subtype (Hans et al., 2004).](image)

THRLBCL is thought to arise from GC B-cells but, contrary to DLBCL, NOS, its histology is dominated by background T-cells and histiocytes, while lymphoid B-cells are scattered. THRLBCL can also represent transformation from nodular lymphocyte predominant HL (Eyre, King, Hatton, & Collins, 2014). A typical patient group comprises middle-aged men. Extranodal involvement of liver, spleen and BM is common. THRLBCL is considered aggressive malignancy with frequent failure from therapy (Swerdlow et al., 2017).

PCNSL is a subtype of DLBCL that affects primarily the central nervous system: brain, spinal cord, leptomeninges and eye. It is more common in males with a median patient age of > 60 years. Aetiology is often unknown (Côté et al., 1996; Villano, Koshy, Shaikh, Dolecek, & McCarthy, 2011). PCNSL is postulated to arise from a late GC B-cell with both GCB and ABC features. Morphology resembles the centroblastic or immunoblastic variant (Montesinos-Rongen et al., 2008). The mutational profile and gene expression profile differ from DLBCL. The expression of human leukocyte antigen class I and II are frequently decreased (Booman et al., 2006; Riemersma et al., 2000). Disease is usually confined to CNS, and extraneural involvement is rare. Testis is the most common dissemination site. (Booman et al., 2007).
Table 1. Genetic characteristics in diffuse large B-cell lymphoma (DLBCL) subtypes.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Frequency</th>
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<tr>
<td></td>
<td>GCB DLBCL</td>
</tr>
<tr>
<td>Rearrangements</td>
<td></td>
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<tr>
<td>BCL2</td>
<td>40%</td>
</tr>
<tr>
<td>BCL6</td>
<td>15%</td>
</tr>
<tr>
<td>MYC</td>
<td>5 – 8%</td>
</tr>
<tr>
<td>CD274</td>
<td>Rare</td>
</tr>
<tr>
<td>CIITA</td>
<td>Rare</td>
</tr>
<tr>
<td>TBL1XR1</td>
<td>5%</td>
</tr>
<tr>
<td>Copy-number aberrations</td>
<td></td>
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<tr>
<td>1p36.32 deletion (TNFRSF14)</td>
<td>30%</td>
</tr>
<tr>
<td>2p16 gain/amplification (REL)</td>
<td>30%</td>
</tr>
<tr>
<td>3q27 gain/amplification</td>
<td>15 – 20%</td>
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<tr>
<td>6q21 deletion (PRDM1)</td>
<td>25%</td>
</tr>
<tr>
<td>9p21 deletion (CDKN2A)</td>
<td>20%</td>
</tr>
<tr>
<td>9p21.1 gain/amplification (CD274)</td>
<td>Uncommon</td>
</tr>
<tr>
<td>18q21.3 gain/amplification (BCL2)</td>
<td>15%</td>
</tr>
<tr>
<td>Recurrent mutations</td>
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<tr>
<td>EZH2</td>
<td>20 – 25%</td>
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<tr>
<td>GNA13</td>
<td>25%</td>
</tr>
<tr>
<td>KMT2D</td>
<td>40%</td>
</tr>
<tr>
<td>TP53</td>
<td>20%</td>
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<tr>
<td>MEF2B</td>
<td>15 – 20%</td>
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<tr>
<td>SGK1</td>
<td>15 – 20%</td>
</tr>
<tr>
<td>CREBBP</td>
<td>30%</td>
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<tr>
<td>TNFRSF14</td>
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<td>5%</td>
</tr>
<tr>
<td>CARD11</td>
<td>10 – 15%</td>
</tr>
<tr>
<td>CD79B</td>
<td>Uncommon</td>
</tr>
<tr>
<td>MYD88</td>
<td>Uncommon</td>
</tr>
<tr>
<td>PRDM1</td>
<td>Rare</td>
</tr>
<tr>
<td>B2M</td>
<td>20 – 25%</td>
</tr>
<tr>
<td>CD58</td>
<td>10%</td>
</tr>
<tr>
<td>Pathway perturbations</td>
<td></td>
</tr>
<tr>
<td>NF-Kb</td>
<td>No</td>
</tr>
<tr>
<td>PI3K/PIKT</td>
<td>Yes</td>
</tr>
<tr>
<td>JAK/STAT</td>
<td>Yes</td>
</tr>
<tr>
<td>Immune escape</td>
<td>Yes</td>
</tr>
<tr>
<td>Prognosis</td>
<td></td>
</tr>
</tbody>
</table>
2.2.3 Diagnostics and treatment

Patients with DLBCL usually develop single or multiple painless and rapidly growing tumour lumps with no other symptoms initially. Up to 40% of patients present extranodal lesions. Specific localizing symptoms are possible and depend on the site of involvement. General symptoms, i.e. B-symptoms such as fever, night sweating and weight loss, can precede clinically detectable tumour masses (Anderson et al., 1998; Armitage, 1997). Diagnosis requires histological evaluation of the tumour where morphology and immunophenotype are determined. Intact lymph nodes and tumour biopsies are preferred over core biopsy. Recommended immunohistochemical stainings include CD3, CD5, CD10, CD20, CD30, BCL-2, BCL-6, MUM-1/IRF-4, Ki-67/MIB-1, and MYC. Hans’s algorithm is commonly used in practise. Gene expression profiling is required to discern ABC from non-CGB and fluorescence in situ hybridization/chromogenic in situ hybridization of BCL-2, BCL-6, and MYC to discern BL and HGBL (Leppä et al., 2017; Swerdlow et al., 2017).

Lymphoma staging is based on clinical examination and imaging with contrast enhanced computer tomography (CT) or fluorodeoxyglucose - positron emission tomography (FDG-PET). BM biopsy is required if FDG-PET is unavailable (Table 2). Cerebrospinal fluid sample is taken in the case of CNS symptoms or if there is high risk for CNS involvement (Swerdlow et al., 2017; The International Non-Hodgkin’s Lymphoma Prognostic Factors Project, 1993).

The International Prognostic Index (IPI) has been used in clinical practise since 1993 for risk evaluation and treatment selection. IPI score scales from 0 to 5 and is calculated for every patient. A patient can score one point from each clinical category: age over 60 years, stage III–IV, elevated blood serum lactate dehydrogenase (LDH), WHO performance status two or more (> 50% of time in bed during the day) and presence of two or more extranodal lymphoma sites (The International Non-Hodgkin's Lymphoma Prognostic Factors Project, 1993).
Table 2. Diffuse large B-cell lymphoma (DLBCL) staging criteria.

<table>
<thead>
<tr>
<th>Ann Arbor stage</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>Lymphoma in one lymph node or lymphatic organ</td>
</tr>
<tr>
<td>Stage II</td>
<td>Lymphoma in more than one lymph node same side of the diaphragm</td>
</tr>
<tr>
<td>Stage III</td>
<td>Lymphoma in lymph nodes both side of the diaphragm</td>
</tr>
<tr>
<td>Stage IV</td>
<td>Disseminated lymphoma in one or more extranodal organs or liver or bone marrow affision</td>
</tr>
</tbody>
</table>

Additional stage specifications: A, no B-symptoms; B, B-symptoms; X, large disease mass: tumour diameter over 10 cm or mediastinum width growth over 1/3; E, single direct extranodal infiltration from lymph node

Modern treatment of DLBCL is based on immunochemotherapy (see the following list). The current standard of care is the R-CHOP regimen (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone). Other regimens have been unable to establish themselves as superior over R-CHOP (Sehn & Gascoyne, 2015). Adding novel agents to R-CHOP appears to improve survival in ABC DLBCL, however (Dunleavy, Erdmann, & Lenz, 2018; Nowakowski et al., 2015; Wilson et al., 2015). Based on IPI score, age, heart ejection fraction, and performance status, patients typically receive R-CHOP or R-CHOEP (R-CHOP with etoposide) for 6–8 cycles every 3 weeks. Risk for CNS relapse is elevated if a patient presents with IPI 3 - 5 disease, HGBL, and/or a disease with testis, adrenal gland, or kidney involvement. CNS prophylaxis with high-dose methotrexate or high-dose cytarabine is recommended for these patients (Kriedel & Dietrich, 2011; Schmitz et al., 2016). Treatment is consolidated with involved-field radiation therapy in the case of bulky disease, bone affision, epidural affision, PET-positive tumour at the end of treatment or large (diameter > 3 cm) PET-negative tumour. However, the exact benefit of consolidative radiotherapy needs further evaluation (Avilés et al., 2005; Held et al., 2014; Pfreundschuh et al., 2017). CT is performed at the middle and FDG-PET at the end of the treatment to evaluate treatment response. Response evaluation is based on tumour size reduction and metabolic activity (Deauville criteria, FDG uptake) (Cheson et al., 2007; Cheson et al., 2014). A new biopsy should be taken if tumour reduction is insufficient or disease progresses during the treatment. Tolerance issues may hinder particularly the use of etoposide, anthracyclines, vinca alkaloids, and CNS prophylaxis (Wunderlich et al., 2003)
R-CHOEP is a valid treatment option for young (age ≤ 60 – 65), high-risk (IPI > 1) patients with improved overall and progression-free survival compared to R-CHOP (Gang et al., 2012; Melén et al., 2016; Wästerlid, Hartman, Székely, & Jerkeman, 2017). Increased toxicity precludes more intensified therapy in elderly patients (Wunderlich et al., 2003). The exact pharmacodynamics of etoposide is unclear. Studies suggest a survival benefit in GCB DLBCL compared to non-GCB DLBCL (Gang et al., 2015) and overcoming the negative impact of double expression of MYC and BCL2 (Pedersen et al., 2017).

A minority of patients suffer a relapse during their follow-up. A new biopsy with histological evaluation is required for diagnosis. Staging and response imaging are made as in de novo disease. Treatment of refractory and relapsed disease overlap. Induction therapy consists typically of 2–3 cycles of platin-based salvage chemotherapy every 2–3 weeks, followed by response CT. If a complete response (CR) or a partial response (PR) is achieved, patients typically proceed to stem cell mobilization and apheresis. After successful stem cell collection, they receive BEAM (carmustine, etoposide, cytarabine, and melphalan) or BEAC (carmustine, etoposide, cytarabine, and cyclophosphamide) as high-dose therapy (HDT) with eventual autologous stem-cell transplantation (ASCT). None of the induction regimens is clearly superior in the whole population, but R-DHAP (rituximab, dexamethasone, high-dose cytarabine, and cisplatin) may be better than R-ICE (rituximab, ifosfamide, carboplatin, etoposide) in GC phenotype. PR confers inferior outcomes compared to CR. (Gisselbrecht et al., 2010; Kewalramani et al., 2004). Patient follow-up time is five years, with a clinical examination every 3–6 months during the first two years and yearly thereafter. Routine radiologic screenings offer no survival benefit (Leppä et al., 2017; Petrausch et al., 2010).

### 2.2.4 Prognosis

Adding rituximab to therapy has significantly improved prognosis (Pfreundschuh et al., 2006). Currently the average 5-year, progression-free survival (PFS) and 5-year overall survival (OS) rates are 60% and 65%, respectively (Swerdlov et al., 2017). High IPI (Sehn & Gascoyne, 2015), large tumour diameter (> 10 cm) (Pfreundschuh et al., 2008), male gender (Müller et al., 2012), concordant BM involvement (Sehn et al., 2011), elevated serum free light chains (Maurer et al., 2011), monoclonal gammapathies (Y. Zhang, Wei, Li, Gao, & Liu, 2018), vitamin D deficiency (Drake et al., 2010), low absolute lymphocyte/monocyte count
(Wilcox et al., 2011), and low body mass index (Carson et al., 2012) contribute to a dismal outcome.

*Curative aimed first-line treatment regimens of diffuse large B-cell lymphoma (DLBCL)*

- **De novo DLBCL**
  - Age < 60 – 65
    - International prognostic index 0-1
      - R-CHOP21 x 6
    - International prognostic index 2-5
      - R-CHOEP14 x 6, consider CNS-prophylaxis
  - Age > 60 – 65
    - International prognostic index 0-1
      - R-CHOP21 x 6-8 or R-mini-CHOP
    - International prognostic index 2-5
      - R-CHOP21 x 8

- **Refractory DLBCL**
  - All patients regardless of age and international prognostic index
    - R-ICE x 2, R-DHAP x 2 or R-GDP x 2 followed by HDT and ASCT (if CR/PR)

- **Relapsed DLBCL**
  - Age < 60 – 65 regardless of international prognostic index
    - R-DHAP x 2 (-8), R-ICE x 2 (-8) or R-GDP x 2 (-8) followed by HDT and ASCT (if CR/PR)
  - Age > 60 – 65 regardless of international prognostic index
    - R-GEMOX x 2 (-8), R-GDP x 2 (-8) or R-MINE x 2 (-8) followed by HDT and ASCT (if CR/PR)

IPI, International prognostic index; R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone); R-CHOEP (R-CHOP with etoposide); R-ICE (rituximab, ifosfamide, carboplatin, etoposide); R-DHAP (rituximab, dexamethasone, high-dose cytarabine, and cisplatin); R-GDP (rituximab, gemcitabine, cisplatin, and dexamethasone); R-GEMOX (rituximab, gemcitabine, and oxaliplatin); R-MINE (rituximab, mesna, ifosfamide, mitoxantrone, and etoposide); HDT, high-dose therapy; ASCT, autologous stem-cell transplantation; CR, complete response; PR, partial response
Primary refractory and relapsed disease present treatment challenges, because they more often display resistance to chemotherapy, leading to a worse prognosis with 5-year OS being 20%. Over 60% of these patients are ASCT ineligible (Sarkozy & Sehn, 2018). Tools to detect high-risk patients for more intensive chemotherapy and CNS prophylaxis are insufficient (Sehn & Gascoyne, 2015).

The impact of immunophenotype and genetics on prognosis is lacking and partly controversial, although COO is established as an important predictive factor (Read et al., 2014; Swerdlow et al., 2017). Other markers include single-hit DLBCL (translocation of BCL2, BCL6 or MYC) (S. L. Barrans et al., 2003; S. Barrans et al., 2010; S. L. Barrans et al., 2002), double-expression of MYC and BCL2 (Sarkozy, Traverse-Glehen, & Coiffier, 2015), CD5 (Chuang et al., 2015), CD30 (Hao et al., 2015), TP53 (Xu-Monette et al., 2012), CDKN2A (Jardin et al., 2010), FOXO1 (Trinh et al., 2013), PDL1 (Kiyasu et al., 2015), stromal signature (Lenz et al., 2008), and microRNA profile (Alencar et al., 2011; Song et al., 2014). Most of these markers confer with aggressive behaviour and inferior outcome. However, their mutual relationship is not established and thus they are not included in routine diagnostics.

### 2.3 Oxidative stress

Reactive oxygen species (ROS) are chemically reactive oxygen compounds that are formed during aerobic metabolism. Common ROS include hydrogen peroxide (H$_2$O$_2$), superoxide anion (O$_2^-$), and hydroxyl radicals (OH$^-$). They are involved in so-called redox signalling that has relevance to immune response and cell growth. Generally, high levels of ROS are harmful, as cellular DNA, proteins and lipids can be damaged. Oxidative stress refers to an unbalanced situation in which ROS are generated to an extent that exceeds the system’s ability to detoxify reactive intermediates (Schieber & Chandel, 2014).

Oxidative stress plays a role in many diseases, including cancer (Szatrowski & Nathan, 1991; Weinberg et al., 2010), cardiovascular disease (Mills, Weiss, Lang, Liu, & Ziegler, 2000; Uchida, 2000), neurodegenerative disorders (Allan Butterfield, Castegna, Lauderback, & Drake, 2002; Jenner et al., 2003), respiratory diseases (Van Der Vliet et al., 2000; Wood, Gibson, & Garg, 2003), diabetes (Hermo et al., 2005; Slatter, Bolton, & Bailey, 2000), autoimmune diseases (Coaccioli et al., 2009; Pérez, Pérez, Rita de Cássia M. Netto, de Lima, D. S. N., & Lima, 2012), obesity (Manna & Jain, 2015) and, possibly, in aging (Schieber & Chandel, 2014).
2.3.1 Oxidative stress markers

Oxidative stress markers are biological substances that result in oxidative reaction with ROS. High levels of markers implicate oxidative stress and damage to cellular structures. These markers are generally used to evaluate oxidative stress, because ROS have shorter half-lives and are harder to measure (Frijhoff et al., 2015).

There are no officially standardised oxidative stress markers. The choice of marker and measurement method is case dependent. Notable choice aspects are in vivo/in vitro nature, system of interest (cell – tissue – organism), molecules of interest and available biological material. Widely used oxidative stress markers include malondialdehyde (lipid), acrolein (lipid), thiobarbituric acid reactive substances (protein), carbonyls (carbohydrate), nitrotyrosine (carbohydrate) and 8-hydroxy-2-deoxyguanosine (8-OHdG) (nucleic acid) (Czerska, Mikolajewska, Zielinski, Gromadzinska, & Wasowicz, 2015; Frijhoff et al., 2015). Allantoin and isoprostanes have been proposed as promising new oxidative stress markers (Czerska et al., 2015).

2.3.2 Antioxidant response

A cell’s natural defences against oxidative stress include antioxidant enzymes (AOE) and various other molecules that prevent the accumulation of ROS. AOEs participate in redox signalling, metabolism, cell cycle and immunity in addition to their antioxidant role. Gene knockdown of AOEs predispose to many oxidative, stress-related ailments (Lei et al., 2015).

Central AOEs are hydrogen peroxide degrading catalase (CAT), glutathione peroxidase (GPX), and peroxiredoxin families (Prx), as well as the superoxide anion-scavenging superoxide dismutase (SOD) family. These families utilise other smaller AOEs and molecules such as thioredoxin (Trx), thioredoxin reductase (TrxR), methionine sulfoxide reductase (Msr), glutaredoxin (Grx), and glutathione (GSH) (Lei et al., 2015) to maintain a functional, reduced state.

Nuclear factor erythroid 2-related factors (Nrf2), and especially Nrf2, are principal inducers of the antioxidant system and regulate basal state production of AOEs (Huang, Li, Su, & Kong, 2015). Nrf2 is marked for degradation in cytoplasm by Kelch ECH associating protein 1 (Keap1) in the absence of oxidative insults. In stress conditions, Keap1 is oxidized, and Nrf2 translocates to the nucleus to exert antioxidant response. The Nrf family is related to the BTB (BR-C, ttk and bab) domain and the CNC homolog (Bach) family that repress Nrf function (Ma, 2013).
Both Nrf2 and Bachs form heterodimers with small musculoaponeurotic fibrosarcoma (Maf) proteins to bind target DNA (Katsuoka & Yamamoto, 2016).

The nuclear factor-kappa B (NF-κB) is an effector of a widely expressed pathway that is activated upon inflammation and immune response. It has canonical and non-canonical pathways. The former can be activated through toll-like receptors, T-cell receptor, B-cell receptor and tumor necrosis factor receptor. The latter participates in lymphoid organogenesis. ROS is a potent stimulator of NF-κB. Many studies have shown an antagonizing relationship between Nrf2 and NF-κB pathways (Buelna-Chontal & Zazueta, 2013). Nrf2 is dominant when oxidative stress is moderate and is replaced by NF-κB in more severe stress (Woods et al., 2009).

### 2.3.3 Oxidative stress in cancer

Sustained proliferation, replicative immortality, resistance to cell death, evasion from growth suppression, angiogenesis, and invasion are listed as hallmarks of cancer, underlined by genomic instability and inflammation (Hanahan & Weinberg, 2011). Aberrant redox signalling can affect all these processes and has indeed been recorded in many types of neoplasms (Sosa et al., 2013) including breast cancer (Matsui et al., 2000; Rothé et al., 2011), lung cancer (Singh et al., 2006), hepatocellular carcinoma (Rothé et al., 2011), prostate cancer (Lim et al., 2005), melanoma (Govindarajan et al., 2007), colon cancer (Vizán et al., 2009), pancreatic cancer (Y. Zhang et al., 2013), renal cell carcinoma (Ganesamoni et al., 2012), and glioblastoma (Kim et al., 2015). Oxidative stress can contribute to both tumour initiation and promotion. In some studies, AOEs and Nrf2 associate with tumour inhibition and better survival, which suggest a more complex interplay (Sosa et al., 2013).

Due to rampant proliferation and increased metabolism, intracellular ROS levels inevitably rise, leading to oxidative stress and even further genomic instability in cancer cells. Coping with this challenge incurs a selection pressure. Resistance to oxidative stress is often gained through Nrf2-mediated AOE overexpression and with metabolic shift to more glycolytic direction from oxidative phosphorylation (OXPHOS) (Sosa et al., 2013). Cancer stem cells, a type of treatment-resilient cell subpopulation responsible for disease relapse, are postulated to be especially glycolytic and possess extensive antioxidant machinery (Diehn et al., 2009).
Rapid growth and inadequate blood supply lead to hypoxia, which activates a hypoxia-inducible factor pathway to promote anaerobic metabolism and angiogenesis (Sosa et al., 2013). Sustained hypoxia leads to mitochondrial dysfunction and ROS production (Chandel et al., 1998; Guzy et al., 2005). Additional ROS is generated through reoxygenation cycles and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases. Concurrent cell damage and cell death are part of the tumour inflammation, which contributes a special tumour microenvironment (Sosa et al., 2013).

Constant ROS production and AOE overexpression can lead to activation of multiple, aberrantly functioning, tumour-enhancing downstream pathways, such as mechanistic target of rapamycin (mTOR) (Heberle et al., 2015), Rat sarcoma (Ras) (Yagoda et al., 2007), and human telomerase reverse transcriptase (hTERT) (Indran, Hande, & Pervaiz, 2010).

### 2.3.4 Oxidative stress in diffuse large B-cell lymphoma

Oxidative stress markers and AOEss have been studied in different lymphomas and have been associated especially with DLBCL in comparison to reactive lymph nodes and more indolent types of lymphoma (Pasanen et al., 2012). Their presence is often linked with unfavourable clinical presentation, such as B-symptoms, higher IPI, and poor prognosis (Table 3). Expression of Nrf2 and Keap1 were also found to correlate with high IPI in a small pilot study (Yi et al., 2018).

The relation of oxidative stress to COO and other biological factors is not clearly delineated. Trx expression has been previously associated with non-GCB phenotype (Peroja et al., 2012) and NF-κB upregulation with ABC subset (Compagno et al., 2009). Based on genome-wide gene expression arrays, two metabolically distinct DLBCL groups have been recognized: DLBCL that derives energy mainly from oxidative phosphorylation (OXPHOS-DLBCL) and glycolytic DLBCL with upregulation of B-cell receptor signalling (BCR-DLBCL). Metabolomics seem to be independent from COO. OXPHOS-DLBCL preferably utilises fatty acids and expresses MnSOD with an abundant GSH pool. BCR-DLBCL is characterised by BCR signalling and constant utilization of anaerobic glycolysis. The level of oxidative stress is attested to be greater in BCR-DLBCL. These insights may have therapeutic implications in the future (Caro et al., 2012).
Table 3. Oxidative stress markers and antioxidant enzymes in diffuse large B-cell lymphoma.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Method</th>
<th>Adverse clinical presentation</th>
<th>Adverse prognosis</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-OHdG</td>
<td>LN/IHC</td>
<td>Yes</td>
<td>Yes</td>
<td>(Pasanen et al., 2012; Peroja et al., 2012)</td>
</tr>
<tr>
<td>2-hydroxybutyrate</td>
<td>BS/NMRS</td>
<td>-</td>
<td>Yes</td>
<td>(Stenson et al., 2016)</td>
</tr>
<tr>
<td>Nitrotyrosine</td>
<td>LN/IHC</td>
<td>Yes</td>
<td>Yes</td>
<td>(Pasanen et al., 2012; Peroja et al., 2012)</td>
</tr>
<tr>
<td>γ-GCS</td>
<td>LN/IHC</td>
<td>-</td>
<td>-</td>
<td>(Pasanen et al., 2012)</td>
</tr>
<tr>
<td>GCL</td>
<td>LN/IHC</td>
<td>-</td>
<td>Yes</td>
<td>(Peroja et al., 2012)</td>
</tr>
<tr>
<td>MnSOD</td>
<td>LN/IHC</td>
<td>-</td>
<td>-</td>
<td>(Pasanen et al., 2012; Peroja et al., 2012)</td>
</tr>
<tr>
<td>Trx</td>
<td>LN/IHC</td>
<td>-</td>
<td>Yes</td>
<td>(Pasanen et al., 2012; Peroja et al., 2012)</td>
</tr>
<tr>
<td>GPX1</td>
<td>LN/DM</td>
<td>-</td>
<td>Yes</td>
<td>(Andreadis et al., 2007)</td>
</tr>
<tr>
<td>GPX2-3</td>
<td>LN/DM</td>
<td>-</td>
<td>-</td>
<td>(Andreadis et al., 2007)</td>
</tr>
<tr>
<td>GPX4</td>
<td>LN/DM, LN/IHC</td>
<td>-</td>
<td>Yes</td>
<td>(Andreadis et al., 2007; Kinowaki et al., 2018)</td>
</tr>
<tr>
<td>GST1-4</td>
<td>LN/DM</td>
<td>-</td>
<td>-</td>
<td>(Andreadis et al., 2007)</td>
</tr>
<tr>
<td>Prx1-5</td>
<td>LN/IHC</td>
<td>-</td>
<td>-</td>
<td>(Kuusisto et al., 2015)</td>
</tr>
<tr>
<td>Prx6</td>
<td>LN/IHC</td>
<td>Yes</td>
<td>Yes</td>
<td>(Kuusisto et al., 2015)</td>
</tr>
</tbody>
</table>

8-OHdG, 8-hydroxy-2-deoxyguanosine; γ-GCS, γ-glutamylcysteine synthetase; GCL, glutamate-cysteine ligase; MnSOD, manganese superoxide dismutase; Trx, thioredoxin; GPX, glutathione peroxidase; GST, glutathione S-transferases; Prx, peroxiredoxin; LN, lymph node; IHC, immunohistochemistry; BS, blood serum; NMRS, Nuclear magnetic resonance spectroscopy; DM, DNA microarray
3 Aims of the present study

Introduction of rituximab to the standard regimen has significantly improved treatment outcomes in DLBCL, and the majority of the patients can be treated curatively. Chemorefractory disease still presents both a treatment challenge and a poor outcome. IPI has been in clinical use since 1993 and is still the foremost prognostic tool. The need exists for more accurate predictive markers and risk stratification to identify high-risk patients who would benefit from more aggressive, first-line therapies. Antioxidant enzymes have recently garnered more studies in DLBCL, but their causal role in disease biology and treatment response should be further explored.

The aims of the present study were to elucidate the clinical significance and prognostic implications of antioxidant enzymes and their upstream regulators in DLBCL. Based on previous findings, the expression of Trx, Prx6, 8-OHdG, Nrf1, Nrf2, Keap1 and Bach1 were the focus of the present study. Trx and Prx6 are antioxidant enzymes that protect cells from oxidative stress. Nrf1, Nrf2, Keap1 and Bach1 regulate antioxidant enzyme production and 8-OHdG is an oxidative stress associated marker that results from DNA damage.

Specific aims of the study were to:
1. investigate the effect of lymphoma on Prx6 and 8-OHdG serum levels and their clinical correlations (I).
2. investigate the immunohistochemical expression of Trx, Prx6, 8-OHdG, Nrf1, Nrf2, Keap1 and Bach1 in DLBCL and their relation to each other in DLBCL (I-III).
3. assess the effects of Prx6, 8-OHdG, Nrf1, Nrf2, Keap1 and Bach1 on clinical disease presentation and prognosis in cancer tissue and blood serum (Prx6, 8-OHdG) (I-II).
4. evaluate the causal role of Trx expression in lymphoma cells and their chemosensitivity and how the Trx expression can be used as a predictive marker in DLBCL for different chemotherapeutics (III)
4 Materials and methods

The present study comprised three publications. All works were done retrospectively during the years 2015 – 2019.

4.1 Patient material

The study included 229 DLBCL patients, 5 PCNSL patients and 20 healthy controls. Patients were treated during years 1991 - 2017 in Oulu University Hospital, Kuopio University Hospital and North Karelia Central Hospital. First-line regimens consisted principally of R-CHOP and R-CHOEP for DLBCL and blood brain barrier disruption (BBBD)-based immunochemotherapy (Kuitunen et al., 2017) for PCNSL, excluding one patient who received CHOD/BVAM chemotherapy (Laack et al., 2011).

Work I included 53 patients with DLBCL, 5 patients with PCNSL, and 20 healthy controls who had diagnostic blood serum samples available. Tumour and blood serum expression of Prx6 and 8-OHdG were correlated between each other and compared to controls.

Work II comprised 76 high-risk DLBCL patients with DLBCL who were treated with first-line R-CHOEP regimen. Their diagnostic biopsy samples were immunostained with Nrf1, Nrf2, Keap1 and Bach1 antibodies.

Work III comprised two DLBCL patient cohorts. Cohort I included 37 patients treated with etoposide containing a high-dose regimen. Cohort II had 63 patients who received the R-CHOEP-regimen. Patients from work II were used as cohort II in this work. Diagnostic tumour biopsies were stained for Trx (Table 4), and results were compared with treatment outcomes. Small interfering ribonucleic acid (siRNA)-mediated knockdown of Trx was also performed in a DLBCL cell culture model.

Table 4. Methods and materials.

<table>
<thead>
<tr>
<th>Article</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunohistochemistry</td>
<td>x¹</td>
<td>x²</td>
<td>x³</td>
</tr>
<tr>
<td>ELISA</td>
<td>x¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell culture</td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Statistical analysis</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>

¹Prx6 and 8-OHdG; ²Nrf1, Nrf2, Keap1 and Bach1; ³Trx
4.2 Immunohistochemistry

Representative tumour tissue samples were fixed in formalin and embedded in paraffin. Sections of 3 µm were cut from the paraffin blocks and placed on SuperFrostPlus glass slides (Menzel-Gläser, Braunschweig, Germany). The slides were incubated at +37°C overnight before deparaffinisation in a clearing agent, Histo-Clear (National Diagnostics, Atlanta, GA, USA), and rehydration in a descending ethanol series. Antigen retrieval was performed in a microwave oven using a 10 mM sodium citrate buffer, pH 6 or a Tris-EDTA buffer, pH 9. Slides were cooled at room temperature for 20 minutes before incubation in a 3% H₂O₂ solution for 5 minutes to block the endogenous peroxidase activity. Staining was continued using the Dako REAL™ EnVision™ Detection System (Dako Denmark A/S, Glostrup, DK) according to the manufacturer’s instructions. Primary antibody incubations were performed as Table 5 outlines. Diaminobenzidine (DAB) was used to detect the immunoreaction, and nuclei were immunostained with Mayer’s haematoxylin (Reagena, Toivola, Finland). Finally, slides were dehydrated and mounted with Histomount (National Diagnostics, Atlanta, GA, USA). All washes between different steps were performed with phosphate buffered saline (PBS) with 0.05% Tween-20.

Analysis of immunohistochemical stainings was performed by three different haematopathologists blinded to clinical data. Immunoreactivity was evaluated separately in cytoplasm and nuclei. Intensity was categorised as negative, low and strong. Modified histoscore (H-score) was used for statistical analysis. H-score was used to divide the patient population into two (low- and high-expression) groups based on the overall mortality-associated receiver operating characteristic (ROC) curve. In work II, staining results were combined pairwise in the survival analysis.

4.3 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was used to measure the blood serum levels of Prx6 and 8-OHdG. The kits used were LF-EK0206 (AbFrontier, Seoul, South Korea) for Prx6 and the Highly Sensitive 8-OHdG Check kit, KOG-HS10E (JaICA, Shizuoka, Japan), for 8-OHdG. ELISAs were performed according to the manufacturer’s instructions.
Table 5. Antibodies and immunostaining methods.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Microwave buffer</th>
<th>Concentration</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prxd6 (LF-MA0018), AbFrontier, Seoul, South Korea</td>
<td>sodium citrate buffer, pH 6</td>
<td>1:6000</td>
<td>0.5 h at room temperature</td>
</tr>
<tr>
<td>8-OHdG (MOG-100P), JaICA, Nikken SEIL Co., Ltd., Shizuoka, Japan</td>
<td>sodium citrate buffer, pH 6</td>
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<td>Nrf1 (HPA065424), Sigma Aldrich, Darmstadt, Germany</td>
<td>Tris-EDTA buffer, pH 9</td>
<td>1:500</td>
<td>overnight at +4°C</td>
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<tr>
<td>Nrf2 (ab76026), Abcam plc., Cambridge, UK</td>
<td>sodium citrate buffer, pH 6</td>
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<td>KEAP1 (NBP2-37431), Novus Biologicals, Oxford, UK</td>
<td>sodium citrate buffer, pH 6</td>
<td>1:100</td>
<td>overnight at +4°C</td>
</tr>
<tr>
<td>BACH1 (NBP2-01904), Novus Biologicals, Oxford, UK</td>
<td>Tris-EDTA buffer, pH 9</td>
<td>1:100</td>
<td>1 h at room temperature</td>
</tr>
<tr>
<td>Human Thioredoxin, product number 70, American Diagnostica Inc., Stamford, CT, USA</td>
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<td>overnight at +4°C</td>
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<td>m/w Tris-EDTA, pH 9</td>
<td>1:400</td>
<td>1 h at room temperature</td>
</tr>
</tbody>
</table>

4.4 Cell culture

4.4.1 Cell lines

Two commercial human DLBCL cell lines, CRL-2630™ and CRL-2632™ (American Type Culture Collection, Manassas, USA), were cultured in RPMI-1640 medium (HyClone Laboratories Inc., Utah, USA), including a 10% fetal bovine serum (FBS) (HyClone UK Ltd., Northumberland, UK), streptomycin at 0.1 mg/ml (HyClone Laboratories Inc., Utah, USA), penicillin at 100 units/ml (HyClone Laboratories Inc., Utah, USA), 1 mM sodium pyruvate (HyClone Laboratories Inc., Utah, USA) and 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid) (HyClone Laboratories Inc., Utah, USA) at +37 °C. Approximate cell confluence was 1.0 × 10⁶ cells/ml.

4.4.2 Transient transfection with siRNA

Sets of 10 × 10⁶ cells were counted with a Bürker chamber (Marienfeld Superior, Lauda-Königshofen, Germany) for siRNA-mediated Trx knockdown with
electroporation. Cells were washed three times with PBS (HyClone Laboratories Inc., Utah, USA) and resuspended into 388 μl of Gene Pulser electroporation buffer (Bio-Rad Laboratories, Inc., USA). 0.6 μM Trx–specific Silencer® Select siRNA, Silencer® scrambled negative control siRNA or Silencer® CyTM3–labelled glyceraldehyde 3-phosphate dehydrogenase (GAPDH) siRNA (all from Ambion, Inc., Austin, USA) was added. Eventual reaction volume was 400 μl. Suspensions were placed in 0.4 cm Gene Pulser® cuvettes (Bio-Rad Laboratories, Inc., USA) and incubated for 20 min at RT. Gene Pulser Xcell™ Electroporation System (Bio-Rad Laboratories, Inc., USA) was used for electroporation with square wave protocol setting: 10 pulses, voltage 250 V, pulse duration 10 ms, and pulse interval 1 s. Cells were then incubated 20 min at RT and transferred to antibiotic-free 20% FBS RPMI-1640 on 6-well plates.

### 4.4.3 Western blotting

Cells were lysed at three time points (24 h, 48 h and 72 h). Samples were washed with PBS and resuspended with 70 μl cell lysis buffer (Cell Signaling Technology, Danvers, USA), including 1 mM phenylmethylsulphonyl fluoride (PMSF) (Sigma-Aldrich, St.Louis, USA). After 5 min incubation at +4 °C, cells were vortexed and centrifuged to harvest samples. Lysates were stored at -70 °C. Protein concentrations were assayed according to the manufacturer’s instructions (Bio-Rad DCMTM Protein Assay, Bio-Rad Laboratories, Inc., USA).

10 μg of protein samples were added 1:1 to Laemmli Sample Buffer (Bio-Rad Laboratories, Inc., USA) with 2.5 μl β-mercaptoethanol (Sigma-Aldrich, St. Louis, USA). Bio-Rad Mini-PROTEAN® Tetra Cell system (Bio-Rad Laboratories, Inc., USA) was used in electrophoresis. Samples were loaded to 15% SDS-gel that was run at 100 V for 90 min in TGS buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). The gel was plated with a PolyScreen® PVDF Transfer Membrane (Perkin Elmer, USA) using Bio-Rad Criterion™ Blotter (Bio-Rad Laboratories, Inc., USA) at 100 V for 60 min at +4 °C in 20% ethanol TG buffer (25 mM Tris, 192 mM glycine). After blocking, the membrane was incubated with Trx rabbit monoclonal antibody C63C6 (Cell Signaling Technology, Danvers, USA) overnight (o/n) at +4 °C. Secondary antibody incubation was performed with horseradish peroxidase (HRP)-linked anti-rabbit IgG antibody (Cell Signaling Technology, Danvers, USA) for 60 min at RT. Washes were done between all phases. Lastly, Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, Billerica, USA) was applied to the membrane. Comparability of protein contents was assessed with β-actin mouse
monoclonal antibody (Cell Signaling Technology, Danvers, USA). Expression results were evaluated with ImageJ1 software (http://imagej.net/ImageJ).

### 4.4.4 Fluorescence-activated cell sorting

After transfection with GAPDH siRNA or water for control, live cells were detected with Trypan Blue (Fluka AG, Switzerland), and 2 million cells were washed with PBS. Cells were resuspended into 1% FBS PBS, and 7-AAD antibody (Becton Dickinson Pharmingen™, New Jersey, USA) was used to separate dead cells. Cells were then incubated for 10 min at +4 °C before fluorescence-activated cell sorting (FACS) analysis (FACS Calibur™, Becton Dickinson Pharmingen™, New Jersey, USA), which was performed on three separate occasions.

### 4.4.5 Chemotherapeutic agent testing

Cells were plated 10 x 10³ cells per well on 96-well plates after transfection. Wells had antibiotic-free 10% FBS RPMI-1640 media. Chemotherapeutic agents were added at 48 h after transfection in three different concentrations (four for doxorubicin). Agents were doxorubicin (Pfizer, Kirkland, Quebec, Canada), etoposide (Bristol-Myers Squibb, Bromma, Sweden), vincristine (Cellpharm GmbH, Hannover, Germany), carboplatin (Teva Nordic, Helsingborg, Sweden) and prednisolone (ACE Pharmaceuticals BV, Zeewolde, Netherlands). The test was repeated twice using five parallel samples for each agent. Mean values were recorded for analysis. Actual cell death was the difference between the untreated control cell line and the treated cell line. Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) was used for cell death evaluation according to the manufacturer’s instructions. 12 h after agent adding, the live cell count was measured spectrophotometrically at 450 nm.

### 4.5 Statistics

IBM SPSS Statistics V23 for Windows (IBM, Armonk, NY, USA) was used for the statistical analysis. Student’s t-test and Mann-Whitney U test were used in analysis of continuous variables based on distribution. P-values of < 0.05 were considered statistically significant.

Overall survival (OS) was calculated from the date of diagnosis to the date of death or last follow-up date. Disease-specific survival (DSS) was calculated from
the date of diagnosis to the date of lymphoma-specific death or last follow-up, and relapse-free survival (RFS) was calculated from the time of diagnosis to the time of relapse. The Kaplan-Meier log-rank test was used in survival estimates. IPI and GC phenotype were included in the subsequent, multivariate analyses with immunohistochemical results in Cox regression analysis.

4.6 Ethical aspects

This study was approved by the Ethics Committee of Northern Ostrobothnia Hospital District (11/2014) and by the National Supervisory Authority for Welfare and Health (Valvira 6622/05.01.00.06/2010). Work was conducted in accordance with The World Medical Association Declaration of Helsinki ethical principles.
5 Results

5.1 Patient demographics

Table 6 presents patient demographics. Due to aggressive first-line therapy (R-CHOP) in works II and III, the majority of patients were under 60 years at the time of diagnosis. Main clinical study findings are also represented in table 7 with odds ratios (OR) if eligible.

Table 6. Patient demographics.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>I (n%), n = 58</th>
<th>II (n%), n = 76</th>
<th>III (n%), n = 99</th>
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<tr>
<td></td>
<td>Cohort I, n = 36</td>
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<td>Diagnosis</td>
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<tr>
<td>GC DLBCL</td>
<td>28/36</td>
<td>24/32</td>
<td>15/40</td>
</tr>
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<td>Non-GC DLBCL</td>
<td>14/18</td>
<td>32/42</td>
<td>14/38</td>
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<tr>
<td>Other DLBCL</td>
<td>11/14</td>
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<td>8/22</td>
</tr>
<tr>
<td>PCNSL</td>
<td>5/6</td>
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<tr>
<td>Control</td>
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<tr>
<td>Median age (years)</td>
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<tr>
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</tr>
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<td>6/17</td>
</tr>
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<td>39/51</td>
<td>8/22</td>
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<tr>
<td>DM</td>
<td>1/2</td>
<td>4/5</td>
<td>29/56</td>
</tr>
</tbody>
</table>
5.2 Prx6 (I)

Cytoplasmic Prx6 expression in lymphoma cells was found in all DLBCL and all five PCNSL samples. There was no nuclear Prx6 expression (Figure 5). Strong cytoplasmic Prx6 expression in cancer cells was associated with extranodal involvement \( (p = 0.039) \) and high IPI score \( (3–5) \) \( (p = 0.035) \). Pre-treatment median blood serum levels of Prx6 were 10.69 ng/ml in DLBCL patients, 8.10 ng/ml in PCNSL patients and 20.94 ng/ml in controls \( (p < 0.001) \). Prx6 serum levels < 14 ng/ml was a statistically significant risk factor for neutropenic fever \( (p = 0.025, \text{OR } 8.615 [1.032–71.933]) \) (Figure 4). Nine months after treatment, Prx6 level among patients was 19.55 ng/ml \( (n = 24) \) compared to an initial 10.69 ng/ml \( (n = 58) \) \( (p = 0.006) \). There were no statistically significant survival associations with Prx6.
Fig. 4. Patients according to Prx6 levels and neutropenic infections. * Fisher’s exact test.

5.3 8-OHdG (I)

8-OHdG was detected in DLBCL and PCNSL cancer cells in nuclei with 80% median expression and to a lesser extent in cytoplasm (Figure 5). Median serum levels of 8-OHdG were 3.35 ng/ml in DLBCL patients, 2.93 ng/ml in PCNSL patients and 2.95 ng/ml in controls (p = 0.048). High Prx6 and 8-OHdG serum values correlated with each other (p = 0.025). Strong nuclear 8-OHdG expression was negatively associated with the presence of 8-OHdG in cytoplasm (p = 0.036) and with low 8-OHdG serum levels (p = 0.015). 8-OHdG had no significant associations with disease presentation or survival.
Fig. 5. Sample stainings from four different DLBCL patients. Clockwise from top left: weak cytoplasmic Prx6 expression, strong cytoplasmic Prx6 expression, strong nuclear 8-OHdG expression and weak nuclear 8-OHdG expression.

5.4 Nrf1 (II)

Nrf1 was detected in cancer tissue both in cytoplasm and in nuclei (Figure 6). Cytoplasmic expression of Nrf1 was correlated with cytoplasmic expression of Keap1 (p = 0.021). There was also a non-significant (p = 0.064), positive correlation between nuclear expressions of Nrf1 and Nrf2. High cytoplasmic expression of Nrf1 in lymphoma cells was linked with the absence of B-symptoms and high nuclear expression of Nrf1 with neutropenic infection after the first treatment cycle. High cytoplasmic expression and low nuclear expression of Nrf1 in lymphoma cells were also associated with poor overall survival (Figure 7).
5.5 Nrf2 (II)

Nrf2 was also seen in both cytoplasm and nuclei in DLBCL cells. The expression pattern of Nrf2 was similar to Nrf1’s (Figure 6). Cytoplasmic Nrf2 expression was associated with favourable clinical features (no B-symptoms, limited stage disease) in contrast to nuclear Nrf2 expression (high IPI score, neutropenic infection, low haemoglobin). High nuclear expression of Nrf2 correlated also with inferior overall survival (Figure 7). In Cox regression analysis, combined low nuclear Nrf1 and high nuclear Nrf2 expression was an independent prognostic factor ($p = 0.036$, OR 4.269 [1.097–16.608]) from IPI and GC-phenotype.

5.6 Keap1 (II)

Keap1 was expressed only in cytoplasm (Figure 6) in cancer tissue. There were no significant associations with disease presentation. High cytoplasmic expression of Keap1 was linked with favourable overall survival in combination with either low cytoplasmic Nrf1 expression or low nuclear Nrf2 expression (Figure 7). Combined high cytoplasmic Nrf1 and low cytoplasmic Keap1 expression was an independent prognostic factor ($p = 0.033$, OR 3.927 [1.116–13.821]) from IPI and GC-phenotype.

5.7 Bach1 (II)

Bach1 was recorded only in nuclei in 33% of cases (Figure 6) in cancer cells. The only statistically significant finding was a positive correlation with advanced stage (III-IV) disease.
Fig. 6. Immunohistochemical stainings from two patients. a-d: Biopsy represents non-GC DLBCL from lymph node. From upper left: Positive Bach1 expression, low cytoplasmic Keap1 expression, high cytoplasmic Nrf1 expression and high nuclear Nrf1 expression, positive cytoplasmic Nrf2 expression and high nuclear Nrf2 expression. e-h: Biopsy represents T-cell rich BCL from lymph node. From upper left: Negative Bach1 expression, high cytoplasmic Keap1 expression, high cytoplasmic Nrf1 expression and low nuclear Nrf1 expression, negative cytoplasmic Nrf2 expression and low nuclear Nrf2 expression.
Fig. 7. Overall survival charts. Survival according to (A) Nrf1 expression (p = 0.035), (B) Nrf2 and Keap1 expression (p = 0.035), (C) Nrf1 and Keap1 expression (p = 0.005) and (D) Nrf1 and Nrf2 expression (p = 0.012). c = cytoplasmic, n = nuclear.

5.8 Trx (III)

The immunohistochemical staining results of Trx were comparable in both patient cohorts. Trx expression in DLBCL cells was seen more often in cytoplasm, and nuclear expression of Trx did not attain any statistical significance. A strong cytoplasmic Trx expression was associated with age under 60 years (p = 0.032) in patient cohort II. Low cytoplasmic expression of Trx was associated with poor
overall survival, disease-specific survival and, non-significantly, with relapse-free survival (Fig 8). Results with patient cohort I were similar but not statistically significant.

Western blotting was performed at 24 h intervals in the Trx knockdown project. Trx activity was seen to decrease by 70% at 48 h and 80% at 72 h compared to a negative control. In chemotherapeutic agent testing, doxorubicin-induced cell death was observed to increase 4.2 – 11.5% (p < 0.001), while etoposide-induced cell death decreased 3.1 – 24.3% (p < 0.001) compared to control cells.

Fig. 8. Trx and survival. (A) Overall survival (p = 0.026), (B) disease-specific survival (p = 0.026) and (C) relapse-free survival (p = 0.076).
Table 7. Studied proteins and transcription factors with odds ratios.

<table>
<thead>
<tr>
<th>Study marker</th>
<th>Effect on clinical presentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>High cytoplasmic Prx6 expression</td>
<td>Extranodal disease [OR = 5.167 (1.134 - 23.548), p = 0.034]</td>
</tr>
<tr>
<td>Low Prex6 serum level</td>
<td>IPI 3-5 [OR = 5.333 (1.194 - 23.829), p = 0.038]</td>
</tr>
<tr>
<td>High cytoplasmic Nrf1 expression</td>
<td>Neutropenic infections [OR = 8.615 (1.032 - 71.933), p = 0.025]</td>
</tr>
<tr>
<td>High nuclear Nrf1 expression</td>
<td>Neutropenic infections [OR = 12.000 (1.433 - 100.514), p = 0.022]</td>
</tr>
<tr>
<td>High cytoplasmic Nrf2 expression</td>
<td>B-symptoms [OR = 0.096 (0.011 - 0.843), p = 0.034]</td>
</tr>
<tr>
<td>High nuclear Nrf2 expression</td>
<td>B-symptoms [OR = 0.286 (0.100 - 0.815), p = 0.019]</td>
</tr>
<tr>
<td>Low haemoglobin</td>
<td>Stage [OR = 0.149 (0.029 - 0.764), p = 0.022]</td>
</tr>
<tr>
<td>High nuclear Bach1 expression</td>
<td>Low haemoglobin [OR = 8.000 (1.537 - 41.637), p = 0.013]</td>
</tr>
<tr>
<td>High cytoplasmic Nrf1 and low nuclear Nrf1 expression</td>
<td>Neutropenic infections [OR = 3.349 (0.390 - 28.762), p = 0.271]</td>
</tr>
<tr>
<td>High nuclear Nrf2 and low cytoplasmic Keap1 expression</td>
<td>Stage [not eligible for logistic regression]</td>
</tr>
<tr>
<td>High cytoplasmic Nrf1 and low cytoplasmic Keap1 expression</td>
<td>Overall mortality [not eligible for logistic regression]</td>
</tr>
<tr>
<td>Low nuclear Nrf1 and high nuclear Nrf2 expression</td>
<td>Overall mortality [OR = 8.609 (1.1015 - 73.024), p = 0.048]</td>
</tr>
<tr>
<td>Low cytoplasmic Trx expression (cohort II)</td>
<td>Overall mortality [OR = 4.031 (1.237 - 13.142), p = 0.021]</td>
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<tr>
<td>Low cytoplasmic Trx expression (cohort II)</td>
<td>Overall mortality [OR = 0.140 (0.030 - 0.659), p = 0.013]</td>
</tr>
<tr>
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<td>Overall mortality [OR = 2.597 (0.689 - 9.797), p = 0.159]</td>
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<tr>
<td>Low cytoplasmic Trx expression (cohort II)</td>
<td>Disease-specific mortality [OR = 3.583 (0.779 - 16.491), p = 0.278]</td>
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<tr>
<td>Low cytoplasmic Trx expression (cohort II)</td>
<td>Relapse [OR = 2.026 (0.500 - 8.207), p = 0.323]</td>
</tr>
</tbody>
</table>
6 Discussion

6.1 Antioxidant enzymes and their regulators in diffuse large B-cell lymphoma

The implications of oxidative stress on unfavourable disease presentation and prognosis in DLBCL have been studied widely in the past. 8-OHdG, Trx and Prx6 have been associated with adverse disease outcomes in three different immunohistochemical retrospective studies with DLBCL (Kuusisto et al., 2015; Pasanen et al., 2012; Peroja et al., 2012). Based on previous observations, these markers in the present study were evaluated with additional methods, including blood serum levels with ELISA. Moreover, the causal association of Trx and adverse prognosis were studied in vitro with Trx knockdown in a cell culture model. Upstream regulators of the antioxidant enzymes, Nrf1, Nrf2, Keap1 and Bach1, were of special interest, because only one small previous study exists on Nrf2 and Keap1 expression in DLBCL (Yi et al., 2018).

Prx6 serum levels were found to be lower in DLBCL patients compared to healthy controls, while controls had lower levels in other studies with solid cancers (Liu, Wang, & Cao, 2014; Tang et al., 2012; X. Zhang et al., 2009). At a median nine months after treatment, serum expression Prx6 rose to comparable levels with the controls, indicating a peculiar disease phenomenon that might relate to tumour burden or immunology. High Prx6 and 8-OHdG serum values correlated with each other, suggesting a reciprocal relationship. However, serum levels of 8-OHdG among DLBCL patients, PCNSL patients and controls were essentially the same, albeit marginally higher in DLBCL. Low serum levels of Prx6 (< 14 ng/ml) were linked with neutropenic infections (OR 8.615), which can reflect susceptibility to treatment-related (oxidative stress) side effects. These patients might benefit from colony-stimulating factors as a prophylactic treatment. Due to the retrospective study setting, the causal role of Prx6 levels with treatment-induced neutropenia remained undetermined. Numerous studies, however, have also shown enhanced Prx6 expression following bacterial and other inflammatory stimuli (Ambruso, Ellison, Thurman, & Leto, 2012; Godahewa et al., 2016; Knoops, Argyropoulou, Becker, Ferté, & Kuznetsova, 2016; Priyathilaka et al., 2016).

Expression of all transcription factors in DLBCL cells had significant associations with either disease presentation or prognosis. Interestingly, the effect of Nrf1 and Nrf2 on overall survival was dependent on localization between
nucleus and cytoplasm in cancer tissue. Based on nuclear expression (transcriptional activity), Nrf1 was associated with better prognosis, in contrast to Nrf2 where results were largely the opposite. Nrf2 has previously been linked with adverse outcomes in various types of malignancies (Hiroshi Kitamura Hozumi Motohashi, 2018), while low expression of its principal cytoplasmic negative regulator, Keap1, has also been associated with a worse prognosis (Barbano et al., 2013; Hanada et al., 2012; Muscarella et al., 2011; R. Wang et al., 2008). Overexpression of Nrf2 in cancer can confer to chemoresistance (Ma et al., 2012) and immunosuppression (J. Wang, Liu, Xin, Wang, & Li, 2017). Many oncogenes also stimulate Nrf2 expression (Ma, 2013). In lymphoma cells, Nrf1 had some significant effects on overall survival in combination with Nrf2 or Keap1. It has been postulated that intracellular Nrf1 and Nrf2 compete for binding to target DNA, and Nrf1 could replace Nrf2 in the nucleus (Chepelev et al., 2013). Nrf1 interacts also with Keap1, but the significance of this interaction is unclear (Biswas & Chan, 2010). Tian et al. have recently suggested Keap1 as a positive regulator of Nrf1 (Tian, De La Vega, M R, Schmidlin, Ooi, & Zhang, 2018). Nuclear Nrf1 and Nrf2 expressions in DLBCL cells were both linked with higher risk for neutropenic infections. The exact cause of this association is unknown. However, Nrf2 has been shown to modulate innate immunity and to have anti-inflammatory effects (Kim, Cha, & Surh, 2010). Bach1 was seen in a minority of samples in cancer nuclei and was associated with advanced stage, which is in line with previous publications in which Bach1 has been linked with aggressive behaviour and chemoresistance (Davudian et al., 2016; Nie et al., 2016; Shajari et al., 2017; Zhu et al., 2018). The exact interplay of these transcription markers remains unknown, but they are all likely to affect expression and effects of each other, at least through Maf proteins (Katsuoka & Yamamoto, 2016).

Strong cytoplasmic Trx expression in lymphoma cells has previously been associated with inferior outcomes in DLBCL patients who received mainly the R-CHOP regimen (Peroja et al., 2012). Survival results were the opposite in our smaller, R-CHOEP-treated patient cohort II. High cytoplasmic Trx expression in cancer tissue related to superior OS and DSS. The results in patient cohort I showed a similar trend, although the difference was not statistically significant. Overall, these results should be appraised critically as OR was insignificant. Trx knockdown in the cell model sensitised the cells to doxorubicin-induced cell death, while the effect of etoposide decreased. In cancer, Trx can contribute to cell growth (Holmgren & Lu, 2010) and a metastasis cascade (T. Zhang et al., 2012). Trx protects cells from the toxic effects of ROS (Berghella, Pellegrini, Del Beato,
Ciccone, & Contasta, 2011; T. Zhang et al., 2012), while doxorubicin induces oxidative stress (Danesi, Fogli, Gennari, Conte, & Del Tacca, 2002). This appears to explain the sensitivity to doxorubicin in Trx knockdown cells. Trx thus seems to be in a causal role to doxorubicin resistance in DLBCL. Moreover, high expression of Trx appears to favour the use of etoposide in clinical settings. Trx could be used as a biomarker to select DLBCL patients for etoposide-containing treatment (R-CHOEP).

6.2 Limitations of the study

The study’s weaknesses included the retrospective nature and relatively small sample size in all works. This was seen especially in work II, in which immunohistochemical stainings had to be combined for statistical analysis. In work I, the number of follow-up serum samples was limited, which prevented further studies with other clinical characteristics. Moreover, Prx6 and 8-OHdG did not predict survival as has been seen in previous larger studies (Kuusisto et al., 2015; Pasanen et al., 2012; Peroja et al., 2012). The works included mainly patients with DLBCL, NOS, and THRLBCL. These are held in the current WHO classification to be biologically different disease entities even though treatment can be the same. Ultimately, aforesaid findings are correlations based on individual data only and would require another independent research data for validation.

6.3 Future prospects

To increase statistical power, possible upcoming works would benefit from utilisation of nationwide or even international patient data and lymphoma tissue samples. This could be achieved through biobanks and collaboration with local haematopathologist.

Follow-up works should expand and correlate current findings with expressions of other aforementioned antioxidant enzymes and Maf proteins. Nrf2 signaling appears to be closely connected to NF-xB pathway and its upstream regulator mTOR pathway. These pathways and metabolomics as a whole could help science community to further understand lymphoma biology and innovation of new remedies.

The most promising and causally proven transcription factors and proteins should be tested in cell models in vitro for gene knockout. Clustered regularly interspaced short palindromic repeats (CRISPR) technology is enabling gene
modification in way that could revolutionise gene therapy and cancer treatment in the current millennium.
7 Conclusions

The present work evaluated oxidative stress markers, antioxidant enzymes and their upstream regulators with immunohistochemistry and other methods. Oxidative stress has an integral role in DLBCL and is associated with several clinical features, including treatment response and prognosis. This is in line with previous findings with antioxidant enzymes and oxidative stress in DLBCL. These results are hypothesis generating and should be treated as such. It should be noted that only expressions of Nrf2 and Keap1 has been recorded in one previous study.

The study’s specific conclusions were:

1. Low serum levels of Prx6 are seen in patients with DLBCL and relate to the risk of neutropenic fever. 8-OHdG did not attain statistical significance regarding clinical presentation.
2. The serum level of Prx6 seems to be a potential marker for selecting DLBCL patients to receive colony-stimulating factors.
3. Lymphoma cells’ expressions of Nrf1, Nrf2, Keap1 and Bach1 appear to be related to each other.
4. Tissue expression of Nrf1, Nrf2 and Keap1 can probably predict overall survival in patients with DLBCL. The effect of Nrf1 and Nrf2 is dependent on nuclear localization in the cell.
5. High tumour tissue expression of Trx is associated with better survival in DLBCL patients treated with R-CHOEP but not with R-CHOP.
6. Trx seems to predict the treatment response to etoposide and could, thus, be used for patient selection regarding R-CHOEP.
References


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**Original publications**

I  

II  

III  

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Original publications are not included in the electronic version of the dissertation.
1542. Hynynen, Johanna (2019) Status epilepticus in mitochondrial diseases and the role of POLG1 variants in the valproic-acid induced hepatotoxicity

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