OULU 2017
D 1421

ACTA
UNIVERSITATIS OULUENSIS


*Siria Lemma*

# MIGRATION AND ADHESION ASSOCIATED MOLECULES IN LYMPHOMA BIOLOGY AND THEIR POTENTIAL ROLES AS BIOMARKERS

*UNIVERSITY OF OULU GRADUATE SCHOOL;*
*UNIVERSITY OF OULU,*
*FACULTY OF MEDICINE;*
*MEDICAL RESEARCH CENTER OULU;*
*OULU UNIVERSITY HOSPITAL*

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*SIRIA LEMMA*

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Academic dissertation to be presented with the assent of the Doctoral Training Committee of Health and Biosciences of the University of Oulu for public defence in Auditorium 7 of Oulu University Hospital, on 1 September 2017, at 12 noon

UNIVERSITY OF OULU, OULU 2017

Acta Univ. Oul. D 1421, 2017

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ISBN 978-952-62-1603-4 (Paperback)
ISBN 978-952-62-1604-1 (PDF)

ISSN 0355-3221 (Printed)
ISSN 1796-2234 (Online)

Cover Design
Raimo Ahonen

JUVENES PRINT
TAMPERE 2017

**Lemma, Siria, Migration and adhesion associated molecules in lymphoma biology and their potential roles as biomarkers.**

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

*Acta Univ. Oul. D 1421, 2017*

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***Abstract***

Lymphomas are a heterogeneous group of malignancies that arise from lymphatic tissues. Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoma sub-type. It is an aggressive malignancy with an increasing incidence. The prognosis of DLBCL has improved significantly, but problems also remain. The clinical significance of central nervous system (CNS) relapses has become increasingly important. As secondary CNSL (sCNSL) and primary CNS lymphoma (PCNSL) are known to have poor prognoses; the prevention of sCNSL is of crucial importance. Peripheral T-cell lymphomas (PTCL) are rare neoplasms and include several lymphoma subtypes that possess complex and also overlapping morphological and immunophenotypic characteristics. The identification of different entities has improved, but the biological knowledge remains scarce when compared to DLBCL. The optimal treatment schemas for PTCLs are still lacking and they have long been treated with the same therapies as B-cell lymphomas, mainly with suboptimal treatment results. The aim of this study was to identify poor prognostic markers in DLBCL and PTCLs and potential biological markers for the prediction of DLBCL CNS relapse. The study material included patients with systemic DLBCL without CNS affision (sDLBCL), sCNSL, PCNSL and PTCLs. The expression of epithelial-mesenchymal transition (EMT) transcription factors (TFs), chemokines and their receptors and adhesion-, migration- and inflammatory responses-associated molecules were studied by means of immunohistochemistry. IEM was used to verify the specific subcellular location of the studied molecules. GEP was performed on 12 PTCL samples in order to compare the poor prognosis group with the good prognosis group and on one sDLBCL and one sCNSL sample from the time of primary diagnosis. The EMT TFs were found to be expressed in both DLBCL and PTCLs, where they ultimately proved to have prognostic relevance as well. In PTCLs, these TFs were able to delineate a disease group with a specific gene-expression profile. CXCR4, CXCR5, ITGA10, PTEN and CD44 were found to be differently expressed between DLBCL cases with CNS affision when compared to those without CNS disease. These molecules seem to play a role in the development of CNS relapse and hopefully, if further verified, will lead towards the identification of biological markers for CNS relapse prediction.

*Keywords:* adhesion, central nervous system lymphoma, central nervous system prophylaxis, chemokine receptors, diffuse large B-cell lymphoma, epithelial-mesenchymal transition, migration, prognosis

**Lemma, Siria, Migraatioon ja adheesioon liittyvät molekyylit lymfoomien biologiassa ja niiden potentiaalinen rooli biomarkkereina.**

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

*Acta Univ. Oul. D 1421, 2017*
Oulun yliopisto, PL 8000, 90014 Oulun yliopisto

## *Tiivistelmä*

Lymfoomat ovat heterogeeninen ryhmä imukudossyöpiä, joista diffuusi suurisoluinen B-solulymfooma (DLBCL) on yleisin alatyyppi. Se on aggressiivinen maligniteetti, jonka insidenssi on noussut viime vuosina. DLBCL potilaiden ennuste on parantunut merkittävästi, mutta yhä osa potilaista menehtyy tautiinsa. DLBCL:n keskushermostorelapsin kliininen merkitys on tänä päivänä aiempaa suurempi. Sekundaarisen keskushermostolymfooman (sCNSL) ja primaarin aivolymfooman (PCNSL) ennusteet ovat nykyhoidoilla huonoja, joten keskushermostorelapsin ennaltaehkäiseminen on tärkeää. Perifeeriset T-solulymfoomat (PTCLs) ovat ryhmä harvinaisia neoplasioita, joka sisältää useita eri alatyyppejä, joiden morfologiset ja immunofenotyyppiset ominaisuudet ovat monimuotoisia ja osin päällekkäisiä. Eri entiteettien indentifiointi on parantunut, mutta PTCL:ien biologinen tietämys on yhä DLBCL:aa heikompaa. PTCL:ien optimaalinen hoito ei ole selvillä ja tätä tautiryhmää on pitkään hoidettu samoilla hoidoilla kuin DLBCL:aa, mutta huonommilla hoitotuloksilla. Tutkimuksen tavoitteena oli löytää huonon ennusteen markkereita, joilla myös pystyttäisiin ennustamaan DLBCL:n keskushermostorelapsia. Aineisto koostui DLBCL, sCNSL, PCNSL ja PTCL näytteistä. Immunohistokemiallisilla värjäyksillä tutkittiin epiteliaalis-mesenkymaalisen transition (EMT) transkriptiotekijöitä (TF), kemokiinireseptoreita sekä adheesioon-, migraatioon ja inflammaatioon assosioituja molekyylejä. Immunoelektronimikroskopialla varmennettiin molekyylien lokalisaatio soluissa. Geeniekspressioprofiloinnilla (GEP) verrattiin kahdentoista hyvän ja huonon ennusteen ryhmään kuuluvan PTCL näytteen välisiä geeniekspressioeroja sekä kahden DLBCL potilaan näytteitä, joista toiselle kehittyi keskushermostorelapsi. EMT TF:ien ekspressiota nähtiin DLBCL ja PTCL näytteissä, joissa niillä myös todettiin olevan ennusteellista merkitystä. PTCL:ssa TF:t pystyivät erottelemaan tautiryhmän, jolla oli oma spesifinen geeniekspressioprofiilinsa. CXCR4, CXCR5, ITGA10, PTEN ja CD44 ekspressio oli erilaista systeemisissä DLBCL tapauksissa verrattuna sCNSL tapauksiin. Edellä mainituilla molekyyleillä näyttää olevan oma roolinsa keskushermostotaudin kehittymisessä ja jos nämä tulokset pystytään vahvistamaan tulevissa tutkimuksissa, johtavat ne toivottavasti kohti keskushermostorelapsiriskin tarkempaa tunnistamista.

*Asiasanat:* adheesio, diffuusi suurisoluinen B-solulymfooma, ennuste, epiteliaalis-mesenkymaalinen transitio, kemokiinireseptorit, keskushermostolymfooma, keskushermostoprofylaksia, migraatio

# Acknowledgements

This work was carried out at the Department of Oncology and Radiotherapy, Institute of Clinical Medicine, University of Oulu from 2011 to 2017.

I am deeply grateful for my supervisors Professor Taina Turpeenniemi-Hujanen, M.D., Ph.D.; Docent Outi Kuittinen, M.D., Ph.D. and Docent Kirsi-Maria Haapasaari, M.D., Ph.D., for their expertise, support and guidance during this process. You have truly managed to create an inspiring and enjoyable environment for our research group and I admire the endless ideas and enthusiasm that you bring to the creation of new research. Thank you Taina for your support and excellent comments during the writing of my thesis. Thank you Outi for all of your support and positivity. I really admire the work you are doing. Thank you Kirsi-Maria for your expertise and your contribution to the histopathology. I also want to thank all my co-authors. Your collaboration has enabled this work and is greatly appreciated. Thank you Professor Ylermi Soini, M.D., Ph.D. and Professor Esa Jantunen, M.D., Ph.D., from Kuopio University Hospital for your skillful collaboration.

Thank you Mirja Vahera and Erja Tomperi, as well as the staff of Biocenter Oulu, for your invaluable technical assistance and contribution to this work. I would like to thank my doctoral training follow-up group members, Docent Jussi Koivunen, M.D., Ph.D., Docent Sakari Kakko, M.D., Ph.D. and Docent Marjaana Säily, M.D., Ph.D. I am most grateful for my reviewers Professor Kaarle Franssila, M.D., Ph.D. and Docent Arto Mannermaa, M.D., Ph.D., for your insightful comments and invaluable expertise.

Special thanks to Mrs. Anne Bisi for your great knowledge with laboratory work and everything else. You have been such a big support and help. I am truly grateful for everyone in our research group, Anne Bisi, my supervisors and fellow researchers. It has been a great pleasure to work with all of you and I have had many rememberable moments with you.

I would like to thank my mother Outi Kuittinen. You have always been so supporting and like a rock in my life. I am forever grateful for many things and I truly admire you. I would also like to thank my father Markos Lemma for pushing me forward ever since I was little. You have taught me strength and perseverance. I am thankful for my sisters Aurora Lemma and Jasmiini Lemma for their love, but also for their criticism. You two are so inspiring. You make me laugh and you broaden my thinking. Many thanks to my grandparents Aune and Toivo Kuittinen – you treat everyone with such love and kindness and I do look up to that. So many thanks to my friends - you make my world and there are not enough words to thank

you. I am grateful to have shared all these different moments of life with all of you. Special thanks to Milla Kosunen for being so easy to be around, on my best days and worst, and for all our trips together, thanks to Riikka Jerkkola for your honesty in everything. You have been a great support in my life. Thanks to Johanna Herajärvi for all our shared research experiences and all the fun times, thanks to Santtu Liikala, Leena-Maija Kimari, Essi Ihanainen, Nelli Perkiömäki, Noora Lahdenperä, Lassi Björnholm, Harri Perämäki, Lasse Pohjola, Juho Huhtala, Pauliina Lahtinen, Laura Jerkkola, Anne Harjuniemi, Hanna Kokko, Jonne Juntura, everyone in the VEG-group and all my other friends for all the laughs and the times spent together. Thank you Jyrki Jauhiainen and Harri Perämäki for your technical support. Last but not least, thank you Antti Mikkonen for dragging me to the library so many mornings this past spring. You are so special to me and I am truly thankful for all your support.

This work was funded by The Cancer Society of Northern Finland, The Finnish Medical Foundation Duodecim, Orion-Famos Research Foundation, Cancer Society of Finland, The Finnish Cultural Foundation and The Ida Montin Foundation.

Oulu, May 2017 Siria Lemma

# Abbreviations

| | |
|---|---|
| ABC | activated B-cell |
| AIDS | acquired immunodeficiency syndrome |
| AIHA | autoimmune hemolytic anemia |
| AITL | angioimmunoblastic T-cell lymphoma |
| ALCL | anaplastic large cell lymphoma |
| alloSCT | allogenic stem cell transplantation |
| AML | acute myeloid leukaemia |
| ASCT | autologous stem cell transplantation |
| ATLL | adult T-cell leukaemia/lymphoma |
| AUC | are under curve |
| BBB | blood-brain barrier |
| BBBD | blood-brain barrier disruption |
| Bcl-2 | B-cell lymphoma-2 protein |
| Bcl-6 | B-cell lymphoma-6 protein |
| BCR | B-cell receptor |
| bHLH | basic helix-loop-helix |
| b-lactoferrin | bovine lactoferrin |
| BM | bone marrow |
| BRD7 | bromodomain-containing protein 7 |
| BSA | bovine serum albumin |
| C-ALCL | primary cutaneous anaplastic large cell lymphoma |
| CD44s | CD44 standard form |
| CD44v | CD44 variant form |
| CDH12 | cadherin-12 |
| CHL | classical Hodgkin lymphoma |
| CI | confidence interval |
| CLL | chronic lymphocytic leukaemia |
| CML | chronic myeloid leukaemia |
| c-myc | cytoplasmic Myc |
| CNS | central nervous system |
| CR | complete response |
| cRNA | complementary RNA |
| CSC | cancer stem cell |
| CSF | cerebrospinal fluid |
| CT | computer tomography |

| | |
|---|---|
| CXCL | CXC chemokine ligand |
| CXCR | CXC chemokine receptor |
| dCLN | deep cercival lymph node |
| DLBCL | diffuse large B-cell lymphoma |
| EATL | enteropathy-associated T-cell lymphoma |
| EBV | Ebstein-Barr virus |
| EMT | epithelial-mesenchymal transition |
| EPHB4 | erythropoietin-producing hepatocellular type-B receptor 4 |
| ERG | ETS-related gene |
| FC | fold change |
| FCM | flow cytometry |
| FL | follicular lymphoma |
| FTCL | follicular T-cell lymphoma |
| GAL1 | galectin-1 encoding gene LGALS1 |
| GC | germinal center |
| GEP | gene-expression profiling |
| GI | gastrointestinal |
| GO | Gene ontology |
| GPCR | G protein coupled-receptor |
| GVHD | graft versus host disease |
| HA | hyaluronan |
| HAART | highly active antiviral therapy |
| HCC | hepatocellular carcinoma |
| HCV | hepatitis C virus |
| HD-MTX | high-dose methotrexate |
| HGBL | high-grade B-cell lymphoma |
| HHV8 | human herpesvirus 8 |
| HIV | human immunodeficiency virus |
| HL | Hodgkin lymphoma |
| HPC | haematopoietic progenitor cell |
| HRS cells | Hodgkin and Reed-Sternberg cells |
| HSC | haematopoietic stem cell |
| HTLV-1 | human T lymphotropic virus type 1 |
| ICAM-1 | intercellular adhesion molecule-1 |
| IEM | immunoelectron microscopy |
| IPI | International Prognostic Index |
| ISF | interstitial fluid |

| | |
|---|---|
| ITGA10 | Integrin alpha 10 |
| IVE | ifosfamide, vincristine and etoposide |
| IVT | in vitro transcription |
| KEGG | Kyoto Encyclopedia of Genes and Genomes database |
| LBCL | large B-cell lymphoma |
| LDH | lactate dehydrogenase |
| LyP | lymphomatoid papulosis |
| MALT | mucosa-associated lymphoid tissue |
| MCL | mantle cell lymphoma |
| MEITL | monomorphic epitheliotropic intestinal T-cell lymphoma |
| MM | multiple myeloma |
| MMP | matrix metalloproteinase |
| MRI | magnetic resonance imaging |
| MSC | marrow stromal cell |
| MUM-1 | multiple myeloma oncogene-1 |
| NFAT | nuclear factor of activated T-cells |
| NF-kB | nuclear factor kappa-B |
| NHL | non-Hodgkin lymphoma |
| NK | natural killer |
| NLPHL | nodular lymphocyte predominant Hodgkin lymphoma |
| NOS | not otherwise specified |
| NSCLC | non-small cell lung cancer |
| OS | overall survival |
| PB | peripheral blood |
| PBS | phosphate-buffered saline |
| PCA | principal component analysis |
| PCNSL | primary central nervous system lymphoma |
| PFS | progression-free survival |
| PI3K | phosphatidylinositol 3-kinase |
| PIP2 | phosphatidylinositol 4,5-biphosphate |
| PIP3 | phosphatidylinositol 3,4,5-triphosphate |
| PTCL | peripheral T-cell lymphoma |
| PTCL NOS | peripheral T-cell lymphoma, not otherwise specified |
| PTEN | phosphatase and tensin homolog |
| qRT-PCR | quantitative real-time polymerase chain reaction |
| RA | rheumatoid arthritis |

| | |
|---|---|
| R-CHOP-21 | rituximab, cyclophospamide, doxorubicin, vincristine and prednisolone every 21 days |
| ROC | receiver operating characteristic |
| RR | risk ratio |
| SCF | stem cell factor |
| SCLC | small cell lung cancer |
| sCNSL | secondary central nervous system lymphoma |
| sDLBCL | systemic DLBCL without CNS affision |
| SLE | systemic lupus erythematosus |
| SLL | small lymphocytic lymphoma |
| SS | Sezary syndrome |
| TF | transcription factor |
| TGF-beta | transforming growth factor-beta |
| TNF | tumour necrosis factor |
| VLA-4 | very late antigen-4 |
| WBRT | whole-brain radiotherapy |
| WHO | World Health Organization |

# List of original publications

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

I Lemma S, Karihtala P, Haapasaari KM, Jantunen E, Soini Y, Bloigu R, Pasanen AK, Turpeenniemi-Hujanen T & Kuittinen O (2013) Biological roles and prognostic values of the epithelial-mesenchymal transition-mediating transcription factors Twist, ZEB1 and Slug in diffuse large B-cell lymphoma. Histopathology 62(2): 326-333.

II Uotila P, Lemma SA, Haapasaari KM, Porvari K, Skarp S, Soini Y, Jantunen E, Turpeenniemi-Hujanen T & Kuittinen O (2017) Epithelial-mesenchymal transition markers Twist, ZEB1 and Slug are associated with progression-free survival and clinical presentation in T-cell lymphomas. Manuscript.

III Lemma SA, Pasanen AK, Haapasaari KM, Sippola A, Sormunen R, Soini Y, Jantunen E, Koivunen P, Salokorpi N, Bloigu R, Turpeenniemi-Hujanen T & Kuittinen O (2016) Similar chemokine receptor profiles in lymphomas with central nervous system involvement - possible biomarkers for patient selection for central nervous system prophylaxis, a retrospective study. Eur J Haematol 96(5): 492-501.

IV Lemma SA, Kuusisto M, Haapasaari KM, Sormunen R, Lehtinen T, Klaavuniemi T, Eray M, Jantunen E, Soini Y, Vasala K, Böhm J, Salokorpi N, Koivunen P, Karihtala P, Vuoristo J, Turpeenniemi-Hujanen T & Kuittinen O (2017) Integrin alpha 10, CD44, PTEN, cadherin-11 and lactoferrin expressions are potential biomarkers for selecting patients in need of central nervous system prophylaxis in diffuse large B-cell lymphoma. Carcinogenesis. In press.

# Contents

# 1 Introduction

Lymphomas are a heterogeneous group of malignancies that arise from lymphatic tissues. The classification of lymphomas has developed greatly throughout the years, mostly due to improved diagnostic techniques and increased biological data. Today in the World Health Organization (WHO) IV classification, there exists various different subtypes with great variation in prognosis and aggressiveness of the diseases. In the forthcoming revised version of the WHO IV classification, significant advances have been made in the classification of nodal and extranodal T-cell neoplasms. There will also be advancements in the classification of diffuse large B-cell lymphoma (DLBCL).

B-cell derived lymphomas represent the most common lymphomas in the Western world and DLBCL covers about 30% of all lymphomas. It is an aggressive malignancy with an increasing incidence. The prognosis of B-cell lymphomas has improved significantly in the past years and especially the introduction of the CD20 antibody, rituximab, has improved the outcomes in all age groups. Today the standard treatment for DLBCL is R-CHOP. Despite the achievements, problems also exist. The drugs included in R-CHOP therapy have a poor penetration through the blood-brain barrier (BBB) and into the central nervous system (CNS), and in the rituximab era, the incidence of CNS relapses has not changed much. Because of the better control of the systemic DLBCL, the clinical significance of CNS relapses is becoming increasingly important. As secondary CNSL (sCNSL) and primary CNS lymphoma (PCNSL) are known to have poor prognoses with the current treatment modalities, the prevention of sCNSL is of crucial importance. Relapsed/refractory, aggressive DLBCL represents another remaining issue and together with CNS lymphomas, these are one of the main causes of morbidity among DLBCL patients. It is known that most of the CNS relapses can be prevented by incorporating prophylactic treatment into the primary treatment of systemic DLBCL. The current recommended CNS prophylaxis includes HD-MTX and HD cytarabine that should be administered during the primary treatment. Unfortunately this treatment is highly toxic and should be limited to high-risk patients only. The identification of the patient population in need of CNS prophylaxis is currently based on the clinical risk assessment, which is not a very specific method. It is suggested that non-GC phenotype, CD5 positivity and double-expressor phenotype may have some prognostic relevance on CNS relapses, but their significance remains unclear. Double hit lymphomas on the other hand are known to have a higher risk of CNS relapse. In the forthcoming WHO revision, this group is

classified as an entity of its own, called high-grade B-cell lymphoma (HGBL), with rearrangements of MYC and BCL2 and/or BCL6, and for this group CNS prophylaxis is recommended. In the future, more specific predictive markers are needed for the selection of the patient group in need of CNS prophylaxis. In addition, the biology behind CNS relapse of DLBCL remains unclear. Chemokines and their receptors have been suggested to play a role in the CNS tropism and one hypothesis is that the malignant cells develop extracranially, but end up localising to the CNS due to highly selective CNS tropism. The changes leading to this remain unclear.

Peripheral T-cell lymphomas (PTCL) are rare neoplasms and together with natural killer (NK)-cell lymphomas, they comprise around 10% of all NHLs in Western countries. PTCLs include several lymphoma subtypes that possess complex and overlapping morphological and immunophenotypic characteristics. The identification of different entities has improved and in the revised WHO classification, new provisional entities are being introduced. These advancements are largely due to the results of transcriptomic studies examining gene-expression profiles (GEP) and the genetic landscape of T-cell malignancies. Due to the rarity of these diseases in the Western world, there are not many clinical trials regarding their optimal therapy and the biological knowledge still remains scarce when compared to DLBCL. As a result of this, PTCLs have been treated with same therapies as B-cell lymphomas, mainly with suboptimal treatment results. Despite the progress made in the recent years in understanding the PTCLs, the optimal treatment schemas are still lacking. In recent years, consolidation with autologous stem cell transplantation (ASCT) have been used in several PTCL subtypes in fit patients. This has led to some improvements in the outcomes. Allogenous transplant represent another option in the treatment of relapsed/refractory cases. A more specific understanding of the PTCL biology is warranted.

# 2 Review of literature

## 2.1 Lymphomas

### *2.1.1 Overview of lymphomas*

Lymphomas are a heterogeneous group of malignancies that arise from lymphatic tissues. The classification of lymphomas has developed greatly throughout the years, mostly due to improved diagnostic techniques and increased biological data. The number of entities has increased in relation to the gained knowledge. Today in the World Health Organization (WHO) IV classification, there exists various different subtypes with a great variation in prognosis and aggressiveness of the diseases (Swerdlow *et al*. 2008). A revised version of the WHO IV classification will be published soon.

Lymphomas can be divided according to the cell line from which they originate. They are either B- or T-/NK-cell derived. Hodgkin lymphoma (HL), although predominantly B-cell derived, is considered a separate entity. These are further classified to precursor diseases and mature peripheral B- and T-cell lymphomas (Swerdlow *et al*. 2008). B-cell derived lymphomas are the most common lymphomas in the Western world. DLBCL represents the most common single lymphoma subtype, covering about 30% of all lymphomas. More about DLBCL will be discussed in detail later.

The second most common lymphoma is a B-cell derived follicular lymphoma (FL), accounting for about 20% of all lymphomas. It is also the most common indolent lymphoma. The median age for occurrence of FL is between 50 and 59. Most follicular lymphoma cases present with a predominantly follicular growth pattern and the tumours are composed of germinal center B-cells. FL is a slow growing disease with the potential to transform to a high-grade lymphoma. This occurs in about 25-35% of patients and usually the disease transforms into DLBCL (Swerdlow *et al*. 2008).

Small lymphocytic lymphoma (SLL)/chronic lymphocytic leukaemia (CLL) represents about 7% of non-Hodgkin lymphomas (NHLs) in biopsies. It usually involves lymph nodes, spleen, bone marrow (BM) and peripheral blood (PB). From all B-cell lymphomas, mucosa-associated lymphoid tissue (MALT) lymphoma accounts for about 7-8%. It is further sub-classified into extranodal, nodal and primary splenic MALT-lymphomas. Mantle cell lymphoma (MCL) is a B-cell

neoplasm that comprises about 3-10% of NHLs. It usually occurs in individuals with a median age of about 60. Rarer subtypes of B-cell lymphomas include Burkitt's lymphoma, lymphoblastic lymphoma and hairy cell leukaemia (Swerdlow *et al*. 2008).

HLs represent around 10% of lymphomas in Finland and most commonly manifests in young adults. It is predominantly B-cell derived and is comprised of five disease entities: four types of classical Hodgkin lymphoma (CHL) and nodular lymphocyte predominant Hodgkin lymphoma (NLPHL). They differ in morphology, immunophenotype, B-cell gene expression and in clinical features and behaviour. The characteristic growth pattern for HLs usually comprises a small number of scattered large multinucleated and mononucleated tumour cells, called Hodgkin and Reed-Sternberg cells (HRS cells) that are surrounded by abundant benign inflammatory and accessory cells. In contrast to NHLs, only a small number of all of the cells are neoplastic, these being the HRS cells (Swerdlow *et al*. 2008).

T-cell and NK-cell lymphomas are more rare, together comprising around 10% of NHLs in Western countries (Finnish Cancer Registry - Institute for Statistical and Epidemiological Cancer Research, Iqbal *et al*. 2014). It is a very heterogeneous group, with complex and also overlapping morphological and immunophenotypic characteristics. The molecular pathogenesis in this disease group is not as well-known as it is in B-cell lymphomas. The characterisation of different entities used to rely significantly on morphological and IHC features, but in recent years significant advances have been made in the classification of nodal and extranodal T-cell lymphomas. These will be included in the forthcoming revised WHO classification (Swerdlow *et al*. 2008, Swerdlow *et al*. 2016). The most common PTCLs are peripheral T-cell lymphoma not otherwise specified (PTCL NOS) and angioimmunoblastic T-cell lymphoma (AITL). Other PTCLs include ALK+ anaplastic large cell lymphoma (ALCL), ALK- ALCL and enteropathy-associated T-cell lymphoma (EATL). ALK+ ALCL has superior prognosis when compared to other PTCLs that generally have clearly worse prognoses than DLBCL (Swerdlow *et al*. 2008). The optimal treatment schemas for different PTCL entities remain somewhat unclear. Remissions are less often achieved and recurrences are much more common when compared to B-cell lymphomas (Casulo *et al*. 2016, Zhang *et al*. 2016). However, not all PTCLs are as aggressive. Most cutaneous lymphomas are T-cell lymphomas and these especially can be very slow-growing, chronic diseases. Due to the heterogeneity of the PTCL group, disease presentations also vary greatly (Swerdlow *et al*. 2008). PTCLs are discussed in more detail from page 37 onwards.

### 2.1.2 Epidemiology of lymphomas

In Finland, NHL comprises about 4.0% of all cancers in men and 3.8% in women. In the year 2014 in Finland, a total of 1506 new lymphomas were diagnosed, 1337 of these being NHLs and 169 HLs (Finnish Cancer Registry - Institute for Statistical and Epidemiological Cancer Research). In recent decades, the incidence of NHLs has increased significantly. Currently NHL is the 7th most common lymphoma among both men and women. In general, lymphomas are a little more common in men than in women (Finnish Cancer Registry - Institute for Statistical and Epidemiological Cancer Research, Swerdlow *et al*. 2008). The average age for lymphoma occurrence depends on the entity. NHL most commonly affects people over 60 years old, HL on the other hand is most common in 20-40 year-olds and then again in people over 60 years old (Finnish Cancer Registry - Institute for Statistical and Epidemiological Cancer Research, Swerdlow *et al*. 2008). Lymphomas are more common in developed countries and the incidence of NHL is increasing worldwide, especially the number of B-cell lymphomas (Swerdlow *et al*. 2008).

### 2.1.3 Etiology of non-Hodgkin lymphomas

There are several risk factors that are known to increase the risk of lymphoma. However, they only explain a small proportion of lymphomas. Most of the etiology remains unknown. Some of the known risk factors include: immunodeficiency, autoimmune diseases and infectious agents (Swerdlow *et al*. 2008). In addition, ionizing radiation and chemotherapy are known to increase the risk of NHLs (Alexander *et al*. 2007, Krishnan & Morgan 2007).

Immunodeficient patients have a significantly increased incidence of B-cell malignancies, especially Burkitt lymphoma and DLBCL. Immunodeficiency can be caused by primary immunodeficiency, infection with the human immunodeficiency virus (HIV) or iatrogenic immunosuppression (Swerdlow *et al*. 2008). In congenital immunodeficiency syndromes, NHL is the most common cancer type (Filipovich *et al*. 1992). Human immunodeficiency virus (HIV) infection gives an explanation to parts of the etiology behind NHL. When compared to the general population, patients with acquired immunodeficiency syndrome (AIDS) have over a 100-fold risk of developing NHL (Goedert 2000). AIDS-related lymphomas are most often high-grade lymphomas. The prognosis used to be very bad and almost all patients succumbed to the disease (Levine *et al*. 1992). Highly

active antiviral therapy (HAART) and the use of modern chemotherapy have significantly improved the prognosis in this patient group (Levine 2008). HAART has also decreased some of the excess incidence of NHL in AIDS patients, the risk still being higher compared to immunocompetent patients. CD4 count is strongly correlated with NHL incidence; both before and after the HAART era and the above mentioned reduced risk of NHL is in concordance with the increased CD4 counts after HAART was taken into use (Biggar *et al*. 2007).

Iatrogenic immunosuppression can be used to prevent allograft rejection or graft versus host disease (GVHD) in the context of organtransplantation (Swerdlow *et al*. 2008). Iatrogenic immunodeficiency causes patients to be at increased risk of developing lymphoma. In general from the malignancies diagnosed after transplant, NHL represents one of the most common ones. The specific lymphoma subtypes are similar as with HIV-infected patients. Both of these groups experience chronic immunosuppression and immune activation. Overall, patients receiving transplants have been shown to have a risk of developing NHL that is six times that of the general population. The risk increase also depends on the organ transplanted, with some of the organs that are not the kidney showing even higher risk for NHL (Clarke *et al*. 2013).

Autoimmune diseases are known to be associated with the incidence of malignancies. This association has been a subject of studies for years. It is known that patients with Sjögren's syndrome, systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) possess an increased risk for developing NHL when compared to the general population (Zintzaras *et al*. 2005). In RA, patients the treatment had an effect on the risk of NHL; cytotoxic and biological treatment showing a higher risk when compared to conventional antirheumatic treatment (Zintzaras *et al*. 2005). An increased risk of NHL also exists for patients with biopsy-verified celiac disease and other autoimmune diseases such as autoimmune hemolytic anemia (Elfstrom *et al*. 2011, Fallah *et al*. 2014).

Infectious agents are also known to play a role in the development of lymphomas. Infectious agents are able to cause immunosuppression, chronic immune stimulation and also to transform lymphocytes. HIV induces AIDS-associated lymphomas of several types, particularly high-grade B-cell NHLs (Hjalgrim & Engels 2008). Hepatitis B and C infections cause chronic immune stimulation and patients infected with the hepatitis C virus (HCV) are more often affected by NHL, specifically DLBCL, marginal zone lymphoma and lymphoplasmacytic lymphoma (de Sanjose *et al*. 2008, Ulcickas Yood *et al*. 2007). Helicobacter pylori bacteria causes chronic immune stimulation and is known to

cause gastric MALT lymphoma (Wotherspoon *et al*. 1991). Other bacteria also seem to associate with the incidence of NHLs. Campylobacter jejuni has been shown to correlate with increased incidence of small intestinal NHL (Lecuit *et al*. 2004). In certain geographical regions such as Italy, Germany and Korea, chlamydia psittaci infection seems to correlate with the incidence of ocular adnexal extranodal marginal zone lymphoma (Ferreri *et al*. 2004, Zhu *et al*. 2013). Borrelia burgdorferi infection has been associated with cutaneous NHL, but contradictory data exists as well and some studies do not support its pathogenic role in NHLs (Hjalgrim & Engels 2008, Ponzoni *et al*. 2011). Lymphocyte transforming agents are also known to promote lymphomagenesis. These include Ebstein-Barr virus (EBV), human T lymphotropic virus type 1 (HTLV-1) and human herpes virus 8 (HHV8) (Hjalgrim & Engels 2008). EBV infection correlates with the incidence of a subset of HL, Burkitt lymphoma, extranodal NK/T-cell NHL and AIDS-associated NHLs, especially CNS DLBCL. In endemic Burkitt lymphoma, EBV DNA and proteins are highly consistently detected in tumour cells (Hjalgrim & Engels 2008). HTLV-1 correlates with adult T-cell leukaemia/lymphoma and HHV8 has been associated with B-cell lymphomagenesis (Hjalgrim & Engels 2008, Swerdlow *et al*. 2008).

## 2.2 Diffuse large B-cell lymphoma

### *2.2.1 Biology, clinical disease presentation and classification of DLBCL*

B-cells originate from the bone marrow hematopoietic stem cells (HSC). Multipotent progenitors commit to the lymphoid lineage by becoming common lymphoid progenitors. These cells can enter the B-cell pathway and further changes are seen in pro-B cells, such as the induction of CD19 expression and complete $D_H$-$J_H$ rearrangement. In late pro-B cells as a part of the pre-B cell receptor (pre-BCR), a cell surface expression of the Igμ proteins occurs due to a productive $V_H$-$DJ_H$ recombination. Further, light-chain gene rearrangement leads to the development of immature B cells that again leave the bone marrow to emigrate into peripheral lymphoid organs (Busslinger 2004).

DLBCL is an aggressive malignancy of mature B-cells. It represents the most common lymphoma subtype, comprising about one third of NHLs. Its incidence has increased in the past decades, as has the incidence of NHLs in general. DLBCL

is slightly more common among men than women. It usually affects elderly people, the median age being between 60 and 70, but it can also affect young people. The prognosis has improved significantly in recent years (Swerdlow *et al*. 2008). DLBCL is a malignancy of large B lymphoid cells with diffuse growth pattern (Swerdlow *et al*. 2008). It can present nodal or extranodal disease. Upon primary diagnosis, about 40% of DLBCL presents an extranodal disease (Harris *et al*. 1994). Extranodal locations include gastrointestinal (GI) tract, CNS, BM, skin, testis, spleen, Waldeyer ring, salivary gland, thyroid, liver, kidney and adrenal gland. Diffuse large B-cell PCNSL represent < 1% of all NHLs (Swerdlow *et al*. 2008). PCNSL and sCNSL will be discussed in more detail later.

The disease presentation depends on the location of the disease. A patient with DLBCL may notice an enlarged lymph node or a lump somewhere else. Then again, B-symptoms can be the first sign of lymphoma. These include weight loss, night sweating and prolonged fever. Itching or variable CNS symptoms, depending on the location of the tumour mass, can also be the first signs of DLBCL. When lymphoma is suspected, a surgical biopsy is needed, however sometimes in clinical practice, a core needle biopsy may be taken instead. The diagnosis is based on a histological evaluation done by an experienced haematopathologist. The tumour sample is stained immunohistochemically for lymphocyte surface antigens and in case of DLBCL, neoplastic cells usually expresss pan B-cell markers such as CD19, CD20, CD22 and CD79a. Other markers are also examined to enable a more specific diagnosis or subtyping of a specific entity (Swerdlow *et al*. 2008).

DLBCL can be subdivided into groups based on morphological, biological and clinical studies (Swerdlow *et al*. 2008). DLBCL subtypes include primary DLBCL of the CNS, primary cutaneous DLBCL leg type, T-cell/histiocyte-rich large B-cell lymphoma and EBV positive DLBCL of the elderly. The DLBCL subtypes that do not belong to a specific subtype or disease entity are included in the DLBCL not otherwise specified (DLBCL NOS) group. In this group, morphologic variants and molecular and immunohistochemical sub-groups are being recognised. Common morphologic variants include centroblastic, immunoblastic and anaplastic variants (Swerdlow *et al*. 2008). Disease entities can also be separated by molecular and IHC analysis. In the revised WHO classification DLBCL NOS will be divided into sub-groups and new categories are introduced (Swerdlow *et al*. 2016). These are presented at the end of this section.

In 2000, Alizadeh et al. identified molecularly different subtypes of DLBCL by GEP. These types are indicative of different stages of B-cell differentiation, called germinal center B-cell-like (GC) and activated B-cell-like (ABC) types

(Alizadeh *et al*. 2000). A third type was also identified and designated as type 3 DLBCL (Rosenwald *et al*. 2002). It proved to be a collection of cases that cannot be classified as the GC- or ABC-subtype (Swerdlow *et al*. 2008). These types correspond to the different stages of B-cell differentiation from which the malignant cells originate. As described in the beginning of this section, after the immature B-cells leave the bone marrow, they enter GCs in the follicles of secondary lymphoid tissues, where somatic hypermutation occurs and B-cells go through antigen selection. In DLBCL, the rearranged Ig genes bear mutations that are characteristic of somatic hypermutations, which in physiological conditions occur only within the GCs. Together this and the results from the above mentioned GEP studies suggest that DLBCL with a GC phenotype arises from the GC B-cells and the ABC subtype from B-cells that are at a later stage of differentiation. The type 3 DLBCL has characteristics of both GC and ABC DLBCLs (Alizadeh *et al*. 2000, Klein *et al*. 1998).

Patients with GC phenotype have been shown to have the best prognosis of the subgroups (Alizadeh *et al*. 2000, Rosenwald *et al*. 2002). In 2004, Hans et al. described a corresponding subtyping that is done by IHC. This characterisation includes a GC and non-GC subtypes that are specified by the expression of GC and non-GC biomarkers in patient samples (Hans *et al*. 2004). These biomarkers include CD10, B-cell lymphoma-6 protein (Bcl-6) and multiple myeloma oncogene-1 (MUM-1) and the subtype division is done as shown in Figure 1 (Hans *et al*. 2004). Also other algorithms for IHC cell-of-origin phenotyping have been proposed, but Hans's algorithm still seems to be the most commonly used one (Choi *et al*. 2009, Meyer *et al*. 2011, Muris *et al*. 2006, Nyman *et al*. 2009). Hans's algorithm or other algorithms are not as accurate as GEP and their ability to separate different prognostic groups remains controversial (Choi *et al*. 2009, Culpin *et al*. 2013, Hwang *et al*. 2014, Meyer *et al*. 2011, Muris *et al*. 2006, Nyman *et al*. 2009, Read *et al*. 2014). Internation Prognostic Index (IPI) is considered to be the best prognostic indicator in DLBCL; it is further discussed in the clinical prognostic factors section (Nyman *et al*. 2007).

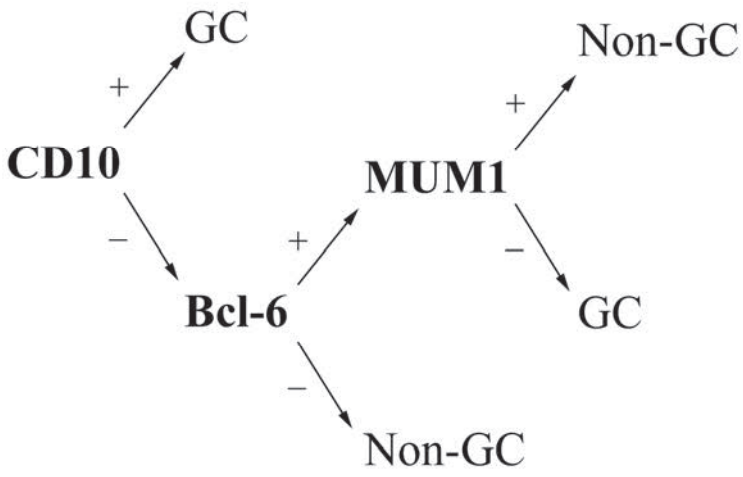


**Fig. 1. Immunohistochemical cell-of-origin phenotyping according to Hans *et al.* (2004).**

Due to the different cells of origin, the different molecular subtypes defined by GEP are often associated with specific genetic alterations. B-cell lymphoma-2 (BCL2) is an oncogene that encodes Bcl-2, a protein with anti-apoptotic properties (Hockenbery *et al*. 1990). Translocation t(14;18)(q32;q21) is known to cause Bcl-2 overexpression and it is more common in the GC subtype than in the ABC type (Huang *et al*. 2002). Bcl-6, encoded by BCL6, is a transcriptional repressor with a central role in B-cell differentiation and it is implicated to play a role in lymphomagenesis (Shaffer *et al*. 2000). BCL6 translocations are more common in the ABC type (Akyurek *et al*. 2012, Iqbal *et al*. 2007). Additionally, an anti-apoptotic nuclear factor kappa-B (NF-kB) pathway is more often constitutively activated in the ABC subtype than in the GC type (Compagno *et al*. 2009, Davis *et al*. 2001). Upregulation of the NF-kB pathway has been found to occur in many lymphomas, including DLBCL. Constitutive NF-kB activation is thought to promote malignant transformation by providing anti-apoptotic and pro-proliferative signals (Compagno *et al*. 2009). In addition, other genetic lesions affecting multiple genes may lead to abnormally prolonged NF-kB activation and responses (Compagno *et al*. 2009, Shaffer *et al*. 2012). In general in DLBCL, constitutive Myc protein expression has been shown to promote lymphomagenesis. Myc is a transcription factor, encoded by an MYC oncogene, which has many

properties, such as cell survival and proliferation-associated ones, which are pivotal for DLBCL pathophysiology (Adams *et al*. 1985).

In the forthcoming revised WHO classification, DLBCL NOS will be divided into two subgroups based on the cell-of-origin. The revised classification requires the identification between GCB versus ABC/non-GC subtypes with the use of IHC algorithms, as GEP is still not used as a routine clinical test. In addition, all large B-cell lymphomas (LBCL) with MYC and BCL2 and/or BCL6 rearrangements will be included in a new category designated as high-grade B-cell lymphoma (HGBL), with rearrangements of MYC and BCL2 and/or BCL6. These do not include cases that fulfill the criteria for lymphoblastic lymphoma or follicular lymphoma. Cases intermediate between DLBCL and Burkitt lymphoma or cases that appear blastoid, that do not harbor MYC and BCL2 and/or BCL6 rearrangements will be included in a category called HGBL, NOS (Swerdlow *et al*. 2016).

### *2.2.2 Clinical prognostic factors*

A lot of research has been done to define prognostic factors for DLBCL. Still to date, IPI is considered as the most important prognostic factor for survival of immunotherapy-treated patients. As the strongest tool for identification of high-risk patients, IPI classification has its shortcomings. It does not offer information on the biological features of the disease nor does it possess predictive value on the subjects' responsiveness to therapies (Nyman *et al*. 2007).

IPI has been used in clinical practice since 1993. It is a scoring method based on clinical factors, with a maximum of five points. One point is gained from each of the following factors: existence of more than one extranodal lesion at diagnosis, elevated serum lactate dehydrogenase (LDH), age over 60 years, Ann Arbor stage III-IV and poor WHO performance status (2-4) (Shipp *et al*. 1993). The patients are classified into four risk groups accordingly: the low-risk group (0-1 points), the low intermediate risk group (2 points), the high intermediate risk group (3 points) and the high-risk group (4-5 points). When IPI scoring was created, before the inclusion of rituximab into the standard therapy, different prognostic groups presented with varying 5-year overall survivals (OS), the low-risk group with 73% and the high-risk group with 26% OS (Shipp *et al*. 1993). After inclusion of rituximab into the treatment, the outcomes in all IPI classes have improved, with the low-risk group showing 91% 3-year OS and the high-risk group showing 59% 3-year OS (Ziepert *et al*. 2010). In addition to its use in predicting patient prognosis, in some hospitals IPI classification is also used in the treatment selection (Leppä *et*

*al*. 2008). In the future, it is desirable that more disease-specific, biological characteristics would be found and used when deciding on the treatment of DLBCL. A better understanding of the biology of such a heterogeneous group as DLBCL would help to define more accurate disease types and again lead to more specified treatments per patient.

### *2.2.3 Biological prognostic factors*

During recent years, a lot of research has been done in search of good biological prognostic factors. There have been many markers suggested for clinical use, however, sufficiently strong validation for routine clinical practice is lacking. The most promising biological prognostic factors for DLBCL are presented here.

#### *Cell-of-origin*

Within the GEP defined cell-of-origin phenotypes, the GC phenotype is shown to have the best prognosis of the subgroups (Alizadeh *et al*. 2000, Rosenwald *et al*. 2002). Before the inclusion of rituximab into the standard treatment, patients also separated into GC and non-GC subgroups by Hans's algorithm seemed to have differences in the prognosis; GC phenotype correlated with a better outcome (Hans *et al*. 2004). After the inclusion of rituximab into the therapy, conflicting results of the prognostic value of immunophenotyping have been published (Culpin *et al*. 2013, Fu *et al*. 2008, Hwang *et al*. 2014, Meyer *et al*. 2011, Read *et al*. 2014). Nyman et al. reported that the combination of rituximab with chemotherapy seems to eliminate the prognostic value of GC- and non-GC phenotypes defined by IHC (Nyman *et al*. 2007). Also in other studies, Hans's algorithm has still been correlated with GEP phenotyping but not as well with prognosis (Culpin *et al*. 2013, Hwang *et al*. 2014, Meyer *et al*. 2011, Read *et al*. 2014). Last year a study by Gang et al. appeared where patients were defined into subgroups by Hans's algorithm and with R-CHOEP treatment patients with GC-phenotype disease did have a better prognosis when compared to the non-GC subgroup. The same differences were not seen in R-CHOP treated patients (Gang *et al*. 2015). In 2016 in a study by Lu et al., chemoimmunotherapy-treated patients that were defined as GC phenotype by Hans's algorithm did have a better OS and PFS when compared to the non-GC phenotype (Lu *et al*. 2016). So the results remain conflicting. Other algorithms have also been proposed, but Hans's algorithm still seems to be the most commonly used one (Choi *et al*. 2009, Meyer *et al*. 2011, Muris *et al*. 2006, Nyman *et al*. 2009). In

a study by Meyer et al., Hans's and Choi's algorithms were the most accurate IHC ones in determining the cell-of-origin that correlates with GEP phenotyping (Meyer *et al*. 2011). When GEP is used in the phenotyping, ABC subtypes still seem to possess markedly lower survival rates in the rituximab era (Gutierrez-Garcia *et al*. 2011, Lenz *et al*. 2008). GEP would definitely be the most accurate method for the identification of these subgroups. However, due to the high costs of GEP, IHC is a more accessible method.

In the revised WHO classification, DLBCL NOS will be divided into two subgroups based on the cell-of-origin and the identification between GCB versus ABC/non-GC subtypes will be done with means of IHC algorithms. Hans' algorithm remains the most popular one based on the WHO recommendations, but other algorithms may also be used (Swerdlow *et al*. 2016).

### *BCL2*

Bcl-2 is an antiapoptotic factor that is encoded by BCL2 oncogene (Hockenbery *et al*. 1990). BCL2 translocation t(14;18)(q32;q21) causes Bcl-2 overexpression, which is often seen in DLBCL. It is more common in the GC subtype and seen in about 20-30% of DLBCLs, although some studies report BCL2 gene rearrangements in only 15% of DLBCL patients as well (Akyurek *et al*. 2012, Hill *et al*. 1996, Huang *et al*. 2002, Rosenwald *et al*. 2002, Swerdlow *et al*. 2008, Weiss *et al*. 1987). Other mechanisms that also cause Bcl-2 overexpression exist (Gascoyne *et al*. 1997, Iqbal *et al*. 2006). The prognostic significance of the above-mentioned BCL2 rearrangement in the rituximab era is controversial (Akyurek *et al*. 2012, Gascoyne *et al*. 1997, Iqbal *et al*. 2011, Visco *et al*. 2013). In 2013, Visco et al. found BCL2 rearrangement to predict poor outcome in the GC subtype but not in the ABC subtype (Visco *et al*. 2013). Additionally, Bcl-2 protein expression, detected by IHC, has been a subject of studies. In a study by Kawamoto et al., BCL2 translocation was not a prognostic indicator for DLBCL but its protein expression showed independent prognostic value (Kawamoto *et al*. 2016). In the era of modern chemoimmunotherapy, contradictory results of Bcl-2 protein expression's prognostic value also exist (Culpin *et al*. 2013, Mounier *et al*. 2003, Wilson *et al*. 2007).

In the new, updated, WHO classification, DLBCLs with high Myc and Bcl-2 protein expression have been named douple-expressor lymphomas. This is not a separate category but as observations from most, but not all, studies suggest that DLBCLs with double expression have a worse outcome than other DLBCL NOS,

double expression should be considered as a prognostic indicator. Most of these cases do not harbor MYC/BCL2 chromosomal alterations and douple-expressor lymphomas are not as aggressive as the new category HGBL, with rearrangements of MYC and BCL2 and/or BCL6. The cut-off point for Bcl-2 expression varies in the literature, but in the forthcoming WHO revision, a figure of >50% is recommended (Swerdlow *et al*. 2016).

### *BCL6*

Bcl-6 is a transcriptional repressor with a central role in normal B-cell differentiation, more specifically germinal center B-cell differentiation. It has also been implicated to play a role in lymphomagenesis (Shaffer *et al*. 2000). Bcl-6 protein is encoded by BCL6 gene. Up to 30% of DLBCL cases manifest with abnormalities of the 3q27 region involving the BCL6 gene, which is the commonest translocation in DLBCL (Swerdlow *et al*. 2008). BCL6 translocations are more common in the ABC subtype (Akyurek *et al*. 2012, Iqbal *et al*. 2007). The prognostic significance of BCL6 gene translocation in the rituximab era remains elusive. In a study by Akyurek et al., BCL6 gene rearrangement was associated with an inferior outcome in univariate analysis, but did not show independent prognostic value in multivariate analysis (Akyurek *et al*. 2012). Bcl-6 protein expression has often been said to associate with a favorable outcome (Horn *et al*. 2013, Iqbal *et al*. 2007). Meanwhile, the significance of BCL6 gene translocation, its impact on Bcl-6 protein expression and the prognostic significance of protein expression on prognosis remain elusive, as conflicting results are being reported in the rituximab era (Copie-Bergman *et al*. 2009, Horn *et al*. 2013, Iqbal *et al*. 2007, Shustik *et al*. 2010, Winter *et al*. 2006).

### *MYC*

MYC is an oncogene that encodes a transcription factor that is known to upregulate a number of genes (Cai *et al*. 2015). In DLBCL, Myc protein participates in processes such as cell survival and proliferation (Adams *et al*. 1985). MYC gene rearrangement is usually associated with a complex pattern of genetic alterations (Swerdlow *et al*. 2008). It is rearranged in 5% to 15% of DLBCLs and frequently associated with BCL2 translocations and less often with BCL6 translocations. In the updated WHO classification, these old prognostic groups, ‘double-hit’ and ‘triple-hit’ lymphomas, are now included in a new category called HGBL, with

rearrangements of MYC and BCL2 and/or BCL6 (Swerdlow *et al*. 2016). It is known that patients with MYC translocations confer a worse prognosis than those without translocations. These results are also seen in studies from the rituximab treatment era (Akyurek *et al*. 2012, Barrans *et al*. 2010, Horn *et al*. 2013, Kawamoto *et al*. 2016, Savage *et al*. 2009). As today in the rituximab era, MYC gene rearrangement is considered a strong prognostic factor of poor survival; the significance of Myc protein expression is not as clear. Several studies demonstrate that Myc overexpression associates with worse prognosis, but in 2016 contradicting results were found in a study by Kawamoto et al. (Kawamoto *et al*. 2016, Perry *et al*. 2014, Valera *et al*. 2013). Myc protein expression is detected in a higher proportion of cases than MYC gene rearrangements are, altogether in about 30-50% of DLBCLs and not surprisingly Myc overexpression can be caused by mechanisms other than translocation (Kawamoto *et al*. 2016, Swerdlow *et al*. 2016). It is also often associated with Bcl-2 expression. These douple-expressor lymphomas have poorer prognosis and will be discussed later in more detail (Swerdlow *et al*. 2016).

### *Double-expressor lymphomas*

As the old DLBCL NOS double- and triple-hit lymphomas with MYC and BCL2 and/or BCL6 rearrangements are now being included in the new category HGBL, with rearrangements of MYC and BCL2 and/or BCL6, these are no longer considered as prognostic indicators within DLBCL NOS (Swerdlow *et al*. 2016). As mentioned earlier, Myc protein expression is detected in a clearly higher proportion of cases than its gene rearrangement and it associates with concomitant Bcl-2 expression in 20%-35% of cases. As most of these cases do not harbor MYC and/or BCL2 gene alterations, they are being called double-expressor lymphomas. Most (however, not all) studies have provided evidence that these lymphomas have a worse outcome when compared to other DLBCL NOS cases. However, they are not as aggressive as the new category HGBL, with rearrangements of MYC and BCL2 and/or BCL6. Double-expression without gene aberrations is thus considered a prognostic indicator in DLBCL NOS. It does not represent an entity of its own (Johnson *et al*. 2012, Molina *et al*. 2014, Swerdlow *et al*. 2016). In most studies the cut-off point used for Myc expression has been 40% but for Bcl-2, the chosen cut-off points have varied in different studies. The recommended cut-off point in the revised WHO classification is >50% (Swerdlow *et al*. 2016).

*Other prognostic factors*

Other prognostic factors have also been studied in DLBCLs and some studies show that, for example, TP53 mutations and CD5 protein expression are associated with poorer prognosis (Swerdlow *et al*. 2008, Xu-Monette *et al*. 2015).

### 2.2.4 Treatment and current issues in the treatment of diffuse large B-cell lymphoma

Today the standard treatment for DLBCL is the R-CHOP-21 regimen (rituximab, cyclophosphamide, doxorubicin, vincristine and prednisolone, given every 21 days). Rituximab is a CD20 antibody that was taken into clinical use at the beginning of the 21st century. It has improved the outcomes in all age groups when added to CHOP treatment (Coiffier *et al*. 2002, Pfreundschuh *et al*. 2006, Pfreundschuh *et al*. 2008). The amount of cycles given depends on the patient. For young patients with a good risk profile (low or low-intermediate IPI), six cycles of R-CHOP-21 is the standard treatment (Ghielmini *et al*. 2013). Some studies suggest the additional use of radiotherapy for a bulky disease. However, at the moment in the rituximab era, no general consensus on the indications prompting the use of radiotherapy exists (Sehn & Gascoyne 2015).

For young patients with a high-intermediate and high-risk IPI profile, there is no standard treatment. R-CHOP-21 may not be sufficient in these cases and R-CHOP including etoposide (R-CHOEP-14) is considered to offer one possible treatment for this patient group (Ghielmini *et al*. 2013, Greb *et al*. 2008, Holte *et al*. 2013). No randomised studies of the number of treatment cycles in this patient group exist. Before the rituximab era, etoposide was shown to benefit young patients (aged <60) but the use of etoposide, that is a toxic regimen, makes the use of the treatment not recommendable for all patients. Older patients (aged >60) especially showed increased toxicity and thus Wunderlich et al. did not recommend the use of etoposide in this age group (Wunderlich *et al*. 2003). There are no randomised trials that study the benefits of adding etoposide to an R-CHOP regimen.

Usually HDT + ASCT is used to treat relapsed and refractory aggressive DLBCL. In a work by Schmitz et al., no survival benefit was gained when HDT + ASCT was used in the primary treatment when compared to R-CHOEP-14 (Friedberg 2011, Schmitz *et al*. 2012).

For elderly patients, R-CHOP-21 for six or eight cycles is considered a standard treatment (Coiffier *et al*. 2002, Coiffier *et al*. 2010, Delarue *et al*. 2013, Leppä *et al*. 2008). Before the inclusion of rituximab into the standard treatment, a more intense treatment schedule for CHOP/CHOEP treatment with a 14-day interval, seemed to be superior to the 21-day interval (Pfreundschuh *et al*. 2004a, Pfreundschuh *et al*. 2004b). However, in the rituximab era, this dose-densification does not seem to offer any benefit, even in specific molecular or clinical subgroups (Cunningham *et al*. 2013, Delarue *et al*. 2013). In 2008, Pfreundschuh et al. compared the efficacy of six cycles of R-CHOP-14 (with eight cycles of rituximab) to eight cycles of R-CHOP-14 in elderly patients (aged >60) and suggested that six cycles of R-CHOP-14 is the preferred treatment (Pfreundschuh *et al*. 2008). Additionally, a study by Habermann et al. suggested that if a complete response (CR) is achieved after four cycles of R-CHOP-21, six cycles may be sufficient in this elderly patient group (Habermann *et al*. 2006).

Due to the toxicity of used treatment regimens, older, frail patients represent a more problematic patient group when it comes to the treatment. Currently, no standard therapy exists. R-miniCHOP that contains lowered doses of CHOP regimens, but a regular dose of rituximab has been suggested to offer a less toxic treatment option for elderly patients (Ghielmini *et al*. 2013, Peyrade *et al*. 2011).

### *2.2.5 Central nervous system lymphomas*

In rare cases, DLBCL shows a tropism to CNS in forms of secondary CNS involvement, called sCNSL or PCNSL. Just recently, CNS was considered an immunoprivileged site, but now there have been studies suggesting that a meningeal lymphatic vasculature exists in the brain that drains cerebrospinal fluid (CSF) and thus interstitial fluid (ISF) from the brain through the glymphatic system, to the deep cervical lymph nodes (dCLN) (Aspelund *et al*. 2015, Louveau *et al*. 2015). Yet, the mechanism explaining why and how lymphoma cells end up homing in the CNS, remains unknown. The most common hypothesis, which is supported by recent studies, suggest that lymphoma cells develop extracranially but develop a high CNS tropism and due to this, end up homing in the CNS (Jiang *et al*. 2010). The pathophysiology of CNS lymphomas is still poorly understood. New studies, where GEP has been used, have shown that there are significant differences in the transcription of several proteins, when normal lymphatic tissue, nodal and extranodal DLBCL and PCNSL are compared (Sung *et al*. 2011, Tun *et al*. 2008).

## *Primary central nervous system lymphoma*

PCNSL is a rare disease and in around 95% of the cases, the histology is DLBCL (Hochberg *et al*. 2007). DLBCL PCNSL represents < 1% of all NHLs (Swerdlow *et al*. 2008). The incidence of PCNSL is increasing, likewise the incidence of DLBCL in general (He *et al*. 2013, Villano *et al*. 2011). The reason for this increase is unknown, both for systemic DLBCL and for PCNSL as well. A prominent known risk factor for PCNSL is immunosuppression, especially HIV infection, but this only explains a small proportion of this increased incidence (Villano *et al*. 2011).

PCNSL is an aggressive disease with a poor prognosis. It is a rapidly progressing disease that will lead to a patient's death without quick initiation of therapy, the prognosis being significantly inferior to systemic DLBCL (Patrick & Mohile 2015). PCNSL usually involves the brain, meninges, spinal cord and cranial nerves and at times intraocular lesions are also seen (Phillips *et al*. 2014). It often presents a perivascular growth pattern in the brain (He *et al*. 2013). The pathophysiology behind PCNSL remains unclear. In a work by Jiang et al., PCNSL cells exhibited a strong CNS tropism, supporting the hypothesis that PCNSL cells originate from extracranial sites (Jiang *et al*. 2010). Recent GEP studies have shown differences in the expression of extracellular matrix and adhesion-associated pathways, when compared to systemic DLBCL and normal lymphatic tissues (Sung *et al*. 2011, Tun *et al*. 2008). Most often PCNSL is considered to represent ABC subtype DLBCL, but overlapping GC characteristics are also seen (Camilleri-Broet *et al*. 2006, Levy *et al*. 2008, Lin *et al*. 2006, Montesinos-Rongen *et al*. 2008, Raoux *et al*. 2010). Thus PCNSL does not represent as clear ABC/GC division as systemic DLBCLs. The symptoms caused by PCNSL can vary depending on the affected location, but usually patients present with focal neurological deficits or progressive neurocognitive dysfunction (Patrick & Mohile 2015). Most often the diagnosis is based on stereotactic biopsy, but at times it can also be based on flow cytometric (FCM) analysis of CSF lymphocytes, gained from lumbar puncture if there is no radiologically apparent tissue for biopsy (Phillips *et al*. 2014). FCM of CSF is more sensitive than CSF cytology, which is not very sensitive and can lead to false-negative results (Patrick & Mohile 2015). In addition, in the presence of ocular disease, diagnosis may be gained from vitrectomy samples as well.

No standard treatment exists for PCNSL, but the discovery of high-dose methotrexate (HD-MTX) has led to the use of HD-MTX containing therapy. High concentrations of MTX have been shown to be able to cross the BBB that represents the greatest challenge in the treatment of PCNSL (Phillips *et al*. 2014). Before this

whole-brain radiotherapy (WBRT) was used in the treatment but its use associates with high frequency of severe late neurotoxicity. In a study by Korfel et al., HD-MTX-based chemotherapy followed by WBRT did not increase survival significantly when compared to the same treatment without WBRT (Korfel *et al*. 2015). Bonn's therapy is currently used as a standard treatment option with variable results and in 2015 Harjama et al. showed that with a long follow-up time of 60 months, constant relapses were observed and only one patient from 54 remained in primary remission (Harjama *et al*. 2015, Pels *et al*. 2003). Currently, according to the IELSG32 study, the recommended standard intravenous therapy is the so-called MATRix regimen including high-dose methotrexate, cytarabine, rituximab and thiotepa. In the same study, consolidation treatments were compared and the second randomisation was done between whole-brain radiotherapy and ASCT and from these HDT + ASCT seems to be a promising consolidative therapy in PCNSL (Ferreri *et al*. 2016).

An interesting treatment option has also emerged called BBB disruption (BBBD) therapy. In this treatment, the problematic BBB that prevents the penetration of most substances into CNS, is momentarily disrupted with mannitol infusion, thus leading to the drugs accessing CNS at therapeutic levels. So far, it has been called an experimental treatment, but in one study it has been shown to lead up to 13.9 years OS in low-risk patients (Angelov *et al*. 2009).

### *Secondary central nervous system lymphoma*

CNS relapse occurs in around 5% of systemic DLBCL cases if prophylactic treatment is not given (Bos *et al*. 1998, Ghose *et al*. 2015, van Besien *et al*. 1998). It is most often a fatal complication. However, in the recent years, new treatments have shown some promises of a long remission time by most often using the treatment schema for PCNSL (Angelov *et al*. 2009, Harjama *et al*. 2015, Tai *et al*. 2011). Since the inclusion of rituximab into the standard therapy, the prognosis of DLBCL patients has improved significantly, but due to the poor penetration of the drugs included in the R-CHOP therapy trough the BBB, the incidence of sCNSL has not changed much, if at all. However, different studies have reported varying incidence rates of CNS relapses (Ghose *et al*. 2015, Tai *et al*. 2011, Yamamoto *et al*. 2010). Today CNS recurrence is usually seen as a solitary CNS disease, without systemic recurrence. This usually occurs within two years of the primary diagnosis, the median time for CNS recurrence being less than 1 year in most series. Earlier relapses may indicate the existence of a subclinical disease that was left undetected

at the primary diagnosis. Late progressions involving the CNS are uncommonly seen (Friedberg 2011, Holte *et al*. 2013, Siegal & Goldschmidt 2012, Zahid *et al*. 2016). The emergence of solitary CNS relapses is likely due to the inclusion of rituximab in the primary treatment, which has led to increased survival of patients with systemic disease, but fails to prohibit the CNS dissemination of the disease (Kridel & Dietrich 2011). The diagnosis of sCNSL is usually based on brain imaging and FCM of CSF (Friedberg 2011).

Most of the CNS relapses can be prevented by incorporating prophylactic treatment as a part of the primary treatment of systemic DLBCL. The optimal prophylactic treatment has been under research in recent years and currently it seems that intrathecal MTX alone is not sufficient and that intravenous HD-MTX should be included or used as a sole administration route (Ferreri *et al*. 2015, Kridel & Dietrich 2011, Tai *et al*. 2011). Currently the recommended prophylactic treatment includes HD-MTX and HD cytarabine combined with standard immunochemotherapy (Holte *et al*. 2013). A study by the Nordic Lymphoma Group showed that the administration of HD-MTX and HD cytarabine after 6 cycles of R-CHOEP-14 prevented about 50% of the anticipated CNS relapses in a 3-year follow-up (Holte *et al*. 2013). This prophylactic treatment is not unproblematic due to its high toxicity. For this reason, it should be limited to high-risk patients only. Currently the identification of high-risk patients is based on clinical risk assessment based on the following clinical factors: high IPI score, advanced stage, elevated LDH, age > 60 years, involvement of two or more extranodal sites and involvement of specific extranodal sites, especially CNS near lesions, bone marrow and/or testicles (Holte *et al*. 2013, Siegal & Goldschmidt 2012, van Besien *et al*. 1998). In addition, other extranodal sites have also been suggested, such as breast, kidney and adrenal glands. The results regarding the significance of breast involvement are controversial. Kidney and/or adrenal gland involvement is taken into account in the CNS-IPI risk model, which is discussed below (Schmitz *et al*. 2016). The problem with the clinical risk assessment is that it is not a very specific method. At best it is able to find a group of patients with around 20% risk for CNS relapse, indicating that around 80% of the patients that receive prophylactic treatment are receiving this toxic treatment in vain (Boehme *et al*. 2007).

In addition to the most commonly used clinical risk evaluation of CNS relapse, CNS-IPI has also been developed. This risk model consists of the five IPI factors and the involvement of kidneys and/or adrenal glands. This risk model has been shown to effectively stratify patients in three risk groups: low risk (0-1 points), intermediate risk (2-3 points) and high risk (≥ 4 points). In a study by Schmitz et

al., these risk groups show 2-year rates of CNS disease of 0.6%, 3.4% and 10.2%, respectively. Patients from their validation data also showed very similar rates of CNS disease (Schmitz *et al*. 2016). Also testicular involvement has been suggested as a risk factor for CNS relapse, though not all studies support this. A study by Kridel et al. reports that patients with testicular involvement represents a high CNS relapse risk group that is not captured by CNS-IPI (Kridel *et al*. 2017, Schmitz *et al*. 2016). In recent years, there has been ongoing studies of biological markers that would be able to indicate a patient group with a high risk for CNS relapse. So far clinical risk assessment seems to represent the best option, but recent studies suggest that CD5 positivity and non-GC phenotype may have some prognostic relevance in this matter (Jain *et al*. 2013, Oki *et al*. 2014, Petrich *et al*. 2014, Savage *et al*. 2016, Snuderl *et al*. 2010). However, in a study by Savage et al., the non-GC phenotype lost its predictive value in a multivariate analysis that included Myc and Dcl-2 double expression (Savage *et al*. 2016). The incidence of CNS relapses in double-expressor lymphomas is not clear, but the same study suggests a higher risk for CNS relapse in this patient group and thus CNS prophylaxis should be considered (Rosenthal & Younes 2016, Savage *et al*. 2016). Double-hit lymphomas are known to have a higher risk for CNS relapse and thus CNS prophylaxis is recommended for patients with HGBL with rearrangements of MYC and BCL2 and/or BCL6 (Rosenthal & Younes 2016).

If CNS relapse occurs, its treatment usually proceeds similarly to PCNSL. The number of studies comparing sCNSL treatment options is limited. There is an unmet need for biological prognostic factors to identify patients with a high-risk for CNS relapse and future studies are warranted.

## 2.3 Peripheral T-cell lymphomas

### *2.3.1 Clinical disease presentation and classification of peripheral T-cell lymphomas*

Peripheral T-cell lymphomas (PTCL) are rare neoplasms and together with NK-cell lymphomas, they comprise around 10% of all NHLs in Western countries, being more prevalent in Asia (Iqbal *et al*. 2014). In Finland, PTCLs alongside NK-cell lymphomas comprise 5-10% of NHLs (Finnish Cancer Registry - Institute for Statistical and Epidemiological Cancer Research). PTCLs as a group include several lymphoma subtypes that possess complex and also overlapping

morphological and immunophenotypic characteristics. In the 2008 WHO classification, the recognition of different entities relied heavily on morphological and IHC features, but in the forthcoming WHO revision, significant advances have been made in the classification of nodal and extranodal T-cell neoplasms. Many of these changes are due to the results of transcriptomic studies examining GEP and the genetic landscape of T-cell malignancies. All of this has led to revisions in classifications and to the introduction of new provisional entities (Swerdlow *et al*. 2008, Swerdlow *et al*. 2016). Due to the rarity of these diseases in the Western world, there are few published phase II studies and even fewer phase III studies regarding their optimal therapy. Because of missing knowledge of their biology, they have been treated with same therapies as B-cell lymphomas, mainly with suboptimal treatment results. Despite the progress made in the recent years in understanding the PTCLs, there is still no optimal single standard of care. As the need for better treatments remains, novel therapeutic options have been evaluated including histone deacetylase inhibitors, pralatrexate and monoclonal antibodies against targets such as CD30 and CD52. Taking into account that conventional chemotherapy alone is not sufficient, consolidation with ASCT and more recently also allogeneic stem cell transplantation (alloSCT) has been used in fit patients (Casulo *et al*. 2016, Schmitz & de Leval 2016, Zhang *et al*. 2016). Compared to the most common NHL DLBCL, PTCLs have clearly inferior outcomes (Casulo *et al*. 2016).

Due to the heterogeneity of the PTCL group, the disease presentations vary from extremely aggressive neoplasms to relatively slow growing, chronic conditions. PTCLs can present nodal and/or extranodal diseases. Most of the cutaneous lymphomas are T-cell lymphomas (Swerdlow *et al*. 2008, Swerdlow *et al*. 2016). In this thesis, we focused on the following peripheral T-cell lymphomas; AITL, PTCL NOS, ALK+ ALCL, ALK- ALCL and EATL. Cutaneous PTCLs were excluded from our studies and thus are not discussed herein.

### *Angioimmunoblastic T-cell lymphoma*

Angioimmunoblastic T-cell lymphoma (AITL) is an aggressive disease that arises from follicular helper T-cells. It is characterised by acute onset and constitutional symptoms. It most often manifests as an advanced stage disease. Patients often present with lymphadenopathy, hepatosplenomegaly, perturbations in the immunesystem and B-symptoms (Iannitto *et al*. 2008, Swerdlow *et al*. 2008). Polyclonal hypergammaglobulinemia is also common (Swerdlow *et al*. 2008). Skin

rash, ascites, pleural effusion, eosynophilia, thrombocytopenia, circulating immune complexes and autoimmune-type phenomena such as autoimmune hemolytic anemia (AIHA), vasculitis and polyarthritis are often seen at the time of diagnosis. Allergic reactions, also to several drugs, are common as well (Iannitto *et al*. 2008, Swerdlow *et al*. 2008). The affected patients are middle aged and elderly people and the incidence of AITL is as common among females as it is among males (Swerdlow *et al*. 2008).

AITL is a systemic disease with a polymorphous infiltrate involving lymph nodes. In most cases, the infiltrates are composed of a malignant monoclonal T-cell population yet some may also be polyclonal. Non-neoplastic reactive CD8+ T-cells are often present within the neoplastic infiltrate. In addition, the neoplastic infiltrate is frequently associated with increased dense meshwork of follicular dendritic cells and an expansion of normal B-cells is also often seen. What is interesting is that EBV positive B-cells are almost always present (Iannitto *et al*. 2008, Swerdlow *et al*. 2008). One of the ponderable features of AITL is the prominent proliferation of high endothelial venules (Swerdlow *et al*. 2008). The almost constant EBV association has suggested a role for EBV in the etiology of AITL. This could occur through antigen drive. It has also been proposed that because many patients with AITL have features of immunodeficiency due to the malignancy, this could provide a background for infection or more likely for reactivation of EBV (Swerdlow *et al*. 2008, Zhou *et al*. 2007). It should be noted that the neoplastic T-cells are EBV negative. Generally the etiology of AITL pathogenesis remains unknown. Secondary B-cell proliferations are seen in forms of plasmacytoma, DLBCL and Hodgkin's lymphoma (Swerdlow *et al*. 2008).

Traditionally, AITL has been treated with CHOP-like chemotherapy regimen with poor outcomes and around 20%-30% 5-year PFS (Schmitz & de Leval 2016). HDT with ASCT has improved the outcomes in this patient group and in 2012 Nordic Lymphoma Group conducted a prospective phase II study that supported the use of HDT + ASCT in PTCLs as a frontline therapy. The study consisted of PTCLs excluding ALK+ ALCL. With the consolidation treatment, the 5-year OS and PFS for AITL were 52% and 49%, respectively. Thus, ACST should be considered the primary treatment in patients eligible for a transplant. The authors also mention some new drugs, such as alemtuzumab, an anti-CD52 antibody that may further improve the treatment results (d'Amore *et al*. 2012).

### *Peripheral T-cell lymphoma, not otherwise specified*

PTCL NOS is a heterogenous category that includes nodal and extranodal mature T-cell lymphomas that do not belong to other specifically defined entities (Swerdlow *et al*. 2008). In the forthcoming revised WHO classification, a new provisional entity, follicular T-cell lymphoma (FTCL), has been proposed (Swerdlow *et al*. 2016). In addition PTCL NOS cases that express at least two or three TFH-related antigens, including CD279/PD1, CD10, Bcl-6, CXCL13, ICOS, SAP, and CCR5, are now being called nodal PTCLs with TFH phenotype. In the revised classification, these together with AITLs and FTCLs are being grouped under an umbrella category to highlight the spectrum of nodal lymphomas. This category is being called nodal T-cell lymphomas with TFH phenotype (Swerdlow *et al*. 2016). The cases remaining in the PTCL NOS group are still greatly cytologically and phenotypically heterogenous. A global GEP signature is close to the one of activated T-lymphocytes (Swerdlow *et al*. 2008, Swerdlow *et al*. 2016).

Because of the heterogeneity of the PTCL NOS group, the clinical manifestations may also vary. However, the majority of patients manifest lymph node enlargement and most have advanced diseases with B-symptoms. Eosinophilia, pruritus and rarely haemophagocytic syndrome are also seen. In addition to the most common peripheral lymph node involvement, any other sites may be affected as well and a widespread disease is often encountered with bone marrow infiltrates. Most patients are adults and PTCL NOS is generally very rare among children. It is almost twice as common among males as it is among females (Swerdlow *et al*. 2008).

The traditional treatment for PTCL NOS has been CHOP or CHOP-like regimen, but the response to therapy has been poor and frequent relapses and low survival rates are seen (Schmitz & de Leval 2016, Swerdlow *et al*. 2008). However, in a study by the Nordic Lymphoma Group, it was shown that patients with PTCL NOS benefit from frontline HDT with ASCT, achieving a 5-year OS and PFS rates of 47% and 38%, respectively (d'Amore *et al*. 2012).

### *Anaplastic large cell lymphoma*

ALCLs are a family of T-cell lymphomas, composed of systemic ALK+ ALCL, systemic ALK- ALCL and primary cutaneous ALCLs (C-ALCL). In the 2008 WHO classification, ALK- ALCL was considered a provisional entity due to the imperfect criteria for distinguishing ALK- ALCL from CD30+ PTCL. GEP studies

have facilitated the distinction between these two types, of which ALK+ ALCL has a superior prognosis, and as improved criteria now exists, ALK- ALCL is no longer considered a provisional entity. GEP studies have also provided evidence that ALK- ALCL and ALK+ ALCL have GEP signatures quite close to each other, providing a genetic rationale for the phenotypic and morphological similarities among these two entities (Swerdlow *et al*. 2008, Swerdlow *et al*. 2016). C-ALCL and lymphomatoid papulosis (LyP) are closely related and may resemble each other and sometimes a definite distinction between C-ALCL and LyP is hard to make. In addition to skin lesions, C-ALCL may also involve extracutaneous sites, mainly regional lymph nodes (Swerdlow *et al*. 2008).

Systemic ALCLs are CD30+ positive and their postulated normal counterparts are activated mature cytotoxic T-cells. ALK+ cases harbour t(2;5) translocations leading to ALK protein expression. ALK+ ALCLs have a board morphological spectrum, yet all cases contain a variable proportion of cells that present specific morphological features, called hallmark cells. These cells are typically large but smaller cells with similar cytological features may also be seen that greatly aid the diagnostic work. In lymph nodes the tumour cells typically grow within the sinuses (Swerdlow *et al*. 2008). ALK+ ALCL occurs predominantly in the first three decades of life and is slightly more common in men. It often involves lymph nodes as well as extranodal sites, most commonly the skin, soft tissues, bone, lungs and liver. Patients often manifest advanced stage disease and B-symptoms, especially high fever (Swerdlow *et al*. 2008). As an exception to most PTCLs, ALK+ ALCL responds excellently to CHOP-based chemotherapies and these are still recommended as first-line therapies. There are also novel agents, such as tyrosine kinase inhibitor of ALK, crizotinib, that are being investigated to establish whether they could increase the efficacy of CHOP-based treatment (Zhang *et al*. 2016).

ALK- ALCL can be distinguished from ALK+ ALCL based on the lack of ALK expression. ALK- ALCL most commonly occurs in adults aged around 40-65 years, but may also occur at any age. It is slightly more common in men. ALK- ALCL involves lymph nodes as well as extranodal sites, the latter including bone, soft tissues and skin. Extranodal involvements are less commonly seen than in ALK+ ALCL. ALK- ALCL most often presents with solid, cohesive sheets of neoplastic cells that efface the tissue architecture. In lymph nodes, when the tissue architecture is preserved, the tumour cells present with intrasinusoidal growth pattern or grow within T-cell areas. The morphological spectrum is similar to ALK+ ALCL although “small cell variant” is not recognized in ALK- ALCL (Swerdlow *et al*. 2008). It must be noted that cutaneous cases must be carefully distinguished from

C-ALCLs. Most patients present with advanced stage disease and B-symptoms (Swerdlow *et al*. 2008). Patients with ALK- ALCLs have a poorer prognosis than those with ALK+ ALCL and they also respond poorly to CHOP-based treatment (Swerdlow *et al*. 2008, Zhang *et al*. 2016). Frontline HDT + ASCT have yielded promising results as in the study by the Nordic Lymphoma Group; ALK- ALCL patients showed 5-year OS and PFS of 70% and 61%, respectively (d'Amore *et al*. 2012).

Brentuximab Vedotin is a new drug, an anti-CD30 monoclonal antibody conjugated with an antitubulin agent monomethyl auristatin E. It has shown excellent results in relapsed and refractory CD30+ ALCLs and if verified in prospective trials in the future, it may turn out to be the preferred therapy modality (Casulo *et al*. 2016).

### *Enteropathy-associated T-cell lymphoma*

EATL is a lymphoma of the intraepithelial T lymphocytes with a varying degree of transformation (Swerdlow *et al*. 2008). Increasing data gained during recent years has led to changes in the categorisation of intestinal T-cell lymphomas, including EATL. It has become clear that the two EATL subtypes that used to be called EATL type I and EATL type II, are two separate entities that will be distinguished in the forthcoming WHO revision. EATL type I will simply be called EATL and EATL type II will be officially designated as monomorphic epitheliotropic intestinal T-cell lymphoma (MEITL). EATL is closely associated with celiac disease and is mainly prevalent among individuals of Northern European origin. MEITL, on the other hand, appears to occur sporadically, independent of celiac disease and shows an increased incidence among people of Asian and Hispanic origin (Swerdlow *et al*. 2016). EATL is much more common than MEITL as before the division EATL type I used to represent 80-90% of EATL cases. EATL is composed of polymorphic medium- to large-sized lymphoid cells and it often has an inflammatory background. Enteropathic changes are present in adjacent intestinal mucosa and epithelial sites and necrosis is common as well. EATL is frequently CD30+ and cytotoxic markers are occasionally expressed. MEITL can be distinguished by its histologic appearance. The lymphoma cells are monomorphic with lymphocytic epitheliotropism and small- to medium-sized. Cells have an activated cytotoxic immunophenotype and the inflammatory background is absent. What is common between these two types is their transmural growth pattern and that both can present with ulceration (Ondrejka & Jagadeesh 2016). Similarly to most PTCLs, there is

no optimal treatment and the prognosis of EATL patients has been quite poor, as CHOP-based therapies have been used. Yet recently ifosfamide, vincristine, etoposide (IVE)/HD-MTX treatment followed by ASCT has shown some promising results (Ondrejka & Jagadeesh 2016).

## 2.4 Epithelial-mesenchymal transition transcription factors

Epithelial-mesenchymal transition (EMT) is a reversible process, that occurs during several physiological and pathological events such as embryonic development, wound healing, fibrosis and cancer progression (Nieto & Cano 2012, Vaquero *et al*. 2016). During EMT, the cells undergo changes that lead to the loss of their epithelial features and gain a mesenchymal-like phenotype. These changes include complex genetic and cellular changes that eventually lead to the loss of cell-cell interactions, certain adhesion-associated proteins such as E-cadherin, and also to the loss of cell polarity. Concomitantly, the cells gain mesenchymal markers and their migratory abilities are increased (Figure 2) (Nieto & Cano 2012). For example, the downregulation of E-cadherin is accompanied by upregulation of cadherins, such as N-cadherin and/or cadherin-11 which are considered mesenchymal-type cadherins. These favor weaker and more transient contacts between the cells (Nieto 2011). EMT has also been associated with increased synthesis of MMPs that enable the degradation of the basement membrane (Horejs 2016). Modifications of the extracellular matrix favor invasion and promote the movements of the cells that have gained mesenchymal-features. The breakdown of the basement membrane has been seen to occur at early stages of the developmental EMT. During embryogenesis, cell migration is a requisite for the generation of tissues and organs (Horejs 2016, Nieto 2011).

The recognized EMT inducers include the TFs of the Snail, basic helix-loop-helix (bHLH) and ZEB families. The Snail family includes SNAI1 and Slug (also known as SNAI2), ZEB family includes ZEB1 and ZEB2 and bHLH family includes Twist (also called Twist1), Twist2; another member of Twist family that has only rarely been associated with EMT when compared to Twist1, and E47/TCF3 (Moreno-Bueno *et al*. 2008, Nieto 2011). These TFs are known to repress E-cadherin expression and to initiate programs that lead to the loss of cell-cell junctions and the cells to gain migratory and invasive properties. They also have several other, TF-specific, functions that will be discussed in more detail later (Nieto 2011).

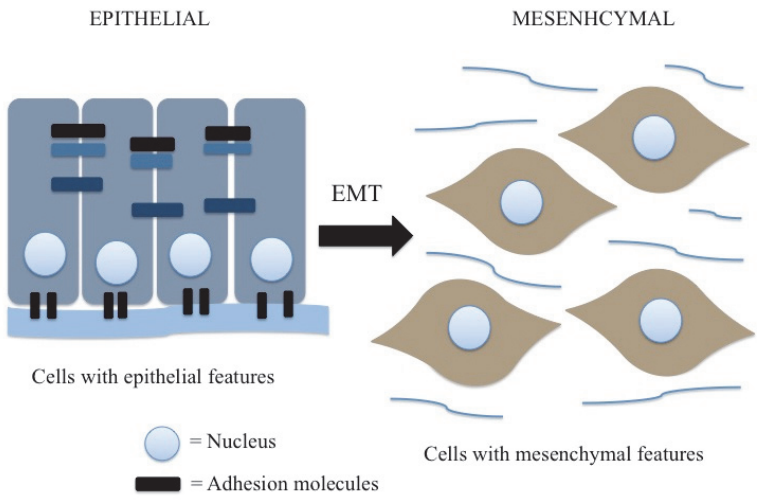


**Fig. 2. During EMT, the cells lose their epithelial features and gain a mesenchymal-like phenotype. These changes lead to the loss of cell-cell interactions, certain adhesion-associated proteins such as E-cadherin and loss of cell polarity. Concomitantly, the cells gain mesenchymal markers and their migratory abilities are increased.**

### *2.4.1 Epithelial-mesenchymal transition and Twist, ZEB1 and Slug in cancer*

EMT is considered a crucial step in cancer progression, especially in the development of metastases. Yet it is not considered the only mechanism causing invasion. In cancers, the EMT signature is seen as an activation of known EMT TFs, downregulation of E-cadherin and as a gain of mesenchymal markers, similarly to physiological EMT. In studies of solid malignancies, EMT signature has been associated with poor prognosis, invasiveness and aggressive cancer types (Nieto 2011). Twist, ZEB1 and Slug are known to induce EMT in epithelial cancers. As mentioned earlier, the downregulation of E-cadherin is considered a hallmark of EMT, also in cancers. Due to its ability to suppress tumour cell invasion and the metastasis CDH1 gene, that encodes E-cadherin, is sometimes called a metastasis suppressor gene (Montserrat *et al*. 2011, Paul *et al*. 1997, Thiery 2002). For example, in breast cancers, high expression levels of Twist are associated with

invasive lobular carcinoma, a highly infiltrating cancer type that has also been associated with loss of E-cadherin cell surface expression (Yang *et al*. 2004).

In addition to invasive and metastatic abilities, by attenuating immune responses, overcoming p53-mediated apoptosis regulation, oncogene addiction and oncogene mediated premature cell senescence, EMT is also able to promote tumour cell escape from the safeguard mechanisms (Nieto 2011). For example, Twist, ZEB1 and Slug are able to repress p53-mediated apoptosis and cell senescence (Cakouros *et al*. 2010, Haupt *et al*. 2006, Vandewalle *et al*. 2009). Twist is able to impair the induction of p53 target genes and can thus inhibit DNA damage-induced apoptosis (Maestro *et al*. 1999). It has been suggested to have a central role in helping cancer cells to gain abilities to resist apoptosis upon treatment with chemotherapeutics (Cakouros *et al*. 2010). In addition to this, it seems that Twist is able to regulate and be regulated by NF-kB. It seems to function as an NF-kB-controlled anti-apoptotic factor that at the same time is able to suppress NF-kB-dependent cytokine expression (Sosic *et al*. 2003). Slug has also been shown to participate in the regulation of p53-induced apoptosis and in this way has also been shown to enhance the proliferation and invasiveness of malignant cells (Haupt *et al*. 2006, Perez-Mancera *et al*. 2005).

### *2.4.2 Twist, ZEB1 and Slug in haematopoietic cells*

EMT has mostly been associated with cells of epithelial origin. Yet there are some studies that provide evidence that EMT TFs play a role in physiological haematopoietic cells as well as in lymphomas. They participate in physiological haematopoietic cell functions, such as cell survival and differentiation, and their roles in haematological malignancies are somewhat concordant. Yet the roles of Twist, ZEB1 and Slug seem to be different from each other, some of their functions being tumour-suppressive and others tumour-promoting. This is in contrast to their role in EMT in solid malignancies, where they are mainly assumed to function towards tumour progression (Brabletz *et al*. 1999, Nieto 2011, Perez-Losada *et al*. 2002, Postigo *et al*. 1997, Vandewalle *et al*. 2009, Wu *et al*. 2005, Zhang *et al*. 2012).

It should be noted that the concept of EMT is not directly applicable to haematological malignancies as it is to solid, epithelial, malignancies. Yet, for example, E-cadherin has also been shown to play a crucial role in lymphoma progression. In a study by Jacobs et al., patients with germline variations in the CDH1 gene had a 4.9-fold increase in the risk of developing a primary gastric

DLBCL. Thus E-cadherin expression could be considered antioncogenic in this lymphoma type (Jacobs *et al*. 2011).

In the hematopoietic system, Twist expression is largely restricted to the CD34+ stem cells. Yet in mouse studies, Twist was also found to function as a key modulator of $T_H1$ lymphocyte pro-inflammatory responses. Its expression was transiently induced following T-cell receptor stimulation through NF-kB and the nuclear factor of activated T-cells (NFAT) and after this, was enhanced and constantly detected under repeated T-cell stimulations. In human studies, Twist expression was found to be low in naive CD4+ T-cells and enhanced in activated effector memory CD4+ T-cells. The highest Twist expression was seen in terminally differentiated effector memory CD4+ T-cells. When CD4+ T-cells from inflamed tissues from patients with chronic inflammation were studied, Twist expression was discovered to be upregulated and its expression persistent, suggesting a role for Twist as a biomarker of chronic inflammation. In a physiological state, Twist2 seems to have functions mainly focused on the inhibition of galectin-1-mediated apoptosis. This occurs during a negative selection in the thymus after NF-kB activation. Both Twist and Twist2 seem to significantly reduce NF-kB binding to galectin-1 receptor, CD7, promoter. Twist2 upregulation thus increases resistance to galectin-1-mediated apoptosis and Koh et al. have demonstrated that this occurs in T-cell lymphomas as well (Koh *et al*. 2009, Merindol *et al*. 2014). Similar antiapoptotic roles have also been suggested for Twist (Merindol *et al*. 2014). In a work by Zhang et al., Twist expression was seen in ALK+ ALCL cells where it contributed to their invasiveness (Zhang *et al*. 2012). Twist knockdown also sensitised these cells to the growth-inhibitory effects of ALK inhibitor Crizotinib (Zhang *et al*. 2012). In a study of MF and SS, Twist protein expression was found to be increased in advanced MF/SS lesions (Goswami *et al*. 2012).

ZEB1 has been shown to take part in the maturation of the hematopoietic system and to be able to repress haematopoiesis through inhibition of c-Myb and Ets (Postigo *et al*. 1997, Vandewalle *et al*. 2009). It is also a physiological regulator of T cell development and differentiation. CD4, which is an important glycoprotein for mature T cell activation and differentially expressed during T cell development, is negatively regulated by ZEB (Brabletz *et al*. 1999). ZEB is also able to represses the GATA3 expression that is required for CD4+ T-cell development (Gregoire & Romeo 1999). In adult T-cell leukaemia/lymphoma (ATLL), ZEB1 has been suggested as a prospective tumour-suppressor gene since it is able to enhance transforming growth factor-beta (TGF-beta) signaling that is again able to induce

growth inhibition. In a work by Nakahata et al., ATLL cells resistant to TGF-beta growth suppression showed downregulation of ZEB1 mRNA (Nakahata *et al*. 2010). Also Hidaka et al. demonstrated that in a majority of ATLL cells, ZEB1 gene was mostly epigenetically deactivated and that in these cells, the transcription of CD4, alpha4 integrin and GATA3 was concomitantly upregulated. Their mouse models also showed that ZEB1 mutant mice frequently developed CD4 + T-cell lymphomas and/or leukaemias. Furthermore, in vitro downregulation of ZEB1 in ATLL cells associated with resistance to TGF-beta, suggesting that the escape from this growth inhibition is an important step in the pathogenesis of ATLL (Hidaka *et al*. 2008). Another mouse model studied the developmental effects of ZEB1. Interestingly, they found ZEB1 mutant mice to have impairment of thymus development. At early stages of development, ZEB1 mutant mice had a reduced T-cell count in the thymus, around 1% from the normal lymphocyte count. ZEB1 mutant mice also showed a significantly lower number of CD8 single positive T-cells in the thymus when compared to CD4 single positive T-cells (Higashi *et al*. 1997). Similar, supporting findings have also been seen in SS, where almost half of the patients diagnosed with SS were detected to have ZEB1 gene deletion (Vermeer *et al*. 2008). Both ZEB1 and ZEB2 are expressed in B-cells (Postigo & Dean 2000). Studies show that ZEB1 participates in silencing the IgH enhancer in B-cells as well as through repression of BCL6 it also participates in GC B-cell functions (Genetta *et al*. 1994, Papadopoulou *et al*. 2010).

Slug plays crucial roles in haematopoietic cell functions. In a work by Perez-Losada et al., haematopoietic developmental defects were seen in Slug mutant mice. They identified Slug as a molecule that contributes to the stem cell factor/c-kit (SCF/c-kit) signaling pathway functions that again seem to play a role in physiological hematopoiesis. One of the conclusions of the study was that Slug might be the factor controlling the migration and survival of c-kit positive cells (Perez-Losada *et al*. 2002). Slug is also known to be able to repress PUMA, a pro-apoptotic protein from the Bcl-2 family, and this way to protect irradiated haematopoietic cells from p53-mediated apoptosis (Wu *et al*. 2005). Thus the activation of Slug might contribute to neoplastic transformation by way of apoptosis inhibition. In chronic myeloid leukaemia (CML), Slug has been shown to contribute to the survival advantage of Bcr-Abl- expressing CML cells. In the same study, Bcr-Abl associated Slug over-expression resulted in a significant E-cadherin reduction at the cell membranes (Mancini *et al*. 2010).

Still the roles of Twist, ZEB1 and Slug in lymphoma progression remain poorly understood.

## 2.5 Chemokines and their receptors

To date there are approximately 50 known human chemokines and 20 G protein-coupled chemokine receptors. Most chemokine receptors are able to bind more than one ligand and most chemokines that act as ligands have more than one receptor (Raman *et al*. 2007). Chemokines and chemokine receptors can be divided into four families according to their pattern of cysteine residues: CXC, CC, C and CX3C, C representing cysteine and X/X3 representing one or three non-cysteine amino acids (Allen *et al*. 2007). When ligand binds to a chemokine receptor, a G protein coupled-receptor (GPCR), it leads to conformational changes in the receptor and intracellular signaling cascades that ultimately lead to the physiological responses, such as chemotaxis, which is the cell movement towards higher chemokine gradients (Allen *et al*. 2007, Marchese *et al*. 2008, Rot & von Andrian 2004). After activation, GPCRs are rapidly desensitized in order to prevent prolonged activity. The ligand-receptor complexes are internalised and are either recycled back to the cell membrane from endosomes or sorted from endosomes into lysosomes for degradation (Marchese *et al*. 2008).

Chemokines can be divided into two groups, inflammatory and homeostatic chemokines based on functional criteria (Zlotnik *et al*. 2011). Homeostatic chemokines are constitutively expressed by specific tissues and cells, whereas inflammatory chemokines are expressed upon activation, for example, from circulating leukocytes, and they participate in the recruitment of lymphocytes to the site of inflammation (Allen *et al*. 2007, Zlotnik *et al*. 2011). Some chemokines may have overlapping features and fall into both categories depending on the biological context (Zlotnik *et al*. 2011). Homeostatic chemokines are known to participate in organogenesis, to have crucial roles in stem cell migration and also to guide the physiological migration of lymphocytes. Certain chemokines are continuously expressed by tissues, such as lungs, skin and intestinal mucosa, where constant immune surveillance is needed. Homeostatic chemokines can also have direct proliferative effects on cells. In contrast to homeostatic chemokines, inflammatory chemokines are expressed temporarily in response to infection, tissue damage or other physiological abnormality. They participate in both innate and adaptive immunity. Both groups of chemokines can participate or promote angiogenesis (Zlotnik *et al*. 2011).

A chemokine-induced migratory pattern of a cell is determined by its specific chemokine receptor profile. The localised concentrations of chemokines act as a directional cue for the cells that migrate towards the chemokines that their receptors

bind (Figure 3). Ligand binding induces intracellular signals that trigger conformational changes in cells. These changes include changes in adhesion molecules that, for example, in leukocyte migration, enable integrin interactions and extravasation of cells from the bloodstream into tissues towards the chemokine gradient (Allen *et al*. 2007). For example, in human hematopoietic stem cells, CXCR4/CXCL12 signaling has been shown to induce integrin and CD44 activation and CD44 localisation to the leading edge during migration (Avigdor *et al*. 2004, Peled *et al*. 2000). Chemokines are also known to be associated with a number of pathologies, for example autoimmune disorders, transplant rejection and cancer (Allen *et al*. 2007).

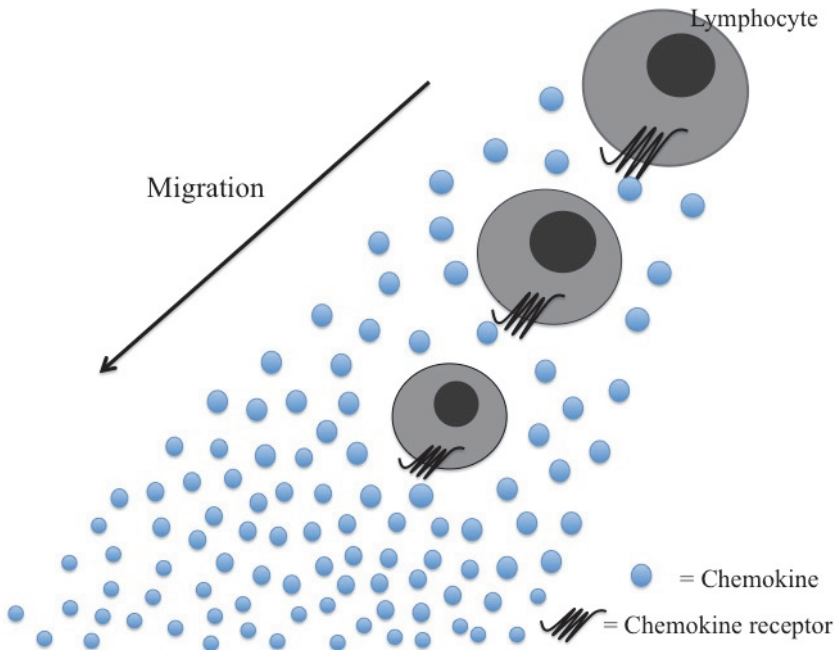


**Fig. 3. A chemokine-induced migration. The localised concentrations of chemokines act as a directional cue for the cells that migrate towards the chemokines. A migratory pattern of a cell is determined by its specific chemokine receptor profile.**

### 2.5.1 Chemokine receptors in lymphocytes homing

CXCR4, CXCR5 and CCR7 have separate and synchronised interactions that are important for the development of lymphoid organs. Their ligands are homeostatic chemokines: CXCL12 is the ligand for CXCR4, CXCL 13 for CXCR5 and CCL19 and CCL21 for CCR7. CCR7-CCL21 and the CXCR5/CXCL13 axis are known to be of importance for lymph node generation (Zlotnik *et al*. 2011). In mice, ectopic CCL21 expression in the pancreas has been shown to induce lymph node formation (Chen *et al*. 2002). Yet signaling through CXCR4 and CXCR5 is able to establish secondary lymphoid structures independently of CCR7 (Zlotnik *et al*. 2011).

CXCL13, the ligand for CXCR5, is mostly produced by lymph nodes and CXCL12, the ligand for CXCR4, by bone marrow, liver, lungs, lymph nodes and the brain. CCL19 and CCL 21, the ligands for CCR7, are mostly produced in secondary lymphoid tissues. In the bone marrow, CXCL12 recruits the CXCR4 expressing hematopoietic stem cells in their niche (Zlotnik *et al*. 2011).

In maturing B-cells, CXCR5 and CCR7 expressions are induced. Their expression enables the cells to exit bone marrow and enter the circulatory system. From there these cells are able to migrate to the sites of chemokine expression (Honczarenko *et al*. 2006). CXCR5 also regulates the recruitment and entry of B cells into lymph nodes. CCR7 is similarly required for T cell entry and recruitment into lymph nodes (Zlotnik *et al*. 2011).

In addition to haematopoiesis, the CXCR4/CXCL12 axis is also essential for the development of CNS and cardiovascular organs (Banisadr *et al*. 2003, Tachibana *et al*. 1998). It has been shown that under normal circumstances, CXCL12 is constitutively produced in the CNS (Kowarik *et al*. 2012). In diseases affecting the CNS, the CXCL13 levels seem to be increased (Kowarik *et al*. 2012, Smith *et al*. 2003). This will be discussed more later.

### 2.5.2 CXCR4, CXCR5 and CCR7 and their ligands in cancer

Chemokines and their receptors also play a role in carcinogenesis. They affect tumour cell proliferation, are able to modulate cell senescence and survival and play a crucial role in angiogenesis and metastasis formation. Chemokines and their receptors also play a role in the inflammation associated with carcinogenesis by promoting inflammatory conditions that may contribute to malignant transformation (Lazennec & Richmond 2010).

The CXCR4/CXCL12 axis has been widely studied in solid malignancies. It has been shown to participate in tumourigenesis and metastasis formation of solid malignancies. In addition to facilitating metastasis to distinct organs expressing CXCL12, the CXCR4/CXCL12 axis also participates in promotion of angiogenesis (Raman *et al*. 2007). CXCR4 seems to be upregulated in tumour cells under hypoxic conditions. Intratumoural hypoxia is a common feature in solid malignancies. This consequence of rapid tumour growth and insufficient vascularisation is an important microenvironmental factor driving cancer towards aggressive behaviour, neovascularisation and metastatic growth. It seems that hypoxia-induced CXCR4 upregulation plays a role in tumour cell migration and invasiveness (Oh *et al*. 2012). Nuclear expression of CXCR4 has been associated with lymph node metastasis and poorer prognosis, in numerous solid cancers such as colorectal cancer, hepatocellular cancer and non-small cell lung cancer (NSCLC) (Na *et al*. 2008, Wang *et al*. 2010, Xiang *et al*. 2009, Yoshitake *et al*. 2008). Cytoplasmic and membranous localisations of CXCR4 are also seen in solid malignancies with variable significances (Ben-Baruch 2008, Na *et al*. 2008, Wang *et al*. 2010, Xiang *et al*. 2009). In a study by Wang et al., CXCL12 binding localised CXCR4 to the nucleus in renal cell carcinoma cells. This nuclear localisation was associated with increased invasive abilities and a metastatic trait. Interestingly, CXCR4 was only found to be located in the nucleus in metastatic renal cell carcinoma cells when compared with the primary disease (Wang *et al*. 2009). The functional role of nuclear localisation of chemokine receptors and chemokines needs further elucidation. It is not certain whether these chemokine receptors are somehow involved in nuclear signaling or whether the receptor located in the nucleus merely represents a newly synthesised protein in endoplasmic reticulum that is located close to the nuclear membrane (Singh *et al*. 2009).

NSCLC's metastatic potential has been associated with CXCR4 expression. NSCLC preferentially metastasizes to organs that constitutively express CXCL12. In mouse models, CXCR4 inhibition was shown to reduce tumour metastases (Su *et al*. 2005). In a study by Hartmann et al., CXCL12 binding to CXCR4 induced integrin activation, which resulted in increased adhesion of small cell lung cancer (SCLC) cells to fibronectin and collagen. CXCR4 antagonists were able to inhibit these changes. In the same study, interactions with stromal cells seemed to protect SCLC cells from chemotherapy-induced apoptosis but this protection could also be antagonised by CXCR4 inhibition. These results imply that activation of chemokine receptors, in this case CXCR4, and integrins are able to promote adhesion and cell survival signals from the tumour microenvironment in addition

to induction of migratory abilities (Hartmann *et al*. 2005). Moreover, overexpression of CXCR4 has been suggested to contribute to brain metastasis in solid malignancies, such as colorectal cancer, NSCL and breast cancer (Chen *et al*. 2011, Lee *et al*. 2004, Mongan *et al*. 2009). In a study by Lee et al., CXCL12 was shown to be able to promote breast cancer cell transmigration through brain endothelial cells. Inhibition of CXCR4/CXCL12 signaling led to decreased transendothelial migration and vascular permeability (Lee *et al*. 2004).

CXCR5/CXCL13 co-expression has been associated with lymph node metastasis. In breast cancer, CXCL13 has been shown to have EMT inducing potential and in GEP, the primary breast tumour samples with CXCR5/CXCL13 co-expression show higher expression levels of mesenchymal markers (Biswas *et al*. 2014). Similar EMT-inducing abilities have been associated with CXCR4/CXCL12 signaling. EMT or an EMT-like process enables cell migration and invasion (Hu *et al*. 2014, Jung *et al*. 2015, Li *et al*. 2012, Roccaro *et al*. 2015, Yang *et al*. 2015). These changes in cancer cells probably attenuate cell migration towards higher expression gradients of chemokines.

In prostate cancer, nuclear CXCR5 expression seems to correlate with advanced Gleason score (Singh *et al*. 2009). Nuclear and membranous CXCR5 expressions have also been registered to be higher in NSCLC cells when compared to non-neoplastic tissues. NSCLC patients also manifest with higher serum CXCL13 levels than their healthy controls. It is not surprising that NSCLC cells with higher levels of CXCR5 migrate more in response to CXCL13 than the cells with lower CXCR5 expression (Singh *et al*. 2014). In addition to this, CXCL13 has been shown to promote the growth, migration and matrigel invasion of colon cancer cells. In the same study, it also stimulated the activation of the PI3K/Akt pathway and increased the expression and secretion of matrix metalloproteinase 13 (MMP-13). CXCR5 knockdown was able to inhibit the above-mentioned CXCL13-induced biological functions (Zhu *et al*. 2015). Even though in healthy tissues, CXCR5 is considered to be expressed primarily by mature B-cells and a small subset of T-cells; the CXCR5/CXCL13 axis clearly has a role in solid malignancies as well (Singh *et al*. 2014).

Similarly to CXCR4 and CXCR5, CCR7 has also been found to be associated with lymph node metastasis in several solid malignancies such as colorectal cancer, gastric carcinoma, esophageal squamous cell carcinoma, NSCLC, cervical cancer and melanoma. In haematological malignancies, CCR7 expression has also been somewhat associated with lymph node involvement. CCR7 has been suggested to play a key role in breast cancer lymph node metastasis. Its activation seems to

induce actin polymerisation, pseudopodia formation, invasion and chemotaxis. In CCR7 expressing thyroid tumour cells, its chemokine stimulation induces actin polymerisation, adhesion, migration and invasion. In NSCLC cells, CCL21 induces integrin-mediated adhesion (Ben-Baruch 2008).

It has been shown that chemokines and their receptors play a crucial role in solid malignancies, especially in enabling metastasis. They participate in the initiation of metastasis and direct cells towards higher chemokine gradients. Necessary steps towards this process require that tumour cells gain the ability to leave the primary tumour, to transmigrate through endothelial cells and the basement membrane, to migrate along blood or lymphatic vessels and that they are able to adhere to again when needed to invade the healthy tissues from the vessels. Cells also need to be able to survive and proliferate in the new environment. All of these changes include conformational changes and changes in the expression of adhesion and migration-associated molecules, such as cadherins and integrins. EMT has been shown to occur in solid malignancies due to chemokine signaling and it plays a major part in enabling many of the above-mentioned changes (Allen *et al*. 2007, Biswas *et al*. 2014, Hu *et al*. 2014, Jung *et al*. 2015, Li *et al*. 2012, Roccaro *et al*. 2015, Yang *et al*. 2015).

### *2.5.3 CXCR4, CXCR5 and CCR7 and their ligands in lymphoma*

Both homeostatic and inflammatory chemokines control lymphocyte migration. They orchestrate both the ongoing migration that includes immunosurveillance and homing of lymphocytes as well as the recruitment of lymphocytes to the sites of inflammation (Allen *et al*. 2007, Zlotnik *et al*. 2011). Synchronised interactions between CXCR4, CXCR5 and CCR7 are important for the development of lymphoid organs. The entry and recruitment of B-cells and naïve T-cells to the lymph nodes is regulated by CXCR5 and CCR7, respectively. CXCL12, on the other hand, recruits the CXCR4 expressing hematopoietic stem cells to their niche in the bone marrow (Zlotnik *et al*. 2011).

CXCR4, CXCR5 and CCR7 have been found to be highly expressed in B-cell malignancies with a widespread nodal disease. In general, chemokines and their receptors are considered to drive the migration of malignant NHL cells, somewhat similarly to the manner employed for their non-malignant counterparts (Lopez-Giral *et al*. 2004, Trentin *et al*. 2004). Different B-cell lymphoproliferative disorders seem to express varying chemokine receptor profiles, depending on the maturational stage of the malignant B-cell population (Durig *et al*. 2001). In

mediastinal large B-cell lymphoma, low expression levels of CCR7 and CXCR5 have been suggested to hinder lymphoma cells from nodal dissemination (Rehm *et al*. 2009).

In 2002 in a work by Husson et al. FL cells were seen to express CXCR5 and to secrete its ligand, CXCL13. CXCL13, alongside CXCL12, produced by stromal cells in lymphoid tissues, were found to have synergistic effects and the secretion of these chemokines was assumed to direct and participate in the accumulation of FL cells to specific anatomical sites (Husson *et al*. 2002). MCL cells have also been found to express high levels of functional CXCR4 and CXCR5 and very late antigen (VLA-4) adhesion molecules. MCL cells are suggested to interact with marrow stromal cells (MSC) in a CXCR4 and VLA-4 dependent way by adhering and migrating beneath them. In work by Kurtova et al. MSCs were found to confer drug resistance to MCL cells, especially to the ones migrating beneath them (Kurtova *et al*. 2009). In a study by Deutsch et al., both MALT lymphoma and extranodal DLBCL originating from MALT lymphoma, were also shown to express CXCR4, CXCR5 and CCR7 (Deutsch *et al*. 2008).

In T-cell NHLs, high CCR7 expression has been correlated with lymphatic and distant dissemination as well as with tumour cell invasion and migration in vitro. PI3K/Akt signaling has been said to be involved in this regulation (Kallinich *et al*. 2003, Yang *et al*. 2011). In Sezary syndrome (SS) it has been suggested that CCL19 and CCL21, along with CXCL13, control the chemotaxis of SS cells and that these chemokines have synergistic impact on cell migration (Capriotti *et al*. 2007, Picchio *et al*. 2008). In addition, the CXCR4/CXCL12 axis has also been suggested to play a role in MF progression and skin homing of SS cells (Daggett *et al*. 2014, Narducci *et al*. 2006).

As the mechanism behind lymphoma CNS tropism remains unclear, a role for chemokines and their receptors in this process have been proposed (Brunn *et al*. 2007, Smith *et al*. 2003, Smith *et al*. 2007, Venetz *et al*. 2010). In leukaemic T-cells, CCR7 is assumed to participate in the direction of T-cells towards the CNS and in an animal model by Buonamici et al., the silencing of CCR7 or its ligand CCL19 inhibited T-cell acute lymphoblastic leukaemia (T-ALL) cells from infiltrating to the CNS (Buonamici *et al*. 2009). In DLBCL, the CXCR4/CXCL12- and CXCR5/CXCL13-axis have both been correlated with CNS tropism and in a study by Rubenstein et al., it was shown that CXCL12 and CXCL13 acted as mediators of CNS lymphoma cell chemotaxis (Brunn *et al*. 2007, Rubenstein *et al*. 2013, Smith *et al*. 2003, Smith *et al*. 2007, Venetz *et al*. 2010). CNS affecting diseases, including CNS lymphoma, have been shown to lead to increased CXCL13

concentrations in the CNS. It seems that the main source of CXCL13 in PCNSL are the malignant B-cells, whereas CXCL12 is expressed in the CNS under normal circumstances (Kowarik *et al*. 2012, Smith *et al*. 2003). CXCL12 seems to be produced by the cerebral endothelial cells, reactive astrocytes and microglial cells but also by the majority of malignant B cells in PCNSL (Brunn *et al*. 2007, Smith *et al*. 2007, Venetz *et al*. 2010). In 2005 Jahnke et al. found that the expression of all three chemokine receptors CXCR4, CXCR5 and CCR7 were seen in PCNSL but the receptors seemed to be localised in the cytoplasm or nucleus with no membranous expression. By contrast, in systemic B-cell lymphomas, both membranous and cytoplasmic expressions were detected. The authors speculated that the receptor microlocalisation might play a role in CNS lymphoma development and disease biology (Jahnke *et al*. 2005). Later in 2008 Tun et al. showed in a GEP study that the CXCL13 expression was upregulated in PCNSL when compared to systemic DLBCL. They concluded that it probably is relevant in PCNSL pathogenesis and involved in B-cell migration (Tun *et al*. 2008).

The chemokine-chemokine receptor-signaling axis, especially CXCR4 and CXCR5 signaling, has been suggested as a potential therapeutic target in lymphomas and leukaemias but as yet no clinical trials on agents blocking the chemokine-chemokine receptor axis have been conducted (Beider *et al*. 2013, Kawaguchi *et al*. 2009, Panjideh *et al*. 2014).

## 2.6 Adhesion-, migration- and inflammatory responses-associated molecules

Cell adhesion is essential for cell organisation and various biological functions, such as cell migration. Cell-to-cell and cell-to-ECM adhesions are complex and often dynamically regulated. The ability to regulate dynamically is crucial for several physiological events, for example, the immune system functions. Cell-to-cell and cell-to-ECM are the two main types of cell adhesion, both consisting of transmembrane adhesion molecules that interact with intracellular signaling proteins and cell cytoskeleton. Adhesion molecules from the cadherin family play crucial roles in the formation and functions of cell-cell adhesions when again the major transmembrane proteins participating in cell-ECM adhesions are integrin heterodimers. Cell adhesions can be altered and are known to be rearranged during cell migration. Dysregulation of cell adhesions has been correlated with pathological processes such as cancer metastasis. For example, carcinoma metastasis is a complicated process that involves cancer cell invasion, migration

and extravasation. These steps require dynamic regulation of cell adhesions through endocytic and transcriptional regulation (Kawauchi 2012). Cell invasion is a crucial step for cancer metastasis and closely related to migration. Invasive abilities enable navigation through adjacent tissues into the secondary sites. It seems that certain cells are capable of clearing a path for themselves, whereas others invade through tissues due to their morphological plasticity rather than tissue degradation (Hanahan & Weinberg 2011, Kawauchi 2012).

Cancer cells exhibit various types of migration, mainly determined by the cell type and adhesion properties. These include collective migration, amoeboid migration and single mesenchymal migration. Amoeboid migration does not depend on adhesions to surrounding cells or ECM. EMT-mediated mesenchymal cell migration on the other hand requires transient adhesions that are dependent on the endocytic recycling of integrin-mediated adhesions. At the cell front, the cells form new adhesions to the ECM and at the end tail cell-ECM adhesions are disrupted (Kawauchi 2012). The integrin endocytosis promotes deconstruction of these adhesions and detachment from the ECM. These processes direct cell migration and especially integrin recycling is known to regulate migration of various cell types, for example fibroblasts, neutrophils and several cancer cell lines. Integrin trafficking has also been correlated with cancer metastases (Kawauchi 2012). Oncogenic mutant forms of p53, a known tumour suppressor, have also been associated with integrin trafficking, purportedly enhancing α5β1-integrin recycling and in this way promoting invasion and migration (Muller *et al*. 2009, Rainero *et al*. 2012). Some studies suggest that certain integrins associate with receptor tyrosine kinases and in this way activate signaling pathways that promote the invasive and mestastatic abilities of cancer cells. Integrin signals enable cancer cells to detach from the primary location and the surrounding cells, re-orientate their polarity, a necessary step during migration, and proliferate in the new environment (Guo & Giancotti 2004). During their journey metastasizing, cancer cells face changing tissue microenvironments, most likely presenting with changing matrix components. These changes demand adaptation. It is suggested that this demand is reached through changes in the expression of integrins (Hanahan & Weinberg 2000).

In a physiological state, adhesion molecules are known to be involved in lymphocyte circulation and homing. These processes are non-random. They are guided by chemokines and by the lymphocyte cell surface adhesion molecules and their counter-receptors that are expressed, for example, on the endothelial cells. Adhesion molecules also participate in mediating the extravasation of lymphocytes.

Lymphocyte-endothelial interactions are central in controlling the entry of specific lymphocytes into particular tissues. During circulation, lymphocytes roll on the endothelial surface. This is a transient process that can be stopped by a signal that leads to the activation of integrins (Drillenburg & Pals 2000, Pals *et al*. 2007). Chemokines, e.g. CXCL12, are known to mediate this activation. Activated integrins cause lymphocytes to get arrested at a specific site, an event that is necessary for extravasation (Allen *et al*. 2007, Drillenburg & Pals 2000). Transmigration is further supported by interactions with junctional adhesion molecules (Pals *et al*. 2007).

Engagement of adhesion receptors with their ligands transduces downstream signals into the cell that can have an impact on cytoskeletal organisation, but also to processes such as cell cycle progression and cell survival. This is called outside-in signaling. Also inside-out signaling occurs, as, for example, chemokine receptor or antigen stimulation can induce cytoplasmic signals that regulate the expression and activity of adhesion receptors (Drillenburg & Pals 2000). Likewise, NHLs often express specific homing receptors and display tissue-specific dissemination patterns. Thus adhesion molecules will most likely play a central role in the dissemination of lymphoid malignancies as well. They also mediate interactions between lymphoma cells and the tumour microenvironment and by promoting cell growth and survival, seem to contribute to lymphoma aggressiveness (Drillenburg & Pals 2000, Pals *et al*. 2007, Shain *et al*. 2015). The fact that lymphoma patients regularly present dissemination of the disease to the sites of inflammation and/or trauma supports this hypothesis. It seems that in the presence of inflammation and/or trauma, lymphoma cells are recruited to the specific site via activated endothelium and locally produced chemokines, similarly to physiological homing processes (Pals *et al*. 2007).

### *2.6.1 Integrin alpha 10*

Integrins are heterodimeric transmembrane glycoproteins that are composed of alpha and beta subunits, which are non-covalently linked together. The integrin family consists of 18 alpha and 8 beta subunits which together are able to form 24 different integrin receptors (Wenke *et al*. 2007). Integrin shifting means changes in the expression of integrin alpha and beta subunits on the surface of the migrating cells. In cultured cells, a forced expression of integrin subunits is able to inhibit or induce an invasive and metastatic behaviour (Hanahan & Weinberg 2000).

Integrin alpha 10 (ITGA10) interacts with beta 1 to form an alpha10-beta1 integrin. It is known to act as a collagen-binding integrin that preferentially binds to collagen type IV, which is the primary collagen type of the basal lamina. Yet it also binds two other collagen types, II and VI, which are the main collagen types in the ECM of cartilage and skeletal muscle, respectively. In a work by Engel et al., ITGA10 was found to be underexpressed in multiple solid tumours at the mRNA level when tumours were compared with their matched normal tissues, including melanoma (Engel *et al*. 2013). On the contrary, in a study by Wenke et al., ITGA10 transcription was induced in all melanoma cell lines when compared to normal epidermal melanocytes. The authors also showed that in melanoma in situ, ITGA10 expression was higher than in melanoma cell cultures. This might be due to the enhancing effects of the microenvironment in vivo. Repression of ITGA10 expression or ITGA10 inhibition hampered the migration of melanoma cells in vitro. Since integrin alpha V-beta 3 has been shown to promote chemotactic mobility of human melanoma cells, its inhibition leading to abolished chemotaxis, the authors of the study speculated that ITGA10 may have similar functions (Wenke *et al*. 2007).

Erythropoietin-producing hepatocellular type-B receptor 4 (EPHB4), a member of the receptor tyrosine kinase family, is overexpressed in several cancers. In prostate cancer, its knockdown has been shown to cause a significant reduction in in vivo tumour growth and cell motility in vitro. Interestingly, EPHB4 knockdown in prostate cancer cells resulted in ITGA10 and ITGB8 downregulation, further supporting a role for ITGA10 in cell migration (Mertens-Walker *et al*. 2015).

In anaplastic meningioma, upregulation of ITGA10 has been seen. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis showed that most of the integrins were upregulated in tumour tissues when compared to arachnoidal tissue. As integrins have a central role in mediating the effects of ECM on cells, it is likely that malignant cells enhance the expression of integrins that favor their survival, migration and proliferation. In concordance with this, the gene cascades that associate with integrin downstream pathways, were also found to be upregulated in anaplastic meningioma. For example, overexpression of FAK gene and total FAK protein was detected. Most integrins activate FAK and its downstream signaling pathways such as PI3K/Akt and Ras/ERK pathways. Many invasive cancers have been found to have elevated FAK levels and it seems to be crucial for cell migration and tumour growth (Wang *et al*. 2012). Additionally, several growth factors and growth factor receptors were also upregulated. There is a lot of crosstalk between growth factor receptor and integrin signaling pathways and some studies suggest

that both are required for cell cycle progression. They share common downstream pathways and it seems that integrin-mediated ECM adhesion promotes both survival and cell cycle progression (Danen & Yamada 2001, Wang *et al*. 2012).

In acute myeloid leukaemia (AML) and T-ALL, overexpression of the oncogene, the ETS-related gene (ERG) is an adverse prognostic factor. In a study by Mochmann et al., ERG overexpression in leukaemic cells promoted a mesenchymal-like gene expression signature, including ITGA10 as well as ITGB5, ITGB3, ITGA2B and CD44 expression, from which the last one will be discussed later in more detail. ERG also promoted chemoresistance. Authors of the study proposed that irrespective of the cell type, high ERG expression might do this by enhancing adhesion to the surrounding stroma that again might increase the cell survival and the likelihood of escaping the chemotherapeutic agents. They also suggested that a mesenchymal-like state also exists in acute leukaemia (Mochmann *et al*. 2014).

The role of ITGA10 in lymphomas is unclear. In a physiological state, only a limited number of integrins are expressed on normal lymphocytes. They mediate the interactions of lymphocytes with ECM components as well as with a variety of other cells, for example endothelial cells, epithelial cells and dendritic cells. Integrins play a crucial role in lymphocyte adhesion and transmigration through endothelium. In germinal centres, integrins mediate some of the interactions of germinal center B-cells with the environment. These integrin-mediated interactions have even been shown to confer protection from apoptosis in in vitro studies. Some integrin subunits have been demonstrated to be expressed in lymphoma cells and they are considered to contribute to the typical tissue distributions of specific entities (Drillenburg & Pals 2000).

### *2.6.2 E-cadherin*

E-cadherin is a calcium ion-dependent cell adhesion molecule that plays a pivotal role in epithelial integrity. It also has a central role in carcinogenesis (Jacobs *et al*. 2011). Its downregulation is considered a hallmark of EMT, both in physiological states and in cancers (Nieto 2011, Thiery *et al*. 2009). E-cadherin is able to suppress tumour cell invasion and metastasis and CDH1, the E-cadherin encoding gene, is thus at times called a metastasis suppressor gene (Paul *et al*. 1997, Thiery 2002). E-cadherin is specifically linked to hereditary diffuse gastric cancer that is caused by germline mutations in the CDH1 gene (Kaurah *et al*. 2007). In addition to mutations, E-cadherin expression can be altered by E-cadherin promoter

hypermethylation. This has been reported in various solid types, indicating that it is one of the key mechanisms involved in E-cadherin silencing in tumour progression. Other mechanisms include epigenetic alterations; loss of trans-activating proteins or over-expression of transcriptional repressors, such as EMT TFs (de Yzaguirre *et al*. 2006). E-cadherin has also been linked to CXCR4 signaling as it has been shown to negatively regulate E-cadherin expression in solid malignancies (Saba *et al*. 2016, Tu *et al*. 2017, Zhu *et al*. 2013). This is in line with the fact that CXCR4/CXCL12 signaling has been proposed to induce EMT or an EMT-like process in malignancies (Hu *et al*. 2014, Jung *et al*. 2015, Li *et al*. 2012, Roccaro *et al*. 2015, Yang *et al*. 2015).

When it comes to the role of E-cadherin in haematological malignancies, it is not as clear as it is in solid malignancies. In 1995, Armeanu et al. found that E-cadherin is expressed in the bone marrow by mononuclear cells. It seems that E-cadherin plays a functional role in the differentiation of erythroid lineage and its expression was restricted to defined maturation stages. E-cadherin inhibition led to diminished formation of erythropoietic cells (Armeanu *et al*. 1995). Intestinal intraepithelial T-cells of the mucosal immune system have also been found to express E-cadherin. In this compartment, the integrins and E-cadherin seem to mediate heterotypic adhesive interactions between epithelial cells and T-cells (Cepek *et al*. 1994). In a study by Ashton-Key et al., only 2 of 6 high-grade B-cell lymphomas expressed E-cadherin. By contrast, 12 out of 13 ALCLs were found to express E-cadherin. The authors concluded that this might be related to ALCL's more cohesive growth pattern (Ashton-Key *et al*. 1996). In HL, HRS cells have been shown to often manifest with loss of 16q22, where also CDH1 gene is located. Concordantly, HRS cells rarely seem to express E-cadherin (Ohshima *et al*. 2001). In leukaemia, CDH1 hypermethylation has been reported (Melki *et al*. 2000). E-cadherin does also seem to play a role in lymphomagenesis and/or lymphoma progression. A study by Jacobs et al. showed that patients with germline variations in the CDH1 gene presented a 4.9-fold increase in the risk of developing a primary gastric DLBCL (Jacobs *et al*. 2011).

Studies of mice have shown that E-cadherin is expressed by the stromal cells of the thymus, fetal thymocytes and by a fraction of T-cells (de Yzaguirre *et al*. 2006, Lee *et al*. 1994, Munro *et al*. 1996). It seems that E-cadherin also participates in thymocyte maturation (Muller *et al*. 1997). In 2006, a study by de Yzaguirre et al. showed that the expression of E-cadherin by the stromal cells and to a lesser extent by thymocytes was significantly reduced in thymic lymphoma. The downregulation of both E- and N-cadherin seem to occur via epigenetic

mechanisms concomitantly with the progression towards more advanced stages of thymic lymphoma, highlighting the role of the microenvironment in lymphomagenesis (de Yzaguirre *et al*. 2006).

### *2.6.3 Cadherin-11*

Cadherin-11 is considered a mesenchymal cadherin, which is upregulated during the so called "cadherin switch" (Cavallaro & Christofori 2004). It is a calcium-dependent cell-cell adhesion molecule that was first identified in mouse osteoblasts (Okazaki *et al*. 1994). In humans, cadherin-11 is expressed by osteoblasts, stromal and mesenchymal cells and cadherin-11 mRNA has been found to be expressed by several organs such as the brain, heart and lung (Kawaguchi *et al*. 1999, Shibata *et al*. 1996). Two different isoforms of cadherin-11 exist; an intact form and a splice variant (Kawaguchi *et al*. 1999).

Cadherin-11 has been shown to be expressed in solid cancers, such as gastric cancer, breast cancer and prostate cancer (Bussemakers *et al*. 2000, Pishvaian *et al*. 1999, Shibata *et al*. 1996, Tomita *et al*. 2000). In breast and prostate cancer, it has been associated with increased invasiveness (Huang *et al*. 2010, Pishvaian *et al*. 1999, Tomita *et al*. 2000). Sobolik et al. showed in their study in 2014 that in breast carcinoma cells, CXCR4 signaling resulted in EMT that was characterised by upregulation of ZEB1, loss of E-cadherin and gain of cadherin-11 (Sobolik *et al*. 2014). In addition, in experimental models, cadherin-11 has been shown to promote breast and prostate cancer cell homing and metastasis to bone (Chu *et al*. 2008, Huang *et al*. 2010, Lee *et al*. 2013, Tamura *et al*. 2008). Studies with contradictory results also exist. These suggest a tumour suppressor role for cadherin-11 in common solid malignancies (Carmona *et al*. 2012, Li *et al*. 2012). In a study by Li et al., cadherin-11 was found to inhibit Wnt/beta-catenin and Akt/Rho A-signaling as well as tumour cell migration and invasion in common carcinomas (Li *et al*. 2012). Also in osteosarcoma, reduced levels of cadherin-11 were associated with poorer patient survival and the authors concluded that the cadherin-11 loss might play a role in osteosarcoma progression and metastasis (Nakajima *et al*. 2008). Some studies suggest that the invasive abilities of cancer cells would depend on the splice variant form of cadherin-11 (Feltes *et al*. 2002).

In gliomas, the results regarding cadherin-11 expression are somewhat controversial (Berghoff *et al*. 2015, Delic *et al*. 2012, Kaur *et al*. 2012, Schulte *et al*. 2013, Zhou & Skalli 2000). In a study by Delic et al., cadherin-11 inhibition promoted glioma cell invasion whereas Kaur et al. showed that cadherin-11

knockdown in glioma cell lines resulted in decreased migration and cell survival (Delic *et al*. 2012, Kaur *et al*. 2012). Concordant with the results from the latter study, Schulte et al. showed that cadherin-11 can promote migration of neural precursor cells as well as glioblastoma cells. In their work, TGF-beta was shown to increase glioblastoma cell motility. Yet this effect could be inhibited by cadherin-11 knockdown, supporting a role for cadherin-11 in the regulation of cell motility and migration (Schulte *et al*. 2013). In addition to these results, cadherin-11 has been shown to be upregulated in glioblastoma in response to Twist, a central EMT activator (Mikheeva *et al*. 2010). Another EMT TF, ZEB2, is also able to induce cadherin-11 transcription and to promote cell invasion (Nam *et al*. 2014). In contrast to most solid malignancies, cadherin-11 has been suggested to have a tumour suppressor role in retinoblastomas (Corson & Gallie 2007, Marchong *et al*. 2004, Marchong *et al*. 2010). Cadherin-11 has also been associated with PI3K/Akt signaling. Kim et al. observed in their study that cadherin-11 induced Akt phosphorylation that led them to propose that cadherin-based cell adhesions are able to activate PI3K/Akt signaling (Kim *et al*. 2014). However, the role of cadherin-11 in lymphomas is unclear.

### *2.6.4 N-cadherin*

Like E-cadherin, N-cadherin is also a calcium ion-dependent cell adhesion molecule and is associated with EMT but contrary to E-cadherin, N-cadherin is a mesenchymal cadherin. For example, Twist is able to mediate N-cadherin upregulation during EMT (Guarino *et al*. 2007). N-cadherin is considered to be crucial for cancer progression of several solid cancers and to participate especially for metastasis formation and chemotherapy resistance (Gumbiner 2005). N-cadherin upregulation has been correlated with induced cancer cell motility (Nieman *et al*. 1999). As mentioned earlier, the loss of E-cadherin is often accompanied by gain of expression of N-cadherin in several solid malignancies. This process is called a ”cadherin switch” (Cavallaro & Christofori 2004, Guarino *et al*. 2007). When E-cadherin is largely expressed by epithelial cells, mesenchymal cadherins are expressed by stromal cells (Cavallaro & Christofori 2004). It is considered that N-cadherin mediates adhesion of malignant cells to the stromal and endothelial cells, both of which express N-cadherin (Cavallaro & Christofori 2004, Qi *et al*. 2005, Sandig *et al*. 1997). N-cadherin mediated adhesion has been shown to be involved in transendothelial migration of melanoma cells and has also been implicated in the interactions of breast cancer cells and vascular endothelial cells

(Hazan *et al*. 2000, Qi *et al*. 2005, Sandig *et al*. 1997). In addition to these, N-cadherin is known to participate in angiogenesis (Nalla *et al*. 2011).

However, the role of N-cadherin is less well known in haematological malignancies. In a study by Groen et al., an elevated N-cadherin expression was seen in plasma cells from about 50% of patients with multiple myeloma (MM) when compared to normal plasma cells. In the same study. it was shown that N-cadherin directly mediates the MM cell localisations to the BM. N-cadherin was shown to potentiate the MM cell motility, CXCL12-induced migration and to mediate the MM cell interactions with the BM, especially with the osteoblasts (Groen *et al*. 2011). Vandyke et al. showed that circulating N-cadherin levels act as a viable prognostic marker for high-risk MM patients (Vandyke *et al*. 2013). N-cadherin has also been shown to be expressed by early haematopoietic progenitor cells in the BM. However, during further differentiation, the expression is lost (Puch *et al*. 2001). Normal leukocytes do not seem to express N-cadherin, but interestingly it has been detected to be expressed by T-cell leukaemia and lymphoma cells (Kawamura-Kodama *et al*. 1999, Tsutsui *et al*. 1996). Nygren et al. concluded in their study that N-cadherin dependent mechanisms might mediate leukaemia cell-stroma interactions (Nygren *et al*. 2009). Kawamura-Kodama et al. proposed that since N-cadherin seems to be expressed on lymphoma cells it could have a role in mediating the frequently seen invasion of lymphoma cells into the mesenchymal tissues in the skin and also the CNS (Kawamura-Kodama *et al*. 1999). N-cadherin is known to have pivotal roles in regulating the neural development and maintaining the normal architecture of the neuroepithelium in the CNS and it is found widely expressed in the nervous system (Ganzler-Odenthal & Redies 1998, Gumbiner 2005, Kadowaki *et al*. 2007). Yet the role of N-cadherin in brain tumours remains elusive. The significance of N-cadherin expression in gliomas has been extensively studied, but the results are somewhat controversial. Several studies suggest that downregulation of N-cadherin correlates with increased invasion and migration (Asano *et al*. 2004, Camand *et al*. 2012, Peglion & Etienne-Manneville 2012, Shi *et al*. 2015, Xu *et al*. 2016). On the other hand, Utsuki et al. showed that N-cadherin was associated with a higher histological grade in astrocytomas (Utsuki *et al*. 2002). Then again in 2013, Zhu et al. showed that in human glioma cells CXCR4 silencing was able to inhibit the invasive and adhesive abilities of these cells as well as the expression of N-cadherin, beta-catenin, vimentin, TGF-beta1, Akt and the activity of TFs such as NF-kB, Snail and Twist. Further, a concomitant E-cadherin upregulation was observed, these results suggesting that N-cadherin is associated with a more invasive phenotype (Zhu *et al*. 2013).

N-cadherin functions have been associated with a PI3K/Akt signaling pathway (Nalla *et al*. 2011, Zhang *et al*. 2013). N-cadherin has been associated with increased Akt activity in prostate cancer and bladder cancer cells, in addition to invasive behaviour (Tanaka *et al*. 2010, Wallerand *et al*. 2010). A study by Zhang et al. showed that in erlotinib-resistant lung cancer cells persistent Akt phosphorylation was able to be overcome by N-cadherin inhibition (Zhang *et al*. 2013). Tran et al. also proposed that in metastatic cancer, N-cadherin adhesion is able to initiate anti-apoptotic signaling that enhances Akt cell survival pathway (Tran *et al*. 2002). Phosphatase and tensin homolog (PTEN), which dephosphorylates PIP3 and thereby inhibits the PI3K/Akt pathway activation, is considered to function as an important tumour suppressor, the loss of function of which is often seen in various cancers (Chalhoub & Baker 2009, Nguyen *et al*. 2014). Tran et al. concluded that cancer progression could be associated with PTEN loss and this could lead to increased responsiveness to extracellular signals such as cadherin mediated adhesions that activate PI3K/Akt pathways (Tran *et al*. 2002).

### *2.6.5 PTEN*

PTEN is a phosphatase that dephosphorylates phosphatidylinositol 3,4,5-triphosphate (PIP3) into phosphatidylinositol 4,5-bisphosphate (PIP2) and thereby opposes the activity of phosphatidylinositol 3-kinases (PI3K), acting as a negative regulator of the PI3K/Akt signaling pathway (Chalhoub & Baker 2009, Chow & Baker 2006). The PI3K/Akt signaling pathway regulates cell growth, survival and proliferation and as PTEN loss enables Akt activation, the active Akt isoforms drive the above-mentioned processes by further phosphorylating downstream signaling proteins. In addition to its physiological functions, PTEN acts as a tumour suppressor in various cancers and its lipid phosphatase activity has been shown to be essential for this function (Chow & Baker 2006, Song *et al*. 2012). It is one of the most commonly mutated tumour suppressors and in addition to mutations, it is often downregulated or suppressed in cancers as well (Song *et al*. 2012).

Somatic PTEN mutations or deletions are frequently found in several cancers such as melanoma, prostate cancer, endometrial cancer and glioblastoma. Lower prevalences of PTEN mutations have also been observed in bladder, colon, ovary and lung cancers as well as in lymphoma (Morris *et al*. 2010). PTEN levels can be regulated through several mechanisms, such as inactivation of a given PTEN allele or repression of PTEN gene expression. Other mechanisms include post-translational modifications and protein-protein interactions that can cause a subtle

or dramatic loss of PTEN function or even have a positive regulatory effect on PTEN function. Also enhanced degradation and aberrant subcellular compartmentalisation of PTEN have been associated with cancer (Naguib & Trotman 2013, Song *et al*. 2012).

PTEN is often characterised to be located in the cytoplasm, from where it is recruited to the cell membrane where it dephosphorylates PIP3 and antagonises PI3K signaling. However, PTEN can also be located in the nucleus. The variations in PTEN distribution may occur due to multiple reasons, for example different cell types, mutations and even technical variations in different studies, i.e. the used antibodies, might explain some of the differences seen. The significance of PTEN's subcellular location has been of interest and some studies suggest a tumour suppressor function for nuclear PTEN whereas other studies demonstrate that nuclear PTEN participates in controlling genomic stability and positively regulates DNA-damage responses and cell cycle progression (Chalhoub & Baker 2009, Song *et al*. 2012). It is suggested that these functions of nuclear PTEN depend on mechanisms other than its lipid phosphatase activity (Song *et al*. 2012). In ischaemic neurons, nuclear translocation of PTEN occurs and it might be that it has two different neuroprotective functions: activation of nuclear-specific functions and deprivation of PTEN from the cytoplasm, which would allow cytoplasmic Akt-dependent cell survival signaling (Naguib & Trotman 2013). With the current knowledge, it seems that the best neuroprotection is reached by blocking cytoplasmic PTEN and retaining nuclear PTEN (Krishnan *et al*. 2016).

What comes to suppressing cancer, PTEN is haploinsufficient (Naguib & Trotman 2013). Reduced PTEN expression increases cell growth and proliferation. Interestingly though, complete loss of PTEN leads to cell senescence. This phenomenon could explain the fact why complete PTEN loss is not a very frequent event in different cancers and why PTEN loss often coincides with functional p53 loss in late-state diseases. PTEN has also been demonstrated to play a role in the self-renewing activity of physiological as well as leukaemic HSC. PTEN loss results in a depletion of physiological HSC, a generation of leukaemia-initiating cells and promotes leukaemogenesis (Song *et al*. 2012).

It seems that PTEN also plays a role in establishing cell polarity and inhibiting cell migration of various cell types. In neurons, PTEN inhibition as well as knockdown has been shown to increase neurite outgrowth. Neural stem cells show an increased proliferation and self-renewal in the presence of PTEN loss (Leslie *et al*. 2008, Zochodne 2014). Christie et al. showed that the effects of depleted PTEN were mediated through PI3K/Akt-signaling (Christie *et al*. 2010). However, there

are also studies that suggest that the effects of PTEN on migration and cell polarity are mediated through mechanisms other than PIP3-dependent pathways (Leslie *et al*. 2008).

PTEN has also been linked to EMT and it seems that SNAI1, one of the EMT TFs, can target PTEN and mediate PTEN repression. PTEN has thus been suggested to participate in SNAI1 mediated loss of cell polarity and survival properties (Moreno-Bueno *et al*. 2008). A study using chicken embryos showed that PTEN was able to modulate the cell migration of mesoderm cells by controlling EMT and the directional motility of these cells. More specifically, the overexpression of PTEN led to EMT inhibition in certain mesoderm cells (Leslie *et al*. 2007). In the development of epithelial-derived cancers, PTEN function is frequently lost and many of these types of cancers are thought to go through an EMT-like process in order to metastasise. As dysregulation of the PTEN-PI3K axis has been linked to the gain of mesenchymal properties, increased cell motility and invasiveness, it is speculated that these functions of PTEN might also contribute to its tumour suppressor abilities (Leslie *et al*. 2008, Song *et al*. 2012). In concordance with this are the findings from a study by Song et al., wherein nasopharyngeal carcinoma the upregulation of Bmi-1, a molecule that is dysregulated in several different cancers, induced EMT and transciptionally downregulated PTEN expression levels. The results of their study suggested that PTEN downregulation might be a crucial event in Bmi-1 induced EMT, leading to activation of PI3K/Akt/Snail pathways (Song *et al*. 2009).

The survival of mature B-cells in the peripheral immune system depends greatly on the B-cell receptor (BCR). The BCR, for example, mediates the interplay between outside-in and inside-out signaling by CXCR4 and integrins. It plays a crucial role in malignant B-cell survival and homing. It also participates in the environment-mediated drug-resistance (Shain & Tao 2014, Srinivasan *et al*. 2009). It seems that in mature resting B-cells, the PI3K signaling acts as a critical survival determinant downstream of the BCR and interestingly Srinivasan et al. showed that B-cells lacking BCR could be rescued by PTEN inhibition (Srinivasan *et al*. 2009). In a work by Suzuki et al., PTEN-deficient B-cells showed resistance to apoptotic stimuli, hyperproliferated and showed enhanced migration (Suzuki *et al*. 2003). Meanwhile, PTEN deficient mice did not show signs of B-cell malignancies, even though PTEN mutations have been reported in human B-cell malignancies (Gronbaek *et al*. 1998, Hyun *et al*. 2000, Nakahara *et al*. 1998, Suzuki *et al*. 2003). Additionally, in T-cells it has been shown that homozygous PTEN deletion in mice led to the development of T-cell lymphomas. PTEN-deficient T-cells also showed

hyperproliferation, autoreactivity, apoptosis resistance and increased phosphorylation of Akt (Suzuki *et al*. 2001).

PTEN has also been linked to chemotaxis and CXCR4/CXCL12 signaling. In prostate cancer cells, PTEN loss resulted in increased CXCR4 and CXCL12 expression, supposedly via PI3K/Akt signaling (Conley-LaComb *et al*. 2013). In NSCLC, PI3K inhibitors were able to prevent CXCL12-induced chemotaxis and PTEN introduction to the cells inhibited CXCR4 upregulation in response to hypoxia (Phillips *et al*. 2005). Further, in T-cells PTEN downregulation has been shown to enhance CXCR4/CXCL12-mediated chemotaxis (Gao *et al*. 2005).

### *2.6.6 Lactoferrin*

Lactoferrin is a protein with an iron-binding ability. It is synthesised by most mammalian tissues and is found in high concentrations in human milk. In addition, it is found in lower concentrations in a variety of other secretions as well. Lactoferrin has several physiological activities, such as anti-inflammatory, antibacterial, antiviral, antifungal and immune regulatory activities. In addition, it has been reported to have anticancer functions (Zhang *et al*. 2014).

Lactoferrin has two isoforms, delta-lactoferrin and lactoferrin, with different intracellular distributions, cytosolic lactoferrin and secreted lactoferrin, respectively. Both isoforms have been reported to enter the nucleus and to contain nuclear targeting signals (Benaissa *et al*. 2005, Zhang *et al*. 2014). They are able to regulate a number of genes by either activating signaling pathways or directly binding to DNA, thus acting as transcription factors (Mariller *et al*. 2007, Oh *et al*. 2001, Oh *et al*. 2004). For example, lactoferrin binding to the intercellular adhesion molecule-1 (ICAM-1) promoter in endothelial cells inhibits tumour necrosis factor-(TNF) alpha-stimulated ICAM-1 expression by competing with NF-kB. As the upregulation of endothelial adhesion molecules plays a central role in the recruitment of leukocytes to the inflammatory site, it has been suggested that this inhibitory effect of lactoferrin is one mechanism by which it is able to reduce inflammatory events (Kim *et al*. 2012).

Lactoferrin has been demonstrated to have a role in modulating the activation of cells involved in innate and adaptive immunity, having either a pro-inflammatory or anti-inflammatory role. It also seems to interact with membrane components on cell surfaces, such as cell receptors, probably having a direct impact on the activity of immune cells, especially on the proliferation, maturation and differentiation of lymphocytes. Yet because lactoferrin is not normally synthesised in lymphoid

tissues, it is unlikely that it plays a role in physiological maturation and differentiation of lymphocytes (Legrand & Mazurier 2010). Mature lymphocytes, on the other hand, do express lactoferrin. In a work by Hoedt et al., both lactoferrin and delta-lactoferrin mRNAs were expressed by lymphocytes. When different tissues were studied, especially leukocytes from fresh human blood expressed very high levels of delta-lactoferrin mRNA, the hippocampus expressed only slightly lower levels. By comparison, the Jurkat cells, leukaemic T-cells, did not express delta-lactoferrin mRNA (Hoedt *et al*. 2010).

Interestingly, bovine lactoferrin (b-lactoferrin) has been shown to induce apoptosis of malignant cells such as B-lymphoma cells. In a study by Furlong et al., immune-deficient mice with B-cell lymphoma that were administered with b-lactoferrin exhibited extended survival (Furlong *et al*. 2010). Yet another study associated high plasma lactoferrin levels with poorer prognosis in AML (Bezwoda & Dajee 1991).

Lactoferrin gene has been suggested to act as a tumour suppressor gene (Zhang *et al*. 2011, Zhou *et al*. 2008). It has been shown that delta-lactoferrin expression and lactoferrin overexpression are able to promote cell cycle arrest (Breton *et al*. 2004, Zhou *et al*. 2008). The silencing or downregulation of lactoferrin or cytoplasmic delta-lactoferrin genes has been found in various cancer cell lines (Benaissa *et al*. 2005, Hoedt *et al*. 2010, Liu *et al*. 2003, Siebert & Huang 1997, Ward *et al*. 2005). It is assumed that genetic and epigenetic deactivations of the lactoferrin gene may provide the tumour cells a needed selective growth advantage (Benaissa *et al*. 2005, Hoedt *et al*. 2010). The lactoferrin gene polymorphism and promoter hypermethylation have been associated with lactoferrin functions and cancer occurrence (Zhang *et al*. 2011, Zhang *et al*. 2014, Zhou *et al*. 2012). In breast cancer, both lactoferrin and delta-lactoferrin have been shown to have prognostic value, with high concentrations correlating with longer overall survival (Benaissa *et al*. 2005). However, conflicting results also exist, as lactoferrin has been shown to increase the migration and invasiveness of breast cancer cell lines (Ha *et al*. 2011).

The exact anticancer mechanisms of lactoferrin remain somewhat unclear. They are thought to include intracellular effects, extracellular effects and immunostimulation. It is also thought that lactoferrin synergises with other anticancer agents or pathways in order to exert its anticancer effects (Zhang *et al*. 2014).

In inflammatory conditions and under oxidative stress, lactoferrin expression is upregulated. In the brain, it is synthesised by the activated microglial cells and

in common neurological disorders, such as Parkinson's and Alzheimer's diseases, lactoferrin concentrations are increased. In these neuropathological disorders, oxidative stress and inflammatory processes that also lead to increased secretion of cytokines contribute to neuronal death. Because lactoferrin is able to inhibit these effects, it may prevent cell damage and, in this way, protect neuronal integrity (Fillebeen *et al*. 2001). Rousseau et al. provided supporting evidence, showing that in different experimental settings modeling neurodegenerative events, lactoferrin provides strong protection of dopamine neurons (Rousseau *et al*. 2013). Lactoferrin has also been shown to provide neuroprotection against prion protein mediated neurotoxicity and to protect ventral mesencephalon neurons against 1-methyl-4-phenylpyridinium ion -induced damages in experimental settings (Park *et al*. 2013, Wang *et al*. 2015). In addition to its above-mentioned effects, lactoferrin-mediated promotion of neuronal survival has been linked to PI3K/Akt-dependent mechanisms. Akt activation seems to be essential for its survival-promoting abilities (Rousseau *et al*. 2013). Then again in solid cancers, where lactoferrin has been associated with tumour suppression, it has been shown to suppress Akt signaling (Deng *et al*. 2013a, Deng *et al*. 2013b, Xiao *et al*. 2004, Xu *et al*. 2010). Only one study exists of the role of lactoferrin in CNS lymphoma and leukaemia. In this work, lactoferrin levels were increased in the CSF among some of the patients with CNS involvement when compared to patients without CNS involvement (Oberg *et al*. 1987).

### *2.6.7 CD44*

CD44 is a transmembrane glycoprotein that was originally identified as lymphocyte-homing receptor and a receptor for hyaluronan (HA). It is known to participate in both physiological and pathological processes such as cell adhesion, inflammation, angiogenesis and tumour development (Xu *et al*. 2015). In addition to HA, CD44 is capable of binding other ECM components as well, such as osteopontin, collagens, MMPs and growth factors, yet HA is its most common and immediate ligand (Thapa & Wilson 2016). CD44 expression can be regulated by both extracellular as well as intracellular factors, one example being the Wnt/beta-catenin-pathway (Wielenga *et al*. 1999, Xu *et al*. 2015). CD44 is also able to regulate the Wnt/beta-catenin signaling pathway as CD44 downregulation has been shown to affect beta-catenin phosphorylation and nuclear accumulation in CML cells (Chang *et al*. 2013, Wielenga *et al*. 1999, Zeilstra *et al*. 2008).

Regarding CD44, there exists a standard form (CD44s) and several variant forms (CD44v) that are results of a tightly regulated alternative splicing and post-translational modification (Thapa & Wilson 2016). CD44s is found in most cells when again the variant isoforms are restricted to more specific cell types (Dzwonek & Wilczynski 2015, Orian-Rousseau 2015, Zoller 2015). For example, haematopoietic cell maturation and activation have been shown to lead to expression of CD44v isoforms (Orian-Rousseau 2015, Zoller 2015). In addition, CD44v isoforms are found expressed in proliferative tissues, such as the intestine and skin (Orian-Rousseau 2015). Further, the presence of variant isoforms has been demonstrated in normal human brains as well as in specific brain tumours, although CD44s presents the predominant form in the nervous system (Dzwonek & Wilczynski 2015).

Many CD44 isoforms have been shown to play a role in various cancers and correlations with metastatic progression have also been seen. However, some of the results are conflicting. This has been considered to occur due to different antibodies used, as they probably have divergence in their ability to target different CD44 isoforms. Further, the different methods, such as PCR vs IHC, may lead to different results (Thapa & Wilson 2016). The standard form has also been associated with aggressiveness and tumour grade in a few solid malignancies, such as hepatocellular carcinoma (HCC) and breast cancer (Xu *et al*. 2015). Still today, the regulation of CD44 alternative splicing in tumourigenesis is not completely understood (Orian-Rousseau 2015, Prochazka *et al*. 2014).

Different isoforms have been reported to have a central role in the regulation of EMT. Both in cancer cells as well as non-tumourigenic epithelial cells, a CD44 isoform switch has been reported to occur as part of the EMT process. This switch from CD44v to CD44s activates Akt signaling that again further activates EMT (Xu *et al*. 2015). In HCC, high CD44s expression levels have been shown to correlate with an EMT expression profile (Mima *et al*. 2013). Yet in other studies, CD44v6, a variant form, has been associated with EMT features (Ni *et al*. 2014, Saito *et al*. 2013). Despite the differences between the different isoforms, extensive research has indeed revealed that CD44 plays a crucial role in EMT and in a variety of cancers (Xu *et al*. 2015). EMT has also been shown to induce a CD44-positive phenotype with high CD44 expression (Mani *et al*. 2008, Xu *et al*. 2015). Twist has been shown to induce CD44 expression and in a work by Li and Zhou, Twist was shown to do this through the activation of the beta-catenin and the Akt-signaling pathways (Li & Zhou 2011, Xu *et al*. 2015). In contrast E-cadherin takes part in the negative regulation of CD44 expression (Deep *et al*. 2014). CD44 expression has

been linked to the activation of PI3K/Akt and by inhibiting the formation of membrane-bound E-cadherin-beta-catenin complexes, it is also able to promote beta-catenin nuclear trafficking and signaling and the downstream activation of invasion and migration-related genes (Cho *et al*. 2012, Xu *et al*. 2015). In CLL, it is assumed that the ECM may provide survival signals to the cells via engagement of CD44. This further activates pathways such as the PI3K/Akt pathway, which is capable of providing protection from apoptosis (Herishanu *et al*. 2011).

Increasing evidence suggests that CD44/HA interaction plays a key role in tumour progression. What is interesting is that all CD44 isoforms are capable of interacting with HA, all sharing a common ligand-binding region for HA (Wang & Bourguignon 2011, Xu *et al*. 2015). HA is a component of the ECM that is found in various cell types, such as endothelium (Jiang *et al*. 2007). CD44/HA interactions promote activation of pathways in malignancies that lead to cancer cell growth, increased invasion and migration and apoptosis resistance (Thapa & Wilson 2016). As CD44 has been shown to promote the invasion and metastasis of multiple cancer types, one mechanism for this is suggested to be the contribution of CD44 to the ability of cancer cells to adhere to endothelium. Enhanced adhesion to endothelial cells has been shown to correlate with the metastatic potential (Xu *et al*. 2015). McFarlane et al. showed in their study that CD44 knockdown in human breast cancer cells attenuated the tumour cell adhesion to endothelial cells and, in this way, reduced invasion. They also demonstrated that elevated CD44 expression enhanced distant metastases in an in vivo mouse model (McFarlane *et al*. 2015). There are also other, supporting studies providing evidence that CD44 knockdown leads to suppressed adhesion and invasion. In addition, CD44 knockdown has been shown to be able to sensitise cancer cells to treatment-induced apoptosis and to suppress progression of certain cancers (Xu *et al*. 2015). Because of these effects, CD44 is an interesting target for cancer treatment studies. Different CD44 targeting treatments have reached clinical trials, but their use still requires more information and studies remain ongoing (Thapa & Wilson 2016, Xu *et al*. 2015).

HA is also the key component of the brain ECM where CD44/HA interactions play a central role in various physiological and pathological processes. Some of these processes include neuronal development and growth, response to injury and neurodegeneration. In addition, CD44 is frequently expressed in brain malignancies. High CD44 expressions have been demonstrated to correlate with poor prognosis in patients with brain tumours and both variant and standard forms associate with invasive abilities of various brain tumours (Dzwonek & Wilczynski 2015). In glioblastoma multiforme, brain tumour stem-like cells express high CD44

levels and their growth has been suggested to depend on CD44v6/Akt signaling (Jijiwa *et al*. 2011). Still, contradictory results exist from neuroblastomas, where advanced tumours often present low CD44 expression levels (Dzwonek & Wilczynski 2015). CD44 also seems to play a role in injury and inflammatory processes of the brain.

The different isoforms of CD44 are stem cell markers that were first described in hematopoietic stem cells (Zoller 2015). Interestingly, CD44 has also been linked to cancer- and leukaemia-initiating cells, so-called cancer stem cells (CSC) (Xu *et al*. 2015, Zoller 2015). CSCs are said to drive tumour initiation and metastasis. Several studies show that in certain cell types, CD44 expression plays a central role in tumourigenesis (Xu *et al*. 2015). It has also been shown that when abundantly expressed, CD44 contributes to the maintenance of stem cell features by participating in the formation and maintenance of a stem cell niche, homing processes into the niche and maintenance of cell quiescence. Abundantly expressed, different forms of CD44 also contribute to a relative apoptosis resistance in CSC (Zoller 2015). CD44 is associated with Wnt/beta-catenin signaling that is known to participate in many ways in the maintenance of HSCs. Some studies show that CD44 has Wnt/beta-catenin-signaling strengthening functions. Importantly, Wnt/beta-catenin signaling dysregulation is also associated with the development of haematological malignancies and CD44v6 binding to the ECM has been shown to activate Wnt/beta-catenin signaling as well as PI3K/Akt pathway (Ashihara *et al*. 2015, Zoller 2015). In addition, it has been found to directly interact with CXCR4, having an impact on the CXCR4/CXCL12 signaling. Some studies suggest that CXCR4/CXCL12 signaling depends positively on CD44 functions, but contradictory data also exists (Fuchs *et al*. 2013, Olofsson *et al*. 2014, Zoller 2015). In human HSCs, CXCR4/CXCL12 signaling can induce integrin and CD44 activation and the localisation of CD44 to the leading edge during migration (Avigdor *et al*. 2004, Peled *et al*. 2000).

In addition to participating in hematopoiesis, CD44 plays a crucial role in lymphocyte activation, migration and homing (Drillenburg & Pals 2000). It seems that peripheral lymphocytes constitutively express CD44s, but that upon activation, the expression of specific variant forms are induced (Naor *et al*. 1997, Salles *et al*. 1993, Sneath & Mangham 1998). It also seems that of the constitutively expressed CD44, there is a functionally activated form that is expressed by activated lymphocytes. These lymphocytes show enhanced binding to HA when compared to naive lymphocytes (McDonald & Kubes 2015).

CD44/HA signaling plays a role in host defense, participating in the rolling and extravasation of lymphocytes to the sites of inflammation (Jordan *et al*. 2015). Extravasation depends on at least three different interactions: CD44/HA interactions, the binding of CD44v to the members of the selectin family and lastly on CD44/VLA-4-mediated transendothelial migration (Hertweck *et al*. 2011). The strength of adhesion to the surrounding HA depends on the amount of CD44 on the cell surface. This can also affect the speed of the rolling (Gal *et al*. 2003). The lymphocyte cell surface CD44 cross-linking co-stimulates integrin activation. It also stimulates antigen-receptor-mediated proliferation and the release of cytokines via so-called outside-in signaling (Drillenburg & Pals 2000).

High levels of CD44 expression have been associated with different forms of NHL and also to poorer prognosis (Sasaki & Niitsu 2000, Sneath & Mangham 1998, Stauder *et al*. 1995). Yet contradictory results also exist and for example Burkitt's lymphoma has been shown to be somewhat CD44 deficient (Attarbaschi *et al*. 2007). CD44 has also been found to be hypermethylated and transcriptionally silenced in certain aggressive NHLs, including Burkitt's lymphoma and parts of DLBCL. These CD44 hypermethylated cell lines show resistance towards anti-CD44-induced apoptosis (Eberth *et al*. 2010). An old work from the year 1991 by Sy et al. on the other hand demonstrated that CD44 was capable of enhancing the growth and metastatic capacity of Burkitt's lymphoma cells in a nude mouse model (Sy *et al*. 1991).

Both CD44s and CD44v have been associated with poorer prognosis in DLBCL, yet some studies show that only CD44v6 expression correlates with poorer survival (Drillenburg *et al*. 1999, Horst *et al*. 1990, Inagaki *et al*. 1999). In GEP, CD44 mRNA expression has been shown to be very low in reactive GC B-cells (Alizadeh *et al*. 2000). Similarly in DLBCL, CD44 is expressed differently in ABC versus GC phenotypes, being expressed at a higher level in the first one (Rosenwald *et al*. 2002). Tzankov et al. have also shown that a specific CD44 variant, CD44v6, was predominantly expressed in the ABC phenotype DLBCL and that its expression correlates with the disease stage. In addition, in CD44s negative cases, its expression was correlated with poorer prognosis. It has been speculated that the expression of CD44v6 might contribute to lymphoma dissemination (Tzankov *et al*. 2003). Although the different CD44 isoforms seem to share a common ligand-binding region for HA, a study by Wallach-Dayan et al. suggested that with regard to lymphoma cells, the CD44 variant, rather than CD44s, supports the rolling of these cells (Wallach-Dayan *et al*. 2001, Wang & Bourguignon 2011, Xu *et al*. 2015). Lymphoma cells that were transfected with CD44v showed

enhanced migration and accumulation in the peripheral lymph nodes (Wallach-Dayan *et al*. 2001). Yet both isoforms have been associated with poorer prognosis in NHLs so the significance of the different isoforms remains unclear (Drillenburg *et al*. 1999, Stauder *et al*. 1995).

Hu et al. identified CD44 as a novel translocation partner of IGH in some mature B-cell NHLs. In DLBCL, the IGHSμ/CD44 translocations were only seen in GC phenotypes. The authors concluded that the translocation caused an overexpression of a CD44 variant, the overexpression of which in CD44 negative GC DLBCL cells resulted in a significant increase in cell growth. What is interesting is that the CD44s was located in the cell membrane, when again the variant form was located in the cytoplasm and nucleus (Hu *et al*. 2010). In follicular lymphoma Higashi et al. also noted that the levels of CD44 expression, both standard and variant forms, were upregulated in concordance to the diffuse evolution of follicular lymphoma (Higashi *et al*. 2009). In vivo experiments have shown that lymphoma cell dissemination can be somewhat controlled or disturbed by injecting anti-CD44 antibodies or hyaluronidase (Naor *et al*. 2008). Yet CD44-positive DLBCL has also been shown to grow while localised to a specific site in contrast to disseminated growth (de Leval *et al*. 2003). The mechanisms of how CD44 could affect lymphomagenesis or the prognosis of the patients remains somewhat unclear. One mechanism leading to worsened survival could be that CD44 augments the DNA repair mechanisms in lymphocytes and in this way enhances resistance to chemotherapy (Chen *et al*. 2005).

### *2.6.8 P-cadherin*

P-cadherin, a cell-cell adhesion glycoprotein is a classical cadherin belonging to the cadherin superfamily. It primarily, but not solely, promotes homotypic interactions (Paredes *et al*. 2007, Vieira & Paredes 2015). P-cadherin participates in the maintenance of tissue architecture as well as embryonic development. It is important for cell differentiation, polarity, shape, growth and migration regulation. P-cadherin is known to be present in various adult tissues, often co-expressed together with E-cadherin. The tissues that it is known to be completely absent from include heart muscle and brain. In addition to its physiological roles, it is also involved in diseases such as cancer and specific hereditary genetic disorders (Vieira & Paredes 2015).

P-cadherin has been reported to be present in embryonic stem cells and it contributes to the biology of mammary gland and hair follicle stem cells. In

mammary cells, P-cadherin is found to be co-expressed with EMT TF Slug. Studies of P-cadherin in mouse mammary glands suggest that P-cadherin has important roles in limiting the growth of mature luminal epithelial cells as well as it seems that it might act as a stem cell marker and participate in modulation of stem cell signaling pathways. P-cadherin null mice also show increased risk of pre-neoplastic lesions (Vieira & Paredes 2015). In malignancies, P-cadherin has been shown to contribute to the survival of aggressive cancer cells that often exhibit stem cell-like properties and a work by Vieira et al. provided evidence that P-cadherin is associated with the expression of breast stem cell markers in breast cancer cells. These include markers such as CD44 and CD49. In addition, the inhibition of P-cadherin was able to sensitise the cancer cells to X-ray induced apoptosis (Vieira *et al*. 2012, Vieira & Paredes 2015).

With regard to the roles of P-cadherin in tumourigenesis, the results are somewhat contradictory, with some studies suggesting tumour promoting and others tumour-suppressive functions. The results also seem to depend on the tumour type, as in the case of breast cancer, where P-cadherin has been extensively studied, it is correlated with poorer prognosis, disease aggressiveness and invasive growth. Interestingly though, P-cadherin has not been associated with lymph node metastasis in breast cancer (Ribeiro & Paredes 2015, Vieira & Paredes 2015). As many studies provide evidence that aberrant P-cadherin expression is correlated with aggressive tumour behaviour and metastasis in several solid malignancies, supporting its tumour-promoting functions, it seems that in melanoma, oral squamous cell carcinoma, hepatocellular carcinoma and NSCLC, it acts like a tumour-suppressor, similarly to E-cadherin. Still these results are not unequivocal, as conflicting results have been reported within some tumour types. The variability of the results might be related to the different subcellular locations, as different results have been reported for membranous and cytoplasmic P-cadherin expressions (Vieira & Paredes 2015).

Studies on breast cancer show that P-cadherin overexpression promotes cell motility, cell migration, invasive abilities as well as induces the secretion of MMPs. Ribeiro et al. showed that the secretion of MMPs led to P-cadherin ectodomain cleavage and that the soluble P-cadherin fragment was associated with pro-invasive abilities (Ribeiro *et al*. 2010). What is interesting is that it seems that in breast cancer P-cadherin needs to be expressed together with E-cadherin, in order for it to promote cancer aggressiveness and in general to have tumour-promoting properties (Vieira & Paredes 2015). Ribeiro et al. studied breast cancer cells and provided evidence that P-cadherin co-localises with E-cadherin. This co-localisation causes

a disruption in the interaction between E-cadherin and cytoplasmic catenins that further promotes cell invasion (Ribeiro *et al*. 2013). However, in other tumour types, a sort of a "cadherin switch" between E- and P-cadherin has been reported (Vieira & Paredes 2015). For example in a study of colon cancer, P-cadherin inhibition induced upregulation of E-cadherin and a concomitant downregulation of beta-catenin and its downstream molecules such as cytoplasmic Myc (c-myc) and survivin. As P-cadherin knockdown was shown to lead to reduced cell proliferation, invasion and motility and decreased liver metastases in nude mice, the oncogenic effects of the Wnt/beta-catenin signaling pathway were concluded to be correlated with the aberrant expression of P-cadherin (Sun *et al*. 2011).

In breast and ovary carcinomas P-cadherin has been demonstrated to have a connection with the activation of integrin molecules. This activation supports attachment of the cancer cells to the ECM, for which P-cadherin seems to be essential. P-cadherin is assumed to be involved in the early steps of metastatic adhesion in specific solid malignancies. P-cadherin has been suggested as a therapeutic target in specific cancer types and the research in this area remains ongoing (Vieira & Paredes 2015).

P-cadherin has been linked to EMT-like features and similarly to its roles in tumourigenesis, the results are contradictory and probably depend on the tumour type as well. In melanoma, P-cadherin downregulation has been associated with Slug expression and an acquisition of a migratory phenotype. Then again, other studies provide evidence that P-cadherin expression can lead to an increase in gene-expression of EMT-associated TFs (Vieira & Paredes 2015).

Whether P-cadherin has roles in lymphomas remains unclear.

### *2.6.9 Cadherin-12*

Cadherin-12 (CDH12) is a type II classical cadherin belonging to the cadherin superfamily. It is sometimes referred to as a brain cadherin as it was first identified in the brain (Selig *et al*. 1997, Zhao *et al*. 2013). CDH12 is presumed to play a role in the development and function of mouse CNS (Mayer *et al*. 2010). In humans it has been associated with the pathogenesis of various neuropsychiatric disorders (Redies *et al*. 2012). CDH12 has been a subject of studies in solid malignancies as well, where it has been studied in NSCLC, salivary adenoid cystic carcinoma and colorectal cancer (Bankovic *et al*. 2010, Ma *et al*. 2016, Wang *et al*. 2011, Zhao *et al*. 2013). NSCLC patients with mutated CDH12, had a shorter survival time when compared to patients without CDH12 mutations, suggesting that CDH12 plays a

role in NSCLC progression (Bankovic *et al*. 2010). These results hint that CDH12 would have tumour-suppressive functions in NSCLC. Then again in colorectal cancer and salivary adenoid cystic carcinoma, CDH12 seems to have tumour promoting functions, as in the latter it was shown by Wang et al. to promote migration and invasion (Ma *et al*. 2016, Wang *et al*. 2011, Zhao *et al*. 2013). In colorectal cancer, CDH12 has even been speculated to act as an oncogene as it seems to promote the proliferation, invasion and migration of cancer cells (Ma *et al*. 2016, Zhao *et al*. 2013). Zhao et al. demonstrated that CDH12 expression is upregulated in colorectal cancer tissues when compared to adjacent normal tissues (Zhao *et al*. 2013). In colorectal cancer, CDH12 has also been associated with angiogenesis, lymph node lesions, poorer prognosis and to EMT (Ma *et al*. 2016, Zhao *et al*. 2013). Ma et al. showed that CDH12 promotes EMT possibly via activation of an EMT TF Snail. Concomitantly, CDH12 downregulation decreased the expression of mesenchymal markers N-cadherin and Vimentin and promoted the expression of E-cadherin, a known epithelial marker (Ma *et al*. 2016). However, the role of CDH12 in lymphomas is unclear.

# 3 Aims of the present study

Lymphomas are a heterogeneous group of diseases. In recent years, the prognosis of specific lymphoma entities, especially DLBCL, has improved. However, problems also remain, as secondary CNS lymphoma and the majority of PTCLs still harbor poor prognoses. In these cases, more personalised treatments would probably benefit the patients. In the future, the incorporation of the evolving biological data and the new treatment methods will lead the treatment choices towards more individually tailored therapies. Personalised medicine should aim to take into consideration the age of the patient, the performance status, comorbities, the biological properties of a specific disease and in the future, possibly the genetic variability of the patients.

As a majority of the CNS relapses can be prevented by incorporating a CNS prophylactic treatment into the standard therapy, biological markers for this patient selection are warranted. However, the biological processes behind CNS relapse in DLBCL remain unknown. There are some suggestions that chemokines and chemokine receptors may have a role in the CNS tropism. Currently, there is a limited amount or no data regarding the clinicopathological role of molecules participating in adhesion and migration or the regulation of inflammatory responses in lymphomas.

The specific aims of the present study were to explore the following questions:

1. Do epithelial-mesenchymal transition transcription factors play a role in diffuse large B-cell lymphoma and if so, what is their role in this disease entity?
2. Do epithelial-mesenchymal transition transcription factors play a role in peripheral T-cell lymphomas and if so, what is their role in these diseases?
3. What is the mechanism behind the central nervous system homing of malignant lymphoma cells?
4. What is the optimal way to indicate the diffuse large B-cell lymphoma patients that have an increased risk of developing a central nervous system relapse?

# 4 Materials and methods

## 4.1 Patient material

Patient material consisted of 286 patients with primary diagnoses of DLBCL, PCNSL and PTCLs including AITL, PTCL NOS, ALK+ ALCL, ALK- ALCL and EATL. Patients were diagnosed and treated at the Oulu University Hospital, Kuopio University Hospital, Tampere University Hospital and Jyväskylä Central Hospital between years 1993-2014. The methods used in this study included IHC, immunoelectron microscopy (IEM) and GEP. Biopsy blocks included in our studies were diagnostic biopsy samples, collected from pathology archives and evaluated to be representative enough for the study. IHC samples were paraffin-embedded tissue samples and IEM and GEP samples were taken in addition to diagnostic tissue samples. IEM samples were fresh tissue samples stored in IEM specimen fixative and GEP samples were formalin-fixed paraffin embedded tissue blocks in Study II and fresh frozen tissue samples in Sudy IV. The preparation of these samples is described in the following sections.

Diagnostic work-up included medical history, physical examination, analysis of blood chemistry, collection and analysis of tumour tissue biopsies, bone marrow aspirate and biopsies and whole-body computer tomography (CT). For patients with PCNSL and for DLBCL patients with a suspected CNS disease, CSF analysis and brain magnetic resonance imaging (MRI) were performed. Detailed patient information was collected retrospectively from the hospital records. These included clinical data such as date of diagnosis, age, the presence of B symptoms, GC/non-GC phenotype, stage, LDH, IPI, the number of extranodal lesions, treatment and treatment responses as well as follow-up data. The clinical data usage varied amongst the different studies.

Patients were treated according to the disease entity recommendations or with BBBD-therapy. These are presented in Table 1. In Studies III and IV, where CNS lymphomas were studied, the patients in the control group with systemic DLBCL had not received CNS prophylactic treatment.

**Table 1. Treatment according to the lymphoma subtype.**

| Lymphoma subtype | Number of samples | Primary treatment |
|---|---|---|
| DLBCL | 178 | R-CHOP or R-CHOEP based on age and IPI points, +/- CNS prophylaxis, +/- involved field radiotherapy |
| PCNSL | 41 | Bonn or BBBD-therapy |
| AITL | 27 | CHOP +/- ASCT depending on the age of the patient </> 70 years old and comorbidities |
| PTCL NOS | 14 | CHOP +/- ASCT depending on the age of the patient </> 70 years old and comorbidities |
| ALK+ ALCL | 3 | CHOP |
| ALK- ALCL | 10 | CHOP +/- ASCT depending on the age of the patient </> 70 years old and comorbidities |
| ALCL, ALK unspecified | 5 | CHOP |
| EATL | 8 | IVE + ASCT or CHOP (elderly patients) |

The original publication number I included 102 systemic DLBCL without CNS affision (sDLBCL) samples and 8 reactive lymphoid hyperplasia samples. IHC was the only method used in addition to histological studies. The median age of patients was 69 years.

Publication number II included altogether 67 samples that were studied with IHC. The material consisted of the following entities: 3 ALK+ ALCL, 10 ALK- ALCL, 5 unspecified ALCL (ALK expression undetermined), 8 EATL, 14 PTCL NOS and 27 AITL. The GEP was performed on 6 AITL and 6 PTCL NOS samples. The median age of patients was 68 years.

Publication number III included 35 PCNSL, 21 sCNSL, 33 sDLBCL and 10 reactive lymph node samples that were studied with IHC. In addition, IEM analysis was performed and it included 2 PCNSL, 1 sCNSL (brain biopsy), 1 sDLBCL and 1 reactive lymph node sample.

Publication number IV included 15 PCNSL, 31 sCNSL and 50 sDLBCL samples that were studied with IHC. In addition, IEM analysis was performed, including 2 PCNSL, 1 sCNSL (brain biopsy), 1 sDLBCL and 1 reactive lymph node sample. GEP included two lymph node fresh frozen tissue samples, one sDLBCL and one sCNSL sample from the time of primary diagnosis. The median age of patients was 65 years.

## 4.2 Ethics

Permission to use patient samples included in the study was granted by the Finnish National Supervisory Authority for Welfare and Health (6622/05.01.00.06/2010) and the studies were approved by the Ethics Committee of Oulu University Hospital (42/2010, 23.6.2010). The principles of the Declaration of Helsinki were also followed.

## 4.3 Immunohistochemistry

The samples that were used for IHC had been fixed in formalin and then embedded in paraffin. Representative tumour areas were cut into 3-µm sections that were then placed on SuperFrostPlus glass slides (Menzel-Gläser, Braunschweig, Germany). The slides were incubated at 37°C for 30 minutes (Studies I and II) or overnight (Studies III and IV) and after this deparaffinised in a clearing agent (xylene; Oy FF-Chemicals Ab, Haukipudas, Finland) and rehydrated in a descending series of ethanol solutions. Antigen retrieval was carried out in a microwave oven with either 10 mM Tris-EDTA solution (pH 9) or 10 mM sodium citrate buffer (pH 6), depending on the used antibody. For two antibodies no antigen retrieval was done (Table 2). In order to block endogenous peroxidase activity, the slides were cooled at room temperature for 20 minutes and incubated in 3% $H_2O_2$ solution for five minutes. The immunostaining procedure was carried out by using the Novolink Polymer Detection System Kit (Novocastra/Leica Biosystems Newcastle Ltd., Newcastle-upon-Tyne, UK), according to the instructions of the manufacturer. The primary antibodies and their concentrations levels are presented in more detail in Table 2. Primary antibody incubations were carried out in a humidity chamber at room temperature for 30 minutes (Studies I and II) or for 1 hour (Studies III and IV). Between all stages of the immunostaining procedures, the slides were washed with either phosphate-buffered saline–Tween (PBS-Tween) (Studies I and II) or Tris-buffered saline (TBS) (Studies III and IV). Lastly, the slides were counterstained with Mayer's hematoxylin (Reagena, Toivola, Finland), dehydrated and mounted with either Tissue-Tek 4770 Coverslipping film (Sakura Finetek USA, Torrance, CA, USA) (Studies I and II) or Histomount (National Diagnostics, New Jersey, USA) (Studies III and IV). Previously known positive control samples were used in all of the staining series. In negative controls, either PBS-Tween or TBS was used to replace the primary antibody.

In order to determine the cell-of-origin in DLBCLs, Hans's algorithm was used. For this, monoclonal antibodies for CD10 (NCL-CD10-270; Novocastra), Bcl-6 (anti-human Bcl-6 antibody; DakoCytomation, Glostrup, Denmark), and MUM-1 (anti-human MUM-1 antibody; DakoCytomation) were used. The staining was performed using the Dako REALTM EnVisionTM Detection System kits (K5007; DakoCytomation).

Micrographs of the IHC staining patterns were obtained using an Olympus BX41 microscope and an Olympus DP11 digital microscope camera (Olympus, Center Valley, PA, USA). To import micrographs an HP Photo and Imaging software (Hewlett-Packard Company, Palo Alto, CA, USA) was used.

**Table 2. The antibodies and immunehistochemical staining methods.**

| Antibody | Concentration | Antigen retrieval | Manufacturer, catalogue number and country | Study number |
|---|---|---|---|---|
| Twist | 1:100 | 10 mM Tris- EDTA solution, pH 9 | Abcam ab50887 Cambridge, UK | I, II |
| ZEB1 | 1:300 | 10 mM Tris- EDTA solution, pH 9 | GenWay Biotech 20-372-60303 San Diego, USA | I, II |
| Slug | 1:150 | 10 mM Tris- EDTA solution, pH 9 | Abgent AP2D53a San Diego, USA | I, II |
| CXCR4 | 1:150 | 10 mM sodium citrate buffer, pH 6 | Abnova H00007852-MO5 Taipei, Taiwan | III |
| CXCR5 | 1:2000 | 10 mM sodium citrate buffer, pH 6 | R&D Systems MAB190 Minneapolis, USA | III |
| CCR7 | 1:100 | 10 mM Tris- EDTA solution, pH 9 | Abnova PAB14776 Taipei, Taiwan | III |
| CXCL12 | 1:25 | 10 mM sodium citrate buffer, pH 6 | Abcam MM0211-9N26 Cambridge, UK | III |
| CXCL13 | 1:100 | No antigen retrieval | Bioss ABIN741960 Atlanta, USA | III |
| ITGA10 | 1:400 | 10 mM Tris- EDTA solution, pH 9 | Millipore AB6030 Temecula, USA | IV |
| CD44 | 1:100 | 10 mM sodium citrate buffer, pH 6 | Cell Signaling Technology Inc. 156-3C11 Danvers, USA | IV |

| Antibody | Concentration | Antigen retrieval | Manufacturer, catalogue number and country | Study number |
|---|---|---|---|---|
| PTEN | 1:150 | 10 mM sodium citrate buffer, pH 6 | Dako M3627 Glostrup, Denmark | IV |
| Cadherin-11 | 1:300 | 10 mM sodium citrate buffer, pH 6 | R&D Systems MAB1790 Minneapolis, USA | IV |
| CDH12 | 1:10 | No antigen retrieval | Abcam ab71055 Cambridge, USA | IV |
| N-cadherin | 1:50 | 10 mM Tris- EDTA solution, pH 9 | Santa Cruz Biotechnology D-4 sc-8424 Santa Cruz, USA | IV |
| P-cadherin | 1:25 | 10 mM Tris- EDTA solution, pH 9 | Santa Cruz Biotechnology, Inc. H-105 sc-7893 Santa Cruz, USA | IV |
| Lactoferrin | 1:100 | 10 mM Tris- EDTA solution, pH 9 | Abcam ab15811 Cambridge, USA | IV |
| E-cadherin | 1:200 | 10 mM sodium citrate buffer, pH 6 | Invitrogen 13-1700 Camarillo, USA | IV |

## 4.4 Immunoelectron microscopy

The tissue samples used for IEM were fixed in 4% paraformaldehyde with 2.5% sucrose in 0.1M phosphate-buffered saline (PBS) for 2 hours (Study III) or 6 hours (Study IV) at room temperature and then immersed in 2.3 M sucrose in PBS at 4°C. The tissue pieces were then placed on specimen stubs and frozen in liquid nitrogen. Ultrathin (70 nm) sections were cut with a Leica EM UC7 cryo-ultramicrotome. For immunolabelling, the ultrathin sections were incubated in 0.1% glycine + PBS followed by incubation in 1% bovine serum albumin (BSA) in PBS. All washes between different stages of the staining procedure and antibody dilutions were done in PBS + 1% BSA. For single immunolabelling, the samples were incubated with the primary antibodies presented in Table 2 for 60 minutes. Rabbit anti-mouse IgG (Zymed, San Francisco, CA, USA) was used as a secondary antibody in Study III with antibodies against CXCR4, CXCR5 and CXCL12 and rabbit anti-mouse IgG (Jackson ImmunoResearch Europe Ltd, UK) in Study IV with all the antibodies. After washing, the samples were incubated for 30 minutes in a protein A-gold complex (size: 10nm).

For double-labelling in Study III, the sections were first exposed to primary antibodies against CXCR4 or CXCR5 for 60 minutes, followed by incubation with rabbit anti-mouse IgG for 30 minutes and then incubated in protein A-gold complex (size: 10 nm). After washing, free binding sites on protein A were blocked by using 1% glutaraldehyde in 0.1M PBS. Samples were then exposed to the secondary antibodies against CXCL12 or CXCL13, followed by a new incubation in protein A-gold complex (size: 5 nm) for 30 minutes.

Control samples were prepared by carrying out the same labelling procedures but without the primary antibodies.

Samples were finally embedded in methylcellulose and examined in a Philips CM100 transmission electron microscope (FEI Company, Eindhoven, the Netherlands) equipped with a Morada CCD camera (Olympus Soft Imaging Solutions GMBH, Munster, Germany) (Study III) or a Tecnai Spirit transmission electron microscope (FEI Company, Eindhoven, the Netherlands) equipped with a Quemesa digital camera (EMSIS GmbH, Münster, Germany) (Study IV). Micrographs were captured by using the above-mentioned cameras.

## 4.5 Gene-expression profiling

The samples used for GEP were formalin-fixed paraffin embedded tissue blocks in Study II and fresh frozen tissue samples in Study IV. In Study II, the RNA was extracted from the samples using the RNeasy FFPE Kit (Qiagen, Hilden, Germany). Following this, the capillary electrophoresis was carried out to check the quality of the RNA. For this the Aglient bioanalyser and Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA) were used. The transcriptome was analysed with GeneChip Human Gene ST Arrays (Affymetrix, Santa Clara, CA, USA) and the WT pico kit (Affymetrix Santa Clara, CA, USA) according to the manufacturer's instructions. The principal component analysis (PCA) was used for the gene-expression data visualisation. In Study IV, the RNA isolation was performed by using the RNeasy Mini Kit (Qiagen, USA), according to the instructions of the manufacturer. A GeneChip protocol was used and the experimental procedures followed the instructions of the Affymetrix GeneChip Expression Analysis Technical Manual. A 5 µg of total RNA was used as a template as double-stranded DNA was synthesised by means of the One-cycle cDNA synthesis kit (Affymetrix, USA) and T7-(dT)24 primer. To purify the DNA, GeneChip Sample Cleanup Module (Qiagen, USA) was used. The *in vitro* transcription (IVT) was performed to produce biotin labelled complementary

RNA (cRNA) by using an IVT labelling kit (Affymetrix, USA) according to the instructions of the manufacturer. Biotinylated cRNA was then cleaned using the GeneChip Sample Cleanup Module (Qiagen, USA), fragmented to 35 to 200 nucleotides and further hybridised to Affymetrix Human Genome U133 Plus 2.0 arrays that contain approximately 55 000 human transcripts. After washing, the arrays were stained with streptavidin-phycoerythrin (Molecular Probes, USA). Following this, the staining signal was amplified by biotinylated anti-streptavidin (Vector Laboratories, USA) and a second staining with streptavidin-phycoerythrin was performed. Finally, the arrays were scanned on the GeneChip Scanner 3000.

### 4.6 Sample analysis

The IHC stained slides were reviewed and analysed by two observers blinded to the clinical data. The analysis was carried out using a multihead light microscope. First the location of positivity in the malignant B-cells was determined, separately for nucleus, cytoplasm and/or cell membrane. After this, the percentage of positive cells was estimated separately for each location, using a scale from 0% to 100%, with 5% accuracy. The intensity of staining was evaluated separately for each location with a scale from 0 to 3, (0 = negative, 1 = light, 2 = moderate and 3 = strong). Staining percentages were evaluated in all studies, staining intensities in Studies I, II and III. Staining percentages and intensities were evaluated with similar scales in reactive lymphocytes from non-malignant control samples, except in Study III the intensity of the expression of chemokines (CXCL12 and CXCL13) was evaluated with a more specific scale from 0 to 4 (0 = negative, 1 = weak, 2 = light, 3 = moderate and 4 = strong).

The cell-of-origin was determined by using Hans's algorithm and for this the IHC expression of CD10, Bcl-6 and MUM-1 was evaluated. The sample slides were graded as either negative or positive for each marker based on the amount of positively stained malignant B-cells by using a cut-off point of 30% (Hans *et al*. 2004).

IEM samples were reviewed using a transmission electron microscope also by two observers blinded to the clinical data. A general assessment was made of expression pattern, taking into account the location (nucleus, cytoplasm and/or cell membrane) and the amount of positivity detected that was roughly classified as either low (1), moderate (2) or high (3) expression. Extracellular labelling was also noted when present. In Study III a more specific analysis was made as 24–26 micrographs were taken of each sample and from these the number of receptors,

ligands and receptor-ligand complexes present on the cell membrane and/or in the cytoplasm were counted. The length of the cell membrane was measured from each micrograph and the density of receptors and ligands and the relative proportion of receptor-ligand complexes expressed per 100 μm of cell membrane were calculated. Also from each micrograph, a cytoplasmic area of 1 $\mu m^2$ was chosen for receptor, ligand and receptor-ligand complex assessment. Similarly as for the cell membrane, the expression densities and proportions were calculated for receptors, ligands and complexes per 100 $\mu m^2$ of cytoplasm. Of the expression densities, mean values were calculated for each sample in order to compare the expressions and distributions among different diagnostic groups.

The expression data gained from GEP was analysed by using the open source platform Chipster (version 3.11.0) (Study II) or the dChip software (Study IV). In Study II, 12 samples were divided into a poor prognosis group and a good prognosis group, based on IHC staining patterns that associated with PFS. The poor prognosis group, with low Twist + strong Slug IHC expression, included 3 AITL and 3 PTCL NOS cases. The good prognosis group, with high Twist + weak Slug IHC expression, included 3 AITL and 3 PTCL NOS cases as well. The inclusion criteria for these IHC combination variables is presented in Table 3. The GEP data was analysed comparing the two prognostic groups. In order to identify the genes with statistically significant changes in the relative mRNA expression, the Rank Product test was performed. A hypergeometric test was used to identify the over-expressed pathways from the Kyoto Encyclopedia of Genes and Genomes database (KEGG) and Gene ontology (GO) biological process terms. The inclusion criterias for the genes included in the pathway analysis were p-value <0.01 and fold change (FC) >1 or <-1. FC was calculated in the log2 scale.

In Study IV, the possible differences and relative fold changes in the expression of the studied molecules were sought out between the two samples (sCNSL and sDLBCL).

## 4.7 Statistics

For statistical analyses, IBM SPSS Statistics software (IBM Corp. Armonk, NY, USA), versions 20.0, 22.0 and 24.0, were used.

To enable statistical analyses, cut-off points for IHC staining percentages and intensities as well as for IPI points and the number of extranodal lesions were chosen. IPI values were divided into two groups, group one including IPI points from 0 to 2 and group two including IPI points from 3 to 5. The number of

extranodal lesions was also divided into two groups, group one including cases with 0 to 1 extranodal lesions and group two including cases with 2 or more extranodal lesions. The staining intensities were divided into two categories: weak and strong, weak including negative and light staining and strong including moderate and strong staining. Receiver operating characteristic (ROC) analysis was used to determine the cut-off points for IHC staining percentages in Studies III and IV. The area under the curve (AUC) shows sensitivity versus specificity and only values greater than 0.5 were taken into account, values closer to 1.0 possessing a better discriminatory power. Due to the small study material, AUCs were relatively low. The used cut-off points were chosen by estimating a good balance between sensitivity and specificity. However, in Study IV, ROC curve-based cut-off points could not be chosen for all of the studied molecules due to the lack of discriminatory power. Thus for the analysis of the associations between protein expression and clinicopathological parameters as well as the associations between the studied proteins, median values were used as cut-off points for all the proteins. These are presented in the results section. The used cut-off points and the relative AUCs with 95% confidence intervals (CI) are presented in Table 3. Molecules with negative staining or non-discriminatory ROC curves are not presented in Table 3. In Study III, a totally negative chemokine receptor staining was evaluated as its own group, in addition to the cut-off point gained from ROC analysis, because biologically the situation where there is no chemokine receptor expression differs significantly from low expression. However, in order to enable the use of a combination variable in Study III, only one cut-off point (≥80%) was used for this purpose and negative staining was being included in the category of low expression. Combination variables were also used in Study II, where it included Twist staining percentage and Slug staining intensity. These are also presented in Table 3. In Study III, ROC curves were analysed by comparing sDLBCL and CNS lymphomas (PCNSL together with sCNSL), when again in Study IV ROC curves were analysed by comparing sDLBCL and sCNSL (without PCNSL).

For the analysis of nominal variables, a Pearson Chi-Square test was used in all of the studies. It was used to study the associations of the studied proteins, clinicopathological parameters and diagnosis. It was also used to study the associations of EMT TFs with each other (Studies I and II) and associations of the studied molecules with the occurrence of CNS relapse (Studies III and IV). In the latter analyses, CNS lymphomas were analysed together (PCNSL and sCNSL) in Study III when again in Study IV only sCNSL was used, sDLBCL being the control group in both studies.

In the original publication number IV, continuous variables were studied using a Kruskal-Wallis test with three different diagnoses (sDLBCL, sCNSL and PCNSL) and a Mann-Whitney U test with two diagnostic groups (sCNSL and PCNSL).

Survival analyses in Studies I and II were performed to study the associations between EMT TFs and patients' survival. These were done using the Kaplan-Meier method. Statistical significances were evaluated using the log-rank test. Risk ratios (RR) and 95% CIs were calculated with Cox regression analysis. Progression-free survival (PFS) was calculated from the date of diagnosis to the date of disease relapse or progression. Overall survival (OS) was calculated from the date of diagnosis to the date of death from any cause. In all of the analyses p-values $<0.05$ were considered statistically significant.

As the original publication number II contained five different entities, the IHC staining results were analysed separately within different disease entities but also as a one group representing PTCLs.

**Table 3. The used cut-off points for the studied proteins.**

| Molecules | Location of staining | AUC (CI) | Cut-off points | Study number |
|---|---|---|---|---|
| Twist | All | | ≥5% | I |
| | | | ≤25%, >25% | II |
| ZEB1 | All | | ≥5% | I |
| | | | ≤25%, >25% | II |
| Slug | All | | ≥5% | I |
| | | | ≤25%, >25% | II |
| Low Twist + strong Slug | Nucleus | | ≤25% + 2-3 | II |
| High Twist + weak Slug | Nucleus | | >25% + 0-1 | II |
| CXCR4 | Nucleus | 0.642 (0.504–0.780) | >0%, ≥80% | III |
| CXCR5 | Cytoplasm | 0.664 (0.543–0.784) | >0%, ≥80% | III |
| CXCR4 + CXCR5 | Nucleus + Cytoplasm | | <80% + ≥80% | III |
| ITGA10 | Nucleus | 0.657 (0.500-0.815) | >10% | IV |
| ITGA10 | Cytoplasm | 0.679 (0.519-0.838) | >10% | IV |
| ITGA10 | Membrane | 0.745 (0.606-0.884) | >0% | IV |
| CD44 | Membrane | 0.668 (0.514-0.821) | >70% | IV |
| PTEN | Nucleus | 0.694 (0.547-0.840) | >0% | IV |
| Cadherin-11 | Nucleus | 0.673 (0.514-0.831) | >70% | IV |

Combination variables are signified with a + mark

# 5 Results

## 5.1 Immunohistochemical expression patterns

### *5.1.1 Epithelial-mesenchymal transition transcription factors*

*Diffuse large B-cell lymphoma*

Due to technical reasons, out of the 102 DLBCL samples, 99 were available for IHC staining with Twist and ZEB1 antibodies and 97 for staining with the Slug antibody. In lymphoma cells, all of the EMT TFs were expressed in the nucleus but cytoplasmic immunoreactivity was only seen with Twist and Slug antibodies. The intensity of nuclear Twist and ZEB1 stainings varied from light to strong when again the intensity of nuclear Slug staining varied between light and moderate. The cytoplasmic staining intensities for Twist and Slug varied between light and moderate. The amount of positive cells per sample is presented in Table 4. The IHC expression profiles of Twist, ZEB1 and Slug with the used cut-off points are presented in Table 1 of Study I and the IHC expression patterns in DLBCL samples are presented in Figure 1 of Study I. In a few DLBCL samples there was a strong endothelial and connective tissue staining with the Twist antibody. In addition, in essentially all of the samples, the ZEB1 antibody stained some of the endothelial cells with a moderate or strong intensity.

**Table 4. The immunohistochemical expression profiles of Twist, ZEB1 and Slug in DLBCL samples.**

| Subcellular location | Antibody staining | Twist | ZEB1 | Slug |
|---|---|---|---|---|
| Nucleus | Positive samples | 83/99 | 96/99 | 38/97 |
| | Positive cells | 0%-30% | 0%-50% | 0%-30% |
| | Staining intensity | Light – strong | Light – strong | Light - moderate |
| Cytoplasm | Positive samples | 2/99 | - | 95/97 |
| | Positive cells | 100% | - | 100% |
| | Staining intensity | light and moderate | - | Light – moderate |
| Membrane | Positive samples | - | - | - |
| | Positive cells | - | - | - |
| | Staining intensity | - | - | - |

Antibody stainings with no immunoreactivity are marked with -

EMT TFs were also studied by IHC in 8 reactive lymphoid hyperplasia samples. In a few of these samples, a strong endothelial and connective tissue immunoreactivity was seen with the Twist antibody. In addition, in all of the samples, a moderate to strong ZEB1 staining intensity was seen in parts of the endothelial cells. The IHC staining of Twist, ZEB1 and Slug in reactive lymphoid tissue is presented in Figure 2 of Study I.

### *Peripheral T-cell lymphomas*

With all of the EMT TFs, a nuclear immunoreactivity in lymphoma cells was seen. The intensity of nuclear staining varied from light to strong with all of the studied antibodies. Cytoplasmic Twist staining was detected in one sample when again no cytoplasmic ZEB1 expression was seen. Slug positivity was detected in the majority of the samples, the intensities varying from light to moderate. The amount of positive cells per sample is presented in Table 5. The IHC expression profiles of Twist, ZEB1 and Slug with the used cut-off points is presented in Table 2 of Study II and the IHC expression patterns in PTCL samples are presented in Figure 2 of Study II.

**Table 5. The immunohistochemical expression profiles of Twist, ZEB1 and Slug in PTCL samples.**

| Subcellular location | Antibody staining | Twist | ZEB1 | Slug |
|---|---|---|---|---|
| Nucleus | Positive samples | 57/67 | 60/65 | 24/63 |
| | Positive cells | 0%-70% | 0%-100% | 0%-70% |
| | Staining intensity | Light – strong | Light – strong | Light – strong |
| Cytoplasm | Positive samples | 1/67 | - | 55/63 |
| | Positive cells | 100% | - | 70%-100% |
| | Staining intensity | Moderate | - | Light – moderate |
| Membrane | Positive samples | - | - | - |
| | Positive cells | - | - | - |
| | Staining intensity | - | - | - |

Antibody stainings with no immunoreactivity are marked with -

### 5.1.2 Chemokine receptors and chemokines

#### *Chemokine receptors*

CXCR4 immunostaining primarily showed nuclear and cytoplasmic staining patterns in malignant cells. CXCR5 on the other hand showed cytoplasmic and membranous expression, but did not show any nuclear immunoreactivity. CCR7 staining results in lymphoma cells were negative in most of the cases. The amount of positive cells per sample and the staining intensities are presented in Table 6. The IHC expression profiles of studied chemokine receptors with the cut-off points used are presented in Table 1 of Study III. Figure 1 in Study III presents the IHC expression patterns in DLBCL samples.

**Table 6. The immunohistochemical expression of chemokine receptors and chemokines in PCNSL, sCNSL and sDLBCL samples.**

| Molecules | Antibody staining | Nucleus | | | Cytoplasm | | | Membrane | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Prim. | Sec. | Syst. | Prim. | Sec. | Syst. | Prim. | Sec. | Syst. |
| CXCR4 | Positive samples | 14/32 | 11/20 | 22/33 | 31/32 | 17/20 | 29/33 | - | 1/20 | 1/33 |
| | Positive cells | 0%-100% | 0%-100% | 0%-100% | 100% | 100% | 100% | | 5% | 10% |
| | Staining intensity | Light - moderate | Light – moderate | Light – moderate | Light – moderate | Light – moderate | Light – moderate | | Moderate | Light |
| CXCR5 | Positive samples | - | - | - | 28/33 | 18/20 | 29/30 | 23/33 | 12/20 | 22/30 |
| | Positive cells | | | | 0%-100% | 0%-100% | 0%-100% | 0%-100% | 0%-90% | 0%-100% |
| | Staining intensity | | | | Light – moderate | Light – strong | Light – strong | Light – strong | Light – strong | Moderate – strong |
| CCR7 | Positive samples | 11/32 | 3/20 | 10/30 | - | - | - | - | 1/20 | 1/30 |
| | Positive cells | 0%-10% | 0%-30% | 0%-10% | | | | | 10% | 10% |
| | Staining intensity | Light – moderate | Light | Light – moderate | | | | | Moderate | Moderate |

Antibody stainings with no immunoreactivity are marked with -, Prim. = PCNSL, Sec. = sCNSL and Syst. = sDLBCL

*Chemokines*

The IHC expression of chemokines CXCL12 and CXCL13 was evaluated in PCNSL and reactive lymph node samples. In PCNSL samples CXCL12 immunostaining was positive in most endothelial cells but negative in glial cells. In endothelial cells, no nuclear immunoreactivity was seen, cytoplasmic staining varying from weak to light and membranous staining varying from light to moderate. Malignant cells in PCNSL as well as B- and T-lymphocytes in reactive lymph node samples did not show any immunoreactivity for the CXCL12 antibody. In PCNSL samples, CXCL13 immunoreactivity was negative in most endothelial cells but in few samples a light cytoplasmic staining was seen. CXCL13 expression varied in glial cells from negative to weak cytoplasmic staining. The malignant cells in PCNSL samples showed mostly cytoplasmic CXCL13 staining and in half of the samples, a nuclear staining was also seen. Cytoplasmic staining was seen in all of the lymphoma cells per sample, intensities varying from weak to light when again the amount of cells with nuclear positivity per sample varied from all negative to all positive, intensities varying from light to moderate. In most reactive lymph node samples, there was no CXCL13 immunoreactivity in the B-cells. However, in a few samples, a weak nuclear staining was observed, the amount of positive cells in these samples being low. T-lymphocytes on the other hand showed a nuclear CXCL13 immunoreactivity, the intensity of staining being light in all of the samples.

### 5.1.3 Adhesion-, migration- and inflammatory responses-associated molecules

In Study IV, where the expression of adhesion-, migration- and inflammatory responses-associated molecules was explored by IHC, only the amount of positive cells was evaluated. The staining intensities were not evaluated. In general, E-cadherin did not show any immunoreactivity. Nuclear immunostaining was seen with all other antibodies used; except for CD44 there was no nuclear immunoreactivity. Cytoplasmic positivity was seen for all of the antibodies used, except E-cadherin. Membranous localisation was seen in immunostainings for ITGA10, CD44, PTEN and CDH12. IHC expressions of the proteins that were studied in the original publication number IV are presented in Table 7. Table 8 presents the median values for the IHC staining frequencies. The IHC expression

patterns of the studied proteins in DLBCL samples are presented in Figure 1 of Study IV.

The distribution of the studied proteins from Studies III and IV among the three diagnoses (PCNSL, sCNSL, sDLBCL) is presented separately for GC and non-GC phenotypes in Table 9.

**Table 7. The immunohistochemical staining of lymphoma cells according to subcellular location in PCNSL, sCNSL and sDLBCL samples.**

| Molecules | Antibody staining | Nucleus | | | Cytoplasm | | | Membrane | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Prim. | Sec. | Syst. | Prim. | Sec. | Syst. | Prim. | Sec. | Syst. |
| ITGA10 | Positive samples | 6/13 | 12/30 | 14/21 | 11/13 | 22/30 | 6/21 | 3/13 | 20/30 | 6/21 |
| | | 46% | 40% | 67% | 85% | 73% | 29% | 23% | 67% | 29% |
| | Positive cells | 0%-100% | 0%-100% | 0%-100% | 0% or 100% | 0%-100% | 0%-100% | 0% or 100% | 0%-100% | 0%-100% |
| CD44 | Positive samples | - | - | - | 5/12 | 4/30 | 8/47 | 12/13 | 30/30 | 47/47 |
| | | | | | 42% | 13% | 17% | 92% | 100% | 100% |
| | Positive cells | | | | 0% or 100% | 0% or 100% | 0% or 100% | 0%-100% | 0%-100% | 0%-100% |
| PTEN | Positive samples | 6/13 | 17/30 | 5/23 | 10/13 | 21/30 | 20/23 | 3/13 | 5/30 | 1/23 |
| | | 46% | 57% | 22% | 77% | 70% | 87% | 23% | 17% | 4% |
| | Positive cells | 0%-100% | 0%-80% | 0%-10% | 0%-100% | 0%-100% | 0%-100% | 0% or 100% | 0% or 100% | 0% or 20% |
| Cadherin-11 | Positive samples | 11/13 | 25/30 | 41/48 | 4/13 | 1/30 | 7/48 | - | - | - |
| | | 85% | 83% | 85% | 31% | 3% | 15% | | | |
| | Positive cells | 0%-100% | 0%-100% | 0%-100% | 0% or 100% | 0% or 100% | 0% or 100% | | | |
| CDH12 | Positive samples | 4/13 | 10/31 | 9/49 | 9/13 | 26/31 | 45/49 | 3/13 | 10/31 | 16/49 |
| | | 31% | 32% | 18% | 69% | 84% | 92% | 23% | 32% | 33% |
| | Positive cells | 0%-100% | 0%-100% | 0%-100% | 0%-100% | 0%-100% | 0%-100% | 0% or 100% | 0% or 100% | 0% or 100% |
| N-cadherin | Positive samples | 4/14 | 4/31 | 12/48 | 7/14 | 16/31 | 30/48 | - | - | - |
| | | 29% | 13% | 25% | 50% | 52% | 63% | | | |
| | Positive cells | 0% or 100% | 0%-100% | 0%-100% | 0%-100% | 0%-100% | 0%-100% | | | |

| Molecules | Antibody staining | Nucleus | | | Cytoplasm | | | Membrane | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Prim. | Sec. | Syst. | Prim. | Sec. | Syst. | Prim. | Sec. | Syst. |
| P-cadherin | Positive samples | 12/13 | 17/31 | 36/48 | 13/13 | 29/31 | 48/48 | - | - | - |
| | | 92% | 55% | 75% | 100% | 94% | 100% | | | |
| | Positive cells | 0%-100% | 0%-100% | 0%-100% | 0% or 100% | 0% or 100% | 50% or 100% | | | |
| Lactoferrin | Positive samples | 11/13 | 22/31 | 18/23 | 12/13 | 9/31 | 5/23 | - | - | - |
| | | 85% | 71% | 78% | 92% | 29% | 22% | | | |
| | Positive cells | 0%-100% | 0%-100% | 0%-100% | 0% or 100% | 0%-100% | 0% or 100% | | | |
| E-cadherin | Positive samples | - | - | - | - | - | - | - | - | - |
| | Positive cells | | | | | | | | | |

Antibody stainings with no immunoreactivity are marked with -, Prim. = PCNSL, Sec. = sCNSL and Syst. = sDLBCL

**Table 8. Median values of the immunohistochemical staining frequencies according to subcellular location in PCNSL, sCNSL and sDLBCL samples.**

| Molecules | Nucleus | Cytoplasm | Membrane |
|---|---|---|---|
| ITGA10 | 5% | 50% | 0% |
| CD44 | 0% | 0% | 70% |
| PTEN | 0% | 100% | 0% |
| Cadherin-11 | 80% | 0% | 0% |
| CDH12 | 0% | 100% | 0% |
| N-cadherin | 0% | 100% | 0% |
| P-cadherin | 10% | 100% | 0% |
| Lactoferrin | 80% | 0% | 0% |
| E-cadherin | 0% | 0% | 0% |

**Table 9. Distribution of the studied molecules according to the cell-of-origin.**

| Cell-of-origin | Molecules | PCNSL | sCNSL | sDLBCL |
|---|---|---|---|---|
| GC phenotype | ITGA10 nucleus | 1/3 (33%) | 5/14 (36%) | 4/9 (44%) |
| | ITGA10 cytoplasm | 3/3 (100%) | 11/14 (79%) | 3/9 (33%) |
| | ITGA10 membrane | 0/3 (0%) | 10/14 (71%) | 3/9 (33%) |
| | CD44 membrane | 2/3 (67%) | 3/14 (21%) | 11/22 (50%) |
| | PTEN nucleus | 2/3 (67%) | 8/14 (57%) | 3/11 (27%) |
| | Cadherin-11 nucleus | 3/3 (100%) | 3/14 (21%) | 17/22 (77%) |
| | CXCR4 nucleus | 1/5 (20%) | 2/8 (25%) | 10/15 (67%) |
| | CXCR5 cytoplasm | 3/5 (60%) | 6/8 (75%) | 5/15 (33%) |
| Non-GC phenotype | ITGA10 nucleus | 1/3 (33%) | 5/16 (31%) | 9/12 (75%) |
| | ITGA10 cytoplasm | 2/3 (67%) | 11/16 (69%) | 3/12 (25%) |
| | ITGA10 membrane | 2/3 (67%) | 10/16 (63%) | 3/12 (25%) |
| | CD44 membrane | 3/3 (100%) | 7/16 (44%) | 17/24 (71%) |
| | PTEN nucleus | 2/3 (67%) | 9/16 (56%) | 2/12 (17%) |
| | Cadherin-11 nucleus | 2/3 (67%) | 4/16 (25%) | 15/25 (60%) |
| | CXCR4 nucleus | 2/26 (8%) | 2/11 (18%) | 8/17 (47%) |
| | CXCR5 cytoplasm | 16/27 (59%) | 6/11 (55%) | 5/14 (36%) |

The numbers and percentages represent the number of cases with high expression from all of the cases. For this division the ROC curve based cut-off points were used.

## 5.2 Immunoelectron microscopy

IEM analysis was used in Studies III and IV. In both studies IEM analysis was performed on 2 PCNSL, 1 sCNSL (brain biopsy), 1 sDLBCL and 1 reactive lymph node samples. In Study III CXCR4, CXCR5, CXCL12 and CXCL13 were studied. Study IV included all of the studied molecules, except E-cadherin that did not show any immunoreactivity in IHC studies. IEM expression patterns are presented in Figure 3 in Study III and in Figure 2 in Study IV.

### *5.2.1 CXCR4, CXCR5, CXCL12 and CXCL13*

#### *CXCR4 and CXCL12*

In the reactive lymph node sample the CXCR4 localisation was mainly nuclear in reactive lymphocytes with few cytoplasmic labels. No membranous expression was detected and CXCL12 staining was negative. Nodal DLBCL sample presented with few CXCR4 and CXCL12 labels randomly located in the nucleus, cytoplasm and cell membranes of the malignant B-cells. In CNS samples CXCR4 and CXCL12 were expressed in high numbers when compared to lymph node samples. Their expression in malignant B-cells in CNS samples was cytoplasmic and membranous, with no clear nuclear expression. In the sCNSL sample CXCR4 and CXCL12 expressions were seen more in the cell membrane whereas in PCNSL samples the expression was more focused to the cytoplasm. In PCNSL occasional nuclear CXCL12 expression was detected but this scarce expression was considered background labelling.

#### *CXCR5 and CXCL13*

In the reactive lymph node sample the IEM showed CXCR5 and CXCL13 to be expressed in the cytoplasms (mostly cytoplasmic vesicles) and in the cell membranes of reactive lymphocytes. In nodal DLBCL sample the expression levels of both CXCR5 and CXCL13 were low in malignant B-cells with few labels located in the cell membrane and even fewer in the cytoplasm. In sCNSL sample CXCR5 and CXCL13 positivity in malignant B-cells was higher and mostly cytoplasmic, with some labels localised in the endoplasmic reticulum and some in the cell membrane as well. In PCNSL samples CXCR5 staining was considerably stronger when compared to other samples. CXCL13 was also detected in higher numbers

but to a lesser extent. CXCR5 expression was mostly cytoplasmic and localised in the endoplasmic reticulum, although a considerable number of CXCR5 signals were also detected in the cell membrane. The cytoplasmic expression density of CXCR5 and CXCR5/CXCL13 complexes was more than twice as high in PCNSL samples when compared to sCNSL. The difference was even higher when compared to sDLBCL and reactive lymph node samples. However, the relative proportion of CXCR5 receptors binding their ligand CXCL13 was highest in reactive lymph node sample. The expression densities of CXCR5 and CXCL13 in different diagnostic groups are presented in Figure 4 in Study III.

### 5.2.2 Adhesion-, migration- and inflammatory responses-associated molecules

*Malignant B-cells and reactive lymphocytes*

In IEM reactive lymph node sample the strongest positivity in reactive lymphocytes was seen for lactoferrin and CDH12. Both were localised to the nucleus, cytoplasm and cell membrane, lactoferrin labels existing in high numbers in all three subcellular locations but CDH12 labels only in the nucleus. The only molecule with no immunoreactivity in reactive lymphocytes was P-cadherin. Other molecules were mostly expressed in low or moderate levels with varying locations. In nodal DLBCL immunoreactivity for all of the molecules was seen to some extent in malignant B-cells, with varying locations. A strong expression was seen for membranous CD44 and nuclear CDH12. In sCNSL N-cadherin was the only molecule with no labelling in malignant B-cells. Otherwise almost all of the molecules presented with solely membranous expression, the only exceptions being lactoferrin and PTEN that were expressed in all three subcellular locations. The strongest and substantial labelling within the malignant cells was seen with membranous ITGA10. In PCNSL samples the strongest expressions were seen for CD44, PTEN, cadherin-11 and N-cadherin. Table III in the Supplementary material of the original publication number IV represents IEM expression patterns more specifically.

*Other cells*

The studied molecules were also detected to have immunolabelling outside of lymphoma cells. Especially lactoferrin was seen outside of lymphoma cells in all samples but with the most abundant expression in PCNSL samples. CDH12 was as well detected outside of the lymphoma cells in sCNSL and PCNSL samples. Low expression of N-cadherin was seen in PCNSL in cells that surrounded the lymphoma cells. In sCNSL N-cadherin expression was seen in similar cells but in higher numbers. Likewise, CD44 was expressed in PCNSL samples on cell membranes of cells that came into contact with lymphoma cells with cell protrusions. In sCNSL sample high cadherin-11 expression was seen in the cell membrane of similar cells. These lymphoma cell-surrounding cells with long cell protrusions also showed membranous PTEN labelling in sCNSL and PCNSL samples. The precise cell type of these cells could not be determined but they possibly represent antigen-presenting microglial cells. In PCNSL samples ITGA10 was as well detected on cells other than lymphoma cells and in addition it was expressed in myelinated and non-myelinated neurons.

## 5.3 Gene-expression profiling

### *5.3.1 Diffuse large B-cell lymphoma*

All of the studied molecules in original publication number IV were found in GEP data but only CD44, cadherin-11, lactoferrin and E-cadherin presented with differences in the gene expression levels. All of them were expressed in higher levels in sDLBCL when compared to sCNSL. The fold changes for CD44, cadherin-11, lactoferrin and E-cadherin were 3.93, 42.04, 1200.21 and 16.90, respectively. The fold change for CD44 is a mean of five different values from five different probes.

### *5.3.2 Peripheral T-cell lymphomas*

By comparing the two prognostic groups (page 88), 59 differentially expressed genes with FC >1 or <-1 and p-values <0.01 were identified. In the poor prognosis group 13 genes were found to be upregulated and 46 genes downregulated. The upregulated genes included beta-catenin encoding gene CTNNB1 (FC = +3.49, p = 0.006) and bromodomain-containing protein 7 (BRD7) encoding gene BP75 (FC

= +2.43, p = 0.003) among others. The downregulated genes included janus kinase 1 encoding gene JAK1 (FC = -1.31, p < 0.001) and galectin-1 encoding gene LGALS1 (GAL1) (FC = -1.43, p = 0.008). The results of gene-expression profiling are visualised in whole in Figure 4 in Study II. The full list of differentially expressed genes is presented in Supplementary material 1 in Study II.

The over-represented pathways included the KEGG term Systemic lupus erythematosus (ID: 5322, expected: 4.42, observed: 23, total: 113, p < 0.001) and the GO biological process terms SRP-dependent cotranslational protein targeting to membrane (GO: 0006614, expected: 2.45, observed: 25, total: 87, p < 0.001), establishment of protein localisation to endoplasmic reticulum (GO: 0072599, expected: 2.73, observed: 25, total: 97, p < 0.001), activation of immune response (GO: 0002253, expected: 6.2, observed: 35, total: 223, p < 0.001) and viral life cycle (GO: 0019058, expected: 4.11, observed: 26, total: 149, p < 0.001). When the inclusion criterion for differentially expressed genes (FC >1 or <-1 and p-values <0.01) was used, the observed count/size ratio in the over-represented pathways was generally low. The full list of over-represented pathways is presented in Supplementary material 1 in Study II.

## 5.4 Prognostic significance and clinical correlations

### *5.4.1 Survival*

#### *Diffuse large B-cell lymphoma*

The IHC staining intensities of the EMT TFs were used for the survival analyses. In DLBCL (Study I) cytoplasmic Slug expression was associated with favourable OS (p = 0.044, RR 6.16, 95% CI 0.81–46.7) and PFS (p = 0.050, RR 5.95, 95% CI 0.78–45.4). The three-year OS and PFS among patients with strong Slug expression were 100.0% and 91.7% and among patients with weak Slug expression 78.6% and 77.8%, respectively. Nuclear ZEB1 expression on the other hand was associated with adverse OS (p = 0.006, RR 0.251, 95% CI 0.087–0.72). The three-year OS among patients with strong ZEB1 expession was 70.6% and that among patients with weak ZEB1 expression was 94.0%. Nuclear ZEB1 expression did not have statistically significant association with PFS and neither nuclear nor cytoplasmic Twist expression did have statistically significant associations with OS or PFS. The Kaplan-Meier curves are presented in Figure 3 in Study I.

*Peripheral T-cell lymphoma*

The IHC staining patterns of the EMT TFs were used for the survival analyses. The results were gained using the staining percentages for Twist and ZEB1 and the staining intensities for Slug. Nuclear Twist expression was associated with favourable PFS in PTCL NOS ($p = 0.044$). The three-year PFS among patients with strong Twist expression was 100% and that among patients with weak Twist expression was 0%. Nuclear ZEB1 expression was associated with favourable PFS when all PTCLs were analysed as a one group and also in PTCL NOS cases separately ($p = 0.018$ and $p = 0.014$, respectively). The three-year PFS among patients with strong ZEB1 expression was 70% and 53% and among those with weak ZEB1 expression 38% and 0%, respectively. Nuclear Slug expression was associated with adverse PFS when all PTCLs were analysed as a one group and also in AITL cases separately ($p = 0.029$ and $p = 0.018$, respectively). The three-year PFS among patients with strong Slug expression was 17% and 0% and that among those with weak expression was 63% and 49%, respectively.

Based on the good prognostic value of the EMT TFs a combination variable including Twist and Slug was created. The used cut-off points for this combination variable are presented in Table 3 (page 91). The expression of these two EMT TFs determined a poor prognosis group and a good prognosis group. The poor prognosis group (low Twist + strong Slug) had a three-year PFS of 21% whereas the good-prognosis group (high Twist + weak Slug) presented a three-year PFS of 86% ($p = 0.012$).

### *5.4.2 Central nervous system relapse*

*Chemokine receptors*

The different chemokine receptor expression profiles for systemic and CNS DLBCLs were gained from the analyses of the IHC staining patterns. Nuclear CXCR4 expression associated with systemic DLBCL whereas cytoplasmic CXCR5 expression was associated with CNS involvement when sCNSL and PCNSL where analysed together as one group ($p = 0.003$ and $p = 0.039$, respectively). When cases with both nuclear and cytoplasmic CXCR4 expression were compared with those that were detected to only have cytoplasmic expression, an association with concurrent nuclear and cytoplasmic CXCR4 expression with systemic disease ($p = 0.032$) was found.

A combination variable, presented in Table 3 (page 91), was discrovered by combining the above-mentioned variables that associated with CNS disease. The combination variable, with low nuclear CXCR4 expression together with high cytoplasmic CXCR5 expression, associated with CNS disease ($p = 0.018$) when compared to rest of the cases. The different expression profiles associated with CNS disease are presented in Figure 2 in Study III.

### *Adhesion-, migration- and inflammatory responses-associated molecules*

There were statistically significant differences between the IHC expression of ITGA10, CD44, PTEN, cadherin-11, P-cadherin and lactoferrin among different diagnostic groups. These results were gained from analyses with Chi-Square test with the chosen cut-off points (sDLBCL versus sCNSL), from the Kruskal-Wallis test (comparing all three diagnostic groups PCNSL, sCNSL and sDLBCL) and from the Mann-Whitney U test that was performed to compare the differences between CNS relapse and PCNSL.

Results from the Kruskal-Wallis test and from the Mann-Whitney U test are presented in Table 10. In these analyses for continuous variables ITGA10 and PTEN associated with CNS lymphomas and lactoferrin expression was strongly associated with PCNSL (Table 10). CD44 and cadherin-11 expressions were also strongest in PCNSL, but lowest in sCNSL (Table 10). Figure 3 in Study IV represents the associations, gained from the Kruskal-Wallis test, between the studied proteins and different diagnostic groups.

**Table 10. Associations of the adhesion-, migration- and inflammatory responses-associated molecules with different diagnostic groups sDLBCL, sCNSL and PCNSL.**

| Protein, Location | Mean rank, sDLBCL | Median, sDLBCL | Mean rank, sCNSL | Median, sCNSL | Mean rank, PCNSL | Median, PCNSL | P-value |
|---|---|---|---|---|---|---|---|
| Kruskal-Wallis test (sDLBCL, sCNSL and PCNSL) | | | | | | | |
| ITGA10, C | 22.62 | 0.00 | 33.20 | 50.00 | 46.85 | 100.00 | <0.001 |
| ITGA10, M | 25.40 | 0.00 | 40.25 | 100.00 | 26.08 | 0.00 | 0.002 |
| CD44, M | 46.22 | 70.00 | 34.82 | 50.00 | 67.54 | 100.00 | 0.001 |
| PTEN, N | 25.72 | 0.00 | 38.37 | 5.00 | 36.04 | 0.00 | 0.024 |
| Cadherin-11, N | 51.80 | 100.0 | 34.02 | 30.00 | 52.23 | 100.00 | 0.006 |
| Cadherin-11, C | 46.64 | 0.00 | 41.52 | 0.00 | 54.00 | 0.00 | 0.048 |
| Lactoferrin, C | 28.50 | 0.00 | 30.18 | 0.00 | 52.85 | 100.00 | <0.001 |
| Mann-Whitney U test (sCNSL and PCNSL) | | | | | | | |
| ITGA10, C | - | - | 18.70 | 50.00 | 29.62 | 100.00 | 0.006 |
| ITGA10, M | - | - | 24.73 | 100.00 | 15.69 | 0.00 | 0.014 |
| CD44, M | - | - | 18.12 | 50.00 | 30.96 | 100.00 | 0.002 |
| P-cadherin, N | - | - | 19.84 | 5.00 | 28.85 | 20.00 | 0.030 |
| Lactoferrin, C | - | - | 18.00 | 0.00 | 33.23 | 100.00 | <0.001 |

Mean ranks and median values are gained from the statistical analyses. N = nucleus, C = cytoplasm and M = cell membrane

With the Chi-Square test cytoplasmic ITGA10, membranous ITGA10 and nuclear PTEN expression associated positively to the occurrence of CNS relapse ($p = 0.002$, $p = 0.007$ and $p = 0.011$, respectively). Then again nuclear ITGA10, membranous CD44 and nuclear cadherin-11 expression associated inversely with CNS relapse ($p = 0.044$, $p = 0.015$ and $p = <0.001$, respectively).

### *5.4.3 Clinical correlations*

#### *Associations of Twist, ZEB1 and Slug with different peripheral T-cell lymphoma entities*

In Study II ZEB1 nuclear staining intensity ($p = 0.017$), Slug nuclear staining percentage ($p = 0.043$) and intensity ($p = 0.002$), as well as Slug cytoplasmic staining percentage ($p = 0.023$) showed statistically significant differences between different entities when all of the diagnoses were compared. In EATL and PTCL NOS ZEB1 nuclear staining intensity was generally strong when again in AITL it tended to be non-existent or light. PTCL NOS samples presented with high nuclear ZEB1 staining percentages when compared to other PTCL entities. The nuclear staining intensity of Slug was relatively strongest in ALK+ ALCL and PTCL NOS cases. In EATL the nuclear Slug staining percentage and intensity were low and weak in almost all of the cases. Then again the cytoplasmic staining percentage of Slug was high in all of the EATL cases. AITL was the only entity presenting with low Slug cytoplasmic staining percentages. These are presented in Table 2 of Study II.

#### *Associations of Twist, ZEB1 and Slug with clinicopathological parameters*

The statistically significant associations between the immunohistochemically studied proteins and the clinical data are presented in Table 11. In Study II all PTCLs were analysed as a one group. Various associations were seen, especially for IPI and the number of extranodal lesions. From the chemokine receptors only CXCR4 associated with clinical markers, showing negative associations with age > 60 years and the number of extranodal lesions ($p = 0.013$ and $p = 0.025$, respectively). CD44 was the only protein that associated with the cell-of-origin, more specifically with non-GC phenotype ($p = 0.049$).

**Table 11. Associations of all of the studied molecules in DLBCL and PTCL samples with clinicopathological parameters.**

| Protein, Location | Diagnosis Study number | Age >60 years | B-sympt. | Stage | Extranodal lesions | LDH | GC/non-GC | IPI |
|---|---|---|---|---|---|---|---|---|
| ZEB1, N | DLBCL, I | | 0.012 posit. intens. | 0.005 posit. | | | | |
| Slug, N | DLBCL, I | | 0.030 posit. | 0.023 posit. | 0.047 posit. | | | 0.026 posit. |
| Twist, N | PTCL, II | 0.038 negat. intens. | | | | | | |
| ZEB1, N | PTCL, II | | 0.010 negat. intens. | | | | | |
| CXCR4, N | DLBCL, III | 0.013 negat. | | | 0.025 negat. | | | |
| ITGA10, C | DLBCL, IV | | | | | | | 0.022 posit. |
| ITGA10, M | DLBCL, IV | | | | 0.006 posit | | | 0.007 posit. |
| CD44, M | DLBCL, IV | 0.034 posit. | | | 0.037 negat. | | 0.049 posit. Non-GC | |
| PTEN, N | DLBCL, IV | | | | | 0.002 posit. | | |
| CDH12, N | DLBCL, IV | | | | 0.026 posit. | | | 0.010 posit. |
| Lactof., C | DLBCL, IV | | | | | | | 0.029 posit. |

The numbers presented in the table represent p-values. Most of the associations are gained by analysing the protein expression percentages but the cases where intensities are used, are marked with an abbreviation for intensity. N = nucleus, C = cytoplasm, M = cell membrane, intens. = the staining intensity, posit. = positive association and negat. = negative association.

*Associations between the studied proteins*

In Studies I and II the immunohistochemically studied EMT TFs showed a number of associations between each other and in most cases the staining percentages correlated positively with the relative staining intensities per location and TF. The statistically significant associations are presented in Table 12.

**Table 12. Associations between Twist, ZEB1 and Slug in DLBCL and PTCL samples.**

| EMT TFs | Twist, N, % | Twist, N, intensity | ZEB1, N, % | ZEB1, N, intensity | Slug, N, % | Slug, C, intensity |
|---|---|---|---|---|---|---|
| Twist, N, % | | | 0.035 posit. DLBCL | | | |
| Twist, N, intensity | <0.001 posit. DLBCL, <0.001 posit. PTCL | | | | | |
| ZEB1, N, intensity | | | 0.010 posit. DLBCL, <0.001 posit. PTCL | | | |
| Slug, N, % | 0.001 posit. DLBCL | 0.036 posit. DLBCL | | | | |
| Slug, N, intensity | | | | | 0.010 posit. PTCL | |
| Slug, C, % | 0.032 posit. PTCL | | 0.024 posit. PTCL | 0.010 posit. PTCL | | 0.014 posit. PTCL |
| Slug, C, intensity | 0.047 posit. DLBCL | | | | <0.001 posit. DLBCL | |

The diagnostic groups (DLBCL and PTCL) are presented in the table. The numbers represent the p-values, posit. = positive association and negat. = negative association. N = nucleus, C = cytoplasm and M = cell membrane.

In Study III there was an inverse association between IHC nuclear CXCR4 and cytoplasmic CXCR5 expressions (p = 0.021). Other associations between the chemokine receptors were not discovered. The statistically significant associations between the immunohistochemically studied molecules in Study IV are presented in Table 13.

**Table 13. Associations between the adhesion-, migration- and inflammatory responses-associated molecules.**

| Proteins | ITGA10, C | ITGA10, M | CD44, M | CDH12, M | N-cadherin, N | P-cadherin, N | Lactoferrin, N |
|---|---|---|---|---|---|---|---|
| ITGA10, N | 0.012 negat. | 0.001 negat. | | | 0.006 posit. | 0.003 posit. | |
| ITGA10, C | | | | 0.001 posit. | | | |
| CD44, C | | | 0.016 posit. | 0.002 posit | | | |
| PTEN, N | <0.001 posit. | | | 0.001 posit. | | | |
| Cadherin-11, N | | 0.024 negat. | | | 0.010 posit. | <0.001 posit. | |
| Cadherin-11, C | | 0.023 negat. | | | | | 0.028 posit. |
| CDH12, N | | | | | | 0.008 posit. | |
| CDH12, C | | | 0.034 posit. | | | | |
| CDH12, M | | | 0.008 posit. | | | | |
| N-cadherin, C | | | | | 0.002 posit. | | 0.038 posit. |
| P-cadherin, N | | | | | | | 0.002 posit. |
| Lactoferrin, C | 0.006 posit. | | | | | | |

The numbers presented in the table represent p-values. Posit. = positive association, negat. = negative association, N = nucleus, C = cytoplasm and M = membrane

### *Associations of clinicopathological parameters and central nervous system lymphoma*

In Study III B-symptoms, increased LDH, >1 extranodal lesions, stage IV and IPI score 3-5 associated with sCNSL when compared to sDLBCL ($p = 0.014$, $p = 0.013$, $p = 0.048$, $p = <0.001$ and $p = 0.013$, respectively). Overall, 85% of PCNSL cases represented the non-GC phenotype, additionally, 60% of the sCNSL cases represented the non-GC phenotype and from the sDLBCLs, the proportion was 53%. PCNSL correlated with the non-GC phenotype ($p = 0.015$), however, the non-GC phenotype did not predict CNS relapse among patients with primary systemic DLBCL.

In Study IV increased LDH, > 1 extranodal lesions, stage IV and IPI score 3-5 associated with sCNSL when compared to sDLBCL ($p = 0.019$, $p = 0.031$, $p = 0.001$ and $p = 0.003$, respectively). 50% of PCNSL cases represented the non-GC phenotype and from the sCNSL cases and sDLBCLs, the proportion was 55%. The non-GC phenotype did not associate with the presence of CNS relapse or PCNSL.

# 6 Discussion

Lymphomas are a heterogeneous group of malignancies. The knowledge of biological properties and the classification of different lymphomas has improved greatly during the past decades. Different lymphoma entities and subtypes are being recognised more and more. These advances are due to improved diagnostic techniques and increased biological data from the ongoing studies in the field of lymphomas (Swerdlow *et al*. 2008, Swerdlow *et al*. 2016). Additionally, the treatment of lymphomas has advanced but problems also exist as, despite the better prognosis of the DLBCL patients, the incidence of CNS relapses has not changed much, if at all. It is known that the drugs included in the R-CHOP therapy have a poor penetration through the BBB and into the CNS (Ghose *et al*. 2015, Tai *et al*. 2011, Yamamoto *et al*. 2010). As sCNSL and PCNSL are known to have poor prognoses with the current treatment modalities, the prevention of sCNSL is of crucial importance (Ghose *et al*. 2015, Leppä *et al*. 2008, Patrick & Mohile 2015, Swerdlow *et al*. 2008, Tai *et al*. 2011, Yamamoto *et al*. 2010).

What comes to the classification of PTCLs, it still remains imprecise although in the forthcoming WHO revision significant advances have been made in the classification of nodal and extranodal T-cell lymphomas (Swerdlow *et al*. 2008, Swerdlow *et al*. 2016). However, the optimal treatments remain unclear. PTCLs are most often treated with the same therapies as B-cell lymphomas, with mostly suboptimal results. Although advances have been made in this field, most PTCLs still have clearly inferior outcomes when compared to DLBCL (Casulo *et al*. 2016, Schmitz & de Leval 2016, Zhang *et al*. 2016).

There is a need for more specific biological understanding of these different malignancies. This and the improving classification will hopefully in the future enable more specific, tailored treatment choices that will further improve the prognosis of lymphoma patients. The studies presented here give new information of the biology behind DLBCL CSN tropism and PTCLs.

## 6.1 The prognostic value and clinicopathological correlations of Twist, ZEB1 and Slug

In solid cancers the activation of EMT TFs has been linked to disease aggressiveness, invasiveness as well as poorer prognosis (Nieto 2011). There are some studies where Twist, ZEB1 and Slug have been studied in haematopoietic cells and in lymphomas. These studies propose that they play a role in physiological

haematopoiesis, including cell survival and differentiation, and probably have concordant roles in haematological malignancies. Different EMT TFs have been shown to have different functions, some being tumour suppressing and some tumour promoting (Brabletz *et al*. 1999, Perez-Losada *et al*. 2002, Postigo *et al*. 1997, Vandewalle *et al*. 2009, Wu *et al*. 2005, Zhang *et al*. 2012). In our studies Twist, ZEB1 and Slug did have prognostic value and were seen to have a number of associations with clinicopathological parameters.

In Studies III and IV the prognostic value of the studied molecules for survival or systemic relapses was not evaluated due to the highly selective nature of the study material.

### *6.1.1 Associations of Twist, ZEB1 and Slug with clinicopathological parameters and each other*

The associations between the clinicopathological parameters and the studied EMT TFs were in line with their prognostic value. Those TFs that were found to correlate with poorer prognosis were also found to correlate with clinicopathological parameters that generally are considered to correlate to disease aggressiveness and poorer prognosis. Vice versa Twist and ZEB that were linked to favorable prognosis in PTCLs associated negatively to age and the presence of B-symptoms. The significance of Slug expression seemed to be dependent on the subcellular localisation similarly as in associations with the prognosis. Both EMT TFs and adverse prognostic markers were associated to specific PTCL gene-expression profile, which will be discussed later.

When the associations between Twist, ZEB1 and Slug were studied, nuclear Twist was associated positively with both nuclear ZEB1 and Slug as well as with cytoplasmic Slug in DLBCL. In DLBCL ZEB1 and Slug did not show any statistically significant associations with each other but in PTCLs cytoplasmic Slug did associate positively with nuclear ZEB1. In addition, nuclear Twist associated positively with cytoplasmic Slug in PTCLs. In PTCLs the mutual correlations between the studied TFs were in line with their prognostic value and as all of the staining percentages showed a positive association with their relative staining intensity, it seems that these both means of measuring the IHC expression are as valid. The only discrepancy in these results is the fact that in DLBCL Slug cytoplasmic staining intensity was associated positively with Slug nuclear staining percentage. As nuclear and cytoplasmic Slug expressions seemed to associate to

contradictory disease features, it could be the balance between these two subcellular localisations that determines the functional role for Slug expression.

### 6.1.2 The biology behind the impact of Twist, ZEB1 and Slug on prognosis

Twist, ZEB1 and Slug did not all associate to poorer prognosis in DLBCL and PTCLs as they mostly do in solid malignancies. This is probably explained by the fact that in physiological haematological cells these TFs participate in the regulation of normal cell growth and differentiation and thus their prognostic role is likely mediated through these effects rather than true EMT-like event (Brabletz *et al*. 1999, Nieto 2011, Perez-Losada *et al*. 2002, Postigo *et al*. 1997, Vandewalle *et al*. 2009, Wu *et al*. 2005, Zhang *et al*. 2012).

Twist has been shown to have antiapoptotic functions in malignancies, which are mediated through mechanisms such as inhibition of c-myc induced apoptosis and antagonisation of the p53 pathway (Cakouros *et al*. 2010, Maestro *et al*. 1999). In addition Twist seems to function as NF-kB-controlled anti-apoptotic factor (Sosic *et al*. 2003). In haematopoietic cells Twist expression is physiologically restricted mainly to CD34+ stem cells but its expression is upregulated in activated memory CD4+ T cells as well (Merindol *et al*. 2014, Zhang *et al*. 2012). In addition Twist2 has been shown to inhibit galectin-1-mediated apoptosis that occurs during negative selection in thymus after NF-kB activation. This is mediated by regulating the expression of galectin-1 receptor, CD7. These antiapoptotic functions have been reported to occur in T-cell lymphomas as well (Merindol *et al*. 2014). In lymphomas Twist expression has been found to be increased in advanced MF/SS lesions and interestingly constitutive NF-kB expression has been described as a characteristic feature of MF, that again might explain the upregulation of Twist protein (Goswami *et al*. 2012, Izban *et al*. 2000). In contrast to previous data, in our studies Twist expression was linked to favorable prognosis in PTCL NOS cases, when again no statistically significant associations were seen in DLBCLs. The association to favorable prognosis was very strong in PTCL NOS when at a three-year follow up point all of the Twist negative patients had experienced disease progression when again none of the Twist positive patients had experienced a disease progression. The mechanism how Twist affects the prognosis of these patients is unclear. It would be interesting to study Twist expression in cutaneous PTCLs as in MF Twist has been associated with disease progression and MF on the other hand to constitutive NF-kB expression that is further linked to Twist expression (Goswami

*et al*. 2012, Izban *et al*. 2000). It might be that Twist has different roles in cutaneous T-cell lymphomas than in PTCLs.

The fact that ZEB1 associated differently to prognosis in DLBCL than in PTCLs may be explained by the fact that ZEB1 has been shown to have separate B- and T-cell specific physiological functions. In B cells ZEB1 participates in the repression of IgH and Bcl-6 (Genetta *et al*. 1994, Papadopoulou *et al*. 2010). In DLBCL Bcl-6 protein overexpression has been associated with favorable outcome in several studies and by repressing the BCL6 gene expression ZEB1 would most likely worsen the outcome (Horn *et al*. 2013, Iqbal *et al*. 2007, Winter *et al*. 2006). Then on the other hand in PTCLs and PTCL NOS cases separately, ZEB1 expression proved out to be a marker of better prognosis. Low ZEB1 expression also correlated with CD4+ diseases that originate from helper T-cells, such as AITL and on the other hand CD4- entities like EATL and a significant number of PTCL NOS cases had a strong ZEB1 expression. These results are in line with what is already known of the role of ZEB1 in both malignant and physiological T cells. ZEB1 is a physiological regulator of T-cell development and differentiation. CD4, an important protein for mature T cell activation, is differentially expressed during T cell development. It also seems to be negatively regulated by ZEB1 and in a study by Hidaka et al. when in a majority of ATLL cells ZEB1 gene was found to be inactivated, the transcription of CD4, alpha4 integrin and GATA3 was concomitantly upregulated (Brabletz *et al*. 1999, Hidaka *et al*. 2008). In a mouse model setting it was seen that ZEB1 mutant mice frequently develop CD4+ lymphomas and/or leukaemias (Hidaka *et al*. 2008). In addition, in ATLL ZEB1 has been suggested to act as a tumour-suppressor gene by enhancing TGF-beta growth inhibition (Hidaka *et al*. 2008, Nakahata *et al*. 2010). ZEB1 indeed seems to have tumour suppressive abilities and in SS as well almost half of the patients have been reported to harbor ZEB1 gene deletion (Vermeer *et al*. 2008). In light of these findings our results in PTCLs are in line with the known data of ZEB1. However, the exact mechanism that mediates the effects of ZEB1 on favorable prognosis in PTCLs remains unconfirmed and should be a target of future studies.

Slug is known to have antiapoptotic functions and for example it has been shown to be able to protect irradiated haematopoietic cells from p53-mediated apoptosis (Wu *et al*. 2005). Slug has crucial roles in haematopoietic cell functions and Slug mutant mice present with haematopoietic developmental defects. Results from a study by Perez-Losada et al. suggest that Slug may be a factor that controls the migration and survival of c-kit positive HSCs (Perez-Losada *et al*. 2002). It might be that Slug contributes to the neoplastic transformation by inhibiting

apoptosis. In a study by Mancini et al. Slug did indeed contribute to the survival advantage of Bcr-Abl-expressing CML cells and its overexpression also resulted in a significant E-cadherin reduction (Mancini *et al*. 2010). Concordantly, in our studies nuclear Slug expression associated with aggressive clinical features in DLBCL and was linked to an adverse clinical outcome in PTCLs. Surprisingly, however, in DLBCL the cytoplasmic expression of Slug was an indicator of favorable prognosis. A possible explanation for this is the fact that because Slug is a TF, its functional target is in the nucleus and its expression in the cytoplasm may imply that its transport/migration to the nucleus is disturbed or that it otherwise represents an inactive form in the cytoplasm.

### *6.1.3 Gene-expression profiling in peripheral T-cell lymphomas and the potential biology behind the findings*

Based on the IHC results a GEP was performed on 6 AITL and 6 PTCL NOS cases, 6 cases belonging to the poor prognosis group and 6 to the favorable prognosis group. The prognostic groups were determined by IHC expression of Twist and Slug. GEP results showed that these two groups had significant differences in their gene-expression profiles. Twist and Slug were able to delineate these two groups better than histological diagnoses.

There were several differentially expressed proteins between these two groups but one of the highest overexpressions in the poor prognosis group was seen for beta-catenin, a key activator of the Wnt-signaling. Interestingly, other regulators of the Wnt signaling were also found with altered expression profiles in the poor prognosis group. BRD7 and galectin-1 were found to be overexpressed and underexpressed, respectively. The data regarding BRD7 is somewhat contradictory, some studies indicating a tumour suppressor role for BRD7 in solid malignancies and a Wnt/beta-catenin pathway signaling suppressing functions (Park *et al*. 2014, Peng *et al*. 2007, Wu *et al*. 2009). However, BRD7 has also been reported to act as a positive regulator of EMT and by inducing dephosphorylation of GSK-3beta at Tyr216, causing a subsequent activation of Wnt signaling dependent transcription (Kim *et al*. 2003, Liu *et al*. 2017). Sole tyrosine phosphorylation at Tyr216 has a critical role in maintaining GSK-3beta activity and in the above-mentioned study its dephospohorylation led to consequent beta-catenin stabilisation and its translocation to the nucleus (Kim *et al*. 2003). It is known that the inhibition of GSK-3beta, which leads to activation of beta-catenin, promotes haematopoiesis (Ashihara *et al*. 2015). As the poor prognosis group was determined by the

combination variable that included high Slug expression, these results regarding GSK-3beta are in line with those from a study by Saegusa et al. that reported a correlation between Slug upregulation with phosphorylated, activated Akt and nuclear beta-catenin. It was shown in the same study that active Akt induced stabilisation of nuclear beta-catenin, through inactivation of GSK-3beta, that led to transactivation of Slug, proposing that Slug overexpression is actually regulated by nuclear beta-catenin (Saegusa *et al*. 2009). In a study by Kim et al. it was demonstrated that BRD7 is able of participating in the subcellular localisation of beta-catenin to the nucleus (Kim *et al*. 2003). In light of these studies the upregulation of BRD7 is understandable in the poor prognosis group that overexpresses beta-catenin. Galectin-1, that was under-expressed in the poor prognosis group, has also been indicated to have contradictory functions. It has been reported to promote Akt activation, beta-catenin nuclear translocation, TCF4/LEF1 transcriptional activity and increased c-myc and cyclin D1 expression. These are changes that suggest the activation of Wnt pathway, although in the same study it was also reported that galectin-1 overexpression led to downregulation of beta-catenin (Bacigalupo *et al*. 2015). Then again a study by Satelli et al. showed that intracellular galectin-1 induced cell cycle arrest and apoptosis in colorectal carcinoma cells with concomitant downregulation of Wnt signaling pathways (Satelli & Rao 2011).

Of specific interest was the Wnt/beta-catenin pathway itself as beta-catenin was markedly overexpressed in the poor prognosis group. Beta-catenin has been linked to Slug expression and EMT in other studies, its signaling is known to represent a crucial pathway in maintaining stem cell properties in HSCs and in addition constitutively active beta-catenin is able of reprogramming myeloid and lymphoid progenitors to multipotent haematopoietic progenitor cells (HPC) (Ashihara *et al*. 2015, Inoue *et al*. 2016, Medici *et al*. 2008). Dysregulation of the Wnt/beta-catenin signaling has been shown to play a major role in the pathogenesis of haematological malignancies, especially myeloid malignancies, and what is specifically of interest is the fact that for example AITL is considered to have a genomic landscape that resembles more closely myeloid diseases than other lymphomas, indicating some common pathogenetic mechanisms. This might help explaining the poor outcomes with treatments originally developed against B-cell lymphomas (Ashihara *et al*. 2015, Odejide *et al*. 2014). Our results give rise to an interesting hypothesis that there might be a specific, so far unidentified, T-cell lymphoma subtype with dysregulated Wnt/beta-catenin signaling pathway that harbors an extremely poor prognosis. As there are ongoing clinical trials of agents

targeting the Wnt-pathway, the possible identification of this potential PTCL group might eventually lead to more targeted treatments (Ashihara *et al*. 2015). Further, if these results can be repeated in a larger patient material, these results might imply that EMT TFs are able of defining this new subgroup among PTCLs. Especially Slug is of interest as its overexpression has already been suggested to be regulated by nuclear beta-catenin (Saegusa *et al*. 2009). Future studies are warranted and it should be kept in mind that our results represent a preliminary data and there are various important questions that remain to be answered and although our GEP included only PTCL NOS and AITL cases, a larger material of PTCLs should be constructed. These are further discussed in the future prospects section.

## 6.2 The biology behind diffuse large B-cell lymphoma central nervous system tropism

DLBCL CNS relapse is most often considered a fatal complication that occurs in around 5% of systemic DLBCL cases if prophylactic treatment is not given. However, varying incidence rates are also reported (Bos *et al*. 1998, Ghose *et al*. 2015, van Besien *et al*. 1998). Understanding the biology behind CNS lymphomas has been a challenge for years as it remains unclear why lymphoma cells end up in the CNS. One hypothesis is that these cells originate from extracranial sites but end up developing a highly selective CNS tropism that directs their homing (Jiang *et al*. 2010). GEP studies have been performed comparing normal lymphatic tissue and nodal and extranodal DLBCL with PCNSL and in these studies differences in transcription of several proteins have been observed. The differences in ECM- and adhesion related pathways have been assumed to take part in CNS lymphoma development and tropism (Sung *et al*. 2011, Tun *et al*. 2008).

### *6.2.1 Associations between the studied molecules with clinicopathological parameters*

In the present studies nuclear CXCR4 seemed to be protective of CNS relapses. It was also negatively associated with the presence of 0 or 1 extranodal lesion and age < 60. Cytoplasmic CXCR5 on the other hand was linked to CNS involvement but it did not have any associations with clinicopathological paramenters. Then again higher IPI points, the presence of more than one extranodal lesions and increased LDH, showed a positive association with cytoplasmic ITGA10, membranous ITGA10 and nuclear PTEN expressions, that were also correlated

with CNS relapse in our series as well as to the presence of CNS lymphoma, including both sCNSL and PCNSL. Given that all of the above-mentioned clinical markers are included in the clinical CNS relapse risk assessment, these results are very concordant (Holte *et al*. 2013, Siegal & Goldschmidt 2012, van Besien *et al*. 1998). Membranous CD44 was associated with age > 60 and the absence or maximum number of one extranodal lesion. It also associated with non-GC phenotype, but then again seemed to be protective of CNS relapse. This is an interesting finding since non-GC phenotype has previously been associated with higher incidence of CNS relapses (Savage *et al*. 2016). In a study by Yuan et al. CD44 was as well associated with non-GC phenotype (Yuan *et al*. 2013). However, in the present studies, the cell-of-origin did not show any associations to CNS relapse.

In DLBCL GEP defined GC phenotype is known to have better prognosis than the non-GC phenotype (Alizadeh *et al*. 2000, Rosenwald *et al*. 2002). Although the results of the prognostic value of IHC determined cell-of-origin remain somewhat conflicting, cases with Hans's algorithm determined non-GC phenotype often have worse prognoses (Gang *et al*. 2015, Lu *et al*. 2016). In the light of these findings the fact that CD44 was associated with age > 60 and non-GC phenotype are in line since both of these clinical markers are included in the IPI classification as markers of poorer prognosis (Shipp *et al*. 1993). It should also be noted that in Study IV CD44 expression was highest in PCNSL and in Study III PCNSL was correlated with the non-GC phenotype. Meanwhile, in study IV there were no statistically significant associations between the cell-of-origin and CNS diseases. Nuclear cadherin-11 was inversely associated with CNS relapse and similarly to CD44, its expression was also highest in PCNSL. The functional role of cadherin-11 nuclear expression is unclear.

### *6.2.2 The biology behind the clinicopathological associations of the studied molecules*

Chemokines and their receptors guide the physiological cell migration and the main regulators of lymphocyte homing are CXCR4, CXCR5 and CCR7 and their ligands CXCL12, CXCL13 and CCL19 and CCL21 (Zlotnik *et al*. 2011). Chemokines and their receptors are considered to drive the migration of malignant NHL cells somewhat similarly to normal lymphocytes (Lopez-Giral *et al*. 2004, Trentin *et al*. 2004). During organogenesis the CXCR4/CXCL12 axis is central for the CNS development and in normal circumstances CXCL12 is constitutively produced in

the CNS but also by lymph nodes, bone marrow, liver and lungs (Banisadr *et al*. 2003, Zlotnik *et al*. 2011). CXCL13 is mostly produced by lymph nodes but in disease affecting the CNS the CXCL13 levels in the brain are increased (Kowarik *et al*. 2012, Smith *et al*. 2003, Zlotnik *et al*. 2011). It seems that in PCNSL the main source of CXCL13 expression are the malignant B cells and GEP results also show an upregulation of CXCL13 expression in PCNSL (Kowarik *et al*. 2012, Smith *et al*. 2003, Tun *et al*. 2008). Majority of malignant cells also seem to produce CXCL12 as well, but it is also produced by endothelial cells, reactive astrocytes and microglial cells of the CNS (Brunn *et al*. 2007, Smith *et al*. 2007, Venetz *et al*. 2010). Our results showed concordant expression patterns for CXCL12 and CXCL13, the first one being expressed by endothelial cells of the CNS and the latter by malignant B cells of the CNS. CXCR4/CXCL12 and CXCR5/CXCL13 axis have been studied and correlated with DLBCL CNS tropism in previous studies (Brunn *et al*. 2007, Rubenstein *et al*. 2013, Smith *et al*. 2003, Smith *et al*. 2007, Venetz *et al*. 2010). Based on the literature, the IHC expression of the chemokine receptors and adhesion-, migration- and inflammatory responses-associated molecules was evaluated in all subcellular locations, in addition to their known functional place. Our studies suggest a role for these chemokine receptors and somewhat similarly to the results by Jahnke et al. we found that the associations to CNS lymphomas were found for nuclearly or cytoplasmically located CXCR4 and CXCR5 (Jahnke *et al*. 2005). Nuclear CXCR4 expression has been correlated with lymph node metastases in solid malignancies and in renal cell carcinoma CXCL12 binding localizes CXCR4 to the nucleus (Na *et al*. 2008, Wang *et al*. 2009, Wang *et al*. 2010, Xiang *et al*. 2009, Yoshitake *et al*. 2008). However, in our studies when CXCR4 was located to the nucleus it was less frequently seen on the cell membrane of same cells and as CXCL12 is constitutively produced by the CNS, it might be that the cells that have membranous CXCR4 migrate towards CNS (Banisadr *et al*. 2003, Zlotnik *et al*. 2011). In addition none or very few CXCL12 molecules were detected in reactive lymph node and nodal DLBCL samples. In line with this are the findings that CXCR4/CXCL12 signaling has been shown to induce an EMT-like process that enhances cell migration and invasion (Hu *et al*. 2014, Jung *et al*. 2015, Li *et al*. 2012, Roccaro *et al*. 2015, Yang *et al*. 2015).

Interestingly CXCR4/CXCL12 signaling has been shown to induce integrin and CD44 activation and CD44 localisation to the leading edge in CD34+ stem/progenitor cells during migration (Avigdor *et al*. 2004, Peled *et al*. 2000). However, the results regarding CD44 were contradictory as its membranous expression was negatively correlated with CNS relapse. The fact that the highest

CD44 expression was seen in PCNSL might be explained by the fact that HA is a primary component of the brain extracellular matrix and CD44 is known to interact with HA (Avigdor *et al*. 2004, Bignami *et al*. 1993). Other studies have also provided evidence that CD44 is expressed by the perivascular cells in PCNSL and in CLL cells CD44 mediated adhesion to HA antagonized their motile behavior (Girbl *et al*. 2013, He *et al*. 2013). The role of CD44 in sDLBCL is logical too, as CD44 has a role in binding lymphocytes to high endothelial venules in the lymph nodes (He *et al*. 2013). Additionally, as the PCNSL samples are brain biopsies and sCNSL primary lymph node biopsies, the different microenvironments are likely to affect these results.

Integrin expression was strongly associated with the presence of CNS disease and secondary CNS lymphoma as well and in the literature integrins are linked to increased cell migration (Guo & Giancotti 2004, Hanahan & Weinberg 2000). In addition, integrin-activated pathways have been shown to confer protection against apoptosis (Guo & Giancotti 2004).

The nuclear localisation of PTEN may not be solely linked to migration and invasion as in injured neurons, nuclear trafficking of PTEN has been shown to lead to cell survival (Goh *et al*. 2014). This probably is due to its depletion from cytoplasm and cell membrane, its place of function, since it is generally considered to act as a tumour suppressor. The fact that PTEN loss has also been reported in B-cell lymphomas supports this idea (Battistella *et al*. 2015, Goh *et al*. 2014, Goschzik *et al*. 2014, Li *et al*. 1997, Ma *et al*. 2015, Marsh Durban *et al*. 2014). In addition nuclear PTEN has been proposed to have important functions in maintaining genome stability and protecting cells against oxidative damage (Chalhoub & Baker 2009, Song *et al*. 2012). None the less PTEN has been linked to CXCR4/CXCL12 singaling as well, as in prostate cancer cells PTEN loss resulted in increased expression of CXCR4 and CXCL12 (Conley-LaComb *et al*. 2013). Similarly in T-cells PTEN downregulation has been shown to enhance CXCR4/CXCL12-mediated chemotaxis (Gao *et al*. 2005). As both CXCR4/CXCL12 signaling and dysregulation of the PTEN-PI3K axis have been linked to the gain of mesenchymal-like properties, increased cell motility and invasiveness, it could be proposed that if nuclear PTEN is not inhibiting PI3K/Akt signaling, it might contribute to CNS tropism of DLBCL cells (Hu *et al*. 2014, Jung *et al*. 2015, Leslie *et al*. 2008, Li *et al*. 2012, Roccaro *et al*. 2015, Song *et al*. 2012, Yang *et al*. 2015).

Cytoplasmic lactoferrin was strongly expressed in PCNSL and in our series its expression was associated with higher IPI points. Lactoferrin is often upregulated in inflammatory conditions and under oxidative stress and its expression levels

probably reflect the disease burden and/or aggressiveness (Fillebeen *et al*. 2001). PCNSL is a very aggressive disease that possess a very poor prognosis (Patrick & Mohile 2015). Lactoferrin expression has previously been associated with neurological diseases affecting the CNS, such as Alzheimer's and Parkinson's diseases, where it is produced by activated microglial cells (Fillebeen *et al*. 2001).

In Study III it was assumed that in CNS lymphomas cytoplasmic CXCR5 might represent a non-functional protein. Meanwhile, some CXCR5/CXCL13 complexes were also seen in PCNSL samples and when malignant cells are already located to CNS it might be that CXCR5 also has roles in maintaining the already gained location. This is supported by the fact that malignant cells in PCNSL are reported to express CXCL13 and as chemokines also have growth promoting abilities, CXCR5/CXCL13 axis might in fact enable lymphoma growth in the CNS (Kowarik *et al*. 2012, Matsuki & Younes 2015, Smith *et al*. 2003, Tun *et al*. 2008). In Hodgkin's lymphomas cytokines and chemokines that are produced by the Hodgkin and Reed-Sternberg cells work in an autocrine and/or paracrine manner and promote the survival of these cells (Matsuki & Younes 2015). The cytoplasmic CXCR5 may represent a non-functioning protein or it might be internalised due to ligand binding. Both scenarios might also occur concomitantly or become altered during disease progression.

NHLs often display tissue-specific dissemination patterns and express specific homing receptors. Thus adhesion molecules are likely to play a major role in the dissemination of malignant cells as well and by promoting cell survival and growth they might also contribute to lymphoma aggressiveness (Drillenburg & Pals 2000). Our results support a role for chemokine receptors CXCR4 and CXCR5 in the biology behind CNS lymphomas and CNS tropism. Especially CXCR4/CXCL12 axis and its downstream effects on the expression of adhesion and ECM-related proteins should be of interest in future studies as our results regarding these molecules are very concordant with the known data.

## 6.3 How to select the patients in need of central nervous system prophylaxis

Most of the CNS relapses can be prevented by incorporating prophylactic treatment into the primary treatment of systemic DLBCL. Currently HD-MTX and HD cytarabine are recommended to be used for CNS prophylaxis but this treatment is highly toxic and should be limited to high-risk patients only. The identification of the patient population in need of CNS prophylaxis is currently based on the clinical

risk assessment (Holte *et al*. 2013, Siegal & Goldschmidt 2012, van Besien *et al*. 1998). Unfortunately this is not a very specific method and the more specific predictive markers are needed (Boehme *et al*. 2007). Recent studies suggest that non-GC phenotype and CD5 positivity may have some prognostic relevance on CNS relapses as well, although the significance of non-GC phenotype is somewhat unclear (Jain *et al*. 2013, Oki *et al*. 2014, Petrich *et al*. 2014, Savage *et al*. 2016, Snuderl *et al*. 2010). The incidence of CNS relapses in double-expressor lymphomas has been suggested to be higher and thus CNS prophylaxis in this patient group should be considered (Rosenthal & Younes 2016, Savage *et al*. 2016). Double hit lymphomas on the other hand are known to have a higher risk for CNS relapse and CNS prophylaxis is recommended for patients with HGBL with rearrangements of MYC and BCL2 and/or BCL6 that is classified as an entity of its own in the forthcoming WHO revision (Rosenthal & Younes 2016, Swerdlow *et al*. 2016).

The studies presented here give new information of the biology behind DLBCL CSN tropism and based on the present results promising biological markers for the selection of patients in need of CNS prophylaxis would be CXCR4, PTEN and ITGA10, but CXCR5 and CD44 should also be included in future studies. As the study materials were relatively small, multivariate analyses could not be performed.

## 6.4 Future prospects

Future studies of EMT TFs in PTCLs should include a large study material where all three TFs could be studied, in order to see which ones have the best ability in separating the cases utilising the Wnt/beta-catenin signaling pathway. The specific entities that harbor these Wnt/beta-catenin signaling pathway using diseases should also be identified. In our forthcoming study IHC will be used to study the expression of Twist, ZEB1, Slug and beta-catenin in PTCLs. In addition beta-catenin expression will be studied by means of PCR. These methods will hopefully identify and further verify the existence of a specific disease group. A cell culture should also be performed in order to study whether there exists a causal relationship between beta-catenin signaling and cell survival.

In a longer run we hope to find biological markers that could be used for selecting patients that are in need of CNS prophylactic treatment. So far CXCR4, PTEN and ITGA10 come across as promising biological markers that associate to CNS relapse. However, CXCR5 and CD44 should also be studied further. Future studies should include a larger patient material in order to perform multivariate

analyses that could not be performed in these studies. This could also enable the creation of a biological CNS prognostic index. The aim is to find more specific methods for patient selection than the currently used clinical risk evaluation and in this way prevent the occurrence of often deadly CNS relapses.

# 7 Conclusions

In the present study the expression of EMT TFs, chemokine receptors and adhesion-, migration- and inflammatory responses-associated molecules were evaluated in DLBCL. In addition, the expression of the EMT TFs was evaluated in PTCLs. The study methods included IHC, IEM and GEP. The EMT TFs were found to be expressed in both DLBCL and PTCLs, where they proved out to have prognostic relevance as well. In PTCLs these TFs were able to delineate a disease group with a specific gene-expression profile. In this group the expression levels of Wnt-signalling pathway associated molecules were altered. These results are especially interesting as there are ongoing clinical trials in haematological malignancies of agents targeting the Wnt-pathway. If these results are further verified, they might suggest that there is a specific PTCL subtype that can be separated based on the expression of these TFs. The biological significance of chemokine receptors and adhesion-, migration- and inflammatory responses-associated molecules were studied in DLBCL with or without CNS involvement. From these molecules especially CXCR4, CXCR5, ITGA10, PTEN and CD44 were found to be differently expressed between DLBCL cases with CNS affision than those with no signs of CNS disease. These results are worthy of further studies and hopefully in the longer run will lead towards identification of biological markers for CNS relapse prediction.

The specific conclusions from the present study are:

1. Epithelial-mesenchymal transcription factors Twist, ZEB1 and Slug were expressed in diffuse large B-cell lymphoma. Nuclear ZEB1 is associated with poorer prognosis and cytoplasmic Slug with better prognosis.
2. Epithelial-mesenchymal transcription factors Twist, ZEB1 and Slug do play a role in peripheral T-cell lymphomas. Nuclear Twist and ZEB1 were associated with better prognosis and nuclear Slug with poorer prognosis. In addition when the immunohistochemical expression levels of Twist and Slug were evaluated together, these transcription factors were able of delineating a group among angioimmunoblastic lymphomas and peripheral T-cell lymphomas, not otherwise specified, that presented with a specific gene-expression signature and activated Wnt-signalling pathway.
3. The results from the present studies suggested that in diffuse large B-cell lymphoma cells the CXCR4 signaling activates a process that is somewhat

similar to epithelial-mesenchymal transition, which leads to the activation of integrins and other downstream molecules. These events facilitate the migration towards CXCL12 expressing central nervous system.

4. Based on the present results it seems that CXCR4, CXCR5, integrin alpha 10, PTEN and CD44 expressions could be potential biomarkers for CNS tropism albeit further studies are warranted as due to the limited sample size our results are so far hypothesis generating.

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# Original publications

I Lemma S, Karihtala P, Haapasaari KM, Jantunen E, Soini Y, Bloigu R, Pasanen AK, Turpeenniemi-Hujanen T & Kuittinen O (2013) Biological roles and prognostic values of the epithelial-mesenchymal transition-mediating transcription factors Twist, ZEB1 and Slug in diffuse large B-cell lymphoma. Histopathology 62(2): 326-333.

II Uotila P, Lemma SA, Haapasaari KM, Porvari K, Skarp S, Soini Y, Jantunen E, Turpeenniemi-Hujanen T & Kuittinen O (2017) Epithelial-mesenchymal transition markers Twist, ZEB1 and Slug are associated with progression-free survival and clinical presentation in T-cell lymphomas. Manuscript.

III Lemma SA, Pasanen AK, Haapasaari KM, Sippola A, Sormunen R, Soini Y, Jantunen E, Koivunen P, Salokorpi N, Bloigu R, Turpeenniemi-Hujanen T & Kuittinen O (2016) Similar chemokine receptor profiles in lymphomas with central nervous system involvement - possible biomarkers for patient selection for central nervous system prophylaxis, a retrospective study. Eur J Haematol 96(5): 492-501.

IV Lemma SA, Kuusisto M, Haapasaari KM, Sormunen R, Lehtinen T, Klaavuniemi T, Eray M, Jantunen E, Soini Y, Vasala K, Böhm J, Salokorpi N, Koivunen P, Karihtala P, Vuoristo J, Turpeenniemi-Hujanen T & Kuittinen O (2017) Integrin alpha 10, CD44, PTEN, cadherin-11 and lactoferrin expressions are potential biomarkers for selecting patients in need of central nervous system prophylaxis in diffuse large B-cell lymphoma. Carcinogenesis. In press.



Original publications are not included in the electronic version of the dissertation.

1405. Lepojärvi, Sannamari (2017) Normal variation of the tibiotalar joint in dynamic computed tomography

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ISBN 978-952-62-1603-4 (Paperback)
ISBN 978-952-62-1604-1 (PDF)
ISSN 0355-3221 (Print)
ISSN 1796-2234 (Online)