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# Clinical and Molecular Studies of Diffuse Large B-cell Lymphoma

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### **Abstract**

Abu Sabaa, A. 2023. Clinical and Molecular Studies of Diffuse Large B-cell Lymphoma. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1970. 52 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-1878-3.

The general aim of this thesis was to study the prognostic clinical and biological markers of Diffuse Large B-cell Lymphoma (DLBCL).

**Paper I:** Utilizing population-based data for patients with DLBCL in Sweden, the study aimed to establish whether event free survival at 24 months (EFS24) was a reproducible milestone. The disease-free survival for lymphoma patients was compared with that of age and sex matched Swedish general population. We demonstrated that overall survival was similar to age and sex matched general population only for younger patients (<60 years of age) achieving EFS24. Patients older than that had worse prognosis. Death was mainly linked to cardiovascular disease and secondary malignancies.

**Paper II:** Plasma samples collected via the bio bank U-CAN were analyzed using multiplex extension assay (PEA) utilizing preselected protein panels to examine the possibility of distinguishing lymphomas, leukemias and controls. The study confirmed that PEA technology could be used not only to effectively screen for large number of plasma protein biomarkers in low plasma sample volumes (1 µL), but even to discriminate between controls and different haematological malignancies.

**Paper III:** Plasma protein pattern evolution in DLBCL patients was highlighted by PEA analysis of plasma proteins at different time points under treatment with Rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP). Significant distinctions in protein patterns at diagnosis compared to controls and striking differences in protein levels before and after treatment in patient who responded to treatment were evident. The three top proteins were TCL1A, CXCL13 and IL2RA.

**Paper IV:** An interesting protein that emerged from the above studies was TCL1A. This plasma protein was analyzed in plasma samples by PEA. Validation by plasma enzyme immunosorbent assay (ELISA) was attempted. The cytoplasm and nucleus bound form of TCL1A were analyzed with the help of immunohistochemistry in tissue microarray samples. The study included 178 patients of which 125 received R-CHOP. Clinical risk factor analysis showed no significant correlation with tissue IHC. Significantly higher levels of plasma TCL1A were seen in male patients (measured by ELISA and PEA) and in patients with Ann Arbor stages II-IV (measured by PEA). Survival analysis showed no statistical significance.

**Keywords:** DLBCL, R-CHOP, EFS24, PEA, Biomarker, ELISA, TCL1A.

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*You are not **a drop** in the ocean.  
You are **the entire ocean**, in a drop.*

***Rumi***



# List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I. **A. Abu Sabaa**, C Mörth, S Hasselblom, G Hedström, M Fløge-gård, M Stern, P-O Andersson, I Glimelius, G Enblad. Br J Haematol. **Age is the most important predictor of survival in diffuse large B-cell lymphoma patients achieving event-free survival at 24 months: a Swedish population-based study.** 2021 Jun;193(5):906-914.
- II. **A. Abu Sabaa**, Q Shen, E Bergfelt Lennmyr, AP Enblad, G Gammelgård, A Hein, E Freyhult, M Kamali-Moghaddam, M Höglund, G Enblad, A Eriksson. N Biotechnol. **Plasma proteome profiling reveals major differences between acute leukemia/malignant lymphoma patients and controls.** 2022 Jun 29;71:21-29
- III. **A. Abu Sabaa**, C Mörth, D Molin, E Freyhult, M Kamali-Moghaddam, A Robelius, G Enblad **Plasma Protein Profiling using Multiplex Extension Assay in Diffuse Large B-cell lymphoma Treated with R-CHOP.A Descriptive Study.** (Manuscript)
- IV. **A. Abu Sabaa**, C. Mörth, Mattias Berglund, Jamileh Hashemi, Rose-Marie Amini, E. Freyhult, M. Kamali-Moghaddam, A. Robelius, G. Enblad. **T-cell Leukaemia/Lymphoma Protein 1A (TCL1A) In Diffuse Large B-cell lymphoma (DLBCL).** (Manuscript)

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## Related Publications

- **Autoimmune disease in patients with diffuse large B-cell lymphoma: occurrence and impact on outcome.** Mörth C, Valachis A, Abu Sabaa A, Marshall K, Hedström G, Flogegård M, Baecklund E, Enblad G. *Acta Oncol.* 2019 Aug;58(8):1170-1177.
- **Does the omission of vincristine in patients with diffuse large B cell lymphoma affect treatment outcome?** Mörth C, Valachis A, Sabaa AA, Molin D, Flogegård M, Enblad G. *Ann Hematol.* 2018 Nov;97(11):2129-2135.
- **Plasma proteome profiling of cardiotoxicity in patients with diffuse large B-cell lymphoma.** Mörth C, Sabaa AA, Freyhult E, Christersson C, Hashemi J, Hashemi N, Kamali-Moghaddam M, Molin D, Höglund M, Eriksson A, Enblad G. *Cardiooncology.* 2021 Feb 3;7(1):6.

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# Abbreviations

<b>aaIPI:</b>	Age adjusted international prognostic index.
<b>ABC:</b>	Activated B-cell-like.
<b>ADAM-TS15:</b>	A disintegrin and metalloproteinase with thrombospondin motifs 15
<b>AML:</b>	Acute myeloid Leukemia.
<b>APL:</b>	Acute pro-myelocytic leukemia.
<b>ALL:</b>	Acute lymphoblastic leukemia
<b>AZU-1:</b>	Azurocidin.
<b>BLM:</b>	Bleomycin hydrolase
<b>BMI:</b>	Body mass index.
<b>CD163:</b>	Cluster of Differentiation 163
<b>CD207:</b>	C-type lectin domain family 4 member K
<b>cHL:</b>	Classical Hodgkin lymphoma
<b>COO:</b>	Cell of origin.
<b>CT scan:</b>	Computed tomography.
<b>CTSD:</b>	Cathepsin D.
<b>CVDIII:</b>	Cardiovascular III
<b>CXCL13:</b>	Cyclin-dependent kinase inhibitor 1
<b>DHL:</b>	Double-hit lymphoma.
<b>DLBCL:</b>	Diffuse large B cells Lymphoma.
<b>DPL:</b>	Double-protein expression lymphoma.
<b>EBV:</b>	Epstein Barr virus.
<b>EC:</b>	Ethics Committee.
<b>ECOG:</b>	Eastern cooperative oncology group.
<b>EFS:</b>	Event free survival.
<b>EFS12:</b>	Event free survival at 12 months.
<b>EFS24:</b>	Event free survival at 24 months.
<b>ELISA:</b>	Enzyme linked immune sorbent assay.
<b>FDG:</b>	Fluoro-2-deoxyglucose
<b>FISH:</b>	Fluorescent in situ hybridization.
<b>GCB:</b>	Germinal center B-cell-like .
<b>GEP:</b>	Gene expression profiling
<b>HGF:</b>	Hepatocyte growth factor.
<b>HIV:</b>	Human immunodeficiency virus.
<b>HL:</b>	Hodgkin lymphoma.
<b>IHC:</b>	Immune histochemistry.

<b>IL-6 :</b>	Interleukin-6.
<b>IL2RA:</b>	Interleukin-2 receptor alpha chain
<b>IPI:</b>	International prognostic index.
<b>LDH:</b>	Lactate dehydrogenase
<b>LOD:</b>	Limit of detection.
<b>MAD:</b>	Mothers against decapentaplegic homolog 5
<b>MAPK:</b>	Mitogen activated protein kinase
<b>MMP-9:</b>	Matrix metalloproteinase-9.
<b>MPO:</b>	Myeloperoxidase.
<b>NHL:</b>	Non-Hodgkin lymphoma.
<b>NPX:</b>	Normalized protein expression
<b>NT-pro BNP:</b>	N-terminal prohormone of brain natriuretic peptide.
<b>Olink:</b>	Olink Proteomics is a Swedish company that manufacture panels for precision proteomics.
<b>ONCII:</b>	Olink Oncology II
<b>OS:</b>	Overall survival.
<b>PAI:</b>	Plasminogen activator inhibitor 1.
<b>PCR:</b>	Polymerase chain reaction .
<b>PDGF subunit A:</b>	Platelet-derived growth factor subunit A.
<b>PEA:</b>	Proximity extension assay
<b>PET:</b>	Positron emission tomography.
<b>PGLYRP1:</b>	Peptidoglycan recognition protein 1.
<b>PLS-DA:</b>	Partial least squares-discriminant analysis.
<b>PTLD:</b>	Post-transplant lymphoproliferative disorder.
<b>R:</b>	Rituximab.
<b>RA:</b>	Rheumatoid arthritis.
<b>R-CHOP:</b>	Rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone.
<b>S100A11:</b>	Protein S100-A11.
<b>SLE:</b>	Systemic lupus erythematosus.
<b>SYND1:</b>	Syndecan-1.
<b>TCL1A:</b>	T-cell leukaemia/lymphoma protein 1A
<b>TGF-<math>\alpha</math>:</b>	Transforming growth factor alpha.
<b>THL:</b>	Triple-hit lymphoma.
<b>TME:</b>	Tumour microenvironment
<b>TNF-R1:</b>	Tumor necrosis factor receptor 1.
<b>TNF-RSF6B:</b>	Tumor necrosis factor receptor superfamily member 6B.
<b>TNFRSF10C:</b>	Tumor necrosis factor receptor superfamily member 10C.
<b>TR:</b>	Transferrin receptor protein 1.
<b>U-CAN:</b>	Uppsala-Umeå Comprehensive Cancer Consortium biobank.
<b>VIM:</b>	Vimentin.
<b>vWF:</b>	von Willebrand factor

# Introduction

## Epidemiology

Lymphoma, the malignant transformation of lymphocytes, was previously conventionally classified into non-Hodgkin lymphoma (NHL), comprising almost 90% of all lymphoma cases, and Hodgkin lymphoma (HL) <sup>1</sup>. However, newer classifications have broadened this stratification considering molecular, cytogenic and genetic elements <sup>2,3</sup>. Diffuse large B-cell lymphoma (DLBCL), the most common aggressive NHL <sup>4</sup>, is thereby now considered a collection of different diseases morphologically and genetically, with distinct clinical presentation and response to treatment<sup>2</sup>. It is the most common mature large B cell NHL in Europe and Sweden constituting 30-58% of all lymphoma series with almost 700 cases/year <sup>5,6</sup>. DLBCL is primarily a disease of the elderly, with the median age at presentation being around 70 years. A slightly increased incidence is observed in males (1.2:1)<sup>7</sup> along with a higher prevalence amongst individuals with Caucasian/European descents and high socioeconomic status<sup>8</sup>.

## Aetiology & risk factors

Immunostimulation, immunosuppression, and inflammation collectively grouped as immune dysfunction is thought to play a major role in the aetiology of DLBCL<sup>9</sup>. There are many autoimmune conditions known to be associated with increased risk of developing DLBCL such as systemic lupus erythematosus, Hashimotos thyroiditis and rheumatoid arthritis<sup>10</sup>. Patients receiving immunomodulatory treatment after organ transplantation have a higher risk of developing DLBCL, known as post-transplant lymphoproliferative disorder (PTLD) especially in the first-year after transplantation<sup>11</sup> where more than 80% are associated with EBV infection<sup>12</sup>. HIV infection is well known to be associated with increased risk of developing DLBCL<sup>13</sup>. There is limited evidence that therapeutic ionizing radiation for solid tumours is linked to the development of NHL<sup>14</sup>. Positive family history in first degree relatives increases the risk of developing DLBCL by almost ten folds<sup>15,16</sup>. Other risk factors include atopy, high body mass index and higher recreational sun exposure<sup>17,18</sup>.

## Clinical Presentation

Patients often seek medical advice because of painless rapidly enlarging lymph nodes. Other symptoms, well-known as B-symptoms, include recurrent fever without an obvious infection focus, drenching night sweats and unexplained weight loss (>10% of body weight within the last 6 months). Other unspecific presenting symptoms include fatigue, gastrointestinal manifestations such as bleeding and intestinal obstruction, skin rashes, neurological defects, and bone pains.

## Diagnosis

Surgical or incisional biopsy is the gold standard to yield tissue enough to establish the diagnosis. Core biopsy, whether or not radiologically guided, is a good alternative when the disease is less accessible. Fine needle aspiration cytology is a less preferred alternative<sup>19</sup>. Routine blood tests include full blood count and differential, hepatic, and renal profiles including lactate dehydrogenase (LDH), and hepatitis and HIV serology. It is recommended to assess baseline cardiovascular function with echocardiography in patients with history of cardiovascular disease and those older than 65 years with cardiovascular risk factors such as smoking, hypertension and hyperlipidemia. Fertility preserving treatment should be considered for the rare eligible young patient<sup>19</sup>. Baseline radiological assessment includes computed tomography(CT) scan with intravenous contrast which is important for accurate staging. Positron emission tomography (PET) scan is more sensitive and specific than CT scan as the 18F-fluoro-2-deoxyglucose-based (FDG) contrast uptake offers better visual assessment of the metabolically active involved nodal and extra nodal regions<sup>20</sup>. The metabolic tumor burden is considered to be an independent prognostic marker<sup>21</sup>. Bone marrow biopsy/aspiration was previously an essential part for the staging process. However, bone marrow involvement with high grade lymphoma is usually evident on PET. There is risk of missing indolent lymphoma in the bone marrow on PET, but this does not change the treatment plan and thus patients can be spared the procedure<sup>22,23</sup>.

## Pathology

DLBCL, as the name implies, is characterized by large lymphocytes with vesicular chromatin and round/ovoid nuclei diffusely infiltrating affected tissues. Flow cytometry and immune histochemistry (IHC) illustrate that DLBCL cells express the pan B-cell surface markers CD20, CD19, and CD79a. DLBCL not otherwise specified comprises the largest entity under the umbrella of the most recent WHO-HAEM5 classification which recognizes

17 more entities as large B cell lymphoma<sup>24</sup>. DLBCL is known to arise at the latter stages of germinal B-cell differentiation as a consequence of recurrent genetic alterations. The Cell of origin (COO) designation is currently used to classify DLBCL into three molecular groups, utilizing gene expression profiling (GEP) and DNA microarrays, namely the germinal center B-cell-like (GCB), activated B-cell-like (ABC), and a third unclassified group with heterogeneous molecular profiles. These groups have different oncogenic backgrounds and subsequently prognosis<sup>25</sup>. GCB DLBCL, accounting for 60% of all cases, have a gene expression profile quite similar to the normal germinal center in secondary lymphatic follicles expressing CD10 and BCL-6 and LMO2. Translocation t(14;18) is found in around 40% of all GCB cases and 20% exhibit histone methyltransferase EZH2 mutations. Both BCL-6 and EZH2 are thought to have important roles in the pathogenesis of lymphoma and have been considered as potential therapeutic targets<sup>26-28</sup>. ABC DLBCL, accounts for about 30% of all cases, and resembles activated post germinal center B cells which are blocked during plasmocytic differentiation. Cells have a gene signature of MUM1/LSIRF and are characterized by NFκB activation which inhibits apoptosis and thus promotes malignant cell survival<sup>29</sup>. MYD88 mutations are observed in >30% of ABC DLBCL<sup>30</sup>. Prognosis is worse for the latter two molecular groups in comparison to the GCB subtype<sup>31</sup>. The two prognostically distinct groups can be readily distinguished by IHC testing for CD-10, BCL-6, MUM1 (in addition to FOXP1, Cyclin D2, and BCL-2) which allows categorization of the tumor as having a GCB origin or a non-GCB<sup>32</sup>. GCB subtype has a better prognosis with a 5-year overall survival (OS) of 76% compared with only 34% for the non-GCB group. BCL-2 and Cyclin D2 are adverse predictors in the non-GCB group. The routine use of these three markers in IHC, namely CD-10, BCL-6, and MUM1 is now established for most hematopathologists and is known as Hans algorithm. IHC analysis however has the limitations of not being always concordant with GEP and the fact that it does not identify the third unclassified molecular group<sup>33,34</sup>. While the above-mentioned classifications have helped in gaining prognostic insight, studies designed to treat patients based on the COO designation have failed to establish enough evidence to introduce a shift in the current treatment guidelines<sup>35,36</sup>. Furthermore, fluorescence in situ hybridization (FISH) analysis distinguishes double rearrangement of MYC and BCL-2 genes termed double-hit lymphoma (DHL) which is a chromosomal breakpoint, affecting the MYC/8q24 locus in combination with another recurrent breakpoint, usually BCL-2 (t(14;18) (q32;q21)). In addition, BCL-6/MYC-positive DHLs and BCL-2/BCL-6/MYC-positive triple-hit lymphomas (THL) have been observed<sup>37</sup>. Moreover, IHC detected high expression of MYC and BCL-2 proteins, however without gene rearrangements in FISH, this variant has been dubbed double-protein expression lymphoma (DPL)<sup>38</sup>. Patients with DHL and THL are known to have poor prognosis as they tend to have refractory disease alternatively very short remission<sup>39</sup>. DPL may have a better outcome compared to DHL and THL, however this

subtype may imply worse prognosis compared with tumours without this expression<sup>38</sup>. Recently, advanced modern day next generation sequencing revealed up to 150 driver genes and this resulted in pinpointing between five and seven different functional subgroups of DLBCL. This is yet to be utilized in day-to-day clinical management of DLBCL patients<sup>40,41</sup>.

In addition to the advances in studying the malignant cells' genetic dysregulation much interest has been directed to the environment where these malignant cells arise and reside. Tumor microenvironment (TME), a combination of non-malignant cells, blood vessels and extracellular matrix, have been hypothesized to promote the growth of lymphoma cells through three processes. Firstly, the recruitment of supportive non-malignant cells. Secondly, the re-education of lymphocytes by altering lymphocytes phenotype and promoting homing to these supportive sites. Lastly the process of effacement where the ratio of malignant cells gradually exceed that of the normal cells in the infiltrated tissues<sup>42</sup>. GEP revealed three different gene profiles, stromal-1 signature which reflected extra cellular matrix deposition and had a good prognosis along with the germinal-center B-cell signature. Stromal-2 signature on the other hand illustrated angiogenesis and was coupled to poor outcome<sup>43</sup>. Interestingly, the signatures can be identified in both GCB and ABC DLBCL and are thus thought to be independent of the COO. When added to IPI, the gene expression-based model added to the predictive power of IPI. The IPI added, in turn, to the predictive power of the model implying that prognosis was affected by both clinical and biological factors.

## Staging and prognostic factors

Staging is conducted according to the Ann Arbor staging system which depends mainly on the anatomical location of the engaged lymph nodes/organs and the presence or absence of B-symptoms (Table:1). Furthermore, patients are evaluated based on the international prognostic index (IPI) and the age-adjusted IPI. Both are based on easily accessible clinical criteria at diagnosis: age, serum lactate dehydrogenase, number of involved sites, Ann Arbor stage, and Eastern Cooperative Oncology Group (ECOG) performance status. The sum of those risk factors divides patients into high, high-intermediate, low-intermediate, and low risk groups that correlate to the 5-year relapse free survival (RFS) and the 5-year OS (tables: 2-3). In the Rituximab (R) era this was updated to a revised IPI with three main risk groups<sup>44</sup>. The National Comprehensive Cancer Network IPI (NCCN-IPI) followed and was reported to be more accurate than the afore mentioned indices<sup>45-47</sup>, however IPI and aa-IPI remain the standard in clinical praxis in Sweden.

Central nervous system (CNS) relapse has a dismal prognosis occurring in around 5% of cases often between 6 and 9 months after the initial diagnosis. Patients at high risk can be identified with the help of CNS IPI which includes

the same risk factors of IPI with the addition of kidney and or adrenal gland involvement. This yields three risk groups; low, intermediate, and high where the latter has a 10.2% 2-year risk of CNS relapse<sup>48</sup>. ABC DLBCL, DHL, and primary testicular DLBCL imply higher risk of CNS relapse<sup>49,50</sup>.

**Table 1:** Ann Arbor staging system.

\*Fever (temperature >38.0°C), drenching night sweats, unexplained loss of >10% of body weight within the past 6 months.

Stage	Description
I	Involvement of a single lymphatic site (i.e., nodal region, Waldeyer's ring, thymus, or spleen) (I); or localized involvement of a single extra lymphatic organ or site in the absence of any lymph node involvement (IE).
II	Involvement of two or more lymph node regions on the same side of the diaphragm (II); or localized involvement of a single extra lymphatic organ or site in association with regional lymph node involvement with or without involvement of other lymph node regions on the same side of the diaphragm (IIE).
III	Involvement of lymph node regions on both sides of the diaphragm (III), which also may be accompanied by extralymphatic extension in association with adjacent lymph node involvement (IIIE) or by involvement of the spleen (IIIS) or both (IIIES).
IV	Diffuse or disseminated involvement of one or more extra lymphatic organs, or isolated extra lymphatic organ involvement in the absence of adjacent regional lymph node involvement. Stage IV includes any involvement of the liver or bone marrow, lungs (other than by direct extension from another site), or cerebrospinal fluid.
A	No B-symptoms*
B	B-symptoms
E	Involvement of a single extranodal site.
S	Splenic involvement.
X	Bulky disease (>10 cm)

**Table 2:** Prognostic factors according to IPI and aa IPI

<p><b><u>IPI Adverse prognostic factors:</u></b></p> <ul style="list-style-type: none"> <li>○ Age &gt;60 years.</li> <li>○ Serum LDH*&gt; normal</li> <li>○ Number of extranodal sites <math>\geq 2</math></li> <li>○ ECOG* Performance status <math>\geq 2</math></li> <li>○ Stage III or IV Disease.</li> </ul>	
<p><b><u>aaIPI Adverse prognostic factors :</u></b></p> <ul style="list-style-type: none"> <li>○ Serum LDH*&gt; normal</li> <li>○ ECOG* Performance status <math>\geq 2</math></li> <li>○ Stage III or IV Disease.</li> </ul>	<p><b>*IPI:</b> International Prognostic Index.  <b>aaIPI:</b> Age adjusted international prognostic index.  <b>LDH:</b> Lactate dehydrogenase.  <b>ECOG:</b> Eastern Cooperative oncology group.</p>

**Table 3:** Predicted Response and survival according to IPI and aaIPI<sup>44</sup>

	Number of risk factors	CR (%) *	RFS 5-years(%)*	OS 5-years (%)*
<b>IPI</b>				
Low	0-1	87	70	73
Low intermediate	2	67	50	51
High intermediate	3	55	49	43
High	4 -5	44	40	26
<b>aa IPI</b>				
Low	0	92	86	83
Low intermediate	1	78	66	69
High intermediate	2	57	53	46
High	3	46	58	32

\*CR: Complete response rate

RFS 5-years: Five-years relapse free survival.

OS 5-years: Five-years overall survival.



## Treatment and follow-up

The standard therapy for DLBCL has long been, and still remains, anthracycline-based chemotherapy; cyclophosphamide, doxorubicin, vincristine, and prednisone, best known as CHOP<sup>51</sup> alternatively CHOEP<sup>52</sup> (CHOP plus etoposide for patients younger than 65 years with high aaIPI score<sup>53</sup>) in addition to the monoclonal antibody rituximab (R)<sup>54,55</sup>. In elderly patients, the addition of R to chemotherapy improved the 10-year overall progression free survival (PFS) and OS by more than 16%<sup>56</sup>. The beneficial effect of R was also confirmed for younger patients with good prognostic factors<sup>57</sup>. In R-CEOP, etoposide replaces doxorubicin for patients who have a contraindication to anthracycline therapy such as cardiovascular disease and previous anthracycline therapy<sup>58</sup> without compromising survival<sup>59</sup>. In elderly patients R-mini-CHOP (50% dose reduction of cyclophosphamide, doxorubicin, and vincristine, standard dose R and prednisolone) provides an acceptable less toxic alternative.<sup>60</sup>

Treatment is given in 6 cycles with an interval of 14 – 21 days<sup>61,62</sup>. CNS prophylaxis with high dose intravenous methotrexate and cytarabine incorporated into the 1<sup>st</sup> line of treatment is recommended for patients with high risk of CNS relapse, but this remains unvalidated in prospective randomised studies<sup>63</sup>. Intrathecal CNS prophylaxis is not part of the current standard of care in Sweden<sup>64</sup>.

Radiological response to treatment is classically assessed after three cycles with CT. Circulating tumor DNA(ctDNA) is currently being investigated as an alternative method for response assessment<sup>65</sup>. The majority of patients tolerate treatment well. Partial or complete remission is confirmed before going through the next three cycles. After the completion of treatment, remission is assessed using CT or PET at least 6-8 weeks after treatment<sup>66</sup>. Patients with PET positive residual disease at locations amenable to radiotherapy should be considered for consolidative radiotherapy<sup>67</sup>. Standard follow-up after completed treatment continued previously up to 5 years but that changed when the 24 months milestone was identified as a sufficient end-point for follow-up<sup>68,69</sup>.

Unfortunately, more than one third of the patients succumb to refractory disease or relapse shortly after treatment completion<sup>70</sup>. Despite current advances, those patients cannot be identified with certainty at diagnosis. The prognosis tends to be dismal<sup>71</sup>. The current standard of care is high dose chemotherapy followed by autologous stem cell transplantation for patients with chemo-sensitive disease deemed fit<sup>72</sup>. Alternatives for the unfit patient include, R-bendamustine in combination with the CD79b antibody-drug conjugate polatuzumab vedotine<sup>73</sup>, lenalidomide and CD19 antibody tafasitamab<sup>74</sup> or palliative chemotherapy such as rituximab, gemcitabine, and oxaliplatin (R-GEMOX) could be considered<sup>75</sup>. Newer therapies in the form of

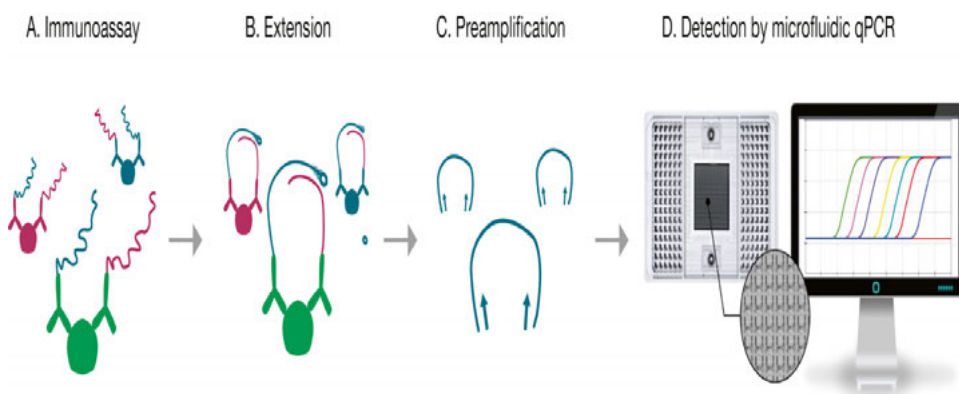
chimeric antigen receptor T-cell therapy (CAR T-cell), employs autologous T-cells genetically modified to target CD19 receptor, have shown promising results with complete response rate of 50-80%<sup>76</sup> that was durable in up to 37% of patients at 27 months<sup>76</sup>. Patient selection, long manufacturing time, potential toxic side effects, requirement of inpatient therapy with access to intensive care and lastly economic considerations<sup>77</sup> are all a few of the hindrances yet to overcome. CAR T-cell therapy is currently available in Sweden for patients who relapse after at least two lines of therapy and are not fit or have relapsed after autologous stem cell transplantation. Promising data supports the use of CAR T-cell therapy in the second line especially for patients with primary refractory disease and patients who relapse within the first year of first line treatment<sup>78,79</sup>. New in the arena are the bispecific antibodies targeting two different antigens, one on the tumor cells and the second on the effector cells, most commonly CD20 and CD3 respectively<sup>80</sup>. Ongoing phase 1 and 2 trials are yet to determine best administration route, scheme, with or without chemotherapy; however, results seem indeed promising<sup>81,82</sup>. There is much debate already ongoing on the appropriate sequence of CAR T-cell therapy and bispecific antibodies as treatment where the first may offer a curative potential and does not seem to interfere with response chances to the second but the reverse is not certain. There is however potential for the bispecific antibodies ending up as part of the first line of therapy.

## Proteomics

Because of the heterogeneity of DLBCL, the hunt for prognostic biomarkers that would eventually translate into innovative new targeted therapies has never been more intense<sup>83</sup>. Proteins constitute the functional units in the living cell, acting as the ultimate determinants of phenotypes. Considering the fact that proteins undergo posttranslational modifications, it is no wonder that the novel field of proteomics carries a significant advantage over genomics. Proteomics refer to the study of the entire protein expression in a tissue or organism<sup>84</sup>. This field has expanded rapidly, due to the current surge in the availability of DNA and protein sequence databases as well as the expansion of computer algorithms allowing database search. A study, for example, that employed data independent acquisition mass spectrometry and antibody array, identified more than 1000 plasma proteins in DLBCL patients (n=147) compared to controls (n=79). Four proteomic subtypes were thereby described. A group with high expression of inflammatory protein markers, in particular metalloproteinase inhibitor-1, was found to have worse PFS and OS. This was confirmed in two validation cohorts (total n=180). Addition of this protein to IPI complemented the prognostic stratification across all cohorts<sup>85</sup>. Another exploratory study investigated immunochemotherapy resistant DLBCL by analysing global protein expression in micro-dissected formalin-fixed paraffin-

embedded tumour tissues from 44 patients with primary refractory/early relapse DLBCL patients and 53 patients who had been in remission for at least 5 years. A total of 2127 proteins were identified. Around one hundred proteins were differentially expressed. Overexpression of ribosomal proteins was noted in the first group while overexpression of proteins related to actin cytoskeleton was observed in the latter group. That could hint to undescribed mechanisms affecting treatment response<sup>86</sup>. Advances in mass spectrometry techniques methods have been enormous, however the sensitivity of the technique can be affected by the sample type, preparation, ionization, in addition to the type of the technique itself and the conducted database search<sup>87</sup>.

Proximity extension assay (PEA) is a targeted immunoassay technique that allows the simultaneous detection of several protein biomarkers in minute amounts of bodily fluids without antibody cross-reactivity<sup>88</sup>. Each protein (antigen) is detected with the help of paired antibodies coupled to complementary oligonucleotides which act as probes. Upon antigen binding the oligonucleotides are brought in close proximity to hybridize to each other. Addition of a DNA polymerase leads to an extension and joining of the two oligonucleotides and formation of a PCR template. Quantitative real time polymerase chain reaction (PCR) measures the formed templates which are proportional to the initial concentration of the target proteins<sup>89</sup>. The technique is very specific and allows quantification below picogram per ml in a single microliter of various biological fluids such as blood, plasma, saliva, urine, or CSF etc<sup>90</sup>. Figure 1.



**Figure 1:** PEA technology. Courtesy of Olink Proteomics AB

Pre-designed panels are currently available for the detection of pre-selected proteins based on their physiological and pathological significance and the respective cellular and biological process. Studies employing this method

coupled with well-designed panels of putative protein markers have indicated its clinical utility in several different disorders including cardiovascular disease, renal failure, in addition to malignant diseases<sup>91,92</sup>. Notably, concurrent tissue analyses may reveal tissue protein biomarker expression. In a Nordic study ELISA was used to analyze a plasma protein sCD163 in two cohorts, a clinical trial cohort (n=119) and a population based one (n=125). In addition to CD163, mRNA levels were measured with Nano String and proportions of CD163+ cells in tumor material were measured using multiplex IHC. The study showed that pre-treatment sCD163 levels were elevated compared to those in healthy controls, and high levels were associated with unfavorable outcomes<sup>93</sup>.

In another study, protein profiling of plasma and tumor tissue protein using PEA in classical HL (cHL) identified a higher number of plasma proteins (30) than tissue proteins (17) in plasma samples and diagnostic lymph node biopsy lysates, respectively, that distinguished cHL patients from age and sex matched controls<sup>94</sup>. Of the 17 tissue proteins, eight proteins had significantly higher plasma levels in cHL patients compared to controls. Six tissue proteins that distinguished cHL were found to significantly correlate with tissue PD-L1 expression which is in line with TME changes in cHL<sup>95</sup>. Another study utilized PEA to examine plasma proteins as well as flow cytometry to analyze single cell suspensions from lymph nodes and peripheral blood samples in cHL patients and matched controls. Immune profile differences related to high inflammation and high tumor burden were observed in comparison to controls. Interestingly, these differences reverted completely in patients achieving complete remission after first line therapy<sup>96</sup>.

# Aims

## Paper I

- To confirm whether event free survival at 24 months (EFS24) is a robust milestone in an unselected population of DLBCL patients in comparison to age- and sex-matched healthy individuals from the general population in Sweden.
- To evaluate factors that influence the achievement of EFS24 and causes of mortality in patients failing to achieve this milestone.
- To evaluate event free survival at 12 months (EFS12) as a possible outcome predictor for patients with low stage (I & II) DLBCL.

## Papers II-IV

- To explore the diagnostic and prognostic role of plasma protein profiling in hematological malignancies, with special interest in DLBCL.
- To explore the proteome profile of DLBCL before, under and after treatment with R-CHOP, with the aim of identifying plasma proteins of prognostic value.
- To further investigate/validate interesting protein biomarkers in the tissues (DLBL biopsy samples).

# Methodology

## Ethical consideration

### Paper I

The study was approved by the Ethical Review Board in Uppsala, Sweden 140-10 (2019-05094).

### Papers II, III and IV

The U-CAN project, including this study, was approved by the Regional Ethics Committee (EC) of Uppsala-Orebro (Ups 2012/198, 210/198/1, 2014/233, 2019-05094). Data collection in the EpiHealth study and usage of the material in this project was approved by the EC of Uppsala (Dnr 2010/402: 2010-12-01, 2011-11-17, 2015/179). The EpiHealth study is approved by the Swedish Data Protection Authority.

## Patients and methods

### Paper I

A retrospective multi-institutional cohort study of 1169 patients ( $\geq 18$  years old) with DLBCL or high-grade malignant B-cells lymphoma treated with R-CHOP or R-CHOP-like regimens with a curative intention were included from the Swedish Lymphoma Registry (SLR). Inclusion was from five Swedish counties; Uppsala, West Gotaland, Gavleborg, Sodermanland and Dalarna. Patients were followed according to the now outdated Swedish lymphoma group guidelines. Recorded baseline data included basic clinical characteristics in addition to the type of treatment (R-CHOP or R-CHOEP), treatment outcome (complete response (CR), partial response (PR), stable disease (SD) or progressive disease (PD)) defined according to International Response Criteria. When applicable, dates of relapse/death, cause of death and the date of last follow-up were outlined. For early stages the grouping was based on the achievement of the EFS12 milestone. Patients who died before reaching the afore mentioned milestones (EFS24 and EFS12 for early-stage lymphoma)

were excluded from the analysis. OS was defined as time from date of diagnosis until date of death or last follow up.

### **Reference age and sex matched population**

Swedish life tables from Statistics Sweden ([www.scb.se](http://www.scb.se)) state the historical individual risk of death depending on sex and age per calendar year. A standard population was generated that matched the study population in terms of age and sex. Subsequently, the survival of the standard population was compared to the survival of the study population.

## **Paper II**

Plasma samples from patients aged  $\geq 18$  years diagnosed with acute leukemia or lymphoma between the years 2010 and 2015, included in the Uppsala-Umeå Comprehensive Cancer Consortium (U-CAN) biobank at the time of diagnosis, as well as from matched healthy controls from the EpiHealth biobank were used to assess plasma protein patterns in patients with the hematological malignancies. A total of 251 patients consisting of 107 patients with acute leukemias (AML: 69, ALL: 29 (including B and T leukemias/lymphomas and Burkitt leukemia), APL: 9) and 144 patients with lymphomas (DLBCL: 95, HL: 49) were included. For comparison, plasma samples from 60 healthy controls (30 male, 30 non-pregnant females) were obtained.

## **Paper III**

EDTA plasma samples (1µl sample/panel) from 95 patients aged  $\geq 18$  years diagnosed with DLBCL between 2010 and 2015 included in the Uppsala-Umeå Comprehensive Cancer Consortium biobank (U-CAN) were analysed and compared to samples from 60 age and gender matched controls obtained from the EpiHealth biobank<sup>97</sup>. Of the 95 patients, 93 patients received R-CHOP, or R-CHOP like regimen as the first line of treatment in a curative intention. Response to treatment was assessed after three cycles of R-CHOP with computed tomography (CT) and 6-8 weeks after treatment completion with CT in all cases and in some cases with PET. Clinical data were obtained from the patients records. Stage was defined according to the Ann Arbor classification and bulky disease as tumour size above 7,5cm. All patients were confirmed histologically to have DLBCL and the subtype, either germinal centre B-cell like (GCB) or non-GCB, was determined using the Hans immuno-histochemistry classification algorithm, based on CD10, BCL6, and MUM1 expression<sup>33</sup>.

Out of the initial 95 samples, 2 samples were removed from the pre-treatment analysis due to mis-labeling. A final number of 93 samples taken before start of chemotherapy were included, 67 taken directly at diagnosis and 26

after corticosteroid treatment. Prednisone 1 mg/kg 3-5 days was given in some patients as a pre-phase therapy to reduce the risk of tumour lysis and improve performance status<sup>98</sup>. Analysis included samples collected midway through chemotherapy (after three R-CHOP cycles) in 29 cases. Samples collected after treatment (n=55) were grouped according to the clinical response (complete remission n=46, progressive disease n=4, and relapse n=5). Age analysis was divided to above and below 60 years based on a previous study that showed that only patients younger than 60 years who achieved event free survival at 24 months had the same overall survival as the general population<sup>99</sup>. Intentional prognostic index (IPI)<sup>45</sup> scores were grouped in to two groups 0-2 and 3-5.

## **Paper IV**

Clinical data and tumour biopsies were available for a total of 178 adult patients, including 107 (60.1%) males, diagnosed with DLBCL between the years 1987-2016. Of the 178 patients 125 were treated with R-CHOP or R-CHOP like regimes in a curative intent, while 53 patients were treated with merely chemotherapy (mostly before the rituximab era) or received palliative symptomatic therapy in the form of radiation or steroids. Only patients treated with R-CHOP or R-CHOP like regimes were included in the survival analyses. Of all 178 patients, 27(15.2%) patients were included in the UCAN biobank and had plasma samples collected at diagnosis. TCL1A was measured utilizing multiplex proximity extension assay (PEA) on samples from a previous proteome study which employed preselected protein panels; OLINK Oncology II and Cardiovascular III panels. ELISA "MyBiosource: MBS2887157" was performed on 19 (10.6%) plasma samples as a validation cohort. To detect tissue bound TCL1A, TMA were stained by immunohistochemistry (IHC) using the antibody TCL1 invitrogen thermofischer 39-4800 clone 1-21 dilution 1:200 which allowed detection of the protein in the cytoplasm and the nucleus. Two reviewers scored the stained TMA, independently, from 0-100%. A third independent reviewer examined the material with almost identical scoring results. The score of the most experienced reviewer was thus used in the analysis.



# Statistics

## Paper I

Categorical variables were expressed as number (%) and continuous variables as median (range). Chi square test was used for bivariate comparisons for categorical variables. Survival curves with 95% confidence interval (CI) were computed using the Kaplan-Meier method. In accordance to statistical methodology no CI was computed for the standard population, since the standard population was based on actual national historical death rates. Overlapping CI-intervals were used to examine survival disparities between different cohorts of the study population and the survival of the derived standard population. Survival at different EFS milestones (12 and 24 months) was calculated (including only patients achieving the specified milestone in the analysis). Standardized mortality ratio (SMR) was calculated for 1 and 5 years after achieving the landmark timepoint. Cox proportional hazard was used for multivariate analysis. A two-sided p-value of  $\leq 0.05$  was regarded as cut-off for statistically significant results in comparisons between groups. Statistical analyses were performed using the R statistical program version 3.4.3 ([www.r-project.org](http://www.r-project.org)).

## Paper II

The difference in protein level between two groups was studied using linear regression, adjusting for age and sex, and evaluated using a likelihood ratio test. Benjamini-Hochberg's false discovery rate method for multiple testing correction was applied and a difference was considered significant if the q-value (the adjusted p-value) was below 0.05. Multivariate partial least squares-discriminant analysis (PLS-DA) classification models were computed using the R function `opls` in the R package `ropls`<sup>100</sup>. For each predefined functional protein subset, a PLS-DA model was constructed to separate between two groups. The overall predictive ability of the PLS-DA models was summarized by the average ER ( $\overline{ER}$ ) (averaged over the 50 test sets). A variable subset selection (VSS) procedure was applied to identify a smaller subset of proteins able to discriminate between groups.

## Paper III

Protein expression was visualized using principal component analyses (PCA). Association between protein levels and clinical parameters of patient groups (patient vs control or between patients with different treatments (early prednisone vs not treated)) were assessed using linear regression, adjusting for age and sex unless otherwise required. Differential expression within a patient

group but between time points were compared using mixed effects linear regression with patient id as random effect. Differential expression was illustrated in volcano plots. Gene set enrichment analysis was performed using the function `fgsea` in the R-package `fgsea` based on the protein groups defined by Olink. Association between protein level and time to event (overall survival, lymphoma specific survival and relapse free survival) was assessed using Cox regression, adjusting for age and sex. Multiple testing correction was performed using Benjamini-Hochberg's FDR (false discovery rate) method adjusting for the number of proteins analysed. The significance level was set to 5% FDR.

## **Paper IV**

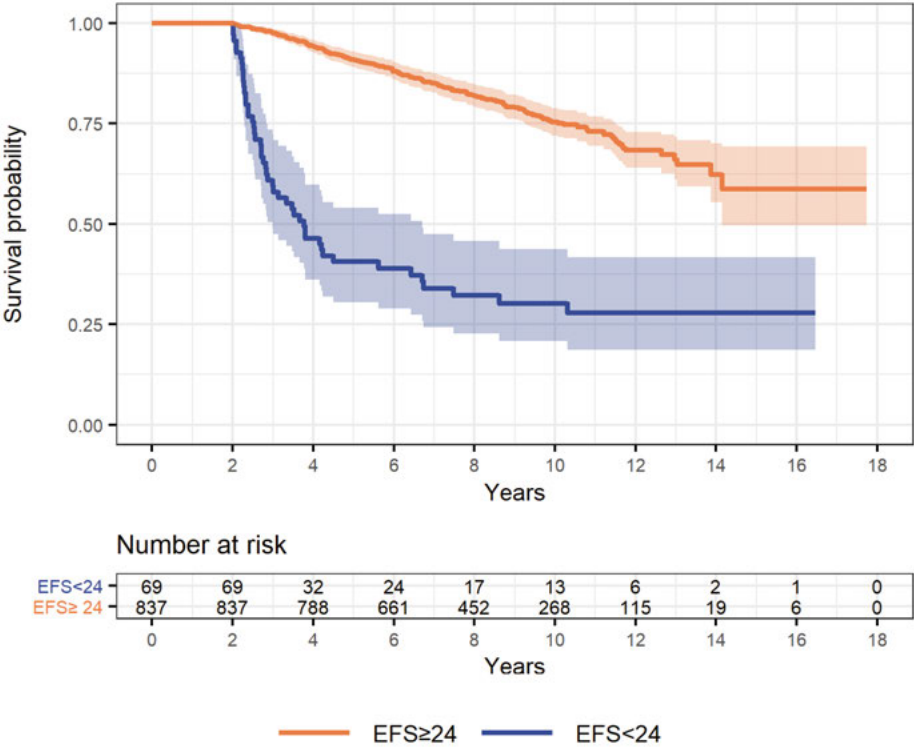
Correlation between variables was calculated using Spearman's rank-based correlation. TCL1A IHC levels were divided into quartiles. Association between TCL1A levels (IHC, PEA and ELISA) and clinical parameters (presence of B symptom, age  $\geq 60$  years or  $< 60$  years, bone marrow involvement, bowel involvement, ECOG performance status (0-1 vs  $\geq 2$ ), IPI, sex, stage (2 groups I-II vs III-IV) was assessed using Mann-Whitney's U test.

Survival time, expressed as Overall survival (OS), lymphoma specific survival (LSS) and progression free survival (PFS) and association with TCL1A was assessed using Kaplan-Meier and log rank test for HC expression and using Cox regression for PEA and ELISA measurements.

# Results

## Paper I

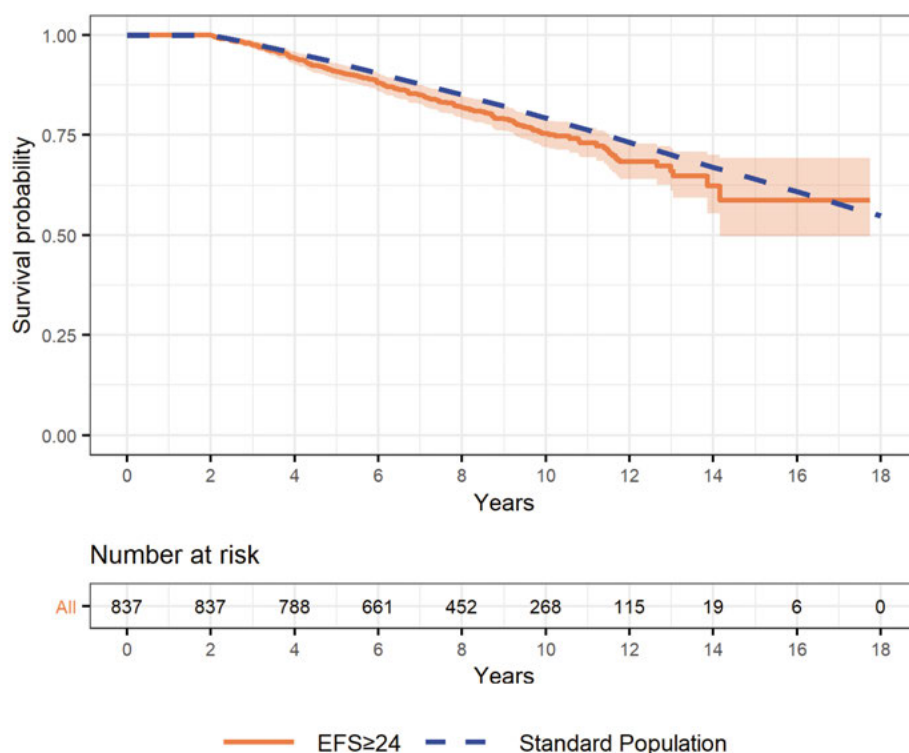
More than two thirds of the patients (n=837, 71.6%) achieved EFS24 with better OS compared with those who did not. (Figure:2).



**Figure 2:** OS for DLBCL patients who achieved EFS24 vs those who did not.

Subjects with event prior to 2 years are excluded from the analysis (only applicable for EFS<24). Patients not achieving EFS24 (n=332, 28.4%) were older (67.6 vs 63.4 years,  $p=0.003$ ), tended to have higher IPI score (3-5) (62% vs 34.2%,  $p<0.001$ ) and were more likely to have B-symptoms (56.6% vs 38.6%,  $p<0.001$ ), bulky disease (31.9 % vs 20.7%,  $p<0.001$ ), and extra-nodal involvement (55.7% vs 42.9%,  $p<0.001$ ). There were no significant

differences in sex, treatment regimen (R-CHOP vs R-CHOEP) or the addition of radiotherapy (RT) following R-CHOP treatment between patients achieving EFS24 and those who did not. OS for all DLBCL patients was only marginally worse when compared with an age- and sex-matched standard population once EFS 24 was reached . SMR at 5 years after EFS24 was 1.23 (95%-CI: 1.02-1.44).(Figure 3)



**Figure 3:** OS for DLBCL patients achieving EFS24 compared with an age- and sex-matched general population.

When dividing the study cohort according to age ( $<$  or  $\geq$  60 years), in patients younger than 60 years of age (n=266) OS was comparable to the standard population with only 9 events occurring up to 5 years after achievement of the EFS24 milestone SMR at 5 years was 2.00 (95%-CI: 0.70 - 3.27).

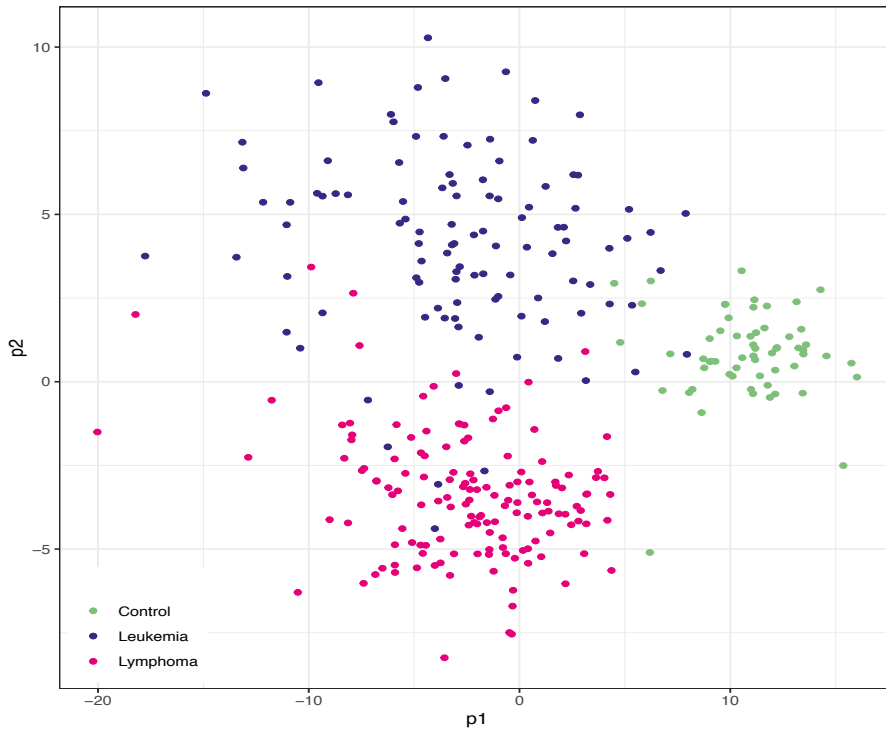
In patients older than 60 years (n=571) however, there were 110 events at 5 years post EFS24 and OS was worse when compared to the standard population though statistically not significant SMR 1.19 (95%-CI: 0.99 - 1.39).

Multivariate Cox regression analyses for patients achieving EFS24, considering risk factors identified by the IPI score revealed that age over 60 years is the only factor significantly affecting survival when compared to other risk factors after EFS24. Patients with early-stage lymphoma (stage I-II) after reaching EFS12 had a worse OS when compared to the matched standard population, SMR at 5 years post EFS12 was 1.35 (95%-CI: 1.07 - 1.62). OS was however better compared with patients not reaching EFS12.

Of all 1169 patients, 501 (42.9%) patients died. In the EFS24 group, a total of 190 patients died, with 38 (20%) of deaths attributed to lymphoma. Causes of death for the remaining 152 patients were as follows: cardiovascular disease 34 (22.4%), cancer 24 (16%), dementia 5 (3.3%), others 6 (4%) and unclear 83 (54.6%).

## Paper II

Highly significant differences were observed using PLS-DA model in plasma protein levels between leukemia and lymphoma patients and healthy controls. Figure 4 is the PCA diagram where every dot is a single control/patient. Principal components (the axis, p1 and p2) are constructed to reduce the number of dimensions without discarding or selecting data.



**Figure 4:** PCA shows highly significant differences in plasma protein levels between leukemia and lymphoma patients and healthy controls.

The PLS- DA model distinguished between leukaemia and lymphoma with very few patients misclassified. In total, 15 samples were misclassified at least once out of the total 10 test rounds. The *ER* for this PLS-DA model was 0.03 and average AUC 0.995. All 4 ALL samples classified as lymphomas at least 5 times out of the 10 test runs came from patients with lymphoblastic lymphomas (LBL) with bone marrow involvement, clinically classified and treated as ALL.

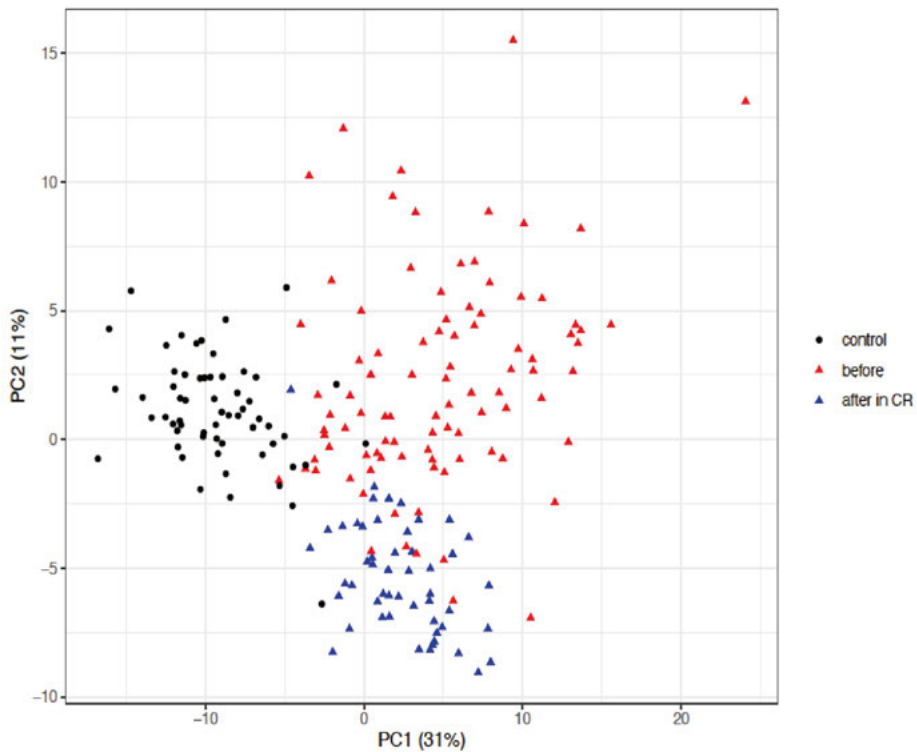
Moreover, significant protein differences were found when DLBCL were compared to HL and controls. To add to the specificity of functional subsets/groups of proteins and for better distinction of patient groups, the *ER* for each of the biological processes as well as all and no proteins, respectively, was computed. Top hits are presented in table 4. All subsets of proteins improved the ability to separate groups, compared to using age and gender only.

**Table 4 :** Protein subsets important for disease separation. Average error rate (ER) for the most informative protein subsets for separating patients with leukaemia from controls, lymphoma from controls and leukaemia from lymphomas. A low (ER) indicate a high ability to separate the different subgroups.

Biological Process	Average Error Rate
<b>Leukaemia vs Controls</b>	
Cell adhesion	0.0060
Cell proliferation	0.0084
Other gene ontology terms	0.0096
Cell differentiation	0.0138
Cell motility	0.0192
<b>Lymphoma vs Controls</b>	
Apoptotic process	0.0000
Cell differentiation	0.0000
Cell proliferation	0.0005
Cell adhesion	0.0015
Response to hypoxia	0.0015
<b>Leukaemia vs Lymphoma</b>	
Cell adhesion	0.0447
Cell proliferation	0.0805
Chemotaxis	0.0770
Proteolysis	0.0820
Blod vesel morphogenesis	0.0869

### Paper III

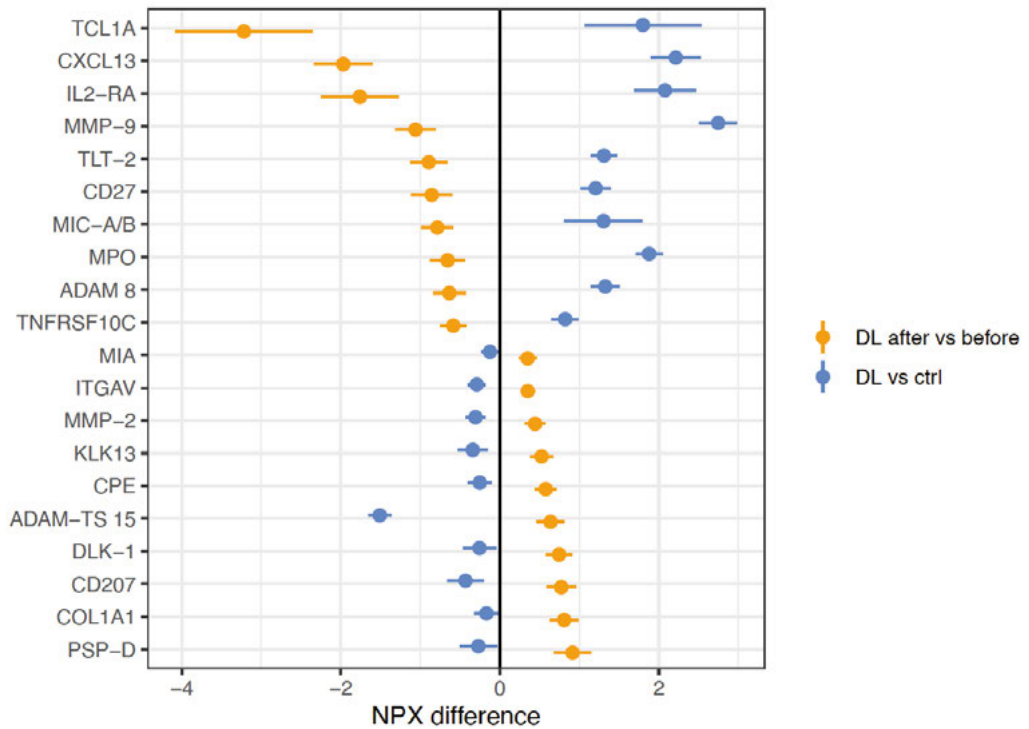
Visualization using principal component analysis (PCA) revealed significant distinctions in protein patterns at diagnosis compared to controls and striking differences in protein levels before and after treatment in patient who responded to treatment. Figure 5.



**Figure 5:** PCA shows clear differences in plasma protein levels between controls and DLBCL patients before and after treatment.

In total 64 proteins had significantly higher levels before treatment in comparison with controls that dropped significantly in patients achieving CR. The three top proteins were TCL1A, CXCL13 and IL2RA (Figure 6 shows the top 20 proteins). Moreover, particular proteins were significantly associated with established clinical risk factors (supplementary table 2 a-h). Interestingly, continual profound differences between patients in CR and controls were observed. Figure 5.





**Figure 6:** Forest plot over proteins that differ significantly between DLBCL at diagnosis (before) and controls (ctrl) and between DLBCL after vs at diagnosis, in opposite directions.

A total of twelve proteins at diagnosis were independently associated with progression free survival and lymphoma specific survival respectively. When adjusting for multiple testing, however, none was significant.

## **Paper IV**

### ***IHC***

TCL1A was expressed in the tumor cells in 117 (65.7%) patients, the expression was cytoplasmic and nuclear. When relating tissue TCL1A to clinical parameters, there was no statistically significant association.

### ***PEA***

TCL1A levels were measured in 27 (15.2%) patients with corresponding IHC available. In 11 samples no tissue expression could be observed. A significant correlation of PEA plasma levels of TCL1A was found with male sex  $P=0.026$  and high Ann Arbor stage (III-IV vs I-II)  $P=0.44$ .

### ***ELISA***

In 19 (10.7%) cases we could measure plasma levels of TCL1A with ELISA, median 0.1245 (0.097-0.163) pg/ml. The only significant association between ELISA plasma levels of TCL1A and clinical presentation was with male sex ( $P=0.016$ ).

### ***Survival analysis and correlation between the three techniques***

No statistically significant associations to time to PFS, LSS or OS were found for any of the three measurement techniques, see Figures (1-3).

There was significant correlation between PEA and ELISA  $P=0.044$  and between PEA and IHC  $P=0.00035$ . No significant correlation was found between ELISA and IHC  $P=0.12$ .

# Discussion

## Paper I

Maurer et al showed in two study cohorts that newly diagnosed DLBCL patients treated with standard immunochemotherapy achieving EFS24, and patients with stage I and II disease who achieved EFS12, had an overall survival similar to the age- and sex-matched standard population<sup>68</sup>. In an unselected patient cohort this could only be confirmed for patients younger than 60 years of age who achieved EFS24. Furthermore, patients with early stages (I and II) who achieved EFS12 still had a worse prognosis in comparison to a matched healthy population.

These findings with regards to EFS24 resemble the findings of a Danish population-based study where, in a total of 1621 patients, EFS24 was calculated for those with CR or CRu after initial treatment and with a follow-up of about 8 years. They found that only patients younger than 50 years of age had a normalised OS comparable to an age- and sex-matched Danish population, regardless of other risk factors such as IPI score<sup>101</sup>.

Established risk factors such as older age, poor performance status at diagnosis, presence of bulky disease, extranodal involvement, high LDH and IPI index, all increased the risk of never reaching EFS24. In part, this has previously been reported in a large study with over 7000 patients, which determined the loss of life expectancy and found that mainly IPI score >2 significantly had an impact on the outcome<sup>102</sup>.

Of the patients achieving EFS24 about one fifth died from cardiovascular disease. Cardiovascular toxicity secondary to treatment with doxorubicin-based chemotherapy is well described both early after treatment and as a long-term sequel after NHL<sup>103,104</sup>. The incidence is largely dependent on the cumulative dose. Long term follow-up of these patients is thus warranted both for clinical assessment and lifestyle counselling with regards to other risk factors such as smoking, obesity, hypertension, and hyperlipidemia. Early intervention should be considered as it has been shown to be crucial in reducing cardiovascular mortality and morbidity<sup>105</sup>. Patients treated for NHL are known to have an increased risk for secondary malignancies such as leukemia, lung cancer, renal cancer, and bladder cancer. Although some studies have shown no

difference in the incidence between patients who received radiation therapy and those who did not<sup>106</sup>, radiation therapy especially in young females is associated with higher risk for breast cancer<sup>107</sup>. In our study 16% of the patients who died without lymphoma, died due to another cancer.

## Paper II

Top protein hits when comparing leukemia samples to control samples showed increased levels of von Willebrand factor (vWF) and FURIN but decreased levels of ADAM-TS15. Other proteins that distinguished leukemia samples from normal control samples included well known mediators of inflammatory response, such as IL-6, Tumor Necrosis Factor superfamily members TNF-RSF6B (also known as Decoy-receptor) and the main receptor of TNF- $\alpha$ , TNF-R1. Another of the ten most prominent proteins when comparing leukemia and control samples was myeloperoxidase (MPO), a central lineage marker for AML.

The disentanglement of malignant hematopoietic cells from the bone marrow is a crucial step in the pathogenesis of leukemia, and the high expression of proteins connected to cell to matrix interactions and cell stability in leukemia samples, such as membrane protein SYND1, VIM and cell adhesion regulator ICAM2 may be attributed to this mechanism. When comparing the different leukemia subgroups, only minor changes in protein expression were detected between samples from patients with AML and APL, possibly influenced by the few APL patients in this study. Comparison of ALL and AML on the other hand, revealed a lymphoid trio: TCL1A, CD27 and CD48, all with higher expression in ALL and well suited to distinguish between the two major leukemia subgroups.

When lymphoma samples were compared to controls, a pattern of consistent upregulation of proteins aiding lymphoma dissemination and TME modifications was seen. At the top of the list, we found PAI, MMP-9, VIM and HGF.

The comparison between lymphoma samples and normal controls also revealed proteins connected to cell motility and differentiation such as S100A11, TGF- $\alpha$  (Transforming growth factor alpha), and PDGF-A (Platelet-derived growth factor subunit A) and MPO.

When DLBCL and HL samples were compared, a new set of proteins emerged, with an overall higher protein expression in HL. Lymphoma cells, especially in HL, rely on various cytokines for survival, growth, and immune escape which can explain why most protein levels were higher in this group. The top marker was DKN1A. Other proteins with higher levels in HL were RET, LYN and TXLNA (IL14).

In summary, samples from patients with acute leukemia had higher levels of proteins associated with hemostasis, inflammation, cell-differentiation, and cell to matrix integration whereas the protein pattern in lymphoma patients tended to reflect altered cell motility and differentiation, matrix invasion and angiogenesis, all aiding lymphoma dissemination.

### Paper III

The aim of the study was to explore the changes in plasma protein levels under treatment with R-CHOP. Distinct differences between patients and controls as well as between samples taken at different time points were observed. As this study drew attention to numerous plasma proteins, only a few were included in the discussion to highlight the implications of the findings and to elaborate on previously known data.

The observed associations between protein expression and clinical factors offered interesting insights. For example, **CD163** is a protein scavenger receptor cysteine-rich type 1 M130. In its membrane bound form it acts as an acute phase induced receptor that facilitates endocytosis of haem complexes by macrophages. The receptor/protein is expressed/secreted by tumour infiltrating macrophages (TAM) which can be classified using double immunohistochemical staining to HLA-DR/CD68 (M1) or CD163/CD68 (M2). Multivariate analysis revealed that the presence of a bulky mass and a higher number of M2 TAMs were significant factors for poor prognosis in DLBCL using IHC<sup>108</sup>. This was confirmed in another study, where an increased number of CD163 (+) TAM and a higher ratio of CD163/CD68 (+) TAM were significantly associated with shorter OS and progression-free survival (PFS) in DLBCL patients using IHC<sup>109</sup>. Moreover, in its soluble form (sCD163) is known parameter for monitoring macrophage activation in inflammatory conditions<sup>110</sup>. In this study sCD163 was the top protein linked to advanced stage in the pre-treatment group. Interestingly a Nordic study ELISA was used to analyse sCD163 in two cohorts, a clinical trial cohort and a population based one. In addition to *CD163*, mRNA levels were measured with Nano String and proportions of CD163<sup>+</sup> cells in tumour material were measured using multiplex IHC. The study showed that pre-treatment sCD163 levels were elevated compared to those in healthy controls, and high levels were associated with unfavourable outcomes<sup>93</sup>.

The top 4 proteins that significantly differed when comparing samples before and after treatment were TCL1A, CXCL13, CXCL17 and IL2RA.

**TCL1A** a well-known proto-oncogene, first identified in T-cell prolymphocytic leukaemia and is implicated T- and B-lymphocyte transformation<sup>111</sup>, encodes the protein T-cell leukaemia/lymphoma protein 1A<sup>112</sup>. In 2005, a study suggested that TCL1A immunodetection is an independent marker of adverse

outcome in DLBCL utilizing gene expression profiling using DNA-microarrays on tumour material<sup>113</sup>. This was further confirmed by immunohistochemistry in a more recent study where high expression was associated with poor prognosis<sup>114</sup>. In the current study the protein was on top of the list of proteins that were significantly lower in remission samples in comparison to pre-treatment samples. It was significantly associated with high IPI. Furthermore, TCL1A was significantly associated with lymphoma specific survival but this was statistically insignificant in multivariate analysis. We plan further studies of this marker both in the soluble and bound forms to further explore its role.

**CXCL1**, a C-X-C motif chemokine 13, which acts as a chemotactic agent for B lymphocytes. In this study it was significantly higher in DLBCL samples compared with controls and the levels were dramatically lower after chemotherapy when patient achieved remission. Higher levels were significantly associated with advanced stage and lymph node engagement. Noteworthy, this confirmed the finding of recent review of the transcriptional levels of different CXCLs in DLBCL using the oncomine database where CXCL13 transcriptional level was higher in DLBCL patients compared to controls<sup>115</sup>. However, CXCL13 was not significantly linked to higher stage at diagnosis in that analysis. Another study using quantitative reverse transcription–polymerase chain reaction (RT-PCR) for CXCL13 and IL-10 in cerebrospinal fluid (CSF) confirmed the diagnostic value in primary and secondary CNS lymphoma<sup>116</sup>.

**CXCL17** is protein C-X-C motif chemokine 17. It is the most recently discovered chemotactic agent for monocytes and of the CXCL family. In addition to its pro-angiogenic activity, its role in the pathogenesis of breast, colon, pancreas, hepatocellular, endometrial and gastric cancers is well described. A review study looking at CXCL transcription and DLBCL survival concluded that CXCL17 appeared of value as a prognostic marker as higher CXCL17 mRNA was associated with better OS<sup>115</sup>. In this study CXCL17 was the top protein significantly associated with age, with significantly higher expression in patients younger than 60 years.

**IL2-RA** or Interleukin-2 receptor subunit alpha has been shown to be an independent prognostic factor for OS and EFS in DLBCL in a Japanese study utilizing sandwich ELISA<sup>117</sup>. In this study this marker was linked to advanced stage and B symptoms and was one of the top proteins that dropped after treatment. This aligned with a study that showed significant association between serum levels of soluble interleukin-2, measured with chemiluminescent enzyme immunoassay (CLEI) in the study cohort and using ELISA in a separate validation cohort, and PET assessment in the form of total metabolic tumour volume (TMTV). The study concluded that IL2RA can be a surrogate marker for estimating tumour burden<sup>118</sup>. Interestingly, the prognostic value of this protein was illustrated in elderly patients (age  $\geq 60$  years) where high serum

levels measured at diagnosis by chemi- luminescence enzyme immunoassay. High levels were significantly associated to poor PFS and OS<sup>119</sup>. In the current study no significant association to age was observed.

An intriguing finding in this study is that the plasma protein levels do not actually normalize after the completion of treatment with complete remission. This calls for continued study of plasma protein evolution after completed first line treatment to establish if the protein levels do normalize or whether the genes and corresponding plasma protein pattern are forever changed.

One of the strengths of this study is the fact that it was based on comprehensive clinical and biological data saved in two high standard biobanks, U-CAN and EpiHealth in Sweden. The PEA technology is very promising in the field of proteomics in terms of sensitivity and specificity in protein identification and quantification in minute samples.

The limitations of this study, however, include the fact that the PEA findings were not validated with any other gold standard method such as ELISA. The original study design did not plan validation and the remaining plasma samples were not enough to continue with a validating test in all samples. Another limitation is the small number of patients even if multiple samples were collected from each patient at different points of time.

## **Paper IV**

TCL1 family proteins have a well-studied role in the normal development of early B- and T-cells. Functioning as oncoproteins, they augment AKT signal activation in a concentration dependent manner and thereby regulate cell proliferation and survival<sup>112</sup>. TCL1A expression is crucial to the normal development of B-cells, but persistent high concentrations confirmed by western blot analysis both in cytoplasmic and nuclear compartments have been incriminated in lymphomas arising from the germinal center and post germinal center cells such as follicular lymphomas, Burkitt's lymphomas, DLBCL and chronic lymphocytic leukemia<sup>112,120</sup>. In addition, the soluble form of the protein detected by PEA was found in higher concentrations in plasma samples taken from DLBCL patients in comparison to controls with levels dropping in patients achieving remission<sup>121</sup>. TCL1 tissue expression in DLBCL was found to correlate with a number of factors including IPI, Ann Arbor stage and primary site. In survival analysis tissue expression was found to independently predict short PFS and OS<sup>114</sup>.

In this study, the role of TCL1A in DLBCL was investigated utilizing IHC on TMA to elucidate the presence of the cytoplasm and nucleus bound form of the protein in tumor tissue. In addition, protein plasma concentrations were measured by PEA and further validated by ELISA. Associations to known prognostic clinical variables were studied for the three different detection

techniques. Significantly higher levels of plasma TCL1A were seen in male patients (measured by ELISA and PEA) and in patients with Ann Arbor stages II-IV (measured by PEA). Survival analysis was however not significant.

The study had a few limitations. As the study cohort included patients treated in the pre-Rituximab era, they were excluded from the analysis. Some of the clinical data was missing due to the absence of electronic patient records. Another limitation is the relatively low number of patients with PEA and ELISA plasma protein measurement in relation to the total number of patients included in the study. Due to depletion of the frozen plasma samples this could not be overcome.



# Conclusions

## Paper I

EFS24 appears to be an attractive end-point for follow-up as most lymphoma-related events occur before this milestone. The Swedish Lymphoma group considers two years of follow-up as satisfactory for relapse-free DLBCL patients. Yet, based on the findings of this study, prolonged follow-up for patients older than 60 years should be considered, at least at the primary care level, with regards to a possibly increased risk for cardiovascular disease and secondary malignancies.

## Paper II

PEA can be used to screen for a large number of plasma protein biomarkers in minute sample volumes, allowing the distinction between controls, acute leukemias and lymphomas. Plasma protein profiling could provide valuable insight in the pathophysiology of acute leukemias and lymphoma and the technique may be a valuable tool in the diagnostics and prognostics of these diseases. Further studies will show its full clinical value in the setting of hematological malignancies.

## Paper III

Plasma protein analysis in DLBCL patients undergoing curative treatment with R-CHOP using PEA have the potential of paving the way to biomarker discovery and may offer deeper understanding of the pathophysiology of NHL. The identified proteins and pathways warrant further exploration as potential prognostic markers and eventual therapeutic targets. Additional scrutiny and validation however are needed. Adjusting for multiple testing and conducting larger-scale studies would strengthen the statistical significance of positive findings.

## Paper IV

TCLA1 appears to play a prominent role in the pathology of DLBCL and may have prognostic impact. Future studies with larger patient cohorts and well-

designed protein panels could achieve a much-awaited biomarker breakthrough.

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