Different chemokine profile between systemic and testicular diffuse large B-cell lymphoma

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Abstract

Although treatment for diffuse large B-cell lymphoma (DLBCL) has taken some notable steps in the 2000s, there are still subgroups of patients suffering from high mortality and relapse rates. To further improve treatment outcomes, it is essential to discover new mechanisms of chemotherapy resistance and create new treatment approaches to overcome them. In the present study, we analyzed the expression of chemokines and their ligands in systemic and testicular DLBCL. From our biopsy sample set of 21 testicular and 28 systemic lymphomas, we were able to demonstrate chemokine profile differences and identify associations with clinical risk factors. High cytoplasmic CXCL13 expression had correlations with better treatment response, lower disease-related mortality, and limited stage. This study suggests that active CXCR5/CXCL13 signaling could overtake the CXCR4/CXCL12 axis, resulting in a better prognosis.

Keywords: Chemokine; diffuse large B-cell lymphoma; testicular lymphoma; systemic lymphoma.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common form of non-Hodgkin lymphoma (NHL) globally, accounting for 30–40% of all new cases [1]. DLBCL may originate virtually from any tissue, but lymph nodes are the most common primary site. In almost half of patients, the disease is also localized in extranodal sites, most commonly in the gastrointestinal track [2]. The revised World Health Organization (WHO) classification divides DLBCL into overlapping

subgroups based on either the cell of origin (COO) or the location of the tumor mass. The two main subtypes of COO classification are germinal center B-cell (GC) type and non-GC or activated B-cell-like (ABC) type. Location-based classification includes primary DLBCL of the central nervous system (PCNSL) and primary cutaneous leg type DLBCL [3].

Primary testicular lymphoma (PTL) represents a rare aggressive form of DLBCL. It accounts for 1–2% of all NHL cases and less than 5% of testicular malignancies [2]. However, it is the most common testicular malignancy in men older than 60 years, with a median age of patients ranging from 66 to 68 years. PTL presentation includes a firm painless mass in one or both testicles. B-symptoms usually indicate an advanced disease [1]. Most PTL cases represent an ABC type. In most studies, the prognosis of patients with ABC DLBCL is inferior compared to GC DLBCL, which might contribute to the worse outcome in PTL compared to systemic DLBCL [1, 4].

Chemokines are small, structurally related proteins acting as chemoattractants for various hematopoietic cells [5]. There are 48 chemokine genes and 20 chemokine receptor genes identified in the human genome, with a wide range of specific and promiscuous interactions [5]. Continuously changing chemokine profile is essential during the development and maturation of lymphocytes [6]. Additionally, several studies have shown that chemokines play a part in cancer dissemination and act as an adverse predictor in various cancers [7-11].

The chemokine system has been increasingly studied in DLBCL and several chemokines and their receptors have been associated with DLBCL pathogenesis. It has been proposed that some chemokines/chemokine receptors could be used as potential biomarkers and even as targets for treatment [12]. In CNS lymphoma, which is a lymphoma presenting at an extranodal site similarly to PTL, it has been shown that certain chemokine receptors (CXCR4, CXCR5) and their ligands (CXCL12, CXCL13) differ in their presentation compared with systemic DLBCL.

Also, the location of these receptors and ligands in tumor cells seems to be important [13, 14]. In the present study, we investigated differences in chemokine profiles between nodal and testicular DLBCL, and their impact on the clinical disease presentation.

Materials and methods

Patient material

The study material consisted of samples from 49 male patients with either confirmed primary testicular lymphoma (n=21) or systemic nodal diffuse large B-cell lymphoma (sDLBCL) (n=28). As control samples there were samples of 10 normal testis, and 10 reactive lymph nodes from our previous study [14]. The patients were diagnosed and treated, and the data were collected from Oulu University Hospital and North Karelia Central Hospital. Clinical information such as the presence of B-symptoms, number of extranodal lesions, age, WHO status, and International Prognostic Index (IPI) were also recorded at the time of diagnosis. Cell of origin was assessed by Hans algorithm. The mean age for PTL patients was 75.2 years and for sDLBCL patients 57.3 years, with an overall median follow-up time of 57.9 months, ranging from 1 to 261 months. For PTL patients the median follow-up time was 15 months (range 1 to 89 months) and for sDLBCL patients 89 months (range 0 to 261 months). The mean age for patients with normal testis removed was 74.3 years. Correlations and percentages of clinical parameters in PTL and sDLBCL subgroups are presented in Table 1.

Treatment regimens for PTL patients included CEOP (cyclophosphamide, etoposide, vincristine, and prednisolone) (n=7), CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisolone) (n=9), CEP (cyclophosphamide, epirubicin, and prednisolone) (n=1), bendamustine (n=1), and MACOP-B (methotrexate with leucovorin rescue, doxorubicin, cyclophosphamide, vincristine, prednisolone, and bleomycin) (n=1). Two patients received no chemotherapy. Sixteen PTL patients received rituximab combined to their chemotherapy

regimen, five did not. Eleven patients received radiotherapy to the contralateral testicle and five also to regional nodal areas. Treatment regimens used for sDLBCL patients included CEOP (n=10), CHOP (n=8), CHOEP (cyclophosphamide, doxorubicin, vincristine, etoposide, and prednisolone) (n=8), CEP (n=1), and carboplatin (n=1). All sDLBCL patients received rituximab. A total of 13 patients in the sDLBCL group received radiotherapy at some point during the treatment. This study was approved by the Ethical Committee of North Ostrobothnia's Hospital District and the National Supervisory Authority for Welfare and Health (Valvira 6622/05.01.00.06/2010). The principles of the Declaration of Helsinki were followed in this study.

[Table 1 near here]

Immunohistochemistry

Tissue samples were originally used for hospitals' diagnostic procedures and were routinely fixed in formalin and embedded in paraffin. The samples were later collected from hospital archives, cut to a thickness of 3.5 μm and placed on SuperFrostPlus glass slides (Menzel-Gläser, Braunschweig, Germany), and then incubated at +37°C for 4 h. Histo-Clear (National Diagnostics, Atlanta, GA, USA) was used as a clearing agent for deparaffinating the slides, and then they were rehydrated in a graded series of alcohol solutions. To retrieve epitopes, the slides were microwaved for 10 to 15 minutes in tris-EDTA or citrate buffer. The slides were cooled for 20 minutes at room temperature. To block the peroxidase activity, the slides were incubated in 3% H202 for 5 min. The primary antibody was then added, and the slides were incubated in a humidity chamber (Table 2). Immunostaining was continued using a Dako REAL™ EnVision™ Detection System (Dako Denmark A/S, Glostrup, DK) according to the manufacturer's instructions. The immunoreaction was detected with diaminobenzidine. PBS-Tween was used to wash the slides between stages of the immunostaining procedure. Mayer's

hematoxylin (Reagena, Toivola, Finland) was used for counterstaining at the end, and then the slides were dehydrated and mounted with Histomount (National Diagnostics, Atlanta, GA, USA). Cell culture and western blotting was performed as described previously [15]. Three DLBCL cell lines were used: OCI-LY1 (ACC 722, DSMZ, Leibniz Institute, German Collection of Microorganisms and Cell Culture GmbH), Pfeiffer (ATCC® CRL-2632™) and SU-DHL-4 (ACC 495, DSMZ, Leibniz Institute, German Collection of Microorganisms and Cell Culture GmbH).

[Table 2 near here]

Review and analyses were performed with a multi-head microscope by an experienced hematopathologist (HRT) with principal investigators (JS, RO), both blinded to the clinical data. The staining data were evaluated from the tumorous cells as one of the following expressions: negative, weak positive, or strong positive. The quantity of each intensity level was recorded (0–100%). Subsequently, a modified Histoscore was used with the following algorithm: 0 × negative expression percentage + 1 × weak expression percentage + 3 × strong expression percentage (range 0–300).

Statistical analysis

IBM SPSS Statistics for Windows (Version 25) was used for statistical analysis. Kaplan–Meier was used for survival analysis, and the log-rank test was used to compare different variables. P-values under 0.05 were considered statistically significant. Overall survival (OS) was calculated from the date of diagnosis to the date of death, and progression-free survival (PFS) was calculated from the date of diagnosis to the last follow-up, when the case was censored from further follow-up or to the date of progression. Chi-square test or Fisher's exact test was used to compare nominal variables. T-test with Levene's test or the Mann–Whitney U test were

conducted to determine differences between two classed variables. Cut-off values for different chemokine histoscores were determined by using a histoscore median.

Results

Expression of immunohistochemical markers in the disease groups

Both CXCR4 and CXCR5 immunostaining revealed only membranous expression patterns in both disease categories. Overall, 44.9% of CXCR4 expression results were assessed as strongly positive, whereas for CXCR5, the same number was 24.5%. CXCR4 was strongly expressed in 26.5% of sDLBCL cases and 38.8% of PTL cases. For CXCR5, the corresponding percentages were 35.7% and 9.5%, respectively.

PTL cases showed cytoplasmic, membranous, and nuclear CXCL12 staining, while all the sDLBCL cases lacked nuclear positivity. Membranous CXCL12 expression was strong in 35.7% of sDLBCL cases but comprised only 4.8% of the PTL group. For cytoplasmic expression, the numbers were 60.7% and 19.0%, respectively. An additional 14.3% of PTL cases had strong nuclear expressions.

CXCL13 demonstrated membranous, cytoplasmic, and nuclear staining patterns in sDLBCL cases, whereas all the PTL cases were negative for membranous staining. Cytoplasmic CXCL13 expression was strong, comprising 89.3% of sDLBCL cases and 57.1% of the PTL group. For nuclear expression, the numbers were 35.7% and 28.6%, respectively. An additional 7.1% of the sDLBCL group showed strong membranous expression. Representation of different expression patterns can be seen in Figure 1.

In Western blot analysis, all the chemokines were expressed also in DLBCL cell culture material [Figure 2].

[Figure 1 and 2 near here]

Expression of immunohistochemical markers in normal testis

There was no CXCR4 staining in the structures of normal testis, except in one sample Sertoli cells showed nuclear positivity. CXCR5 demonstrated some positive nuclear staining in seminiferous tubules and all the Leydig cells were weakly positive. In a few samples endothelium was strongly positive for CXCR5. CXCL12 showed cytoplasmic and/or membranous staining in some seminiferous tubules and in most Leydig cells. Intravascular leukocytes were negative for CXCL12, but strong endothelial staining was seen. Weak CXCL13 staining was seen in the cytoplasm of all seminiferous tubules and strong staining in Leydig cells' cytoplasm. There were intravascular white blood cells that showed dot-like cytoplasmic staining for CXCL13.

Differences in the expression of chemokines and their ligands according to disease group

sDLBCL had higher expressions of membranous CXCR5 ($p = 0.004$), membranous CXCL12 ($p = 0.002$), cytoplasmic CXCL12 ($p = 0.004$), and cytoplasmic CXCL13 ($p = 0.016$) compared to PTL. There was no statistically significant difference between sDLBCL and PTL in the expression of membranous CXCR4, nuclear CXCL12, membranous CXCL13, or nuclear CXCL13.

Correlation of study parameters with clinical disease presentation

Higher CXCR4 expression was associated with lower lactate dehydrogenase (LDH) ($p = 0.04$) in the sDLBCL group and with higher WHO ($p = 0.002$) in the PTL group. Higher CXCR5 expression was associated in the PTL group with higher LDH ($p = 0.028$).

In sDLCBL, cytoplasmic CXCL13 was associated with higher frequency of the germinal center phenotype ($p = 0.082$), fewer lymphoma-related deaths ($p = 0.083$), and lower stage ($p = 0.083$). In the PTL group, it was associated with fewer progressions and with fewer lymphoma-related deaths ($p = 0.046$). Nuclear CXCL13 was associated with older age.

Membranous CXCL13 and cytoplasmic CXCL12 showed no statistical significance between chemokines and clinical markers in either group. CXCL13 results are presented in Table 3.

[Table 3 near here]

Survival correlations

In the whole study population, low cytoplasmic CXCL13 was associated with inferior progression-free survival (PFS) (log-rank p = 0.021) with 2-year PFS rate (41.7% standard error [SE]: 14.2% vs. 72.0% SE: 7.5%) and 5-year PFS rate (27.8% SE: 14.8% vs. 62.9% SE: 8.2%) and inferior OS (p = 0.005) with 2-year OS being (41.7% SE: 14.2% vs. 73.0% SE: 7.3%) and 5-year OS being (33.3% SE: 13.6% vs. 63.9% SE: 8.1%).

In the PTL group alone, PFS had borderline significance when comparing between high and low cytoplasmic CXCL13 expression (log-rank p = 0.069). Five-year PFS was 33.3% (SE: 15.7%) for the low group and 50.9% (SE: 15.8%) for the high group. There were no other significant survival correlations.

In the whole study population, the hazard ratio (HR) for death in the high cytoplasmic CXCL13 group was 0.498 (p = 0.121; 95% confidence interval [CI]: 0.210–1.181). In the testicular lymphoma group, the HR for death in the high expression group was 0.430 (p = 0.97; 95% CI: 0.158–1.166) and in the nodal group 0.858 (p = 0.886; 95% CI: 0.105–7.011), respectively.

[Figure 3 near here]

Discussion

This is the first study to investigate the differences in chemokine profiles between primary testicular lymphoma and systemic DLBCL. We were able to show that there was more cytoplasmic CXCL13, membranous CXCR5, and membranous and cytoplasmic CXCL12

expression in systemic lymphoma compared to PTL. Higher cytoplasmic CXCL13 expression correlated with a favorable prognosis.

Despite similar histological features with sDLBCL, testicular lymphoma represents a biologically distinct disease. Regarding its genetic aberrations and gene expression profiling, PTL has been shown to be closer to PCNSL than to sDLBCL [1, 4]. Indeed, patients with PTL have an increased risk of CNS dissemination and relapses in the contralateral testis even after long periods of time [2]. This could be due to more indolent progenitor cells in the bone marrow (BM), which may serve as a cell reservoir for a relapse to the CNS and testis. PCNSL patients have been shown to harbor monoclonal B-cell lymphocytosis in the BM, and analysis of the IGHV gene suggested that cell populations in the CNS and BM had the same progenitor [16]. The environment in which PTL and PCNSL develop are thought to be immune privileged, which could contribute to the similarities between these two malignancies and may help to explain the immune escape phenotype that is common especially for PTL [2, 17].

Lymphocyte chemokine receptor profile changes throughout lymphocytes lifecycle from a hematopoietic stem cell (HSC) to a mature lymphocyte. Chemokines have promiscuous interactions with their seven transmembrane G-protein coupled receptors, as one ligand can interact with several receptors [5]. C-X-C ligand 12 (CXCL12) is expressed and secreted by mesenchymal-lineage progenitor/stem cells in the hematopoietic niche and thymic stromal cells. It acts as a chemoattractant for lymphocytes, monocytes, double negative and double positive CD4/CD8 thymocytes and CD34+ HSCs. Its function is to home precursor B-cells from circulation to the BM and control HSC development in the BM [6, 18]. CXCL12 has been shown to induce proliferative effects on stem cells in in vitro studies [6, 19], yet contradicting findings have also been reported in which CXCR4 deletion has resulted in increased proliferation of HSCs in vitro [20, 21]. CXCL12 can induce pro-survival signals by either disabling pro apoptotic Bcl-2 protein via activating antagonistic MAPK, ERK, and PI3K-

pathways or by upregulating pro-survival genes [22]. CXCR4 can be upregulated through hypoxia or by nuclear factor Kappa b transcription factor. After the pre-B-cell stage, the CXCR4 pathway loses its importance, and B-cell receptor and B-cell activating factor signaling become more important for B-cell survival and development [6].

CXCR4 receptor is overexpressed in several cancers; it has been shown to be able to induce the epithelial-mesenchymal transition and work as a pro-angiogenic, pro-migratory, pro-survival factor, and it is associated with worse prognosis and accelerated metastasis [23]. In DLBCL active CXCR4/CXCL12 axis induces reduced immune surveillance and increases tumor proliferation. In line with these findings, blocking CXCR4 activity inhibits DLBCL cell line proliferation and prolongs survival of tumor-bearing mice [24, 25]. Indeed, it is not surprising, that high pretreatment CXCR4 protein expression level is associated with an adverse treatment outcome [26].

CXCL13 is a selective B-cell chemoattractant produced physiologically by follicular dendritic cells in secondary lymphatic tissue, like lymph node germinal centers [5, 18]. Its function is mediated by CXCR5 receptor that is expressed in mature B-lymphocytes, follicular helper T-cells and skin-derived migratory dendritic cells [27]. CXCR5/CXCL13 axis has an integral role in lymph node neogenesis and CXCR5 deficient mice fail to develop inguinal nodes and have severely compromised primary and secondary follicle formation in the spleen and Peyer´s patches [28]. Accordingly, CXCL13 can be used as a plasma biomarker for germinal center activity [29].

In the context of malignancies CXCR5/CXCL13 axis orchestrates cell-cell interactions regulating lymphocyte infiltration in tumor microenvironment and formation of tertiary lymphoid structures, a phenomenon integral for tumor immunity [30]. It also induces proliferation, growth, invasion, and migration of malignant cells [27]. CXCL13 correlates with

poorer prognosis, more metastasis, and larger tumor size in multiple myeloma [7], neuroblastoma, leukemias [8], renal cell carcinoma [9], hepatocellular carcinoma [10], and gastric cancer [11]. In ovarian cancer, high CXCL13 expression was associated with better OS, suggesting an anti-tumor role of this chemokine rather than a tumor-progressing role [31].

Chemokine receptors are membrane proteins, which, after coupling with their ligands, are internalized and undergo either degradation or subsequent recycling back to membrane. In general, membranous location of CXCR4 implies an active receptor [32]. For chemokine ligands, membranous staining probably represents active ligand/receptor interaction, while cytoplasmic staining may represent an internalized ligand/receptor complex or also endogenous protein production. We find that our results, demonstrating less membranous CXCR5, more membranous CXCR4 and lack of membranous CXCL13 expression in PTL, nicely explain the extranodal location preference of this rare DLBCL subtype.

In sDLBCL CXCL13 positivity correlated with GC-phenotype and limited stage disease. In the whole study group and in sDLBCL high CXCL13 expression correlated also with favorable PFS. In PTL there was a trend to an improved PFS albeit not statistically significant. This is the first report describing this association of CXCL13 expression level with prognosis. In contrast to previous reports, we were not able to demonstrate association of CXCR4 expression and survival, which might be explained by the relatively small sample size. Strong CXCL13 expression in the cytoplasm may indicate that either the malignant cell has been in a CXCL13-rich environment, or it may implicate that the cell produces it as autocrine growth factor. Later phenomenon has been shown to occur in PCNSL, as CNS lymphoma cells express both CXCL13 and its receptor independently from tumor necrosis factor receptor 1 and lymphotoxin-β receptor, proteins that induce CXCL13 expression in follicular dendritic cells [33]. We found an association between GC-phenotype and high cytoplasmic CXCL13 expression. However, from this material we cannot draw any conclusions regarding the

causality of this phenomenon. It may be that CXCL13 drives the differentiation of the malignant lymphocyte into the GC-phenotype or the genetic lesion associated with GC-phenotype induces CXCL13 synthesis.

As mentioned earlier, CXCR4/CXCL12 could be an important factor in the initiation of cancer, and it is associated with stem cell–like disease and an adverse treatment outcome in DLBCL [12]. In other hematological malignancies it has been shown to be associated with chemoresistance as well [34]. Here we found that CXCR5/CXCL13 pathway implies a favorable prognosis irrespective of CXCR4 expression. From our results, we speculate that the availability of an active CXCR5/CXCL13 pathway overrides the CXCR4/CXCL12 pathway regarding both the tumor cell migration and response to chemotherapy. There are also other data supporting the idea of this hierarchic order of these two systems during normal lymphocyte maturation [6, 35].

This is the first report comparing chemokine profile expression between systemic and testicular DLBCL and demonstrating that extranodal location is explained by different patterns of their expression. One strength of our work includes detailed analysis of cellular location, which enabled to demonstrate differences in the function of CXCR5/CXCL13 axis between these two subgroups. The importance of location has been presented also in previous works [13, 14]. Our research dataset was rather small due to the rarity of the disease in question, and our results should be regarded more as preliminary hypothesis generating ones and should be further studied in a larger population. Anyway, our results support the idea that impairment in the function of CXCR5/CXL13 axis drives lymphomas to an extranodal location and is associated with adverse treatment outcome.

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Disclosure of interest

The authors report no conflicts of interest.

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Tables

	Total (%)	PTL (%)	DLBCL (%)	P-value (F)
B-symptoms				0.072
Yes	19 (38.8)	5 (23.8)	14 (50)	
None	27 (55.1)	15 (71.4)	12 (42.9)	
NA	3 (6.1)	1 (4.8)	2 (7.1)	
Extranodal lesion				0.714
0–1	40 (81.6)	18 (85.7)	22 (78.6)	
>1	9 (18.4)	3 (14.3)	6 (21.4)	
Age				0.001
≤60	18 (36.7)	2 (9.5)	16 (57.1)	
>60	31 (63.3)	19 (90.5)	12 (42.9)	
LDH				0.204
Elevated	22 (44.9)	6 (28.6)	16 (57.1)	
Normal	20 (40.8)	10 (47.6)	10 (42.9)	
NA	7 (14.3)	5 (23.8)		
WHO				0.475
0–1	37 (75.5)	18 (85.7)	19 (67.9)	
>1	10 (20.4)	3 (14.3)	7 (25)	
NA	2 (4.1)		2 (7.1)	

GC-phenotype				0.003
Yes	11 (22.4)		11 (39.3)	
Non-GC	25 (51.1)	13 (61.9)	12 (42.9)	
NA	13 (26.5)	8 (38.1)	5 (17.8)	
IPI				0.068
0–2	31 (63.2)	17 (81)	14 (50)	
3–5	16 (32.7)	4 (19)	12 (42.9)	
NA	2 (4.1)		2 (7.1)	
Stage				<0.001
I–II	27 (55.1)	18 (85.7)	9 (32.1)	
III–IV	22 (44.9)	3 (14.3)	19 (67.9)	

Table 1. Patient demographics. PTL = Primary testicular lymphoma, DLBCL = diffuse large B-cell lymphoma, LDH = lactate dehydrogenase, WHO = WHO performance status, GC = germinal center, IPI = International Prognostic Index. F = Fisher's exact test. NA = Data not available

Antibody	Source of antibody	Concentration	Incubation	m/w
CXCR4	#97680, CXCR4 (D4Z7W) Cell Signaling Technology, Inc., Danvers, MA, USA	1:500	1 h RT	Citrate buffer pH 6, 10 min
CXCR5	MAB190, Human CXCR5 ab, clone 51505 R&D Systems, Minneapolis, MN, USA	1:1000	1½ h RT	Tris-EDTA pH 9, 15 min
CXCL12	#97958, SDF1 (D8G6H) Cell Signaling Technology, Inc., Danvers, MA, USA	1:50	Overnight at +4°C	Tris-EDTA pH 9, 15 min
CXCL13	NBP2-16041, CXCL13/BCL/BCA-1 ab Novus Biologicals USA, Littleton, CO, USA	1:800	1 h RT	Tris-EDTA pH 9, 15 min
*CXCR4	#97680, CXCR4 (D4Z7W) Cell Signaling Technology, Inc., Danvers, MA, USA	1:500 / anti-Rabbit 1:3000		
*CXCR5	MAB190 R&D Systems, Minneapolis, MN, USA	1:500 / anti-Mouse 1:3000		
*CXCL12	#3740, SDF1/CXCL12 Cell Signaling Technology Inc., Danvers, MA, USA	1:500 / anti-Rabbit 1:3000		

*CXCL13	NBP2-16041, CXCL13/BCL/BCA-1 Novus Biologicals, Cambridge, UK	1:1000 / anti-Rabbit 1:4000

Table 2. Antibodies used in immunohistochemistry and Western blot analysis. RT = room temperature, m/w = microwave conditions, *=antibodies in western blot analysis.

	sDLBCL			PTL	
	CXCL13c	CXCL13n	CXCL13m	CXCL13c	CXCL13n
B-symptoms	0.469	0.968	0.165	-	0.308
>1 extranodal lesion	0.611	0.71	0.462	0.735	0.729
Age (<60)	0.397	0.085 ↑	-	0.84	0.544
Elevated LDH	0.854	0.157	0.74	0.806	0.565
WHO (<1)	0.8	0.523	0.464	0.735	0.364
GC phenotype	0.082 ↑	0.124	-	-	-
Treatment response	0.337	0.658	0.566	0.023 ↓	0.143
Relapse	0.931	0.902	0.163	0.108	0.659
Lymphoma death	0.083 ↓	0.8	0.462	0.046 ↓	0.674
IPI (>2)	0.469	0.8	0.166	0.763	0.55
Stage (>II)	0.083 ↓	0.374	0.163	0.393	0.782

Table 3. Prognostic associations of CXCL13 immunohistochemical expression. Values represent T-test P-values. Membranous CXCL13 was negative for the whole PTL group. LDH = Serum lactate dehydrogenase, WHO = WHO performance status, GC phenotype = Germinal center phenotype, IPI = International Prognostic Index. Up-arrow indicates

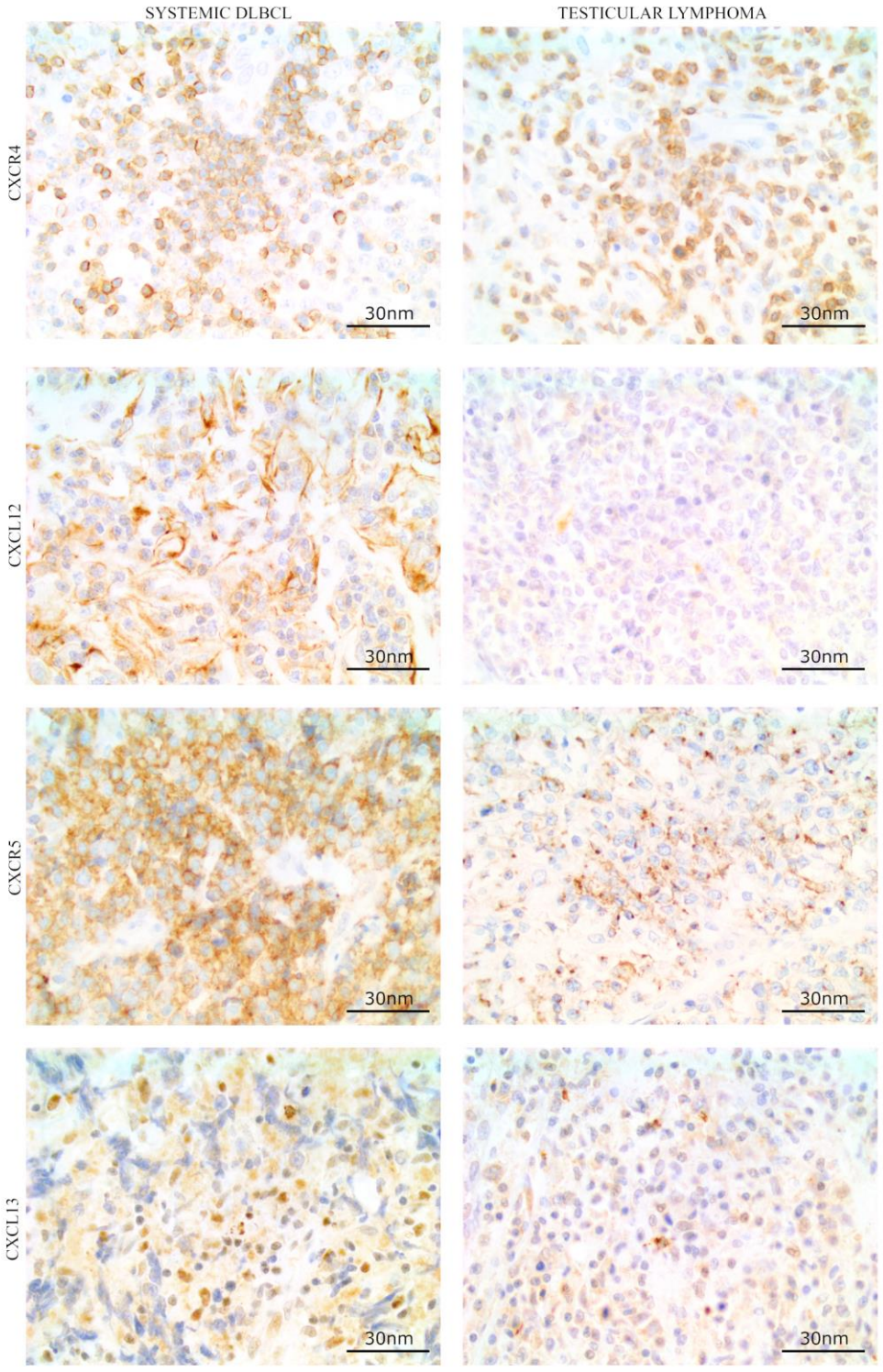
positive correlation and down arrow inverse correlation between expression and prognostic parameter.

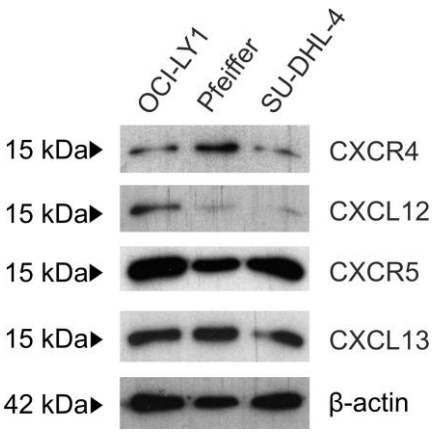
Figure captions (Figures themselves are in separate files)

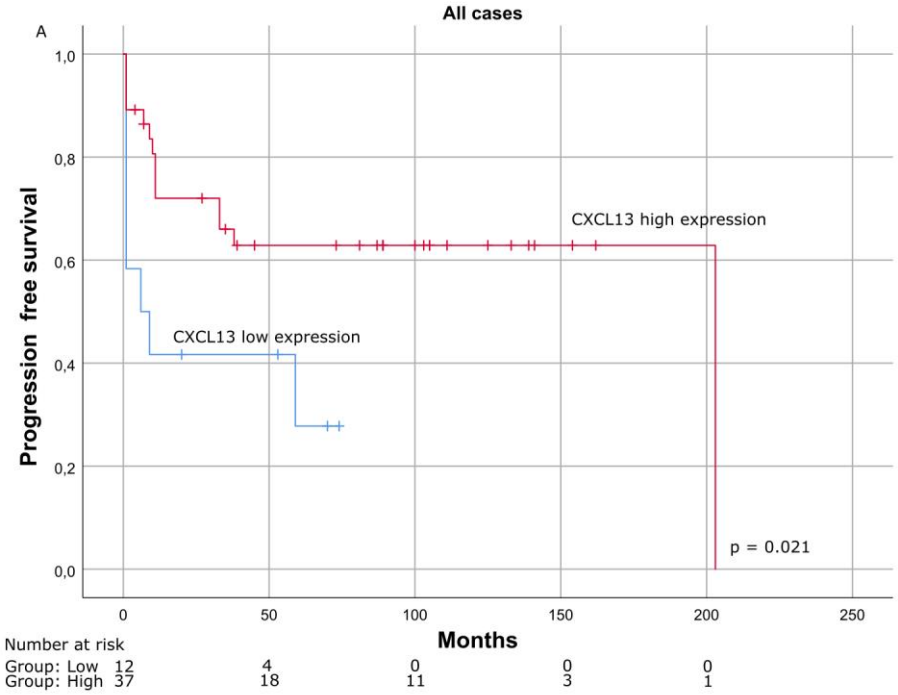
Figure 1. Immunohistochemistry image at x40 magnification, showcasing the different staining patterns across the chemokine pool under investigation. Images represent cases with median modified IHC scores for each group, ranging from weak (cytoplasmic testicular CXCL12) to strong (cytoplasmic systemic DLBCL CXL13). Strong membranous staining is demonstrated with systemic DLBCL CXCR4 imaging.

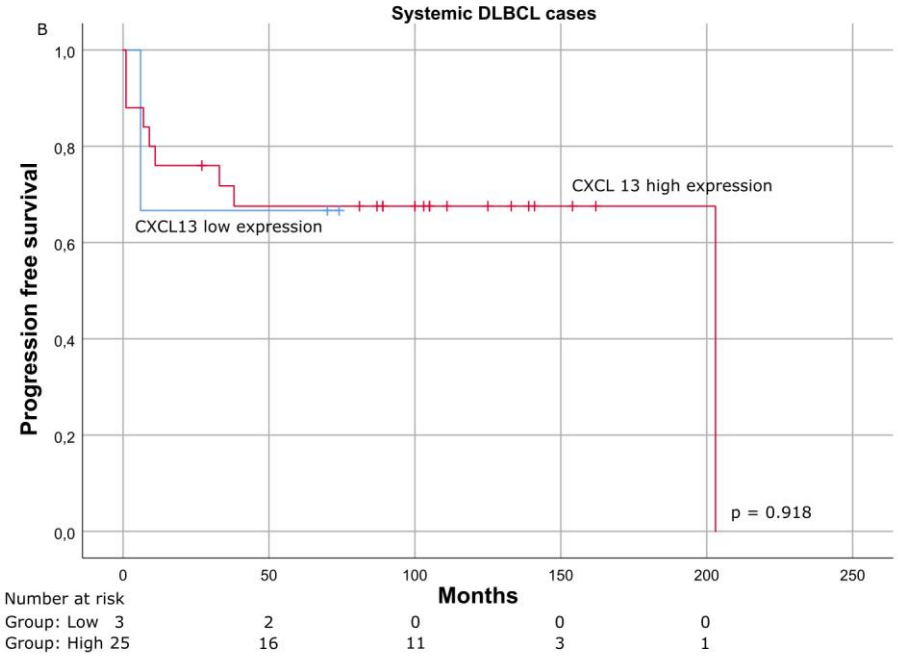
Figure 2. The expression of chemokines CXCL12 and CXCL13 and their receptors CXCR4 and CXCR5 in three DLBCL cell lines.

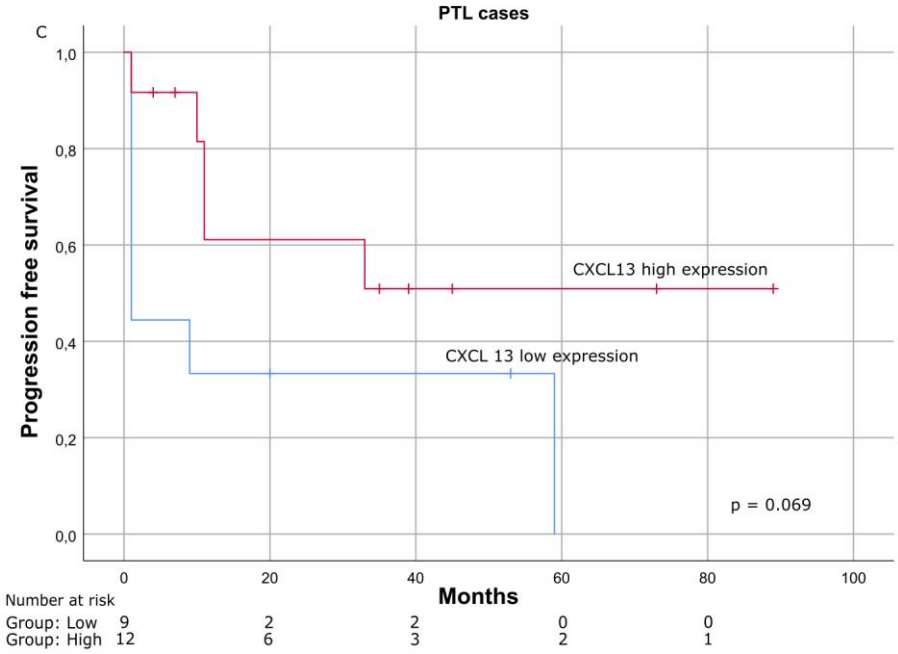
Figure 3. PFS survival correlations between CXCL13 histoscore index. Red line corresponds to (A) both nodal and testicular cases combined, (B) only nodal cases, and (C) only testicular cases. Significance was tested with a log-rank test.


SYSTEMIC DLBCL
TESTICULAR LYMPHOMA
CXCR4
CXCL12
CXCR5
CXCL13
30nm
30nm
30nm
30nm
30nm
30nm
30nm
30nm


OCI-LY1
Pfeiffer
SU-DHL-4
15 kDa
CXCR4
15 kDa
CXCL12
15 kDa
CXCR5
15 kDa
CXCL13
42 kDa
β-actin


All cases
A
Progression free survival
1,0
0,8
0,6
0,4
0,2
0,0
CXCL13 high expression
CXCL13 low expression
p = 0.021
0
50
100
150
200
250
Months
Number at risk
Group: Low 12 4 0 0 0
Group: High 37 18 11 3 1


B
Systemic DLBCL cases
Progression free survival
1,0
0,8
0,6
0,4
0,2
0,0
CXCL 13 high expression
CXCL13 low expression
p = 0.918
0
50
100
150
200
250
Months
Number at risk
Group: Low 3 2 0 0 0
Group: High 25 16 11 3 1


C
PTL cases
Progression free survival
1,0
0,8
0,6
0,4
0,2
0,0
CXCL13 high expression
CXCL 13 low expression
p = 0.069
0
20
40
60
80
100
Months
Number at risk
Group: Low 9 2 2 0 0
Group: High 12 6 3 2 1