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Plasma protein biomarker profiling reveals major differences between acute leukaemia, lymphoma patients and controls

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ABSTRACT

Aiming to accommodate the unmet need for easily accessible biomarkers with a focus on biological differences between haematological diseases, the diagnostic value of plasma proteins in acute leukaemias and lymphomas was investigated. A multiplex proximity extension assay (PEA) was used to analyze 183 proteins in diagnostic plasma samples from 251 acute leukaemia and lymphoma patients and compared with samples from 60 healthy controls. Multivariate modelling using partial least square discriminant analysis revealed highly significant differences between distinct disease subgroups and controls. The model allowed explicit distinction between leukaemia and lymphoma, with few patients misclassified. Acute leukaemia samples had higher levels of proteins associated with haemostasis, inflammation, cell differentiation and cell-matrix integration, whereas lymphoma samples demonstrated higher levels of proteins known to be associated with tumour microenvironment and lymphoma dissemination. PEA technology can be used to screen for large number of plasma protein biomarkers in low μL sample volumes, enabling the distinction between controls, acute leukaemias and lymphomas. Plasma protein profiling could help gain insights into the pathophysiology of acute leukaemia and lymphoma and the technique may be a valuable tool in the diagnosis of these diseases.

Introduction

Comprehension of disease biology and interaction between host and tumour cells is of great importance for devising diagnostic, prognostic and predictive biomarkers. Identifying patterns of plasma proteins could add a dimension of knowledge and understanding of malignant diseases [1–4]. The proximity extension assay (PEA) measures a large number of proteins simultaneously in small volumes of liquid tissues. Studies using this method have indicated its clinical utility in various malignant diseases such as glioma, gastric, and ovarian cancer [5–9].

In this study, three types of acute leukaemia were investigated, i.e. acute myeloid leukaemia (AML), acute promyelocytic leukaemia (APL), and acute lymphoblastic leukaemia (ALL), and two common types of lymphoma, i.e. diffuse large B-cell lymphoma (DLBCL) and Hodgkin lymphoma (HL). Based on proteins detected in low μL plasma samples taken from untreated leukaemia and lymphoma patients and healthy controls, the aim was to explore the possibility of distinguishing these haematological malignancies using well-designed protein detection panels. The patterns of these distinct proteins and their related biological processes may be indicative with regard to the biology of these

Abbreviations: AML, Acute myeloid leukaemia; APL, Acute pro-myelocytic leukaemia; ALL, Acute lymphoblastic leukaemia; CVDIII, Cardiovascular III; DLBCL, Diffuse large B cells Lymphoma; EC, Ethics Committee; HL, Hodgkin lymphoma; IHC, Immunohistochemistry; LOD, Limit of detection; NHL, Non-Hodgkin lymphoma; NPX, Normalized protein expression; ONCII, Olink Oncology II; PEA, Proximity extension assay; PLS-DA, Partial least squares-discriminant analysis; PTLD, Post-transplant lymphoproliferative disorder; TME, Tumour microenvironment; U-CAN, Uppsala-Umeå Comprehensive Cancer Consortium biobank; VSS, Variable subset selection.

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malignancies.

With pancytopenia and maturation arrest as key disease features, it was expected that leukaemia patients would have altered levels of proteins associated with haemostasis and stem cell differentiation and that AML, APL and ALL would differ in markers of lymphoid and myeloid development. It was postulated that patients with lymphoma would have increased levels of inflammatory proteins and that the difference in tumour microenvironment (TME) between HL and DLBCL may be reflected in the protein profiles.

Material and methods

Study design & population

EDTA plasma samples from 107 patients aged ≥ 18 years with acute leukaemias (AML: 69, ALL: 29 (including B and T leukaemias/lymphomas and Burkitt leukaemia), APL: 9) and 144 patients with lymphomas (DLBCL: 95, HL: 49) from the Uppsala-Umeå Comprehensive Cancer Consortium biobank (U-CAN) were included. U-CAN abides by the highest standards of samples collection and storage and collected material has been used for many high impact studies[10]. Samples were taken at the time of diagnosis between 2010 and 2015 after written informed consent.

Clinical patient characteristics are presented in Table 1a-b. Plasma samples from 60 healthy age and gender matched controls (30 male, 30 non-pregnant females) were obtained from the EpiHealth biobank [11]. U-CAN is a high-quality longitudinal biobank with the sequential collection of clinical data as well as blood and tissue samples from currently over 22,000 cancer patients. The EpiHealth cohort is a large population-based study cohort for investigation of gene-lifestyle interactions and comprises a total of 25,104 individuals. For both cohorts, blood samples are retrieved according to a standardized schedule specified for each study. For U-CAN biobank, the time between blood sampling and freezing, which is usually less than four hours, was noted for each sample. Samples are marked according to Standard PREanalytical Code (SPREC) standard [12]. The study was approved by the Regional Ethics Committee (EC) of Uppsala-Örebro (2012/198, 210/198/1, 2014/233). Data collection in the EpiHealth study and usage of the material in this project was approved by the EC of Uppsala (2010/402: 2010–12–01, 2011–11–17, 2015/179). The EpiHealth study is approved by the Swedish Data Protection Authority.

Multiplex PEA

Multiplex PEA technology was utilized to assess plasma samples (1 μL sample/panel) using the Olink Oncology II (ONCII) and Cardiovascular III (CVDIII) protein panels (Olink™ Proteomics, Uppsala Sweden, https://olink.com). A total of 183 different proteins was measured, where each panel includes 92 proteins involved in diverse biological processes and 4 technical controls. Analyses were performed at the Clinical Biomarker Facility at SciLifeLab, Uppsala.

In multiplex PEA, each target protein is recognized by a pair of proximity probes consisting of two antibodies conjugated to single stranded DNA oligonucleotides that in proximity are hybridized to each other allowing DNA polymerization and amplification[13]. The process creates a unique signature quantitatively proportional to the initial concentration of each target protein. The PEA analysis was carried out according to the manufacturer's instruction (details in supplementary material). Results were obtained as normalized protein expression (NPX) which is an arbitrary unit presented on a log2 scale, where one increased NPX corresponds to a two-fold higher protein concentration. Limit of detection (LOD) was determined for each biomarker based on three times standard deviation beyond the NPX value of the negative controls in each run.

Table 1
a-b Clinical characteristics. Clinical characteristics for acute leukaemia (a) and lymphoma (b) patients included in the study.Table 1b. Patient Characteristics Lymphoma.

	DLBCL	HL
	(N = 95)	**(N = 49)**
Gender - N (%)		
Male	54 (56.8)	30 (61.2)
Female	41 (43.2)	19 (38.8)
Age (years)		
Mean (SD)	63.6 (13.8)	45.8 (20.3)
Performance status (WHO) - N (%)		
0	73 (76.8)	N.A.
1	8 (8.4)	N.A.
2	6 (6.3)	N.A.
3	4 (4.2)	N.A.
4	0 (0)	N.A.
Misssing	4 (4.2)	N.A.
Stage - (Ann Arbor)		
1	26 (27.4)	5 (10.2)
2	18 (18.9)	21 (42.9)
3	14 (14.7)	12 (24.5)
4	37 (38.9)	11 (22.4)
Bulky Disease - N (%)		
No	66 (69.5)	39 (79.6)
Yes	28 (29.5)	6 (12.2)
Missing	1 (1.1)	4 (8.2)
B symptoms		
No	63 (66.3)	31 (63.3)
Yes	32 (33.7)	18 (36.7)
Lactate Dehydrogenase (LD) (μkat/L)		
Median (min-max)	4.75 (1.9–39)	3 (2.1–9.7)
IPI[a] Score N (%)		
0	17 (17.9)	N.A.
1	24 (25.3)	N.A.
2	21 (22.1)	N.A.
3	20 (21.1)	N.A.
4	10 (10.5)	N.A.
5	1 (1.1)	N.A.
Missing	2 (2.1)	N.A.
IPS* * Score N (%)		
0	N.A.	3 (6.1)
1	N.A.	6 (12.2)
2	N.A.	4 (8.2)
3	N.A.	10 (20.4)
4	N.A.	7 (14.3)
Missing	N.A.	19 (38.8)

* *IPS: International Prognostic Score for advanced Hodgkiṅs lymphoma
[a] IPI:International Prognostic Index for non Hodgkiṅs lymphoma

Statistical analysis

Samples were randomly distributed in six 96-microwells plates (Sarstedt, Nümbrecht, Germany) with up to 80 patient samples and 10 healthy controls on each plate. The NPX values were normalized with respect to plate based on the healthy controls using linear regression (using lm function in the stats R-package) with NPX as dependent variable and plate as independent variable.

Univariate tests

Differences in protein level between two groups were studied using linear regression, adjusting for age and gender (NPX as the dependent variable and group identity, age and gender as independent variables), and evaluated using a likelihood ratio test. One protein, NT-pro BNP (N-terminal prohormone of brain natriuretic peptide), had a high level of values below LOD (48%) and was therefore excluded; all other proteins had <10% values below LOD. The Benjamini-Hochberg 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 < 0.05.

Multivariate modelling

Multivariate partial least squares-discriminant analysis (PLS-DA) classification models were computed using the R function opls in the R package ropls[14]. The number of latent variables (predictive components) was determined by an internal cross-validation or, if less than two predictive components were found, set to two. The model was evaluated using 10 5-fold cross validations where the samples are divided into 5 equally sized subsets of which 4 are used to train the model and the fifth subset used to test the model and for each fold computing the error rate (ER) – i.e., the fraction of incorrect classifications. The overall predictive ability of the PLS-DA models was summarized by the average ER ($\overline{ER}$and average AUC (area under the receiver operator curve (ROC)) averaged over the 50 test sets.

Variable subset selection

A variable subset selection (VSS) procedure was applied to identify a smaller subset of proteins able to discriminate between groups. The VSS procedure was implemented as follows, (i) Selection of all variables with variable importance value (VIP) ≥ 1 or, if many fulfil this criterion, the 25 variables with the highest VIP. (ii) Forward subset selection. Starting with the single variable that alone had the highest performance measure, stepwise adding the variable improving the predictivity the most until no improvement achieved., (iii) Backward subset selection. From the set of selected variables, stepwise removal of the variable that, by being excluded, improved the predictivity the most. Stop when removing a variable did not improve the predictivity. The VSS was implemented inside the cross-validation procedure and applied to the training data; thereafter, a PLS-DA based on the selected protein subset was constructed and evaluated on the test data. The VSS procedure was also applied to the full data set.

Functional groups

For each predefined functional protein subset, a PLS-DA model was constructed to separate between two groups. The predictive ability for the subset of proteins was measured by the $\overline{ER}$.

Results

The PLS-DA model distinguished the different samples with high precision.

Sample comparison

To further validate the reproducibility of PEA, a set of 32 samples was analysed in replicates. Results with correlation R-squares over 0.98 for all of the duplicates demonstrated high reproducibility of the technology (Supplementary Figure 2).

Leukaemia vs lymphoma vs controls

Highly significant differences were observed between leukaemia, lymphoma and healthy controls (Fig. 1). The top differentiating proteins in each comparison are presented in Fig. 2 and the full protein list in Supplementary Tables 3a-c.

Leukaemia vs controls

The 10 most differentiating proteins between leukaemia and healthy controls were: vWF, SYND1, TNF-RSF6B, MPO, VIM, TNF-R1, IL-6, CTSD, ADAM-TS15, and FURIN; all had higher levels in leukaemia except for ADAM-TS15, which had a lower leukaemia/control ratio (the full protein list is presented in Supplementary Table 3a). The $\overline{ER}$was 0 and average AUC 1.

Lymphoma vs controls

The top 10 differentiating proteins between lymphoma and healthy controls were: PAI, MMP-9, VIM, AZU1, MPO, HGF, PDGF-A, S1000A11, TGF-α, and ADAM-TS15, all with higher levels in lymphomas except for ADAM-TS15, which had a lower ratio (the full protein list is presented in Supplementary Table 3b). The $\overline{ER}$ was 0 and average AUC 1.

Leukaemia vs lymphoma

The top 10 identified proteins distinguishing leukaemias and lymphomas were: MMP-9, PDGF-A, SPARC, PGLYRP1, vWF, TNFRSF10C, PAI, TR, TGF-α, and GPNMB. In general, they were less expressed in leukaemic samples compared to lymphomas, but with certain exceptions

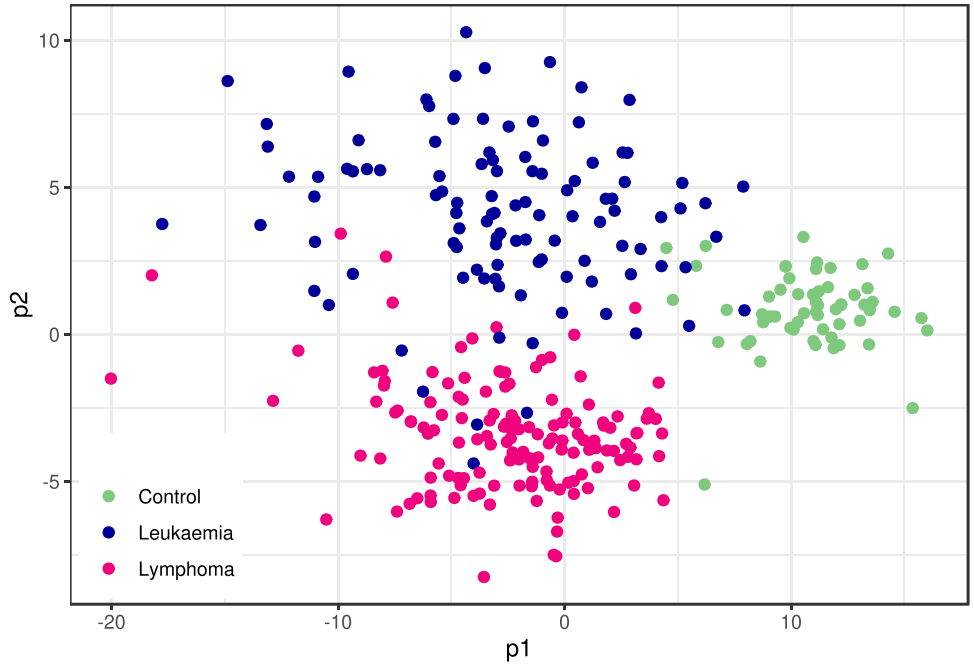

Fig. 1. Highly significant differences in plasma protein separating leukaemia and lymphoma patients and healthy controls. Partial least squares discriminant analysis (PLS-DA) score plot comparing leukaemia, lymphoma and controls. The cross-validated average error rate is 0.029.

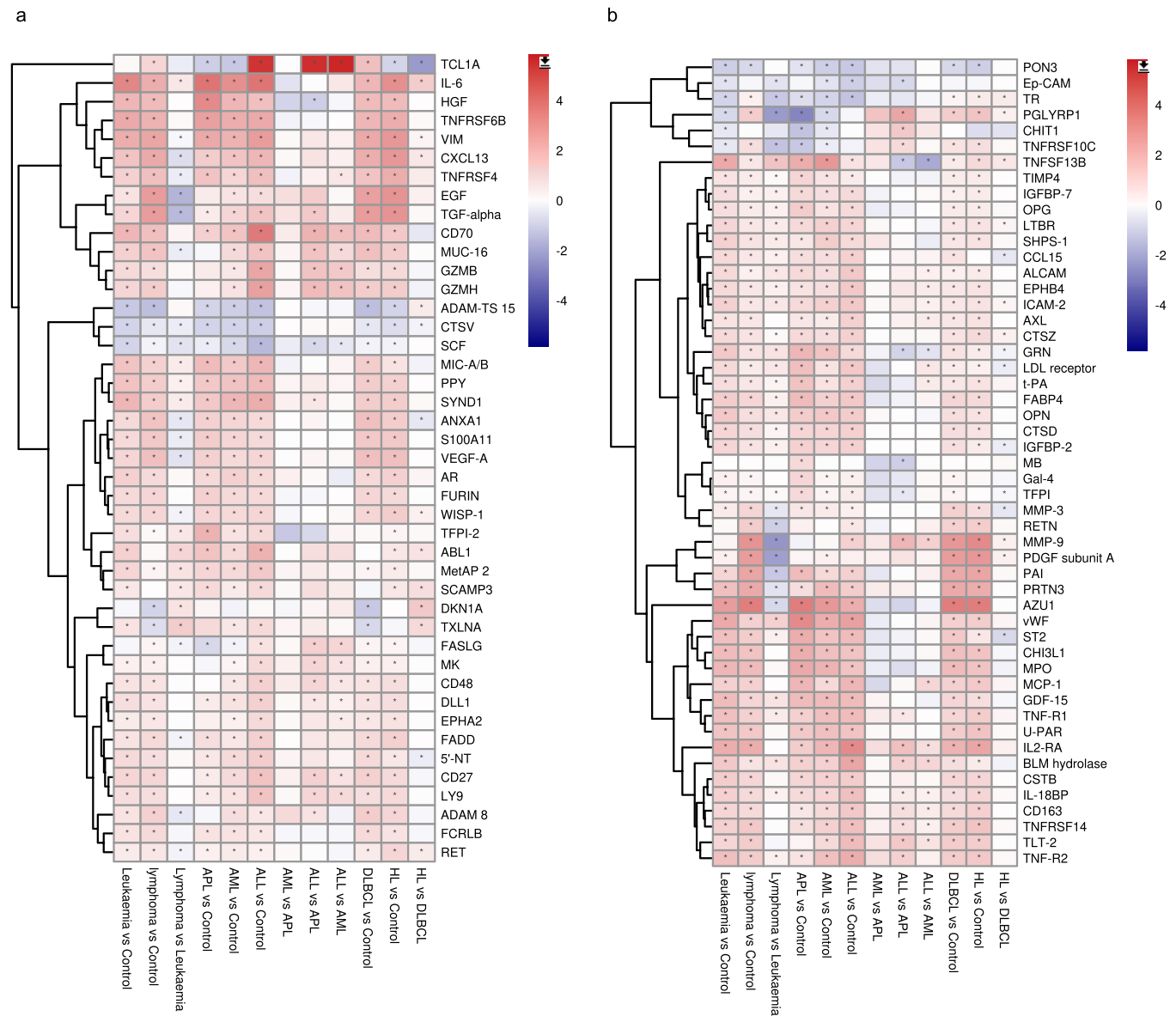

Fig. 2. Association between protein level and patient groups. The heatmap shows log2(fold change) for each of the a) ONCII and b) CVDIII proteins for the pairwise comparisons; Leukaemia vs Control, lymphoma vs Control, Lymphoma vs Leukaemia, APL vs Control, AML vs Control, ALL vs Control, AML vs APL, ALL vs APL, ALL vs AML, DLBCL vs Control, HL vs Control, and HL vs DLBCL. Only proteins with a significant difference with a fold change > 2 for at least one of the comparisons are included in the figure.

such as vWF which was higher (a full protein list is presented in Supplementary Table 3c). Ten 5-fold cross-validations were performed in which each sample was predicted as a test example 10 times. 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 $\overline{ER}$ 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.

AML vs APL vs ALL vs controls

The changes in protein levels remained significant between the different leukaemia subgroups vs controls, respectively (Fig. 2, full protein list in Supplementary Tables 4a-f). AML being the largest subgroup in the analysis had 7 of the 10 top hit proteins common with the whole leukaemia group, while ALL and APL shared respectively 3 of the top 10 proteins. Three of the most differentiating proteins between ALL and AML/APL included TCL1A, CD27, and CD48.

DLBCL vs HL vs controls

Many of the 10 top plasma proteins were re-detected when comparing samples separately from HL and DLBCL vs healthy controls (Fig. 2, full protein list in Supplementary Tables 5a). A totally different set of proteins emerged when comparing HL vs DLBCL with higher protein levels in HL (Supplementary Table 5c).

Variable subset selection

A VSS procedure was used to select a subset of proteins resulting in the best PLS-DA model distinguishing the two groups. The VSS is summarized in Fig. 3 as the number of times that each variable is selected.

Functional groups

To add to the specificity of functional subsets/groups of proteins (as

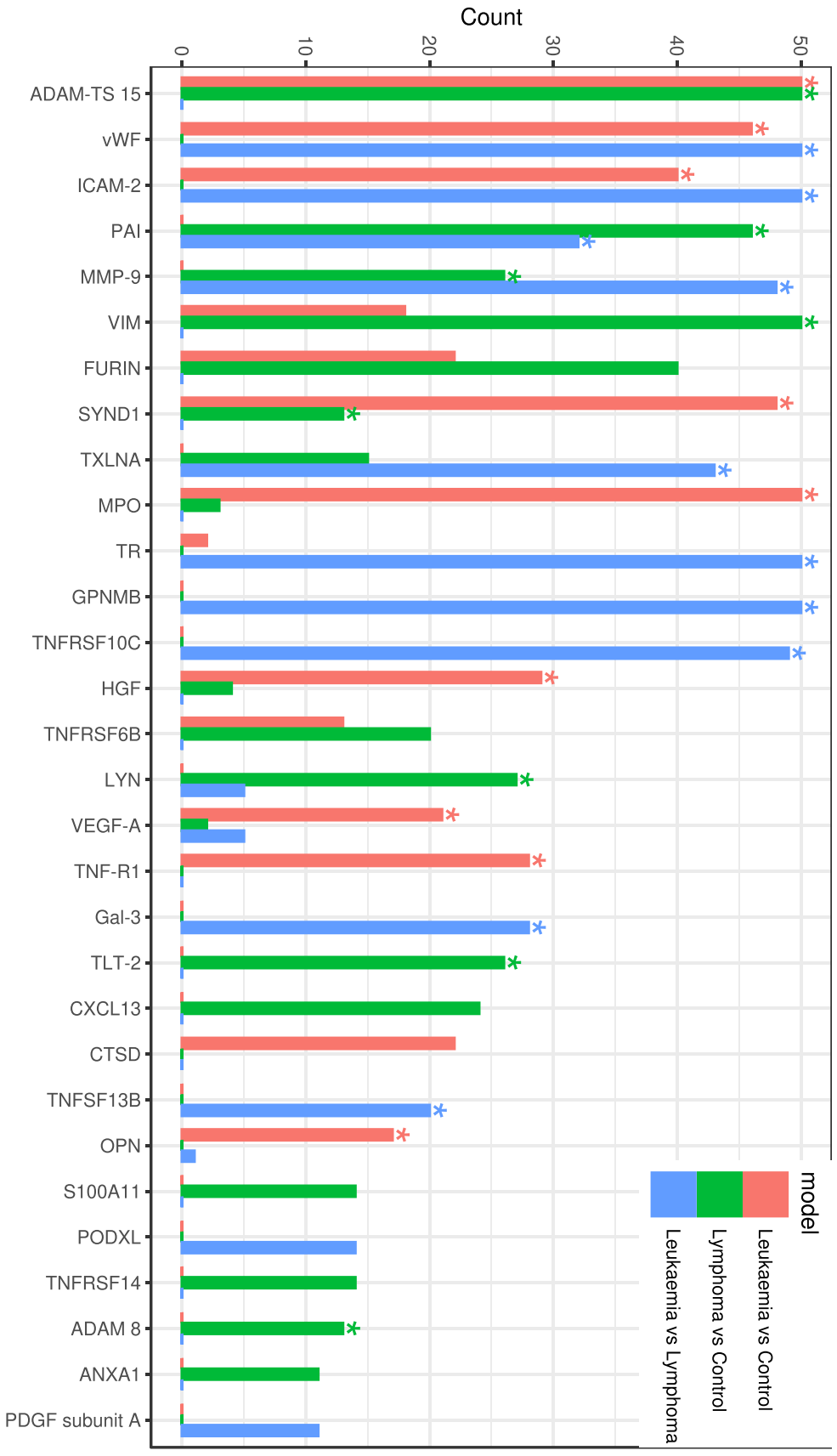

Fig. 3. Variable subset selection to find small subsets of proteins discriminating between groups. The number of cross validation folds in which the protein is selected. The total number of cross validation folds is 50, this means that the variable is selected in every fold if the count is 50. The variables selected in the full model (based on all data) are indicated by * . Only variables selected at least ten times in any of the three models are included in this figure.

predefined by Olink, Supplementary Figure 1 and Supplementary Table 2), which can be important for distinction of patient groups, the $\overline{ER}$ for each of the biological processes as well as all and no proteins, respectively, was computed. Top hits are presented in Table 2 and the $\overline{ER}$ for all biological processes in Supplementary Table 6. All subsets of proteins improved the ability to separate groups, compared to using age and gender only.

Discussion

The aim of this study was to investigate if PEA could differentiate between heterogenous haematological malignancies and controls. It was possible to demonstrate that PEA could discriminate not only between control and disease samples, but also between various haematological diseases. The performance of the PEA has previously been reported by [13], while others [15] have reported validation of selected proteins from the CVDIII panel using Enzyme-Linked Immunosorbent Assay (ELISA). All proteins are classified according to class, disease area, and tissue expression based on public-access bioinformatic databases such as Uniprot and Human Protein Atlas.

The proteins tested for in the panels were pre-selected and many patterns seen here confirm previously reported individual studies, which can be considered as clinical validation. However, in attempting to recognise themes explaining the protein motives which the data illustrated, evidence was found that PEA offered a deeper understanding of the biology of haematological diseases. All proteins discussed are presented in Supplementary Table 1, the complete protein list in Supplementary Table 2 and the biological processes involved in Supplementary Figure 1.

The interesting findings of this study are to be viewed in the light of two limitations. One is the unbalanced number of patients in the different disease groups. It was not possible to overcome this, as the samples were already collected. Another is the lack of validation using a standard method such as ELISA, which was not part of the original study design and samples remaining did not suffice.

While leukaemia samples showed as expected altered levels of proteins associated with haemostasis[16] and stem cell differentiation [17], an inflammatory theme was also prominent. Prevalent markers of cell differentiation discriminated between ALL and AML samples. As for lymphoma, the dominant themes were of altered TME, lymphoma dissemination and increased activity of the myeloid compartment.

Table 2
Protein subsets important for disease separation. Average error rate ($\overline{ER}$) for the most informative protein subsets for separating patients with leukaemia from controls, lymphoma from controls and leukaemia from lymphomas. A low ($\overline{ER}$) indicate a high ability to separate the different subgroups.

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

Haemostasis

Thrombocytopenia and disturbances of blood coagulation are pivotal clinical features of acute leukaemia [16]. Von Willebrand factor (vWF) levels are higher in leukaemia and lymphoma patients using analysis of procoagulant markers and gene mutations[18]. The present data indicate increased levels of both vWF and FURIN, but decreased levels of a disintegrin and metalloproteinase with thrombospondin motifs 15 (ADAM-TS15). vWF, secreted by endothelial cells and which is important for the adhesion of platelets to sites of vascular injury [19], is activated after a proteolytic reaction mediated by FURIN [19,20]. ADAM-TS15, well known to inhibit angiogenesis and promote vascular apoptosis[21], belongs to the same family of metalloproteases as ADAM-TS13, which normally decreases the activity of vWF[22]. However, there is no current data indicating that the lower levels of ADAM-TS15 reflect decreased degradation of vWF multimers.

Inflammation

In agreement with these results, several previous studies reported high levels of circulating inflammatory cytokines such as interleukin-6 (IL-6) and transforming growth factor alpha (TGF-α) in patients with leukaemia[23,24]. Members of the TNF-R superfamily are central to the inflammatory response, but also contribute to cell differentiation and survival [25]. One of the most prominent proteins in leukaemia was myeloperoxidase (MPO), confirming previous reports of its paramount role as a central lineage marker for myeloid cells and a prognostic marker in AML, where high expression in leukaemic cells is well known to be indicative of therapy sensitive disease[26]. The higher expression of MPO seen also in lymphomas strengthens previous evidence of the substantial role of cells of the myeloid compartment. For example, infiltration by tumour associated neutrophils (TAN) in patients with DLBCL is an important marker for lymphoma progression and prognosis [27]. It is well known that myeloid derived suppressor cells (MDSC) are increased in the peripheral blood at diagnosis in HL as well as non-Hodgkin lymphoma[28]. Furthermore, leucocytosis and an increased neutrophil to lymphocyte ratio in the blood have consistently been related to a worse outcome in HL [29,30].

Cell to matrix integration

The disentanglement of malignant hematopoietic cells from the bone marrow is a crucial step in the pathogenesis of leukaemia. In this study a high expression of proteins connected to cell to matrix interactions and cell stability in leukaemia samples was observed. Syndecan-1/ CD138 (SYND1) has been found to be expressed in AML using electron microscopic IHC [31]. Using ELISA in a study of 24 patients, SYND1 determined in both serum and leukocytes correlated with prognosis in the soluble form [32]. Furthermore, in the present study, high levels of the cell adhesion regulator intercellular adhesion molecule 2 (ICAM2) were noted, the role of which well documented in AML[33,34].

Vimentin (VIM) is a protein contributing to the cytoskeleton of mesenchymal cells with a well-documented role in the process of epithelial to mesenchymal transition (EMT) which is vital in the dissemination of solid cancers and haematological malignancies [35]. VIM expression in AML using RNA sequencing shows that upregulation is linked to poor clinical outcome [36].

VIM is also a well-studied marker of lymphoma. More than two decades ago, using IHC with monoclonal antibodies, discrepancies were found in the staining pattern depending on the antibody used [37]. Nevertheless, differential in-gel electrophoresis proteomics implicates VIM in DLBCL cell lines resistant to standard chemotherapy in DLBCL [38].

Tumor microenvironment (TME)

TME is an important contributor to pathogenesis and prognosis of B cell lymphomas[39]. Plasminogen activator inhibitor-1 (PAI) is an inhibitor of fibrinolysis and matrix metalloproteases known to promote angiogenesis and inhibit apoptosis contributing to therapy resistance and worse prognosis in cancer [40]. The higher PAI levels seen in HL here is supported by a study using ELISA on plasma samples in untreated HL patients [41].

Lymphoma dissemination

Activation of matrix metalloproteinases such as MMP-9 is thought to facilitate dissemination of lymphoma by degradation of the extracellular matrix [42]. and the finding here of higher levels in lymphoma substantiated a study using immunoassay in HL and Non-HL patients vs controls [43]. Calgizzarin or metastatic lymph node gene 70 protein (S100A11) is crucial in cytoskeleton-membrane dynamics and over-expression has been reported in a variety of epithelial tumours by a positive feedback loop with members of the epidermal growth factor (EGF) family [44,45]. Moreover, using a DLBCL gene expression microarray to analyse immune related genes aiming to build an immune related prognostic profile, expression of S100A11 in TME has been related to prognosis [46].

Platelet-derived growth factor subunit-A (PDGF-A) is a member of the receptor tyrosine kinase (RTK) family, through which activated signalling has been known to have a central role in oncogenesis. The high levels of PDGF-A in HL samples verified previous studies, using identifying antibodies, that showed expression of several RTK family member including PDGF-A by Reed-Sternberg cell lines (RSC) and activated PDGF-A in untreated classical HL cases[47,48].

Lymphoma cells, especially in HL, rely on various cytokines for survival, growth, and immune escape, which may explain why most protein levels were higher in this group compared to DLBCL. Interactions with tissue fibroblasts or other components of the stroma as well as myeloid-derived suppressor cells (MDSC), have been shown to modulate immune reactions in lymphoma patients[28,49]. The top marker discriminating lymphoma subtypes was cyclin-dependent kinase inhibitor 1 (DKN1A or p21), a key regulator of cell cycle progression [50] P21, regulated by cellular tumor antigen p53 (p53), which can arrest the cell cycle or sustain its progression. The present findings corroborate studies where dysfunctional p53 and p21 was reported in both HL and non-HL lymphomas using IHC and gene sequencing [51, 52]. Proteins associated with B-cell regulation were also seen at higher levels in HL vs DLBCL. LYN is a non-receptor tyrosine kinase inhibitor important for innate and adaptive immune responses and haematopoiesis and plays an important role in B-cell development, activation and inhibition[53]. A study reported intracellular LYN expression in RSC in HL using IHC to Epstein-Barr virus (EBV) infection[54]. Alpha-taxilin (TXLNA or IL-14) is a cytokine detected in aggressive lymphoma effusion fluid by western blot and is involved malignant B-cell regulation [55]. The exact mechanism behind the differences in protein profiles between HL and DLBCL is elusive, but could be related to different properties of the malignant cells and the TME interaction.

Only minor changes in protein expression were detected between samples from patients with AML and APL, possibly influenced by the low number of APL patients in this study. On the other hand, although few and of different phenotypes, comparison of the ALL cohort with the AML group revealed a lymphoid trio: T-cell leukaemia protein 1 A (TCL1A), B-lymphocyte activation marker (CD 48 or BLAST-1), and CD27 antigen, all with higher expression in ALL. Activating mutations of TCL1A are well known in T-cell leukaemia, but high expression of the TCL1 gene is also reported in aggressive pre-B-ALL [56,57]. CD48 is indicative of transformed B-lymphoblasts, but downregulation of this NK-cell receptor is described as a mechanism for AML cells to evade the immune system [58,59]. CD27 has a B-cell activation regulatory function via its mediator CD70, but is also connected to the unfavourable stem cell signature in AML [60].

For the most part, the data in this study has confirmed previous published results regarding various protein attributes. However, when the misclassified samples were considered, the fact that all four ALL samples classified as lymphomas five times or more came from patients with LBL was particularly noteworthy. Lymphoblastic leukaemia and lymphoma are not separated in the WHO classification, but represent different manifestations of the same disease with clonal expansion of lymphoblasts in bone marrow and/or extramedullary sites [61]. The clustering of the LBL cases as lymphoma rather than leukaemia is consequently correct from biological point of view, which strengthens the belief that the PEA technology can identify genuine biological hallmarks of these diseases.

Conclusion

The PLS-DA model distinguished with high precision between leukaemia, lymphoma, and healthy controls. Further studies are required to elucidate the full clinical value of multiplex PEA in identifying prognostic and predictive biomarkers in haematological malignancies. Based on the promising results of this study, it is plausible to take this technique closer to the clinic by attempting to correlate protein levels with clinical prognostic markers and eventually treatment effect. Another prospect would be to use the PEA technique as a tool for detection of measurable residual disease. Identifying markers able to detect early re-emerging leukaemia or lymphoma clones in a blood sample is yet another promising feasibility.

Data Availability

Additional data is provided in the supplementary file. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request..

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Authorship contribution

Amal Abu Sabaa: data acquisition, draft of and final approval of manuscript. **Qiujin Shen**: study design and sample analysis, revision and final approval of manuscript. **Emma Bergfelt Lennmyr**: data acquisition, revision and final approval of manuscript. **Anna Pia Enblad:** data acquisition, revision and final approval of manuscript. **Gustav Gammelgård:** data acquisition, revision and final approval of manuscript. **Daniel Molin:** data acquisition, revision and final approval of manuscript. **Anders Hein:** data acquisition, revision and final approval of manuscript. **Eva Freyhult:** statistical analysis, revision and final approval of manuscript. **Masood Kamali-Moghaddam:** study design, sample analysis, revision and final approval of manuscript. **Martin Höglund:** study design, revision and final approval of manuscript. **Gunilla Enblad:** study design, draft of and final approval of manuscript. **Anna Eriksson:** study design, data acquisition, draft of and final approval of manuscript.

Conflict of interest

Daniel Molin: Honoraria from Roche, Merck, Bristol-Myers Squibb, and Takeda. All other authors have no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2022.06.005.

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