Plasma protein analysis of patients with different B-cell lymphomas using high-content antibody microarrays

Christoph Schröder[1], Harish Srinivasan[1], Martin Sill[2], Jakob Linseisen[3], Kurt Fellenberg[4], Nikolaus Becker[5], Alexandra Nieters[6] and Jörg D. Hoheisel[1]

[1] Division of Functional Genome Analysis, Deutsches Krebsforschungszentrum, Heidelberg, Germany
[2] Division of Biostatistics, Deutsches Krebsforschungszentrum, Heidelberg, Germany
[3] Institute of Epidemiology, Helmholtz Zentrum München, German Research Centre for Environmental Health (HMGU), Neuherberg, Germany
[4] Bioinformatics, Forschungszentrum Borstel, Borstel, Germany
[5] Division of Cancer Epidemiology, Deutsches Krebsforschungszentrum, Heidelberg, Germany
[6] Molecular Epidemiology, Center of Chronic Immunodeficiency, University Medical Center Freiburg, Freiburg, Germany

Purpose: In this study, plasma samples from a multicentric case-control study on lymphoma were analyzed for the identification of proteins useful for diagnosis.

Experimental design: The protein content in the plasma of 100 patients suffering from the three most common B-cell lymphomas and 100 control samples was studied with antibody microarrays composed of 810 antibodies that target cancer-associated proteins. Sample pools were screened for an identification of marker proteins. Then, the samples were analyzed individually to validate the usability of these markers.

Results: More than 200 proteins with disease-associated abundance changes were found. The evaluation on individual patients confirmed some molecules as robust informative markers while others were inadequate for this purpose. In addition, the analysis revealed distinct subgroups for each of the three investigated B-cell lymphoma subtypes. With this information, we delineated a classifier that discriminates the different lymphoma entities.

Conclusions and clinical relevance: Variations in plasma protein abundance permit discrimination between different patient groups. After validation on a larger study cohort, the findings could have diagnostic as well as differential diagnostic potential. Beside this, methodological aspects were critically evaluated, such as the value of sample pooling for the identification of biomarkers that are useful for a diagnosis on individual patients.

Correspondence: Dr. Christoph Schröder, Division of Functional Genome Analysis, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 580, D-69120 Heidelberg, Germany
E-mail: christoph.schroeder@dkfz.de
Fax: +49-6221-424687

Abbreviations: CLL, chronic lymphocytic leukemia; **DLBCL**, diffuse large B-cell lymphoma; **FL**, follicular lymphoma; **HGNC**, human genome nomenclature consortium; **LIMMA**, linear models for microarray data; **logFC**, log-fold change; **PBSTT**, PBS supplemented with Tween-20 and Triton X-100

1 Introduction

Proteins are the molecule class that executes most cellular functions, and many regulative processes take place at the protein level. The latter is reflected by the fact that 5–10% of mammalian genes encode for proteins that modify other proteins. Also, 98% of all current therapeutic targets are proteins. Their obvious central role in cellular activity promotes the search for protein biomarkers as a means for diagnostics.

Variations that are critical to disease manifestation occur at the levels of amino acid sequence, protein structure, concentration, location, and interaction. This variety and the direct link to function represent the principal advantages of protein-based diagnostics compared to analyses of nucleic acids. In a single-molecule class, classifiers can be defined by a combination of different types of variations, thus facilitating standardization. To date, however, only a very small percentage of the large number of existing proteomic disease biomarkers is used in clinical practice [1–3].

This lack of translation is mainly caused by bottlenecks in marker validation [4, 5]. Validation requires an assessment in a standardized manner with a sufficiently large number of samples as well as appropriate controls [6–9]. The majority of current protein biomarkers were identified by MS. This technology is not easily adaptable to an analysis of large sample numbers with sufficient sensitivity [1, 10]. In addition, MS cannot analyze the many different types of protein alteration in one assay format and the transition of the biomarker information into an assay, which can be implemented in a clinical routine setting, such as an immunoassay, remains difficult [10–12]. These obstacles could be overcome by directly using an immunoassay in biomarker discovery and the validation phase [1, 9]. Antibody microarrays are a multiplexed form of immunoassay. In a single assay format, variations in the abundance of proteins, structural differences in form of protein isoforms or protein modifications, and the occurrence of protein interaction can be analyzed. Although in complex analyses a sandwich assay format is made impossible by the number of antibodies displayed on the array, sensitivity can be similar to that of ELISA [13, 14] or better, down to the level of single-molecule detection even in the absence of a signal amplification scheme [15]. In terms of technical performance, processes have been established, which permit analyses with a degree of specificity, robustness and reproducibility that meets the requirements of clinical applications [16]. Concomitantly, the corresponding cost per analyte is considerably lower than in ELISA because of the simultaneous analysis of very many analytes and much less consumption of the expensive binder molecules and the precious clinical sample material.

Many immunobased proteome analyses performed to date have concentrated on proteins from human body fluids, in particular serum and plasma. One reason was the relative ease of access, although robust protocols for the analysis of tissue proteins exist [17]. Any diagnosis based on serum or plasma would be of only minimally invasive nature and thus widely applicable. Already analyses on arrays of limited complexity performed with relatively few samples demonstrated the technique's potential [18–21]. Concerning lymphoma, Belov et al. [22] used antibody microarrays directed at 82 surface proteins to profile lymphoma and leukemia cells by a cell-capture technique. Here, we looked at 200 plasma samples taken from patients suffering from the three most common B-cell lymphomas and age- and sex-matched healthy controls. The microarray used for the analysis consisted of 810 antibodies that target 741 different cancer-related proteins and six nonantibody binders—modified forms of receptors as well as ligands involved in apoptosis [16]. The plasma samples were collected as part of a population-based multicentric case–control study on lymphoma [23], assuring high-quality and matching epidemiological background of lymphoma cases and controls. In a two-phase approach, we initially analyzed sample pools for the identification of relevant markers, followed by individual analyses of the samples. By this process, we did not only identify common markers but could evaluate immediately their value for the diagnosis of individual patients. The markers enable a precise discrimination of the lymphoma entities based on their plasma profile. Moreover, we were able to identify subtypes of considerably differing profiles within each of the lymphoma classes.

2 Materials and methods

2.1 Array production

Microarrays were produced and analyzed using protocols and strict quality control procedures as reported earlier [16, 24]. In brief, a set of 668 target proteins was selected on the basis of transcriptional studies of different cancer entities. Affinity-purified polyclonal antibodies were produced in rabbits by Eurogentec (Seraing, Belgium). Additionally, 142 antibodies were purchased from different sources or provided by collaborating partners. Lastly, six other proteins were added, which are known to exhibit specific binding. A complete list of binders is given in the Supporting Information Table 1. They were characterized by different techniques, such as Western blotting, immunohistochemistry, or fluorescence imaging. In addition, the array has been used to analyze about 2000 protein samples from different sources (tissues, cell culture, secretome, serum, urine), providing also information about their quality [16, 25, 26].

The array composition and layout can be accessed at the public repository ArrayExpress (A-MEXP-1939). All molecules were immobilized on epoxysilane slides (Schott Nexterion, Jena, Germany) at a concentration of 1 mg/mL in spotting buffer made of 10 mM sodium borate, pH 9.0, 125 mM $MgCl_2$, 0.005% w/v sodium azide, 0.25% w/v dextran, 0.0005% w/v [octylphenoxy]polyethoxyethanol, using a Microgrid microarraying robot (BioRobotics, Cambridge, UK) and SMP3B pins (Telechem, Sunnyvale, CA, USA). All arrays used in this study were from a single production batch of some 1400 microarrays. Each array comprised 1800 features. All antibodies were spotted at least twice in a randomized pattern in different array sectors. For control purposes, antibodies were spotted, which bind the proteins beta-actin, human IgM, glyceraldehyde-3-phosphatase dehydrogenase, and albumin. For the same purpose, a polyclonal antibody was added, which is directed against whole human serum protein. Negative controls consisted of spotting buffer as well as further control antibodies, for example, molecules directed

against mouse IgG. All these controls were spotted in 8–18 copies across the entire array to ensure an even distribution of the controls. After printing, the slides were kept at 4°C overnight, washed ten times in PBS supplemented with 0.05% w/v Tween-20 and 0.05% w/v Triton X-100 (PBSTT), and blocked by an incubation in 4% w/v skimmed milk powder and 0.05% w/v Tween-20 in PBS overnight. After blocking, the slides were washed four times with PBSTT, twice with 0.1× PBS, and dried by an air stream prior to storage in a humidity chamber at 4°C. While the analyses reported here were done within a few weeks of array production, the arrays could be stored for up to 2 years without a change in performance (data not shown).

2.2 Plasma samples

The plasma samples were derived from cases and controls recruited into a population-based multicentric case–control study on lymphoma conducted in Germany between 1999 and 2003 [23]. Written informed consent was given by all donors and full ethics approval was obtained. In total, 61 samples of diffuse large B-cell lymphoma (DLBCL), 19 samples of chronic lymphocytic leukemia (CLL), and 20 follicular lymphoma (FL) samples as well as 100 age- and gender-matched controls were studied. All samples were derived from non-smokers to prevent additional sources of bias in this study. None of the patients received any cancer-related therapy prior to sampling.

Since depletion of high-abundance proteins introduces a strong bias in protein representation [16], the samples were subjected to labeling without further processing. A commonly used reference sample was prepared by mixing 5 μL of all 200 plasma samples. In addition, sample pools specific for the four respective sample types (DLBCL, CLL, FL, healthy controls) were generated accordingly. For all samples and pools, the final protein concentration was measured by the bicinchoninic acid assay (Thermo Scientific, Dreieich, Germany).

2.3 Sample labeling

Label reactions were performed at a protein concentration of 4 mg/mL with 0.4 mg/mL of the NHS-esters of the fluorescence dyes Dy-549 or Dy-649 (Dyomics, Jena, Germany), respectively, in 100 mM sodium bicarbonate buffer, pH 9.0, 1% w/v Triton X-100 on a shaker at 4°C. After 1 h, the reaction was stopped by addition of hydroxylamine to a final concentration of 1 M. Unreacted dye was removed 30 min later and the buffer changed to PBS using Zeba Desalt columns (Thermo Scientific). Subsequently, Complete Protease Inhibitor Cocktail (Roche, Mannheim, Germany) was added as recommended by the manufacturer. All labeled protein samples were stored in aliquots at −20°C until used.

2.4 Incubation

Incubations were performed as described in detail previously [16,24]. Homemade incubation chambers were attached to the array slides. The arrays were blocked in a casein-based blocking solution (Candor Biosciences, Weißensberg, Germany) on a Slidebooster instrument (Advalytix, Munich, Germany) for 3 h. A volume of 30 μL labeled sample and 30 μL labeled reference were mixed with 540 μL blocking buffer supplemented with 1% w/v Tween-20 and 1× Complete Protease Inhibitor Cocktail. After incubation for 15 h, the slides were thoroughly washed with PBSTT, rinsed with 0.1× PBS as well as water, and dried in a stream of air. In order to control for possible dye-specific effects, incubations with reference samples were performed, which had been labeled with Dy-549 or Dy-649, respectively. Pearson's correlation coefficients of the two color channels were in the range of 0.92 ≤ r ≤ 0.98 throughout. To assess if there were any differences between the results from different days of incubation, 10% of the samples were repeatedly incubated at different dates. No significant variations could be observed; in a hierarchical clustering, the incubations with identical samples matched perfectly (data not shown).

2.5 Data analysis and statistical testing

Slide scanning was done on a ScanArray 4000 XL unit (Packard, Billerica, MA, USA) using identical instrument laser power and PMT. Relevant parameters, such as the concordance of the two color detection channels, were carefully validated throughout. Spot segmentation was performed with GenePix Pro 6.0 (Molecular Devices, Union City, CA, USA). Resulting data were analyzed using the linear models for microarray data (LIMMA) package [27] of R-Bioconductor after uploading the mean signal intensities. For normalization, a specialized invariant Lowess method was applied as described before [28]. In the analyses, duplicate spots were accounted for [29]. For analysis of the sample pools and individual samples, a one-factorial or two-factorial linear model, respectively, was fitted with LIMMA resulting in a two-sided t-test or F-test based on moderated statistics. All presented p values were adjusted for multiple testing by controlling the false discovery rate according to Benjamini and Hochberg [30]. In the M-CHiPS [31, 32] analysis, the mean signal intensities for each spot were used. Data were normalized by log-linear regression. Correspondence analysis resulted in a biplot of both differentially abundant proteins and the samples. In the plot, only proteins are shown, which exhibited significant variations ($p < 0.05$) as determined by LIMMA (multiclass).

3 Results

In this study, 200 plasma samples from individual study participants were analyzed. Stringent selection criteria were

applied in order to minimize the risk of unaccounted factors that could influence the results. All biomaterial originated from a carefully designed national case–control study on lymphoid neoplasms [23]. Only plasma of nonsmoking study participants was used. None of the lymphoma patients had undergone therapy prior to sampling. In addition, samples from a group of healthy controls were analyzed, who matched the lymphoma patients in age and gender. Also for the array analyses, strict quality measures were applied as reported in detail earlier [16, 24]. Only antibody microarrays of a single production batch of 1400 arrays were used. Based on dual-color detection, each sample was incubated in presence of an aliquot of a common reference for normalization. Overall, the process met the quality requirements set forth by regulative agencies for DNA-microarray analyses.

3.1 Pool analyses revealed distinct protein profiles for the three B-cell lymphomas

In a first analysis, pools made from all the plasma samples of each lymphoma entity and the controls were studied, each in four technical replicas. To visualize differences among protein profiles, correspondence analysis was applied as implemented in the M-CHiPS software package [31, 32]. It is a computational method for investigating associations between variables, such as proteins and patient samples. Multidimensional data are projected into two dimensions for visual inspection, thus revealing associations between them. In the resulting biplot (Fig. 1), each pool sample is depicted as a colored square. Proteins that exhibited strong differential abundance levels are shown as black dots. The closer the colocalization of two squares, the higher is the degree of association between them. Samples located in the same direction from the plot centroid exhibit a similar expression pattern. The further a protein is located in the same direction as a set of samples, the more specific it is for this set. All experimental repetitions produced well-defined discrete clusters, indicating the reproducibility of the analysis. The three disease pools were clearly distinct from each other and the healthy control group, illustrating significant variations in protein content. For each of the three lymphoma types, specifically associated proteins could be determined.

In addition, this dataset was analyzed by LIMMA to screen for proteins with a strong differential signal between the groups and a low technical variability. The resulting p values were used for screening purposes only. In contrast to p values computed from the analysis with individual biological samples, they cannot be used to draw conclusions about the underlying population of B-cell lymphoma, since the technical replicates of the pooled samples are not statistically independent. The analysis revealed a set of 234 proteins (corresponding to 31% of the studied proteins) with differential levels among the pools of lymphoma cases and healthy controls.

Matching to the correspondence analysis, a heatmap of the respective expression levels (Fig. 2) showed highly distinct patterns. In both analysis types—M-CHiPS and LIMMA—pooled CLL plasma samples differed more from the pool of healthy controls than DLBCL and FL did. The last two were also more similar to each other. This correlates with the protein numbers that were found to be associated with the respective disease type (Fig. 2D). For CLL, 218 proteins exhibited differential values, corresponding to more than a quarter of the proteins under analysis. In DLBCL and FL samples, 22 and 13 proteins were present at differential protein levels, respectively. The complete list can be found in the Supporting Information Table 2.

For CLL and DLBCL, very strong differences of abundance were recorded by some of the non-antibody features. For DLBCL, significantly lower signals were recorded by a fusion protein of tumor necrosis factor receptor superfamily member 6 (TNR6), also known as FAS or CD95, with an Fc fragment of IgG. Signals at this feature are derived from binding ligands in the plasma samples such as the Fas ligand (TNFL6). Contrary, in CLL there was significantly stronger binding to this receptor fusion protein. In addition, higher signals were recorded by fusion proteins of three other receptors of the TNF family: TNR1B, TR13B, and TNR21. Lower signals were obtained on a fusion protein of the ligand TNF13, indicating that its binding partners are less abundant in the CLL samples. For CLL, a strong binding to four different receptors of the TNF family demonstrated stronger binding of ligands in the plasma of CLL patients compared to healthy controls. Consistently, for the ligands TNF10 and TNF14, higher signals were obtained by antibody features in the respective samples.

3.2 Individual analysis of 130 plasma samples

Subsequently, protein profiles of the samples were analyzed individually in order to confirm the usability of markers identified from the pooled samples for the diagnosis of individual patients. In contrast to the pooled sample approach, here the variation of protein abundance within the four subgroups can be estimated. The complete set of 100 lymphoma samples as well as 30 samples derived from the healthy controls were labeled and incubated in a dual-color assay competitively with the reference (pool of all samples). The healthy control samples were chosen from the total of 100 samples in a random fashion, but with the restriction that they matched the gender and age distribution of the diseased group. Twenty plasma samples had signal background ratios smaller than two, most probably due to protein degradation during sample storage. Therefore, these samples were not considered in the data analysis. Consequently, 110 incubations were analyzed, corresponding to 52 DLBCL, 18 FL, and 15 CLL patients as well as 25 healthy controls with a matching age and gender distribution (Table 1). Using LIMMA, an eight-factorial model was
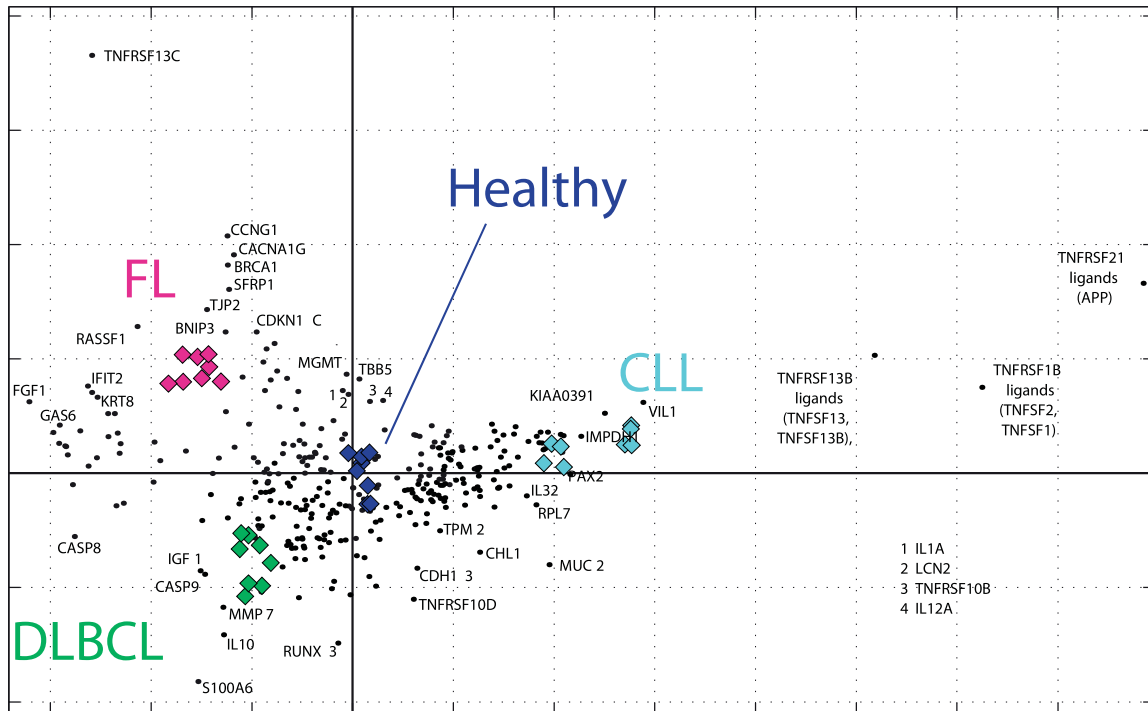
Figure 1. Definition of disease-specific protein profiles with sample pools. Correspondence analysis resulted in a biplot of both differentially abundant proteins and the samples; the two axes represent the first and second principal component, respectively. Sample pools are depicted as squares that are colored according to disease status; black spots stand for differentially expressed proteins. Each pooled sample is represented by eight measurements from incubations on four arrays each as well as two intra-array replicates. Measurements located in the same direction from the centroid exhibit a similar protein level pattern. The smaller the distance between two samples the higher is the concordance of their expression profiles. In addition to more gradual variations, proteins were found, which are particularly associated with the different sample groups. This is indicated by localization in the same direction off the centroid as the respective sample type; the further the distance to the centroid the better is the association. A name tag is attached to the most prominent proteins.

fitted to the data, representing the combinations of gender and the four disease states. In a combined analysis of male and female samples, 21 and 5 differentially abundant proteins, respectively, were identified in CLL and DLBCL plasma as compared to plasma from healthy controls, while no significant variation was found in FL samples (Fig. 3; Table 2). The variations of TR10B and IL12A in CLL patient plasma were observed independently with two different antibodies for each molecule.

3.3 Distinct subtypes in plasma profiles

The comparably low numbers of differential proteins in the individual analyses as well as the lack of a clear separation in hierarchical clustering indicates heterogeneity of the protein profiles within each sample class. To assess the impact of gender, we performed a gender-specific analysis and found the protein AGR2 to be upregulated in the male subgroup of FL patients, while WDR1 was found to be up- and BAX to be downregulated in the female subgroup only (Table 3).

For DLBCL, a gender-based analysis identified two additional proteins, BHE40 and ETS2 to be differentially expressed in males only. For women with CLL, there were another three protein markers, while six others affected only men. In general, a gender-stratified analysis revealed that a major part of the identified proteins was restricted to either the female or the male samples.

No clear correlation with variables, such as gender, age, body mass index, or technical factors, such as date of experiment or label reaction, was found. In individual hierarchical clusterings for the lymphoma types, two subgroups were found for FL (Supporting Information Fig. 1) and CLL (Supporting Information Fig. 2), and three subgroups could be defined for DLBCL (Fig. 4). In addition, two outlying samples were identified in the healthy sample set, two for FL and one for CLL. Distinct sets of differentially expressed proteins were identified for the subgroups and in comparison to the expression in the healthy controls (Fig. 4, Supporting Information Figs. 1 and 2). Interestingly, almost no differentially expressed protein is present in more than one of the subgroups.
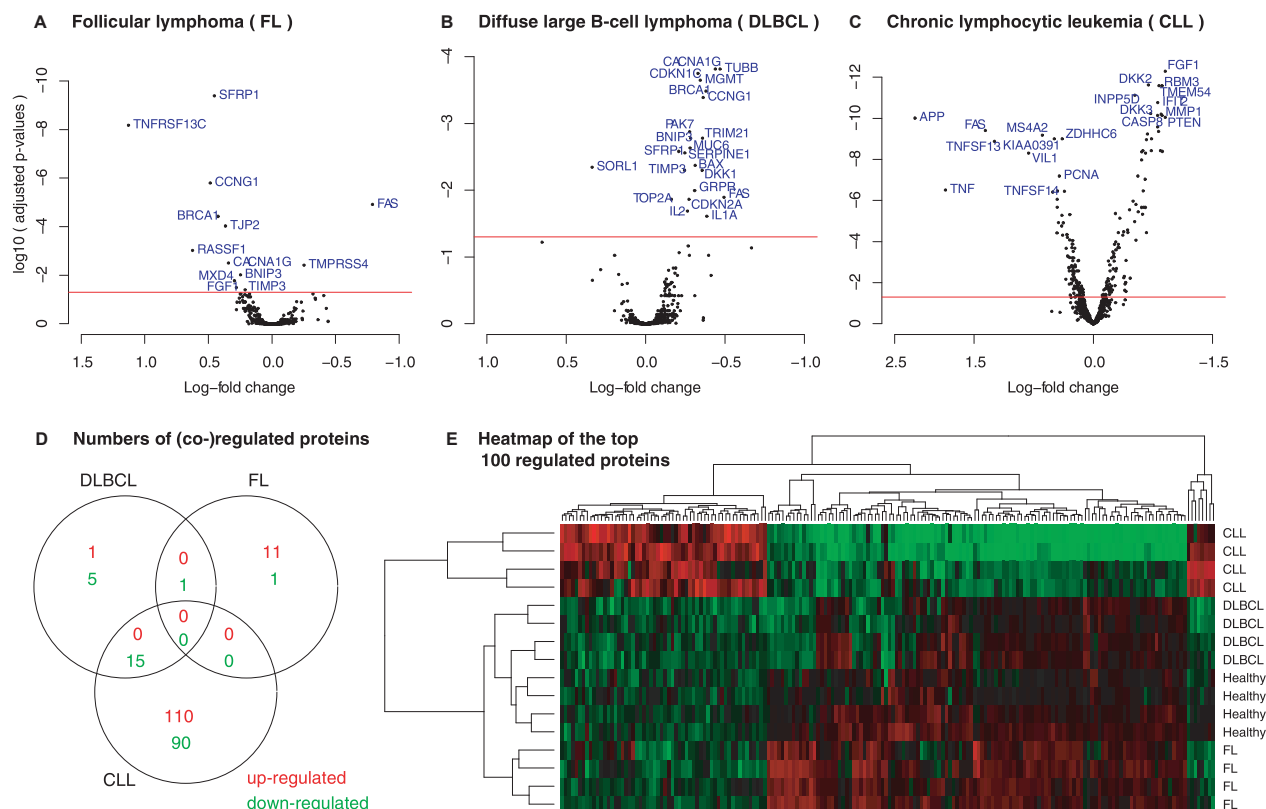
Figure 2. Results of the LIMMA analysis. In the volcano plots (A–C) the adjusted *p* values as well as the corresponding log-fold changes are summarized. Each red line indicates a significance level of *p* = 0.05. (D) The number of proteins with higher or lower abundance in the three lymphomas compared to the healthy controls as well as the degree of coregulation are visualized in a Venn diagram. (E) A heatmap shows the profiles of the 100 most significant proteins (columns) in the sample pools (rows). As indicated by the dendrogram, CLL exhibits a profile that is the most diverse from healthy controls.

3.4 Classification of patients

Due to the high heterogeneity of the plasma profiles within each cancer type, a classification was impossible for individual patient samples, if each lymphoma entity was defined as one group (overall accuracy <50%). When taking the above subgroups into account, however, better results were achieved. A classificator selected by PAM (prediction analysis for microarrays) allocated the samples into the previously defined subgroups with an overall accuracy of 65.5%; 67% of the samples were matched with the appropriate lymphoma type. While only 30% of the healthy samples were correctly classified, a good sensitivity was reached with this classifier. The sensitivity was 71% for CLL, 85% for DLBCL, and 60% for FL.

4 Discussion

Here, we analyzed the protein content of plasma samples that were obtained from cases and respective controls from an epidemiological case–control study of lymphoid neoplasms of patients with different types of B-cell lymphoma and compared them to samples of appropriate healthy controls. By application of stringent selection criteria, utilizing material from an epidemiological study, we made sure that the disease and control samples matched on many aspects, in particular age, gender, and smoke status. Plasma of the cases had been collected prior to any cancer-related treatment. A large proportion (31%) of the studied proteins exhibited a different protein abundance between the different groups compared. This high proportion of identified proteins was

Table 1. Age distribution of samples and controls represented in the analysis of individual samples

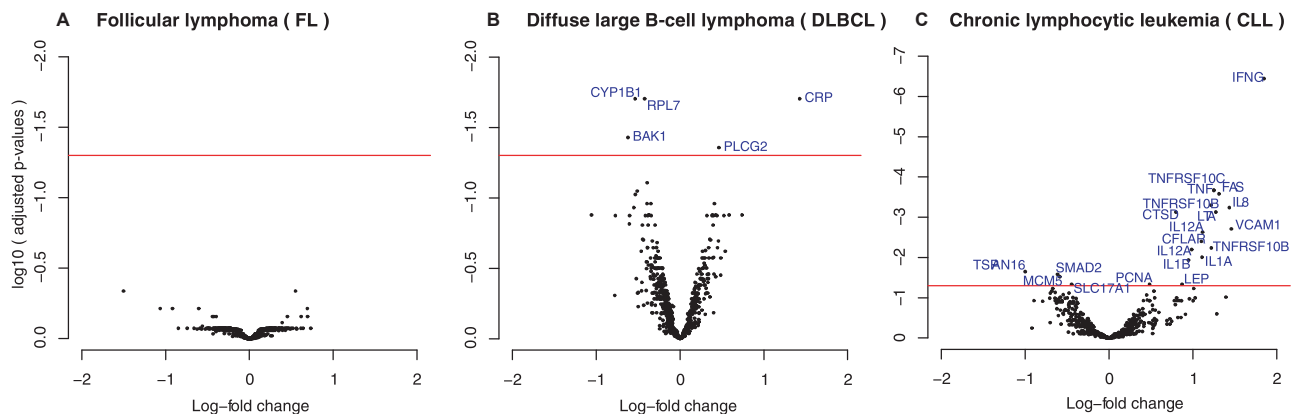| Samples		Min.	First quartile	Mean	Third quartile	Max.
Females	Cancer (*n* = 45)	29.3	52.4	61.8	73.0	80.7
	Control (*n* = 13)	32.6	48.0	60.9	74.5	80.5
Males	Cancer (*n* = 40)	20.1	53.5	58.4	69.6	79.7
	Control (*n* = 12)	25.4	46.1	57.1	72.5	78.1

Figure 3. Individual sample analysis. In an analysis of the individual plasma samples from patients with DLBCL (B) and CLL (C), several proteins exhibited distinct abundance variations as compared to plasma from healthy controls. The volcano plots visualize the *p* values (adjusted for multiple testing) and corresponding log-fold changes. A significance level of *p* = 0.05 is indicated as a horizontal line. Due to high biological variability, no distinct proteins were identified in the FL samples (A).

Table 2. Significant variations in protein levels as elucidated from the analyses of individual samples

Uniprot entry	Uniprot accession	HGNC symbol	logFC	Adjusted *p*-value
CLL associated proteins				
TSN16_HUMAN	Q9UKR8	TSPAN16	−1.00	2.2×10^{-2}
SMAD2_HUMAN	Q15796	SMAD2	−0.61	2.6×10^{-2}
MCM5_HUMAN	P33992	MCM5	−0.59	3.0×10^{-2}
NPT1_HUMAN	Q14916	SLC17A1	−0.45	4.6×10^{-2}
PCNA_HUMAN	P12004	PCNA	0.48	4.6×10^{-2}
CATD_HUMAN	P07339	CTSD	0.80	7.4×10^{-4}
LEP_HUMAN	P41159	LEP	0.87	4.6×10^{-2}
IL1B_HUMAN	P01584	IL1B	0.95	1.2×10^{-2}
IL12A_HUMAN	P29459	IL12A	0.98	6.3×10^{-3}
CFLAR_HUMAN	O15519	CFLAR	1.10	4.0×10^{-3}
IL1A_HUMAN	P01583	IL1A	1.11	9.8×10^{-3}
IL12A_HUMAN	P29459	IL12A	1.11	2.3×10^{-3}
TR10B_HUMAN	O14763	TNFRSF10B	1.21	5.0×10^{-4}
TR10B_HUMAN	O14763	TNFRSF10B	1.22	5.8×10^{-3}
TNFA_HUMAN	P01375	TNF	1.25	2.1×10^{-4}
TR10C_HUMAN	O14798	TNFRSF10C	1.25	2.1×10^{-4}
TNFB_HUMAN	P01374	LTA	1.27	7.4×10^{-4}
TNR6_HUMAN	P25445	FAS	1.31	2.6×10^{-4}
IL8_HUMAN	P10145	IL8	1.43	5.8×10^{-4}
VCAM1_HUMAN	P19320	VCAM1	1.46	1.9×10^{-3}
IFNG_HUMAN	P01579	IFNG	1.85	3.6×10^{-7}
DLBCL associated proteins				
BAK_HUMAN	Q16611	BAK1	−0.62	3.7×10^{-2}
CP1B1_HUMAN	Q16678	CYP1B1	−0.54	2.0×10^{-2}
RL7_HUMAN	P18124	RPL7	−0.42	2.0×10^{-2}
PLCG2_HUMAN	P16885	PLCG2	0.46	4.4×10^{-2}
CRP_HUMAN	P02741	CRP	1.43	2.0×10^{-2}

and are therefore more likely to bind tumor-specific proteins than a randomly selected set of binders.

Clearly, the number of proteins targeted by a microarray contributes to the success, information content, and quality of discovery analyses. Also, adding for each protein, a second antibody that targets a different epitope would strengthen the quality further. Due to the direct labeling protocol, upscaling the number of analytes on the microarray does not interfere with procedural aspects and does not affect quality parameters either, such as sensitivity and specificity. Future microarray releases will consist of more antibodies and cover also protein isoforms.

Among the three investigated lymphoma subtypes, CLL exhibited the most different pattern in both the analysis of the sample pools as well as the analysis of the individual samples. This is not unexpected. CLL is a hematological disease; part of the tumor cells are circulating in the blood stream [33], while FL and DLBCL are solid tumors of lymphoid cells. In transcriptional studies on purified B cells, similar proportions of regulated transcript were found [34]. In plasma, cellular proteins of circulating malign B cells cannot be analyzed, since cellular particles are removed by centrifugation. However, the proteins secreted by circulating CLL B cells and the lymphatic system are present [35, 36]. In addition to tumor cell derived proteins, other changes in the plasma composition are likely to occur as a consequence of an immune response against the tumor.

For CLL, a gene ontology (GO) analysis with the DAVID bioinformatics resource [37] revealed that 16% of the differentially expressed proteins are involved in the immune response (GO:0006955), whereas 13% are connected to cell-cycle regulation (GO:0051726) and 23% to the regulation of cell proliferation (GO:0042127). The most dominant GO-term—for 24% of the proteins—is the regulation of programed cell death (GO:0043067). In particular, a stronger signal transduction of the TNF family was observed, indicated by increased levels

probably caused by the biased selection process of the antibodies. They had been picked on the basis of transcriptional variations observed in pancreatic, colon, and breast cancer

Table 3. Gender specificity

Uniprot entry	Uniprot accession	HGNC symbol	Combined		Females		Males	
			logFC	Adj. *p*-value	logFC	Adj. *p*-value	logFC	Adj. *p*-value
CLL								
CXCR5_HUMAN	P32302	CXCR5	0.45	0.086	0.06	1.000	**0.39**	**0.026**
CDC5L_HUMAN	Q99459	CDC5L	−0.46	0.130	−0.03	1.000	**−0.44**	**0.026**
RL18_HUMAN	Q07020	RPL18	−0.44	0.140	−0.02	1.000	**−0.42**	**0.031**
ETS2_HUMAN	P15036	ETS2	−0.43	0.099	−0.09	0.950	**−0.34**	**0.036**
IRS2_HUMAN	Q9Y4H2	IRS2	−0.61	0.120	−0.12	0.960	**−0.49**	**0.036**
PLCG2_HUMAN	P16885	PLCG2	0.37	0.240	−0.02	1.000	**0.39**	**0.036**
TNFA_HUMAN	P01375	TNF	0.69	0.450	−0.35	0.900	**1.04**	**0.036**
LYAM2_HUMAN	P16581	SELE	0.52	0.200	**0.59**	**0.042**	−0.07	0.830
IL13_HUMAN	P35225	IL13	0.59	0.230	**0.64**	**0.030**	−0.04	0.920
CASP8_HUMAN	Q14790	CASP8	0.81	0.100	**0.82**	**0.002**	−0.01	0.970
DLBCL								
BHE40_HUMAN	O14503	BHLHE40	0.40	0.260	−0.10	0.940	**0.50**	**0.013**
ETS2_HUMAN	P15036	ETS2	−0.35	0.110	−0.02	0.990	**−0.33**	**0.013**
FL								
AGR2_HUMAN	O95994	AGR2	0.47	0.840	−0.27	0.930	**0.74**	**0.020**
BAX_HUMAN	Q07812	BAX	−0.85	0.840	**−0.88**	**0.002**	0.03	1.000
WDR1_HUMAN	O75083	WDR1	0.69	0.700	**0.96**	**4.4×10^{-15}**	−0.27	0.920

An analysis for gender-specific variations revealed proteins that were regulated either in female or male probands only. Significant results are marked in bold letters.

of TNF receptors and ligands. This can lead to apoptosis via induction of a cascade of caspases [38]. However, the lower concentration of the essential caspases, CASP3, CASP8, and CASP9, in the CLL plasma samples indicates that apoptosis is repressed. This might be caused by a CFLAR-mediated resistance, which was observed in CLL cells in previous studies [39, 40]. Accordingly, increased levels of CFLAR were observed in CLL cases of this epidemiological study, indicating a

prevention of apoptosis and an induction of cell proliferation via the NFKB and MAPK signal cascades [41–43].

An increased proliferation in the CLL blood samples was also indicated by proteins that are directly involved in the regulation of the cell cycle. CDC2, a protein essential for the start of S-phase and mitosis, and the positive regulator proteins CDK4 and CK5p3 were observed at higher levels. Conversely, inhibitors of the cell cycle such as CCNG1, CD2A2,
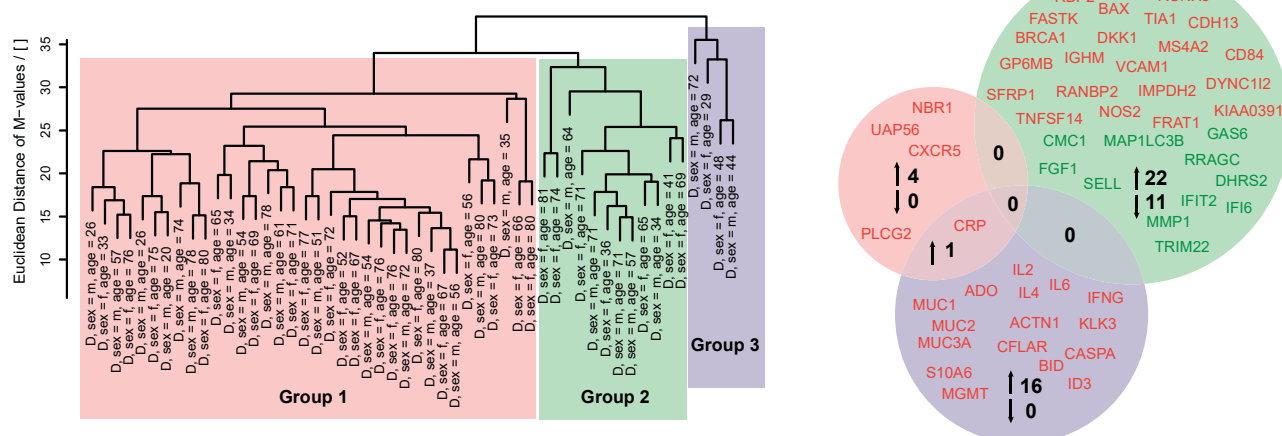

Figure 4. Identification of subtypes within DLBCL samples. Hierarchical cluster analysis revealed heterogeneous protein abundance within each of the different lymphoma entities. For DLBCL, three subclasses were defined from the hierarchical cluster analysis. Each of these subclasses was compared with the healthy control group individually. This led to individual lists of differential proteins with almost no overlap.

Clinical Relevance

An analysis of 100 plasma samples from B-cell lymphoma patients and the same number of controls led to the identification of several biomarkers. In addition, subgroups with partly common and partly discrete plasma protein profiles were identified. Based on this information, different lymphoma entities could be classified with high accuracy by a noninvasive immunobased assay. After clinical validation, such markers could support physicians in disease prognosis and the selection of an appropriate treatment scheme.

CDN1A, and CDN1C were found at reduced levels. Also, PCNA levels were increased, which are well known to correlate with the proliferation of CLL cells and thereby prognosis [44]. In addition, the oncoproteins FRAT1 and c-fos were identified at higher levels, which are known to be involved in lymphoma progression [45, 46], while the tumor suppressor proteins PTEN, BRCA1, RUNX3, and MAD4 were detected at lower levels, in accordance with prior studies [47, 48].

Next to an insight into biological function, the study pointed out interesting features as well as pitfalls of analyses of pooled and individual samples. The experiments based on sample pools led to the identification of a comparably high number of potential biomarkers. Even relatively small differences in the protein abundance of the pools could be detected. In the pooling process, individual variations between the samples are averaged out. However, for the molecules TSN16, NPT1, PCNA, CATD, TNFA, TNR6 (FAS), TR10C, IL8, and VCAM1, relevance for a diagnosis of CLL could be confirmed also in the analysis of the individual patient samples. Only one protein (IL1a) produced contradictory results on the very same antibody: it exhibited slight upregulation in CLL in the individual sample analysis, while downregulation was seen in sample pools. For detecting IFNG, we used three different antibodies. One antibody showed upregulation in the pooled samples, while the other two indicated a lower expression in the individual samples. These discrepancies might have occurred due to the different number of healthy samples in the pooled ($n = 100$) and the individual analysis ($n = 25$). Also, the binders may have different epitopes. Overall, the majority of proteins, for which differences were seen in the pools, exhibited a similar expression pattern in the analysis of individual samples, although often at low significance due to the biological heterogeneity. Thereby, the analysis of sample pools is a cost-efficient approach for a first screening. In this study, it led to a preselection of more than 200 potential marker proteins. Only 16 microarrays were needed for pooled analysis as compared to 130 microarrays required for the analysis of individual samples.

Analyzing individual samples has other advantages. It enables the analysis of the distribution and heterogeneity of each marker in the sample sets. The clinical usability of a biomarker can only be proven by a validation on the level of individual samples. Furthermore, only individual sample data allow the stratification by sample-specific parameters, such as sex, age, or other variables that have emerged as influential at the level of data analysis. In an analysis of individual samples, expression levels can be correlated to any available sample annotation.

In earlier work (unpublished data), we analyzed blood samples from a population-based study and identified smoking behavior and sex as two factors, which correlated strongly with differences in subgroups. To avoid such bias, we selected only samples from nonsmokers in this study. The effect of sex on the protein biomarker set was controlled by a gender-specific analysis of the individual samples. Herein, particular proteins were identified, which differed considerably between lymphoma patients and controls restricted to either men or women. As a matter of fact, the majority of the proteins found to be disease-specific was stronger affected in either male or female. This underlines the importance of considering gender effects in studies for the detection or validation of biomarkers. This had been observed before in an antibody microarray study on urine samples comparing pancreatic cancer patients with healthy controls [16] as well as in other studies [49, 50].

The strongest advantage of analyses on individual samples, however, is the possibility to identify unknown sources of variation. In this study, we identified subgroups within the DLBCL, CLL, and FL patient groups by a hierarchical cluster analysis. Between each other, these subgroups had a very heterogeneous protein profile in blood, for which no correlation was found with any available clinical (age, gender, body mass index) or technical annotation (batch of incubation or sample labeling). Each of these subgroups was compared to the healthy control group. Although the sample number within each subgroup was low, more differential proteins with higher significance levels were obtained in this comparison. This is caused by the fact that the protein profiles of the individual subgroups are less heterogeneous than that of the different lymphoma disease entities. FL, DLBCL, and CLL are well known not to be a homogeneous disease but have a very heterogeneous tumor biology as well as prognosis [51–54].

Based on DNA-microarray analysis, it was possible to identify the source and stage of B cells in the oncogenesis of DLBCL [51]. This information can be used for prognostic purposes and to decide on targeted therapy. Also IHC classifiers have been developed [52]. In addition, it was proposed to use the phenotype of circulating cells for diagnosis and

prognosis [53]. However, there is still the need for an easy, noninvasive, and robust methodology for a differentiation between tumor types, which can be implemented in clinics. While other options for an efficient diagnosis of lymphoma do exist, we demonstrated herein an efficient classification of three investigated lymphoma entities at the protein level with antibody microarrays. Also, within the lymphoma entities, samples could be grouped into subtypes on the basis of common and distinct blood protein profiles. While the investigation of 200 plasma samples is comparably large as a discovery study at the protein level, the numbers within each sample group and subgroup are too small for a proper cross-validation. Therefore, in order to assess clinical implications, the findings need to be validated in a larger study population.

The authors have declared the following potential conflict of interest: Christoph Schröder and Jörg D. Hoheisel are founders of the spin-off company Sciomics GmbH. It offers services based on the use of antibody microarrays.

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