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Identification of plasma protein profiles associated with risk groups of prostate cancer patients

Malin Nordström[1,2], **Christer Wingren**[1,2], **Carsten Rose**[2,3], **Anders Bjartell**[4], **Charlotte Becker**[5], **Hans Lilja**[5,6,7], and **Carl AK Borrebaeck**[1,2]

[1]Dept. of Immunotechnology, Lund University, Medicon Village, Lund, Sweden

[2]CREATE Health, Lund University, Medicon Village, Lund, Sweden

[3]Division of Oncology and Hematology, Skåne University Hospital, Lund, Sweden

[4]Department of Urology, Skåne University Hospital, Malmö, Sweden

[5]Dept. of Laboratory Medicine, Skåne University Hospital, Lund University, Malmö, Sweden

[6]Depts. of Laboratory Medicine, Surgery (Urology), and Medicine, Memorial Sloan-Kettering Cancer Center, New York, USA

[7]Nuffield of Dept. of Surgical Sciences, University of Oxford, Oxford, United Kingdom

Abstract

Purpose—Early detection of prostate cancer (PC) using prostate-specific antigen (PSA) in blood reduces PC-death among unscreened men. However, due to modest specificity of PSA at commonly used cut-offs, there are urgent needs for additional biomarkers contributing enhanced risk classification among men with modestly elevated PSA.

Experimental design—Recombinant antibody microarrays were applied for protein expression profiling of 80 plasma samples from routine PSA-measurements, a priori divided into four risk groups, based on levels of total and %free PSA.

Results—The results demonstrated that plasma protein profiles could be identified that pinpointed PC (a malignant biomarker signature) and most importantly that showed moderate to high correlation with biochemically defined PC risk groups. Notably, the data also implied that the risk group with mid-range PSA and low %free PSA, a priori known to be heterogeneous, could be further stratified into two subgroups, more resembling the lowest and highest risk groups, respectively.

Conclusions and clinical relevance—In this pilot study, we have shown that plasma protein biomarker signatures, associated with risk groups of PC, could be identified from crude plasma samples using affinity proteomics. This approach could in the longer perspective provide novel opportunities for improved risk classification of PC patients.

Corresponding author: Prof. Carl Borrebaeck, Department of Immunotechnology and CREATE Health, Lund University, Medicon Village, SE-22381 Lund, Sweden. carl.borrebaeck@immun.lth,se. Phone: +46-46-2229613. Fax: +46-46-2224200.

Disclosures

Dr. Hans Lilja holds patents for free PSA, intact PSA, and hK2 assays.

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Keywords

Prostate cancer; diagnosis; biomarker; antibody microarray; affinity proteomics

Introduction

Prostate cancer (PC) is the second leading cause of cancer related deaths in western countries [1] and in order to improve the prognosis of PC patients, early and specific diagnosis is crucial. The blood-based biomarker prostate specific antigen (PSA) was introduced in the clinics in the late 1980's and is today used as an indicator of risk for PC and as one parameter for further testing of patient biopsies [2, 3]. The introduction of PSA has resulted in an increased number of early diagnosed PC cases, but the moderate specificity of PSA for malignant disease has raised key concerns regarding the cost and potential side-effects of unnecessary biopsies as well as the risk of over-diagnosis and over-treatment [2–4]. In fact, 65–75 % of the men selected for biopsy based on total serum PSA levels (tPSA) (≥ 4 ng/ml), do not have PC (www.cancer.org). Hence, in order to improve the risk classification enabling clinicians to select adequate patients for biopsy testing, additional and/or more specific biomarkers needs to be defined.

In order to improve the specificity when testing for PC, several attempts have been made to combine the tPSA value with other parameters, such as PSA change over time (PSA velocity), PSA in relation to prostate volume (PSA density), or age specific ranges of PSA [2, 3]. However, no or only modest improvements of the diagnostic power of the tPSA assay have so far been observed [2]. In contrast, differentiating between tPSA and free (unbound) PSA has proven to enhance the assay performance, especially for men with mid-range (4–10 ng/ml) levels of tPSA. In fact, men having a ratio of free PSA to tPSA (%fPSA) below 18–25% have shown to be associated with a significantly higher risk of having PC [5–7]. Still, 25–50% of this particular patient group does not have PC, but they are all selected for biopsy testing [7, 8]. Recently, a panel of four kallikrein markers has been indicated as potential predictors of biopsy outcome [9, 10], and that the combination of tPSA, free PSA, with the free PSA sub-fraction called 2proPSA might also improve diagnostic accuracy [11]. Still, there is a significant unmet clinical need for additional, more specific biomarkers that could be used to stratify this particular risk group prior to biopsy testing and subsequent potential therapy intervention.

Major efforts have also been pursued in order to define additional, novel serum biomarkers associated with PC, initially using various classical biochemical technologies. To date, human kallikrein 2 [12, 13], urokinase-type plasminogen activator [14], [11] transforming growth factor β1 (TGF-β1) [3, 15, 16], and interleukin-6 (IL-6) [16–18] have in particular been indicated as potential serum markers. Albeit promising, further validation studies will be required to explore and confirm the prognostic capabilities of these markers. [2–4]. Moreover, additional potential PC markers have also been indicated using traditional proteomic techniques [19, 20], but these observations remains to be validated using independent patient cohorts [21]. In addition, serious technical issues have also been raised regarding the assay sensitivity, dynamic range, and/or throughput [22]. To this end, affinity

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proteomics have been established as a high-throughput alternative capable of targeting non-fractionated proteomes, e.g. plasma and serum, in a multiple, sensitive, and rapid manner [23, 24].

In this context, we have previously designed a recombinant single chain Fragment variable (scFv) antibody microarray platform for multiplex protein expression profiling of complex proteomes in a high-throughput manner [23, 25, 26]. Using this affinity proteomic technology platform, we have been able to profile a wide range of crude, directly labeled proteomes, including serum, plasma, urine, intact cells, cell lysates, and tissue extracts in a rapid, sensitive and reproducible manner [23]. We have demonstrated that the platform could be used to identify candidate biomarker signatures for e.g. diagnosis, prognosis, classification, and for evidence-based therapy selection [27–30].

In this pilot study, we have investigated whether affinity proteomics could be used to validate and even further refine risk group classification targeting plasma samples from routine PSA measurements with the patient samples being stratified into four biochemically defined risk groups according to one current established clinical practice [31–34]. The data showed that we could identify candidate plasma protein signatures that were able to pin-point PC (“a malignant biomarker signature”) and to stratify patients into current as well as new subgroups related to PC risk, which in the long-term has the potential to contribute to more individualized treatment of PC.

Material and Methods

Clinical samples

We used de-identified EDTA anti-coagulated blood samples from 80 men aged 50–70 years referred for routinely performed PSA testing at the Dept. of Clinical Chemistry, Skåne University Hospital, Malmö, Sweden. No clinical information or patient identifiers were retained for these samples, and samples were stored at −80°C until use (Table 1). The levels of free and tPSA were determined using the routine clinical dual-label DELFIA Prostatus© total/free PSA-assay (Perkin-Elmer, Turku, Finland, which is calibrated against the WHO 96/670 (PSA-WHO) and WHO 68/668 (free PSA-WHO) calibrators. Detectable ranges were from 0.10 to 250 ng/ml for tPSA and from 0.04 to 250 ng/ml free PSA. Coefficients of variation (CV) was ≤10.6% for tPSA, and ≤7.3% for free PSA. The samples were strictly divided into four groups based on the levels of %free and total PSA: A (n=20) had tPSA ≤0.70 ng/ml; B (n=20) had tPSA of 2.1–8.0 ng/ml with %fPSA ≥27.9%; C (n=20) had tPSA of 5.0–10.3 ng/ml with %fPSA ≤12.6%; and D (n=20) had tPSA of 24.6–724 ng/ml. The information about %free and tPSA per individual sample was not available after sample division. These four groups of de-identified samples reflect highly different categories of risk of PC diagnosis or outcome according to current established clinical practice [31–34]. In more detail, group A have very low long-term risk of significant PC, group B have modestly increased risk of prostate disease but low likelihood of clinically significant PC, group C with importantly increased risk of PC, and group D having very high risk of clinically significant or advanced stages of PC [31–34]. The procedures followed were in accordance with the Helsinki Declaration of 1975.

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Labeling of plasma samples

EDTA anti-coagulated plasma samples were labeled according to a previously optimized protocol [25, 26] for serum proteomes with one minor adjustment. In order to prevent the plasma samples from coagulating, EDTA was added to a final concentration of 4 mM to the PBS buffers used throughout the protocol. Briefly, the samples were centrifuged at 16 000 x g for 20 minutes at 4°C, and 5 μl of the samples were then diluted 45 times in 4 mM EDTA PBS. Diluted samples were incubated with 0.6 mM EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL, USA) for 2 hours on ice, after which unreacted biotin was removed by dialysis against 4 mM EDTA-PBS for 72 h at 4°C. Finally, samples were aliquoted and stored at −20°C prior to use in microarray experiments.

Production and purification of scFv

One hundred sixty-two human recombinant scFv antibody fragments directed against 62 different analytes, mainly involved in immunoregulation, were selected from the n-CoDeR library [35], and kindly provided by Bioinvent International AB (157 scFv clones; Lund, Sweden) and Prof. M. Ohlin (5 Mucine-1 specific clones; Dept. of Immunotechnology, Lund University, Lund, Sweden) (Supporting Information Table 1). The specificity, affinity (in the 1–10 nM range), and on-chip functionality of these phage-display derived scFv antibodies was ensured by using i) stringent phage-display selection protocols [35], ii) multiple clones (≤4) per target analyte (directed against different epitopes) and iii) a molecular design adapted for microarray applications [23, 24]. In addition, the specificity/reactivity pattern of several of the antibodies have previously been validated using well-characterized serum samples, and orthogonal methods, such as ELISA, Meso Scale Discovery (MSD), cytometric bead array (CBA) and mass-spectrometry (MS), as well as using spiking and blocking experiments (Supporting Information Table 1) [36] and references therein. The biotinylation of the sample might result in impaired (masked) epitopes, why some of the antibodies might lose their reactivity. By including several antibodies directed against the same protein but against different epitopes, this issue can be minimized. All scFvs were produced in 100 ml *E. coli* cultures and purified from expression supernatants using affinity chromatography on Ni^{2+}-NTA Agarose (Qiagen, Hilden, Germany). Bound molecules were eluted with 250mM Imidazole (Saveen Werner, Malmö, Sweden), dialysed against PBS, and then stored at 4°C prior to use in microarray experiments. The integrity and purity of the scFvs were evaluated by 10% SDS-PAGE (Invitrogen, Carlsbad, CA, USA). The protein concentration was determined by measuring the absorbance at 280 nm.

Production and processing of antibody microarrays

Briefly, scFv microarrays were produced using a non-contact dispenser (SciFlexarrayer S11, Scienion, Berlin, Germany) and processed according to a set-up previously optimized [25, 26]. In total, 180 scFvs and controls were arrayed in 8 replicates onto Blank Polymer Maxisorp slides (NUNC A/S, Roskilde, Denmark), with one drop (300 pL) in each position, at a scFv concentration of 0.05–0.4 mg/ml. The 180 probes were arrayed into three columns, each comprising of 60 rows (Fig. 1A). AlexaFlour-647 labeled streptavidin (10 ug/ml) was printed as a position control, and printing buffer (PBS) was included as a negative control.

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After printing, the slides were allowed to dry and blocked in 5% (w/v) fat-free milk (Semper AB, Sundbyberg, Sweden) in PBS o/n. The slides were then placed in a Protein Array Workstation (Perkin Elmer Life & Analytical Sciences, Wellesley, MA, USA), and all incubation steps were performed at 20°C. The slides were first washed for four minutes with 0.5% (w/v) Tween-20 in PBS (PBS-T). Next, 70 μl of the labeled samples, diluted 1:2 in 1% (w/v) fat-free milk powder and 1% (v/v) Tween-20 in PBS (PBS-MT), were injected and incubated with agitation for 60 minutes. After a second wash, the arrays where incubated for 60 minutes with 350 μl of 1 μg/ml Alexa-647 conjugated streptavidin in PBS-MT. After washing, the arrays were dried under a stream of nitrogen gas and scanned with a confocal microarray scanner (ScanArray Express, Perkin Elmer Life & Analytical Sciences) at 5 μm resolution, using five different scanner settings (50% PMT gain and 70% laser power (50/70), 70/70, 80/80, 80/90, and 90/90). Signal intensities were quantified using the ScanArray Express software version 4.0 (Perkin Elmer Life & Analytical Sciences). The local background was subtracted, and to compensate for any possible local defects, the two highest and lowest replicates were automatically excluded. Presented signal intensities represent the mean value for the remaining four replicate spots. Only unsaturated spots were considered for analysis.

Microarray data normalization

Chip-to-chip normalization of the data sets was performed, using a semi-global normalization approach [27, 37], similar to the normalization developed for DNA microarrays. The coefficient of variation (CV) was first calculated for each analyte and ranked. The fifteen percent of the analytes that displayed the lowest CV-values over all samples were identified, corresponding to 24 analytes, and used to calculate a chip-to-chip normalization factor for each sample. The normalization factor Ni was calculated by the formula $Ni = Si/\mu$, where Si is the sum of the signal intensities for the 24 analytes for each sample and μ is the sum of the signal intensities for the 24 analytes averaged over all samples. Each data set generated from one sample was divided with the normalization factor Ni. Log2 values of signal intensities were used for further analyses.

Data analysis

The 80 patient samples were divided into four groups (n = 20) based on values of tPSA and %fPSA (Table 1). For the initial classification analysis, each of the four risk groups was divided into a training set (n=15) and a test set (n=5). In order to classify the samples, we used the support vector machine (SVM), a supervised learning method in R [38]. The supervised classification was performed using a linear kernel, and the cost of constraints was set to 1, which is the default value in the R function SVM, and no attempt was performed to tune it in order to avoid overfitting. The SVM model was trained using the training set (75% of the samples) and then frozen, and applied to the test set (25% of the samples). The training and test sets were randomly selected. No filtration of the data was performed before training the SVM, i.e. data from all antibodies on the array were included in the analysis in order to avoid overfitting. Further, a receiver operating characteristics (ROC) curve, was constructed using the SVM decision values, and the area under the curve (AUC) was calculated. Using unsupervised hierarchical clustering in Cluster and Treeview [39], the C group could be divided into two subgroups denoted C1 (n = 10) and C2 (n=10). Due to the

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smaller sample number, a division into training and test sets was in this case not possible, and the SVM was therefore trained using a leave-one-out cross-validation procedure. Significantly up- or down-regulated plasma proteins ($p < 0.05$) were defined based on the relative protein levels and identified using Wilcoxon's signed-rank test. The samples were visualized using a principle component analysis (PCA) software program (Qlucore Omics Explorer, Lund, Sweden) and/or Cluster and Treeview. PCA involves a mathematical procedure that transforms the number of correlated variables (the microarray data based on 162 antibodies) into 3 uncorrelated variables, so called principle components, enabling the data to be visualized in a plot of 3 dimensions. In all heat-maps, the list of differentially expressed analytes were made non-redundant, i.e. only one antibody per analyte was included. The antibody with the best p-value was included as representative data point. Notably, all differentially expressed sets of antibodies (≥ 2 clones) directed against the same antigen, showed similar pattern of deregulation, except for only three antibody pairs in one comparison (LDL, TNF-β, and MCP-4) (Fig. 2A). In the latter case, the discrepancy is indicated by retaining both antibody clones in the heat map. The discrepancy might be explained by reasoning that these antibodies, directed against the same protein but different epitopes, might recognize different forms of the same protein, i.e. intact protein vs. partly degraded protein vs. free protein vs. complexed protein etc.

Validation experiments

In an attempt to validate the antibody microarray results, a human Th1/Th2 10-plex MSD (Meso Scale Discovery, Gaithersburg, MD, USA) assay was run on all 80 EDTA-plasma samples. Each well of the MSD 96-plate had been pre-functionalized with antibodies against IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p70, IL-13 and TNF-α in spatially distinct electrode spots. The assay was run according to the protocol provided by the manufacturer and the electrochemiluminiscence-based readout was performed in an MSD SECTOR® instrument. The limit of detection was defined as a signal 2.5 times the standard deviation over the zero point in the standard curve. It should be noted that only IL-8 and TNF-α were above the limit of detection of the MSD assay in a majority of all samples, why only these proteins could be used for validation.

Results

In this pilot study, we have performed protein expression profiling of non-fractionated, biotinylated EDTA-plasma samples from 80 patients with various risk of having PC, using our in-house developed recombinant antibody microarrays. The samples had been a priori divided into four risk groups based on tPSA and %fPSA reflecting different categories of risk of PC diagnosis or outcome (Table 1).

Evaluation of scFv microarray

A representative microarray image is shown in Figure 1A, demonstrating that homogenous spot morphologies, high signal-to-noise ratios, and dynamic signal intensities were obtained. All 80 samples were successfully profiled; hence, array data from all 80 samples could be used for the subsequent statistical analysis. The intra-assay reproducibility was assessed by analyzing the spot-to-spot variation, resulting in an average coefficient of determination (R^2)

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of 0.95 (Fig. 1B). The inter-assay reproducibility, i.e. array-to-array variation, was evaluated by analyzing a single sample on independent arrays, resulting in an R^2–value of 0.96 (Fig. 1C).

Classification of risk groups

First, we determined a focused (62 analyte) plasma proteome profile of the four prostate cancer risk groups, denoted A – D (Table 1), with group A displaying the lowest risk and group D the highest risk. Using Wilcoxon's signed-rank test, 3 to 44 differentially expressed ($p < 0.05$) plasma analytes were identified, which are shown as a heat map in Figure 2A. The data showed that only 3 analytes were differently expressed between the two lowest risk groups, A and B. Hence, as could be expected from a clinical point of view, the data implied only small differences between the two lowest risk groups. In contrast, 37, 44, and 30 deregulated analytes were observed for groups A, B, or C versus the highest risk group D, indicating on large(r) differences. In more detail, several complement proteins (e.g. C3, C4, C1q, Factor B and Properdine) were found to be down-regulated in the high risk group D, while the up-regulated analytes displayed a complex pattern of both TH1 (e.g. IL-2, IL-3 and INF-γ) and TH2 (e.g. IL-4, IL-10) cytokines. It should be noted that we could not differentiate whether the observed up- and down-regulated levels of an analyte was because of an increased or decreased consumption or an increased or decreased production. Most importantly, the biomarker signature differentiating group A (very low PC-risk) versus group D (very high risk of clinically significant or advanced stages of PC) could be viewed as a malignant biomarker signature pin-pointing PC.

Next, we evaluated the ability of the array platform to classify the four risk groups (A to D), based on the observed protein expression profiles. To this end, each patient group was divided into a training set (75% of the samples, n=30) and a test set (25% of the samples, n=10) (Fig. 2B). Hence, the SVM model was trained on the training set and then applied on the independent test set. The results showed that the risk groups could be distinguished with different accuracy, with groups B and D displaying an AUC of 0.68, groups A and B an AUC of 0.84 (only three analytes were differentially expressed), and groups A and D an AUC of 0.72 (Fig. 2C and Supplementary Fig. 1). Thus, the data showed that malignant signature could be used to well discriminate group A (very low risk) versus group D (very high risk of clinically significant or advanced stages of PC) (cfs. Figs. 2A and 2C). In contrast, the C group could not be differentiated from any of the three other risk groups (A, B, and D), as illustrated by ROC AUCs of 0.5 in all cases. In this context, it should be noted that the C group represents a heterogeneous patient group (mid-range tPSA, low %fPSA) of which only 25–50% actually have PC, although all patients are selected for biopsy testing. Hence, the known heterogeneity of group C could be argued to represent one plausible explanation to why group C could not be differentiated from groups A, B, or D. Further stratification of this heterogeneous patient group could thus potentially be a key instrument for identifying patients at higher (i.e. more resembling group D) or lower (i.e. more resembling groups A and B) risk of developing PC.

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Stratification of risk group C

In order to investigate whether the C risk group could be further stratified, we performed an unsupervised hierarchical clustering based on unfiltered data, i.e. using data from all antibodies included on the array. The result showed that the C risk group could indeed be stratified into two distinct subgroups, denoted C1 (n=10) and C2 (n=10) (Fig. 3A), with a ROC AUC value of 0.99 (Supplementary Fig. 2). Similarly, a clear subdivision of the C cohort was also observed, using principle component analysis (PCA) (Fig. 3B). Moreover, 49 significantly differentially ($p<0.05$) expressed plasma analytes were observed for C1 versus C2 (Fig. 3C). In more detail, three complement proteins were up-regulated in C1 (C1q, Factor B and C4), while the remaining differentially expressed proteins were down-regulated in C1. The latter group of analytes included a number of cytokines (e.g. IL-6 and IL-4), complement proteins (e.g. C1-INH and C1s), as well as cell surface proteins (e.g. ICAM, HLA-DR, Mucine-1 and CD-40). Taken together, the data showed that the heterogeneous risk group C could be stratified into two distinct subgroups, C1 and C2, with a large number of deregulated analytes. The known heterogeneity of risk group C (only 25–50% that actually have PC) could thus be argued to represent one plausible explanation to the observed sub-classification.

In an attempt to validate the array data, an independent 10-plex cytokine sandwich antibody microarray (MSD) was applied (Figs. 3D and 3E). However, in a majority of all samples, only IL-8 and TNF-α of the 10 targeted plasma molecules were above the limit of detection of the MSD assay. Still, the observed down-regulation of TNF-α (Fig. 3D) and IL-8 (IL-8) (Fig. 3E) in C1 versus C2 could be validated by the MSD data.

Risk classification of subgroups C1 and C2

In order to assess the biological impact of the stratified subdivision of risk group C, the protein expression profiles of subgroups C1 (n=10) and C2 (n=10) were compared to those of the other three original risk groups, A, B and D (n=20 in each group). In case of C1, the results showed that C1 could be well differentiated from risk group D (AUC = 0.82), but not from risk groups A and B (AUC = 0.5 in both cases) (Fig. 4A and Supplementary Fig. 3A). Furthermore, 47 differentially expressed analytes were observed for C1 versus D, but only 16 and 15 for C1 versus A and B, respectively (Fig. 4B). In the former case, a pattern of up-regulated complement proteins (e.g. C1q, C3, and Factor B) and down regulated cytokines (e.g. TGF- β1, IL-1ra, IL-6 and MCP-1) and cell surface markers (e.g. ICAM, CD40 and Lewis^X) were observed in C1 versus D.

In contrast, the results showed that C2 could be reasonably well differentiated from both risk group A (AUC=0.72) and B (AUC=0.75), but not from risk group D (AUC=0.57) (Fig. 4C and Supplementary Figs. 3B to 3D). In this case, 22 and 28 de-regulated analytes were observed for C2 versus A and B, respectively, but only 5 for C2 versus D (Fig. 4D). While only two down-regulated analytes (C1q and IL-4) were observed in C2 versus A, a pattern of numerous up-regulated cytokines (e.g. IL-1ra, MCP-1, and IL-6) and cell surface markers (e.g. CD40, Lewis^X and Sialyl Lewis^X) were observed in C2 versus both A and B. Taken together, the results showed that C1 was more similar to the lowest risk groups A and B, while C2 displayed a higher similarity to the highest risk group D. Considering the known

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heterogeneity of risk group C, it might be argued that the C1 group represented more low risk patients and the C2 group higher risk patients.

Finally, to further highlight the biological relevance, we compared the overlap of the malignant signature (33 biomarkers) discriminating risk group A (very low risk) versus risk group D (very high risk of clinically significant or advanced stages of PC) (Fig. 2A) with the expression levels of the corresponding markers of the above signatures (Fig. 4E). The data showed that a significant part of the malignant biomarker signature was also differentially expressed in the case of C1 (indicated as low risk group) versus C2 (indicated as high risk group) (27 of 33 biomarkers) and C1 versus D (high risk) (29 of 33 biomarkers). In contrast, the overlap was significant smaller with the signatures differentiating A (low risk) versus C2 and B (low risk) versus C2. Hence, the data further indicated the biological relevance of the malignant biomarker signature pin-pointing PC.

Discussion

Blood plasma is a minimally invasive and clinically well-established sample format that would be the ideal source of biomarkers for early detection and risk classification of PC. Consequently, it has also been assessed for this purpose in numerous large-scale proteomic efforts [19, 20]. However, due to the inherent complexity of plasma samples, with respect to number of proteins and dynamic range, it has been argued that classical proteomic analysis of non-fractionated samples would be unlikely to be useful for classifying PC [21]. Although pre-fractionation reduces the sample complexity, it has in turn been associated with serious issues regarding skewed protein yield/recovery, as well as reproducibility and sensitivity [22]. In this context, we have recently shown that affinity proteomics, represented by recombinant antibody microarrays, could be used to profile high- as well as low-abundant analytes in crude proteomes [23, 24]. Previously, a single serum marker thrombospondin-1 (TSP-1), was shown to be able to differentiate between benign and malignant prostatic disease using antibody microarrays [40]. In this pilot study, we have used affinity proteomics targeting crude plasma samples in order to decipher the first multiplexed candidate plasma protein biomarker signatures associated with risk groups for PC. Hence, recombinant antibody microarray-based analysis of PC demonstrated a potential route toward defining the next generation of PC-associated biomarkers.

The blood plasma proteome consists of both classical plasma proteins, as well as tissue leakage proteins. The intrinsic ability of the human immune system to sense even subtle changes in the body’s homeostasis, ranging from a bacterial infection to a growing tumor, provides a unique opportunity of using the immune system as a remote and early sensor of disease [41, 42]. In fact, immunosignaturing has gained significant interest [43], in particular due to recent studies showing immunosurveillance as an important factor in tumor development [44]. To this end, we have designed and applied a recombinant antibody microarray targeting mainly immunoregulatory analytes [23, 24]. Previously we have demonstrated the potential of our array design for cancer diagnosis [27], evidence-based therapy selection [28], as well as predicting the risk for breast cancer recurrence [30]. In this discovery study, we have extended the range of applications, and indicated its potential use

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for risk group stratification in PC, clearly differentiating the two lowest versus the highest risk groups.

Risk classification of potential PC patients can currently be performed in the clinic, using serum PSA assays (tPSA and %fPSA) [45], one of a few models for risk classification [3, 46, 47]. Although this approach enables detection of many tumors, the low specificity for malignant disease results in several patients being subjected to unnecessary biopsy testing [3]. This number could be significantly decreased if the clinicians had access to more adequate tools for risk classification. In particular, the patient group with mid-range tPSA (4–10ng/ml) and low %fPSA is known to be heterogeneous, including a mix of PC and benign prostatic hyperplasia [7, 45]. In our attempt to classify the four pre-determined risk groups, the results showed in fact that the patient groups with mid-range tPSA and low %fPSA (risk group C) could not be differentiated from any of the other three risk groups (AUC of 0.5). Hence, our data further supported the current notion of group C being a very heterogeneous patient group.

However, our data rather implied that the C group could be divided into two distinct subgroups, C1 and C2, which, as anticipated from the current literature [2, 3, 5, 45], more resembled the two lowest and the highest original risk groups, respectively. Of note, it has so far not been possible to further refine group C based solely on PSA values. When aiming for personalized treatment of cancer patients, the ability to stratify heterogeneous patient groups into more accurate subgroups of higher and lower risk of having (developing) a certain cancer will be instrumental. In the long run, this could provide novel opportunities for managing PC cancer patients. However, this discovery study was in part limited by the fact that full clinical documentation of the patients included was not at hand. Hence, the observed candidate biomarker signatures will be properly validated in follow-up studies, targeting larger, independent cohorts of well-characterized patient samples. Notably, the two cytokines, IL-8 and TNF-α, that could be detected with the orthogonal method (MSD) used for initial validation, supported our discrimination of C1 versus C2.

When examining the candidate biomarker signatures in more detail, novel as well as a number of biologically relevant analytes already known to reflect high and low risk of PC were observed. Briefly, TGF-β1 has been shown to be associated with PC, e.g. promoting cell progression in PC models, higher tumor grade, and metastasis, and has subsequently been proposed as a tentative biomarker [3, 4, 48]. Accordingly, we found TGF-β1 to be down-regulated in the two lowest risk groups, A and B, versus the highest risk group, D. In addition, it was also down-regulated in A and B versus C2 (high risk), C1 (low risk) versus D, as well as in C1 versus C2. In the case of IL-6, this cytokine has also been suggested as a potential biomarker for PC [3, 4]. Our microarray data showed that IL-6 was down-regulated in the various comparisons of the low risk groups (A, B, or C1) versus the high risk groups (D or C2), respectively. Furthermore, other cytokines known to be down-regulated in the low risk groups, such as IL-1ra and MCP-1 [49, 50], was also found to be down regulated in the different comparisons of the low risk groups (A, B or C1) versus the high risk groups (D or C2), further supporting our reported observations. In addition, the observation that $Lewis^X$ was down-regulated in the low risk groups was in accordance with previous results, indicating $Lewis^X$ as a prognostic parameter in prostate cancer [51]. It should be noted that

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these observations also supported the candidate malignant biomarker signature observed to pinpoint PC. To date, complement proteins have not been significantly reported in the context of PC. Our data showed that different combinations of several complement proteins, such as C1q, C3, C4, properdin, and/or C1-INH, were up-regulated in the various low risk groups versus the high risk groups, respectively. Although previously not reported in clinical studies, C1q has been shown to display a protective role in prostate cancer cell lines [52].

Taken together, we have delineated candidate plasma biomarker signatures associated with PC risk groups using affinity proteomics. Although the data will be validated in larger prospective studies, targeting independent well-characterized patient cohorts, the results indicated that the conventional risk groups, a priori defined based on clinical parameters could be stratified, and even further sub-stratified, potentially outlining novel, refined PC risk groups. In a longer perspective, this might provide novel opportunities for improved risk classification of PC based on a minimally invasive plasma sample (and thus a reduced number of biopsies required) and subsequently a more personalized management and therapy tailored to reflect the need of each individual PC patient.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

%fPSA	ratio of free to total PSA
AUC	area under curve
PC	prostate cancer
PCA	principle component analysis
PSA	prostate specific antigen
ROC	receiver operating characteristics
scFv	single-chain fragment variable
SVM	support vector machine
tPSA	total serum PSA

References

1. Ferlay J, Shin HR, Bray F, Forman D, et al. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer. 2010; 127:2893–2917. [PubMed: 21351269]

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2. Parekh DJ, Ankerst DP, Troyer D, Srivastava S, Thompson IM. Biomarkers for prostate cancer detection. J Urol. 2007; 178:2252–2259. [PubMed: 17936845]
3. Shariat SF, Semjonow A, Lilja H, Savage C, et al. Tumor markers in prostate cancer I: blood-based markers. Acta Oncol. 2011; 50(Suppl 1):61–75. [PubMed: 21604943]
4. Steuber T, O'Brien MF, Lilja H. Serum markers for prostate cancer: a rational approach to the literature. Eur Urol. 2008; 54:31–40. [PubMed: 18243505]
5. Catalona WJ, Partin AW, Slawin KM, Brawer MK, et al. Use of the percentage of free prostate-specific antigen to enhance differentiation of prostate cancer from benign prostatic disease: a prospective multicenter clinical trial. Jama. 1998; 279:1542–1547. [PubMed: 9605898]
6. Bjork T, Lilja H, Christensson A. The prognostic value of different forms of prostate specific antigen and their ratios in patients with prostate cancer. BJU Int. 1999; 84:1021–1027. [PubMed: 10571627]
7. Gann PH, Ma J, Catalona WJ, Stampfer MJ. Strategies combining total and percent free prostate specific antigen for detecting prostate cancer: a prospective evaluation. J Urol. 2002; 167:2427–2434. [PubMed: 11992051]
8. Cancer Diagnostic Testing World Markets. TriMark Publications, LLC; 2008.
9. Vickers AJ, Cronin AM, Aus G, Pihl CG, et al. A panel of kallikrein markers can reduce unnecessary biopsy for prostate cancer: data from the European Randomized Study of Prostate Cancer Screening in Goteborg, Sweden. BMC Med. 2008; 6:19. [PubMed: 18611265]
10. Vickers AJ, Gupta A, Savage CJ, Pettersson K, et al. A panel of kallikrein marker predicts prostate cancer in a large, population-based cohort followed for 15 years without screening. Cancer Epidemiol Biomarkers Prev. 2011; 20:255–261. [PubMed: 21148123]
11. Jansen FH, van Schaik RH, Kurstjens J, Horninger W, et al. Prostate-specific antigen (PSA) isoform p2PSA in combination with total PSA and free PSA improves diagnostic accuracy in prostate cancer detection. Eur Urol. 2010; 57:921–927. [PubMed: 20189711]
12. Stephan C, Jung K, Lein M, Sinha P, et al. Molecular forms of prostate-specific antigen and human kallikrein 2 as promising tools for early diagnosis of prostate cancer. Cancer Epidemiol Biomarkers Prev. 2000; 9:1133–1147. [PubMed: 11097220]
13. Becker C, Piironen T, Pettersson K, Bjork T, et al. Discrimination of men with prostate cancer from those with benign disease by measurements of human glandular kallikrein 2 (HK2) in serum. J Urol. 2000; 163:311–316. [PubMed: 10604382]
14. Piironen T, Haese A, Huland H, Steuber T, et al. Enhanced discrimination of benign from malignant prostatic disease by selective measurements of cleaved forms of urokinase receptor in serum. Clin Chem. 2006; 52:838–844. [PubMed: 16543389]
15. Thompson TC, Truong LD, Timme TL, Kadmon D, et al. Transforming growth factor beta 1 as a biomarker for prostate cancer. J Cell Biochem Suppl. 1992; 16H:54–61. [PubMed: 1289674]
16. Shariat SF, Kattan MW, Traxel E, Andrews B, et al. Association of pre- and postoperative plasma levels of transforming growth factor beta(1) and interleukin 6 and its soluble receptor with prostate cancer progression. Clin Cancer Res. 2004; 10:1992–1999. [PubMed: 15041717]
17. Hobisch A, Eder IE, Putz T, Horninger W, et al. Interleukin-6 regulates prostate-specific protein expression in prostate carcinoma cells by activation of the androgen receptor. Cancer Res. 1998; 58:4640–4645. [PubMed: 9788616]
18. Nakashima J, Tachibana M, Horiguchi Y, Oya M, et al. Serum interleukin 6 as a prognostic factor in patients with prostate cancer. Clin Cancer Res. 2000; 6:2702–2706. [PubMed: 10914713]
19. Matharoo-Ball B, Ball G, Rees R. Clinical proteomics: discovery of cancer biomarkers using mass spectrometry and bioinformatics approaches--a prostate cancer perspective. Vaccine. 2007; 25(Suppl 2):B110–121. [PubMed: 17916461]
20. McLerran D, Grizzle WE, Feng Z, Thompson IM, et al. SELDI-TOF MS whole serum proteomic profiling with IMAC surface does not reliably detect prostate cancer. Clin Chem. 2008; 54:53–60. [PubMed: 18024530]
21. Goo YA, Goodlett DR. Advances in proteomic prostate cancer biomarker discovery. J Proteomics. 2010; 73:1839–1850. [PubMed: 20398807]
22. Hanash S. Disease proteomics. Nature. 2003; 422:226–232. [PubMed: 12634796]

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23. Borrebaeck CAK, Wingren C. Design of high-density antibody microarrays for disease proteomics: key technological issues. J Proteomics. 2009; 72:928–935. [PubMed: 19457338]
24. Borrebaeck CAK, Wingren C. Recombinant antibodies for the generation of antibody arrays. Methods Mol Biol. 2011; 785:247–262. [PubMed: 21901605]
25. Ingvarsson J, Larsson A, Sjoholm AG, Truedsson L, et al. Design of recombinant antibody microarrays for serum protein profiling: targeting of complement proteins. J Proteome Res. 2007; 6:3527–3536. [PubMed: 17696517]
26. Wingren C, Ingvarsson J, Dexlin L, Szul D, Borrebaeck CAK. Design of recombinant antibody microarrays for complex proteome analysis: choice of sample labeling-tag and solid support. Proteomics. 2007; 7:3055–3065. [PubMed: 17787036]
27. Ingvarsson J, Wingren C, Carlsson A, Ellmark P, et al. Detection of pancreatic cancer using antibody microarray-based serum protein profiling. Proteomics. 2008; 8:2211–2219. [PubMed: 18528842]
28. Carlsson A, Persson O, Ingvarsson J, Widegren B, et al. Plasma proteome profiling reveals biomarker patterns associated with prognosis and therapy selection in glioblastoma multiforme patients. Proteomics Clin Appl. 2010; 4:591–602. [PubMed: 21137077]
29. Carlsson A, Wuttge DM, Ingvarsson J, Bengtsson AA, et al. Serum protein profiling of systemic lupus erythematosus and systemic sclerosis using recombinant antibody microarrays. Mol Cell Proteomics. 2011; 10:M110 005033. [PubMed: 21350050]
30. Carlsson A, Wingren C, Kristensson M, Rose C, et al. Molecular serum portraits in patients with primary breast cancer predict the development of distant metastases. Proc Natl Acad Sci U S A. 2011; 108:14252–14257. [PubMed: 21844363]
31. Lilja H, Cronin AM, Dahlin A, Manjer J, et al. Prediction of significant prostate cancer diagnosed 20 to 30 years later with a single measure of prostate-specific antigen at or before age 50. Cancer. 2011; 117:1210–1219. [PubMed: 20960520]
32. Lilja H, Ulmert D, Vickers AJ. Prostate-specific antigen and prostate cancer: prediction, detection and monitoring. Nat Rev Cancer. 2008; 8:268–278. [PubMed: 18337732]
33. Thompson IM, Pauler DK, Goodman PJ, Tangen CM, et al. Prevalence of prostate cancer among men with a prostate-specific antigen level < or =4. 0 ng per milliliter. N Engl J Med. 2004; 350:2239–2246. [PubMed: 15163773]
34. Vickers AJ, Cronin AM, Bjork T, Manjer J, et al. Prostate specific antigen concentration at age 60 and death or metastasis from prostate cancer: case-control study. BMJ. 2010; 341:c4521. [PubMed: 20843935]
35. Soderlind E, Strandberg L, Jirholt P, Kobayashi N, et al. Recombining germline-derived CDR sequences for creating diverse single-framework antibody libraries. Nat Biotechnol. 2000; 18:852–856. [PubMed: 10932154]
36. Pauly F, Dexlin-Mellby L, Ek S, Ohlin M, et al. Protein expression profiling of formalin-fixed paraffin-embedded tissue using recombinant antibody microarrays. J Proteome Res. 2013; 12:5943–5953. [PubMed: 24063262]
37. Carlsson A, Wingren C, Ingvarsson J, Ellmark P, et al. Serum proteome profiling of metastatic breast cancer using recombinant antibody microarrays. Eur J Cancer. 2008; 44:472–480. [PubMed: 18171612]
38. Ihaka RRG. R: A language for data analysis and graphics. J Computational and Graphicla Statistics. 1996; 5:299–314.
39. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A. 1998; 95:14863–14868. [PubMed: 9843981]
40. Shafer MW, Mangold L, Partin AW, Haab BB. Antibody array profiling reveals serum TSP-1 as a marker to distinguish benign from malignant prostatic disease. Prostate. 2007; 67:255–267. [PubMed: 17192876]
41. Tan EM, Zhang J. Autoantibodies to tumor-associated antigens: reporters from the immune system. Immunol Rev. 2008; 222:328–340. [PubMed: 18364012]
42. Lin MW, Ho JW, Harrison LC, dos Remedios CG, Adelstein S. An antibody-based leukocyte-capture microarray for the diagnosis of systemic lupus erythematosus. PLoS One. 2013; 8:e58199. [PubMed: 23516448]

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43. Chaudhuri D, Suriano R, Mittelman A, Tiwari RK. Targeting the immune system in cancer. Curr Pharm Biotechnol. 2009; 10:166–184. [PubMed: 19199949]
44. Chow MT, Moller A, Smyth MJ. Inflammation and immune surveillance in cancer. Semin Cancer Biol. 2011
45. Shariat SF, Karam JA, Margulis V, Karakiewicz PI. New blood-based biomarkers for the diagnosis, staging and prognosis of prostate cancer. BJU Int. 2008; 101:675–683. [PubMed: 17941930]
46. Bastian PJ, Carter BH, Bjartell A, Seitz M, et al. Insignificant prostate cancer and active surveillance: from definition to clinical implications. Eur Urol. 2009; 55:1321–1330. [PubMed: 19286302]
47. D'Amico AV, Whittington R, Malkowicz SB, Schultz D, et al. Biochemical outcome after radical prostatectomy, external beam radiation therapy, or interstitial radiation therapy for clinically localized prostate cancer. JAMA. 1998; 280:969–974. [PubMed: 9749478]
48. Reis ST, Pontes-Junior J, Antunes AA, Sousa-Canavez JM, et al. Tgf-beta1 expression as a biomarker of poor prognosis in prostate cancer. Clinics (Sao Paulo). 2011; 66:1143–1147. [PubMed: 21876965]
49. Ricote M, Garcia-Tunon I, Bethencourt FR, Fraile B, et al. Interleukin-1 (IL-1alpha and IL-1beta) and its receptors (IL-1RI, IL-1RII, and IL-1Ra) in prostate carcinoma. Cancer. 2004; 100:1388–1396. [PubMed: 15042672]
50. van Golen KL, Ying C, Sequeira L, Dubyk CW, et al. CCL2 induces prostate cancer transendothelial cell migration via activation of the small GTPase Rac. J Cell Biochem. 2008; 104:1587–1597. [PubMed: 18646053]
51. Jorgensen T, Berner A, Kaalhus O, Tveter KJ, et al. Up-regulation of the oligosaccharide sialyl LewisX: a new prognostic parameter in metastatic prostate cancer. Cancer Res. 1995; 55:1817–1819. [PubMed: 7728744]
52. Hong Q, Sze CI, Lin SR, Lee MH, et al. Complement C1q activates tumor suppressor WWOX to induce apoptosis in prostate cancer cells. PLoS One. 2009; 4:e5755. [PubMed: 19484134]

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Statement of clinical relevance

Prostate cancer (PC) is the most common malignancy and the second leading cause of cancer related deaths among men in western countries. Early PC detection using prostate specific antigen (PSA) measurements in blood reduces PC-death among unscreened men. However, due to modest specificity of PSA at commonly used cut-offs, there are urgent needs for additional biomarkers contributing enhanced risk classification among men with modestly elevated PSA. In this pilot study, we have identified candidate plasma biomarker signatures associated with distinct risk groups of PC patients using affinity proteomics. Of note, the data also implied that the risk group with modestly elevated total PSA and %free PSA, a priori known to be heterogeneous, could be further stratified into two subgroups, each resembling the lowest and highest risk groups, respectively. Furthermore, an additional malignant biomarker signature pin-pointing PC was also indicated. Once replicated in carefully ascertained clinical cohorts, these results might provide novel opportunities for improved risk classification of PC patients based on plasma signatures that subsequently contribute personalized management and therapy tailored to reflect the need of each individual PC patient.

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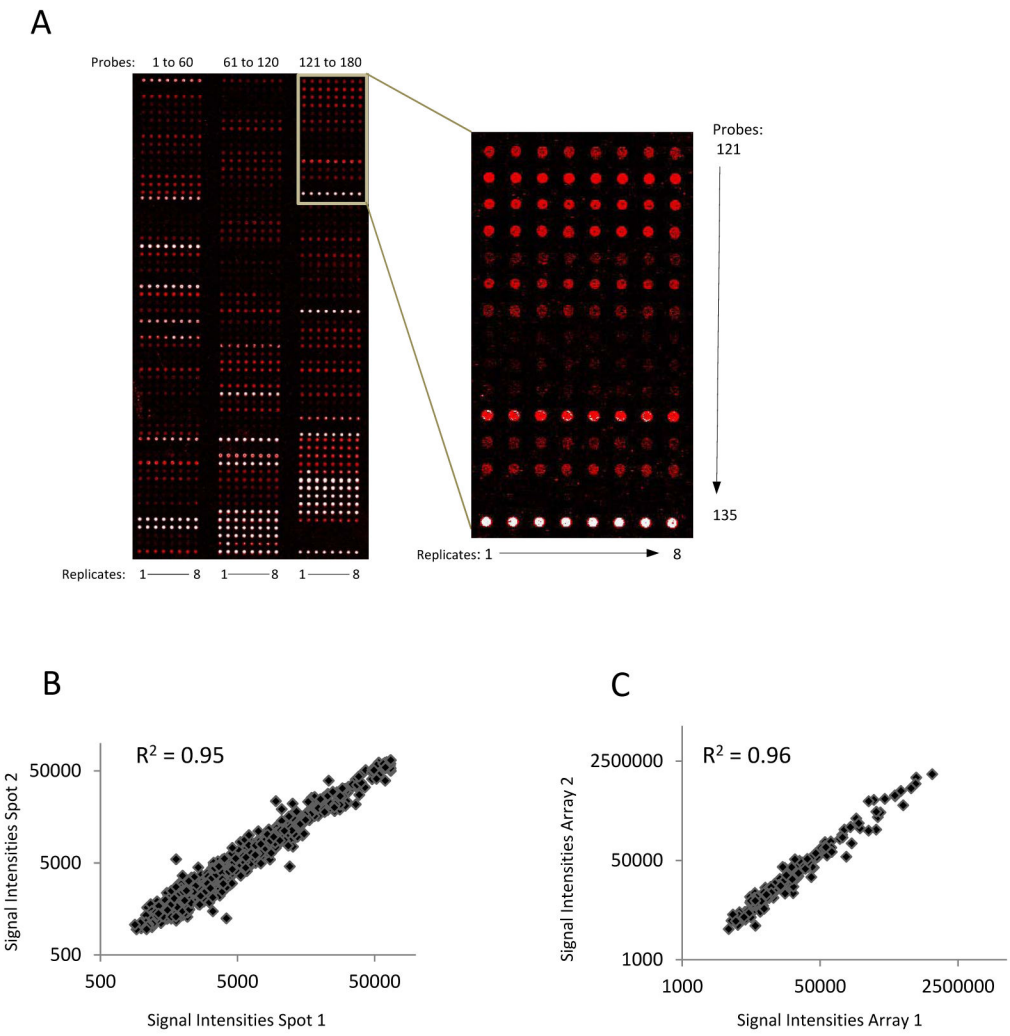


Figure 1.

Evaluation of recombinant scFv antibody microarray. A) Scanned image of a representative microarray containing 1440 data points (180 probes including controls x 8 replicates). The image was scanned at 80% laser power and 80% PMT-gain. B) Intra-assay reproducibility, i.e. spot-to-spot variations. Data based on 162 different antibodies and 8 spot replicates (the 8 replicates were set into 4 random pairs, i.e. each analyte has 4 corresponding dots in the plot). C) Inter-assay reproducibility, i.e. array-to-array variations. Data based on one sample analyzed on two independent arrays, each including 162 different antibodies.

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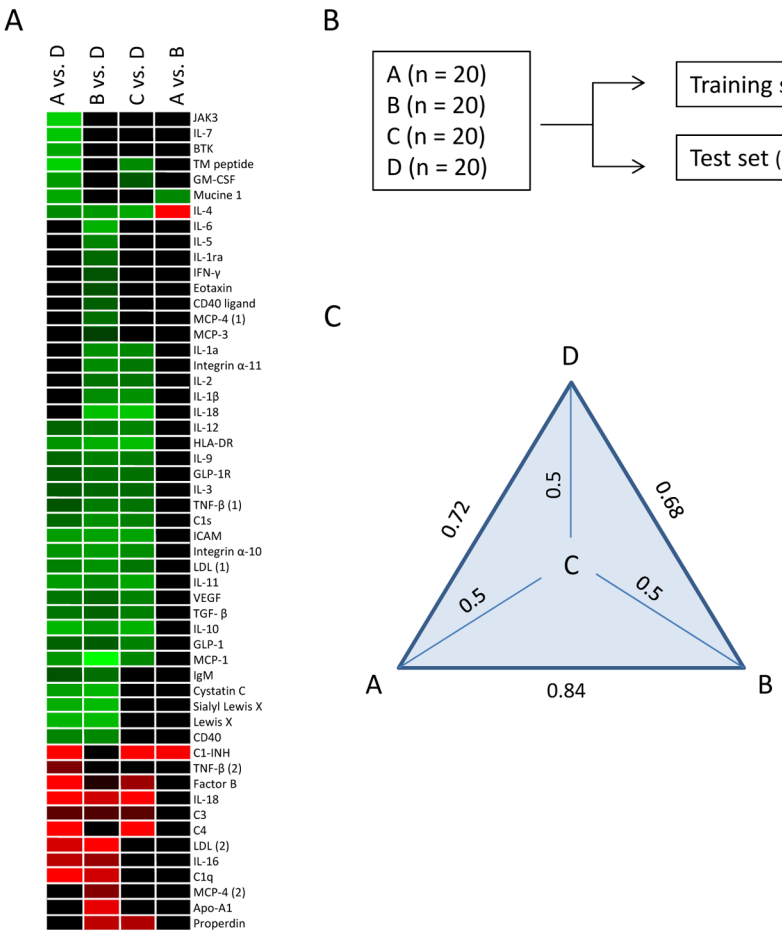


Figure 2.

Protein expression profiling of patient groups with increased risk of having PC. Four risk groups, denoted A (lowest risk) to D (highest risk), were a priori defined based on tPSA and %f PSA. A) Significantly differentially expressed analytes ($p<0.05$) were identified using Wilcoxon's signed-rank test and presented in heat maps; green – down-regulated, red – up-regulated, and black – equal levels (i.e.. for A vs B, green means down-regulated in A etc). B) Each patient cohort was divided into a training set (75% of the samples) and a test set (25% of the samples). C) Classification of the patient cohorts A to D using an SVM based approach using all 162 antibodies, i.e. unfiltered data. The SVM model was trained on the training set and tested on the independent test set, and expressed in terms of ROC AUC values.

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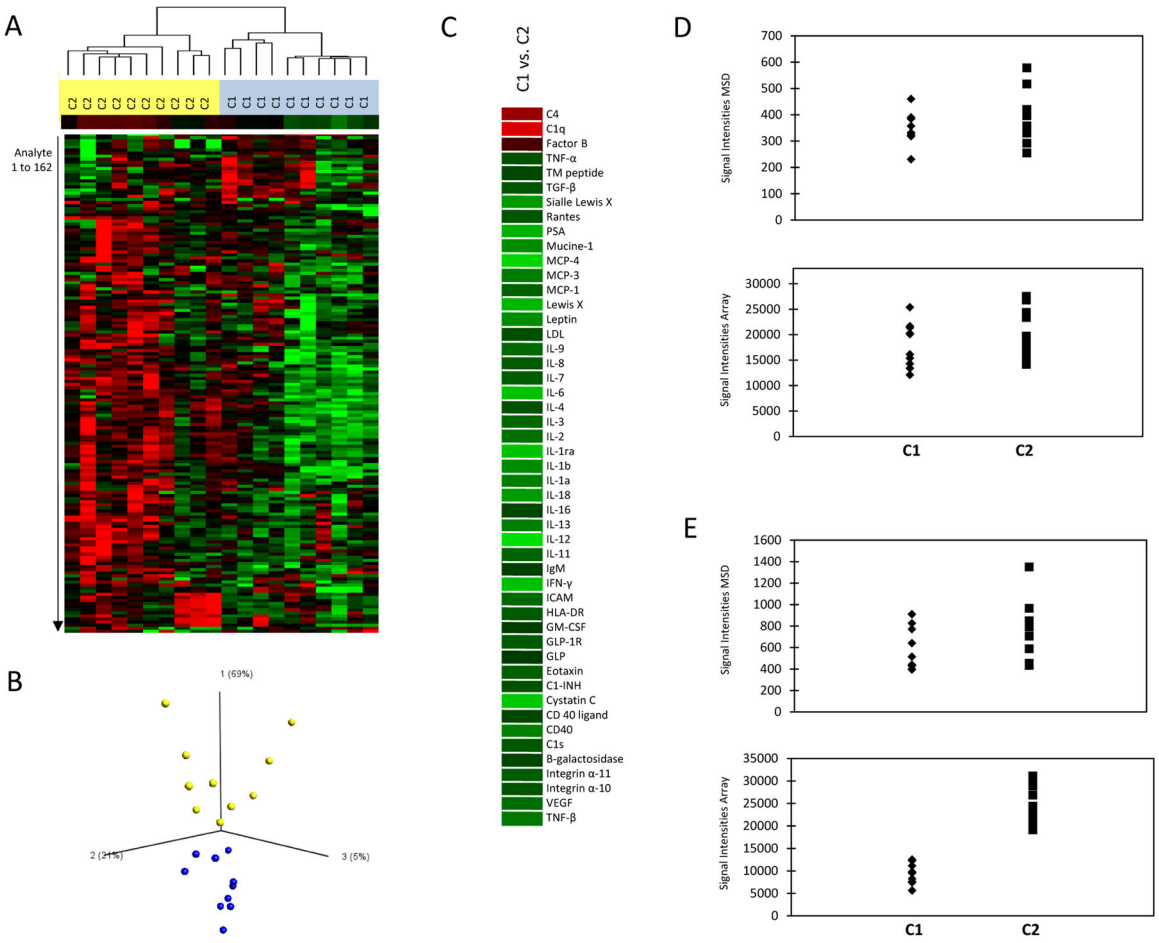


Figure 3.

Stratification of risk group C (mid-range tPSA and low % free PSA). A) Unsupervised hierarchical clustering based on all 162 antibodies, i.e. using unfiltered data, resulted in a division into two subgroups, denoted C1 (yellow) and C2 (Blue). B) Principle component analysis (PCA) based on unfiltered data, confirmed the stratification into subgroups C1 (yellow) and C2 (blue). C) Significantly differentially expressed analytes ($p<0.05$) were identified using Wilcoxon's signed-rank test and presented in heat maps; green – down-regulated in C1, red – up-regulated in C1 and black – equal levels. D–E) Validation of the antibody microarray data of selected molecules using a 10-plex cytokine sandwich antibody assay (MSD). Data is only shown for those two analytes TNF-α (D) and IL-8 (E), for which a majority of the observed signals were above the lower limit of detection for the MSD assay. The MSD data is compared with the corresponding microarray data in those cases (C1 versus C2) where these two analytes were indicated to be differentially expressed.

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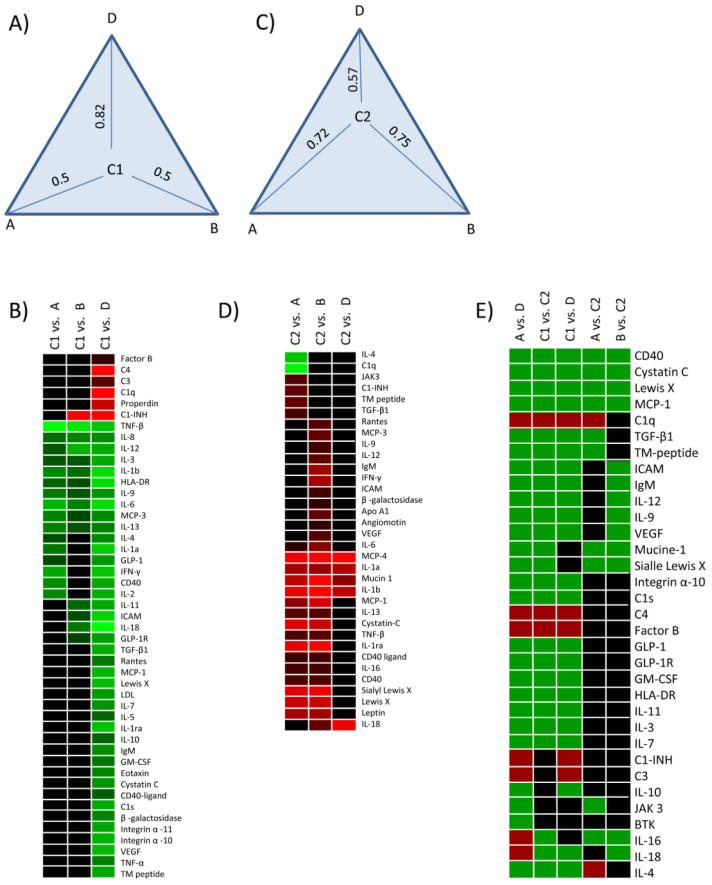


Figure 4.

Protein expression profiling of subgroups C1 and C2 versus the original risk groups A, B, and D. The samples were classified using a SVM-LOO cross-validation and ROC AUC-values were determined. Significantly differentially expressed biomarkers ($p<0.05$) were identified using Wilcoxon's signed-rank test, and presented in heat maps; red – up-regulated, green – down-regulated and black – equal levels (i.e. for C1 vs A, green means down-regulated in C1 etc). A) SVM-LOO cross-validation of C1 vs. A, B and D, respectively. B) Heat map displaying significantly differentially expressed analytes ($p<0.05$) for C1 versus A, B, and D, respectively. C) SVM-LOO cross-validation of C2 vs. A, B, and D, respectively. D) Heat map displaying significantly differently expressed analytes ($p< 0.05$) for C2 versus A, B, and D respectively. E) Comparison of the malignant signature discriminating A versus D with the expression levels of the corresponding markers for C1 vs. C2, C1 vs. D, A vs. C2, and B vs. C2.

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Table 1

Clinical laboratory parameters of patient samples included in the study.

Group	Number of samples	tPSA (ng/ml)	%f PSA (free/total)
A	20	< 0.70	n/a[a]
B	20	2.1 – 8.0	≥ 27.9 %
C	20	5 – 10.3	≥ 12.6 %
D	20	24.6 – 724	n/a[a]

[a] n/a = not applicable