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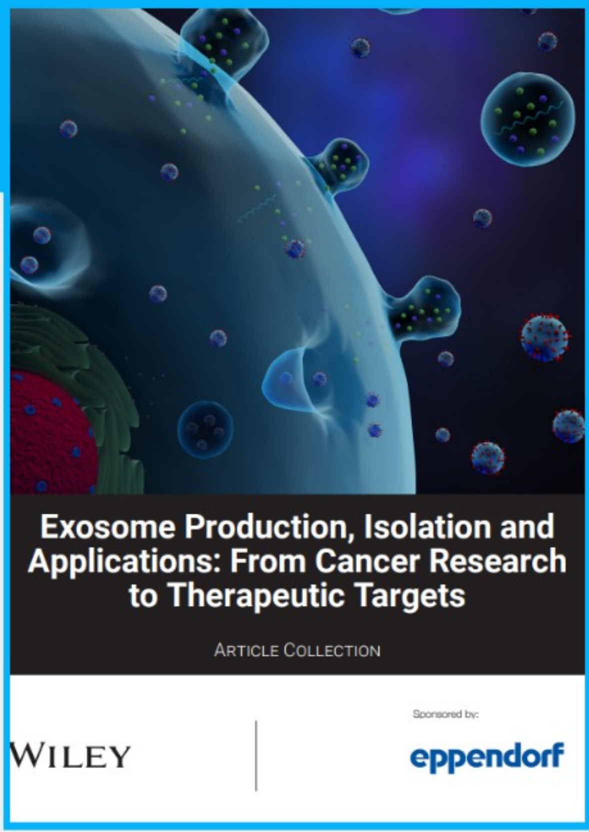

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ORIGINAL ARTICLE

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Multiplex protein analysis and ensemble machine learning methods of fine needle aspirates from prostate cancer patients reveal potential diagnostic signatures associated with tumour grade

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Abstract

Background: Improved molecular diagnosis is needed in prostate cancer (PC). Fine needle aspiration (FNA) is a minimally invasive biopsy technique, less traumatic compared to core needle biopsy, and could be useful for diagnosis of PC. Molecular biomarkers (BMs) in FNA-samples can be assessed for prediction, eg of immunotherapy efficacy before treatment as well as at treatment decision time points during disease progression.

Methods: In the present pilot study, the expression levels of 151 BM proteins were analysed by proximity extension assay in FNA-samples from 16 patients, including benign prostate lesions ($n = 3$) and cancers ($n = 13$). An ensemble data analysis strategy was applied using several machine learning models.

Results: Twelve potentially predictive BM proteins correlating with International Society of Urological Pathology grade groups were identified, among them vimentin, tissue factor pathway inhibitor 2, and integrin beta-5. The validity of the results was supported by network analysis that showed functional associations between most of the identified putative BMs. We also showed that multiple immune checkpoint targets can be assessed (eg PD-L1, CD137, and Galectin-9), which may support the

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selection of immunotherapy in advanced PC. Results are promising but need further validation in a larger cohort.

Conclusions: Our pilot study represents a "proof of concept" and shows that multiplex profiling of potential diagnostic and predictive BM proteins is feasible on tumour material obtained by FNA sampling of prostate cancer. Moreover, our results demonstrate that an ensemble data analysis strategy may facilitate the identification of BM signatures in pilot studies when the patient cohort is limited.

KEYWORDS

biomarkers, fine needle aspiration biopsy, immune signalling, machine learning, prostate cancer, proximity extension assay

1 | INTRODUCTION

Prostate cancer (PC), the most common type of cancer in men, increases continually worldwide and was responsible for 1,414,259 new cases in 2020.[1] To combat this complex disease, it is essential to overcome several challenges associated with the sampling, diagnosis, the stratification of patients prior to treatment, and the ability to monitor the effects of oncological treatments.[2] We have addressed several of these challenges within the pilot study presented here. Transrectal core needle biopsy (CNB), albeit a standard diagnostic method, is associated with risk for complications such as sepsis. Accordingly, attempts should be made to minimise the number of CNBs. A new supposedly safer method, transperineal CNB, is on the rise but is not yet established in clinical practice.[3]

Transrectal fine needle aspiration (FNA) of prostate was introduced during the 1960s and was frequently used during the 1970s and 1980s, since it did not cause complications and did not require prophylactic antibiotics.[4] Nevertheless, FNA-based cytology was gradually replaced by CNB during the 1990s, although it was shown to support diagnosis at a similar accuracy and at a lower risk for complications.[5] One reason for the decrease in the use of FNA-based cytology was the introduction of the Gleason scoring system, which is only possible on CNB.[6] Thereby, prognostic grading of PC was facilitated and currently CNB is the golden standard diagnostic method prior to therapy decisions. Another reason for the decline in FNA-based diagnosis was an increasing deficit of cytology expertise. In recent decades, research in cancer biomarkers (BMs) has been very promising and may, by the multiplexing for example of proteins, open up possibilities for FNA-based molecular cytology diagnostic and prognostic methods.[7,8] Promising studies show that molecular cytology for PC diagnosis is possible and should not be ruled out.[9]

To achieve successful FNA-based molecular diagnostics, one has to overcome the challenges of sampling error, the scarcity of material obtained, and the lack of a standardised sample processing.[10] We have previously addressed these challenges in breast cancer diagnostics and showed that a minimal amount of FNA material can be used for the profiling of hundreds of BM candidates at the protein level.[11,12] In addition, use of this methodology makes it possible to monitor expression levels of key BMs and target proteins for cancer immunotherapy in lung cancer.[13]

In recent years immunotherapy has revolutionised the therapy of advanced cancers, and lately it has also been introduced for PC.[14] Some of the main challenges are for example to monitor molecular drivers including putative targets for modern oncological treatments in the tumour as well as in the tumour microenvironment, which can support optimal selection of treatment combinations via predictive BM signatures.[14,15] PC has been regarded as immunologically "cold"; however, recent studies offer new hope to patients with advanced castration-resistant PC, but it will require identification of specific BM signatures for personalised therapy selection.[16,17] Breast cancer has also been regarded as immunologically "cold," but it has been shown that immune profiling via FNA sampling provides information on immunophenotype which can be analysed as a predictive tool in the treatment of advanced breast cancer.[18] Within the frame of the present pilot study on PC, we have used advanced data analysis to extract putative diagnostic and predictive BM signatures. For the analysis of large datasets in biomedicine, various machine learning (ML) approaches have proven to be very useful.[19] Due to a rather limited patient cohort, we therefore explored a palette of ML methods for data analysis. By an ensemble approach, ie, protein signatures that repeatedly showed predictive value, we were able to obtain a functional network analysis relevant for both diagnostics and prediction of molecular determinants coupled to what is assumed to be needed for the efficacy of therapy.[19,20] This ensemble approach per se may be very valuable for multiplex analysis of limited clinical cohorts to indicate if further collection of a larger validation cohort has the potential to be cost effective.

Taken together, the aim of this pilot study was to demonstrate the feasibility of analysing protein BM candidates and immune target molecules via "precision placement" (magnetic resonance imaging/transrectal ultrasound [MRI/TRUS] fusion imaging-guided) FNA sampling of prostate lesions. Our aim was also to investigate if potentially predictive BMs associated with Gleason scores could be identified via an ML technology-based ensemble approach. In this context, we focused analyses on the identification of BM signatures for differential analysis of benign hyperplasia vs cancer, and also

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low-grade vs high grade cancer, since these diagnostic issues are important to the subsequent selection of therapy. We also analysed the potential for BM signatures predictive for immunotherapy.

2 | MATERIALS AND METHODS

2.1 | Patient inclusion and sampling

Patients were selected for fusion biopsy according to the following criteria: clinical suspicion of PC (elevated prostate-specific antigen [PSA] level, palpable lesion, suspect MRI findings, ie, Prostate Imaging Reporting and Data System [PI-RADS] score ≥3 according to the PI-RADS V2.0 classification). All patients underwent MRI following the European Society of Urogenital Radiology guidelines; These include the use of T2-weighted images (axial, coronal, and sagittal), T1 with large field of view, apparent diffusion coefficient and high trace value (b1000–2000 s/mm^2). All consecutive cases were sampled at the Urology Clinic, Akademiska Sjukhuset, Uppsala, Sweden. The study was approved by the Regional Ethical Board (Uppsala Dnr 2012-165 with amendment 2016 and 2019-00534) and conducted accordingly, including the securing of informed consent.

The fusion biopsy was performed by an experienced urologist with the Artemis device (Artemis™; Eigen Health, Grass Valley, CA, USA), which allows precision biopsy site tracking on ultrasound and fusion of real-time ultrasound with MRI. Subsequently, an FNA sample was taken using a 220 mm needle (Cameco handle, TG Instrument, Helsingborg, Sweden) via the same probe and from the exact same position as the ensuing routine CNB samples. A fraction of each FNA sample was used for cytology (with May-Grünwald-Giemsa staining of smears for microscopic verification of tumour cells and quality control) and residual material was snap-frozen on dry ice within a few minutes for storage (at −70°C) until sample preparation. CNB samples were processed and diagnosed according to current histopathology practice and classified using the Gleason score and International Society of Urological Pathology (ISUP) grade group (*WHO Classification of Tumours*, 2016) by a senior uropathologist. Figure S3 shows haematoxylin and eosin-stained sections from CNB samples representing three cases corresponding to sampling locations shown in Figure S2, and cytology material is shown in two of these cases (#9 and #13, lesions A and B, Figure S4).

In cases where a histopathological diagnosis could be established and the corresponding FNA sample quality was adequate, the sample was prepared for molecular analysis. In cases where two different lesions in the prostate were identified, CNB and FNA samples were taken from both sites (labelled A and B).

2.2 | Sample preparation

FNA-samples were prepared as described earlier in Franzén et al.[13] Each frozen needle with material was thawed at 0°C and the content extracted using 25-50 μL of ice-cold RIPA buffer (R0278, Sigma Aldrich Sweden AB, Stockholm, Sweden) supplemented with protease inhibitors (Protease Inhibitor Cocktail tablet, No. 0469311600, Roche, Sigma-Aldrich Sweden AB, Stockholm, Sweden). After sample lysis on ice, insoluble material was removed by centrifugation at 4°C and the protein concentration was determined. Samples, diluted with RIPA buffer to a concentration of 1 mg/mL, were analysed by proximity extension assay (PEA; https://www.olink.com/) with support from the Clinical Biomarker Facility, Science for Life Laboratory, Uppsala University, Uppsala. Two different multiplex panels were used, the Olink® Immuno-Oncology and Oncology II. Each panel consists of pairs of antibodies (providing high specificity and sensitivity) against 92 proteins and four internal controls (for detailed information, see: https://www.olink.com/products/immuno-oncology/ and https://www.olink.com/products/oncology/). Results were pre-processed according to standard operating procedures at the Clinical Biomarker Facility and log transformed (Log_2), generating Normalised Protein eXpression (NPX) values which were used for subsequent data analyses.

2.3 | Bioinformatic analysis of data

The Gleason score and corresponding ISUP grade group from the CNB histopathology report were used for statistical analysis. The benign samples were given an arbitrary ISUP grade value equal to 0.1 in order to permit regression analysis of all samples.[21] Further, CNB samples were classified as B (benign), C1 (ISUP grade group 1), or C2 (ISUP grade groups 2-5). Classification analysis was based on two alternatives, B vs C (C1 + C2), and C1 vs C2.

Proteins expressed below the limit of detection (LOD; as defined by the manufacturer, https://www.olink.com/) were excluded from the study. As the patient cohort is limited, comprehensive and fully validated models are difficult at this stage. To address this shortcoming, an ensemble approach was applied using various ML techniques to nominate predictive BM candidates. The MLs used in this context were logistic regression, partial least square regression, eXtreme Gradient Boosting (XGBoost), and random forest. BM candidates pointed out by these models were derived by Boruta, Shapley Additive exPlanations (SHAP), and BorutaShap (Figure S1). For a more detailed description of methods, including references, see the Supporting Information. BM candidates nominated at least two times by the different methods were submitted to functional analysis using the STRING database online tool (https://string-db.org/) to explore possible biological interactions between nominated BM candidates, which if positive may support findings and future validation efforts. Multiple statistical analyses were applied including univariate statistical analysis of variance (ANOVA), Pearson correlation, unsupervised methods as principal component analysis, and t-distributed stochastic neighbour embedding (t-SNE) to provide further insight related to BMs of importance. A two-sided *P* value of <0.05 was defined as statistically significant.

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Since the cohort data were imbalanced, ie, few benign vs cancer samples, the Synthetic Minority Over-Sampling Technique (SMOTE) was applied. ML methods were used both on the original and SMOTE-processed data sets. Software used for analysis included Qlucore Omics Explorer (https://www.qlucore.com/), Scikit-Learn (https://scikit-learn.org/stable/index.html), and other open-source Python libraries.

3 | RESULTS

3.1 | Sampling and patient characteristics

Via MRI/TRUS fusion imaging-guided biopsies, 32 consecutive FNA samples were collected from 23 patients (Figures S2–S4). However, after cytology-based quality control and exclusions due to blood contamination, 20 samples from 16 patients were analysed by PEA and subjected to data analysis (Table 1). In four cases (#6, #8, #9, #13), samples from two different lesions (A and B) of the same patient were obtained. The subsequently collected CNB samples were used for routine diagnostics from the corresponding MRI/TRUS-defined locations (for an overview of the workflow, see Figure 1).

3.2 | Protein analysis reveal 12 biomarker candidates

In total, 151 different proteins were assessed by PEA to have a value above the LOD in at least one sample. Only 12 proteins were below the LOD in all samples and therefore excluded from subsequent data analysis. Unsupervised hierarchical cluster analysis based on all proteins that were expressed over the LOD and all samples indicated adjacent clustering of benign samples (#11, #12, #25) and functionally related proteins (eg, CD4/CD5, CCL3/CCL4, CXCL9/CXCL10, GZMB/GZMH; Figure S5). This observation was considered supportive in that biologically relevant results were obtained and in support of the ensuing data analyses.

We used an ensemble approach of analysis; ie, several relevant methods for modelling and combined strategies were applied to generate models. Results from this approach showed that 12 proteins (BM candidates) were nominated at least twice, and seven proteins were nominated most frequently (ie, more than two times: vimentin [VIM], tissue factor pathway inhibitor 2 [TFPI-2], integrin beta-5 [ITGB5], R-spondin-3 [RSPO3], transforming growth factor β1 [TGFβ1], tumour necrosis factor receptor superfamily member 12A [TNFRSF12A], and TNFRSF21; Table 2). Out of the top seven BM candidates, only RSPO3 was expressed below the LOD in 30%

TABLE 1 Patients and characteristics

Patient No.	Age (years)	PSA (ng/mL)	MRI PI-RADS	Prostate volume (mL)	CNB classification[c]	ISUP grade [d]
3	64	4	4	40	C2	3
4	68	4.6	3	63	C1	1
6[a]	55	6.3	4[b]	46	C1	1
7	55	7	3	21	C2	2
8[a]	70	11	5	65	C1	1
9[a]	59	4.6	4[b]	38	C2	2
11	69	8.6	3	63	B	"0.1"[d]
12	59	2.6	3	84	B	"0.1"[d]
13[a]	74	35	5[b]	70	C2	4
16	52	9.3	3	30	C1	1
17	46	6.3	3	20	C2	2
21	67	11	5	50	C2	2
24	65	18	3	90	C1	1
25	60	6.5	3	37	B	"0.1"[d]
26	71	5.2	5	35	C2	2
27	66	6	5	25	C2	3

Abbreviations: CNB, core needle biopsy; ISUP, International Society of Urological Pathology; MRI_PI-RADS, Prostate Imaging Reporting and Data System for magnetic resonance imaging; PSA, prostate-specific antigen.

[a]FNA-samples were obtained from two locations within the prostate.

[b]PI-RADS for different lesions is described in Figure S1.

[c]Benign samples = B. Cancer samples were divided into C1 (ISUP = 1) and C2 (ISUP ≥ 2). Defined as "ISUP2grp" in the analysis

[d]Samples from benign lesions were assigned ISUP grade "0.1" arbitrary value to facilitate data analysis; see Section 2.3.

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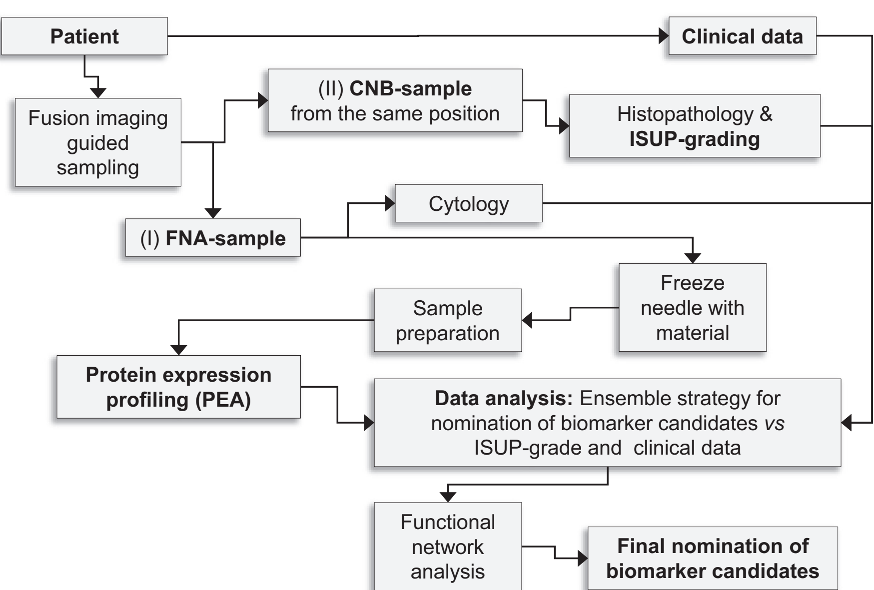

FIGURE 1 Study overview. Patients were included according to criteria described in Section 2 (Materials and Methods). Samples were obtained and analysed as described in the work flow. Protein expression levels were analysed using several multivariate and machine learning algorithms and biomarker candidates were nominated by an ensemble strategy (for a detailed description, see Supplementary methods). Finally, frequently nominated biomarker candidates were evaluated using a functional network analysis (using the STRING database). CNB, core needle biopsy; FNA, fine needle aspiration; ISUP, International Society of Urological Pathology; PEA, proximity extension assay.

of the samples; all other proteins were above the LOD in all samples. Therefore, the raw data were considered robust enough for the generation of prediction models. To further explore the validity of nominated BM candidates, functional network analysis using the STRING database showed functional relations between several of the BM candidates and a strong association with apoptosis and/or necroptotic processes (ie, 5 out of the top 10, Gene Ontology, PPI enrichment *P* value: 3.84 e−13; Figure S7).

One example of a model generated by the XGBoost classifier (groups B, C1 and C2, cross validation, 200 iterations) is shown in Figure 2. This model identified a five-protein signature (VIM, TFPI-2, ITGB5, RSPO3, and TNFRSF12A) that separated group B from C1 and C2 ($P \leq 0.05$, $q \leq 0.03$). The signature did not show associations with prostate volume, PSA, or MRI-based PI-RADS data (Figure 2A). Hierarchical clustering showed higher relative levels of ITGB5 and VIM in C2 than in C1, and the reverse expression pattern was observed for TFPI-2, RSPO3, and TNFRSF12A, with one exception (ie, sample #26, ISUP = 2). Benign samples seem to express intermediate levels of these five proteins. Using a t-SNE plot of the model, the relative positions of all samples showed good separation between groups, despite some overlap between C1 and C2 (Figure 2B).

3.3 | Biomarker candidates nominated via ensemble models and STRING database analysis

As the analysed cohort contained rather few cases, we next focused on results representing the most frequently nominated BM candidates. These proteins include TFPI-2, RSPO3, and VIM. TFPI-2 is involved in the regulation of matrix remodelling. Moreover, TFPI-2 is suggested as suppressor of tumour growth and has been shown to decrease invasiveness of PC cells in vitro.[22,23] However, putative roles for TFPI-2 have not been reported for PC in vivo. Univariate analysis of variance (ANOVA) of TFPI-2 expression levels showed a >4-fold increase compared to benign cases in both low-grade cancers (C1, $P < 0.1$) and high-grade cancers (C2, $P < 0.01$). RSPO3, which has been implicated in the promotion of Wnt signalling and angiogenesis, was also frequently nominated and showed a similar expression profile with a significant increase in C1 cancers vs benign samples ($P < 0.05$). RSPO3 is reported as a prognostic marker in PC and involved in tumour progression in several other types of cancer.[24] VIM, an intermediate filament protein found in mesenchymal cells, in contrast to TFPI-2 and RSPO3, showed elevated levels in C2 vs C1 cancer samples ($P < 0.01$) and benign samples ($P < 0.05$). VIM is known to be increased in different types of aggressive cancers including PC.[25]

Using STRING database analysis, functional associations were mapped between VIM, ITGB5 (a receptor for fibronectin), and TGFβ1 (a multifunctional protein that controls, eg, proliferation and differentiation) (Figure S7). Interestingly, all three proteins were frequently nominated by several analysis models (Table 2, Table S1) and ITGB5 has been reported to have a key role in TGFβ1-induced epithelial-mesenchymal transition[26(p5),27] TGFβ1 may together with the chemokine C-X-C motif chemokine 13 (CXCL13) play a significant role in tumour microenvironment (TME) immune responses.[28] CXCL13 was nominated in several models and is reported to mediate PC cell proliferation through JNK (C-Jun N-terminal kinase)

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TABLE 2 Nomination of biomarker signatures and candidates by multivariate modelling and an ensemble approach

Nominated biomarker candidate recurrent variables 2 times or more (protein name)	UniProt/assay name[a]	UniProt ID number	Recurrence, by rank in prediction models (rank order[b])
Integrin beta-5	ITGB5	P18084	B vs C (1) Rank B-C1-C2 (2)
Tissue factor pathway inhibitor 2	TFPI-2	P48307	C1 vs C2 (2) Rank B-C1-C2 (1)
Vimentin	VIM	P08670	C1 vs C2 (1) Rank B-C1-C2 (3) Rank+clin.[c]
R-spondin-3	RSPO3	Q9BXY4	B vs C (2) Rank B-C1-C2 (5)
Tumour necrosis factor receptor superfamily member 21	TNFRSF21	O75509	B vs C (3)
Tumour necrosis factor receptor superfamily member 12A	TNFRSF12A	Q9NP84	C1 vs C2 (3) Rank B-C1-C2 (6)
Transforming growth factor β1	TGFβ1	P01137	B vs C (4)
C-C motif chemokine 7	CCL7 (MCP3)	P80098	C1 vs C2 (4)
Wnt inhibitory factor 1	WIF1	Q9Y5W5	C1 vs C2 (5)
C-X-C motif chemokine 13	CXCL13	O43927	B vs C (5) Rank B-C1-C2 (4)
FAS-associated death domain protein	FADD	Q13158	B vs C (6) Rank+clin.[c]
Methionine aminopeptidase 2	MetAP2	P50579	C1 vs C2 (6) Rank B-C1-C2 (7)

Note: Several models and variants thereof (see Supporting Information) were applied for analysis of proximity extension assay (PEA) data from the fine needle aspiration (FNA) samples. Two major types of models were used to identify possible association with International Society of Urological Pathology (ISUP) grade groups: classification models and regression models. The machine learning techniques used are described in Section 2.3, and in more detail in the Supporting Information under "Methods" More detailed results from all models are also described in Table **S1**. Table 2 presents biomarker candidates that were nominated by at least two models (recurrence frequencies) and were explored by functional network analysis.

[a]For abbreviations of protein names, see https://www.olink.com/ or Table **S2**.

[b]Rank order from most (1) to less (7) frequent biomarkers is shown.

[c]Rank+clin. = A rank model which included the clinical variables MRI-PI-RADS and Age.

signalling.[29] In general, analysis of chemokines such as CXCL13 and C-C motif chemokine 7 (CCL7) in the TME may be of high significance for patients with PC.[30]

Finally, we found three members of the tumour necrosis factor receptor superfamily to be frequently nominated (ranks 3-6) and within the functional network, i.e., FADD (Fas-associated death domain protein), TNFRSF12A (cytokine tumour necrosis factor-like weak inducer of apoptosis [TWEAK] receptor), and TNFRSF21 (death receptor 6), the latter of which activates the JNK and nuclear factor kappa B (NF-κB) pathways. TWEAK is also implicated in NF-κB signalling, and the biofluids levels of TWEAK from PC patients are reported to correlate with the ISUP grade group.[31] TWEAK has also been detected in the TME of PC[32] and was in addition suggested to be an immunotherapy target in gliomas.[33] Moreover, FADD was included in a prognostic PC signature, and TNFRSF21 promotes metastasis.[34,35]

3.4 | PEA profiling of FNA from PC to reveal potential immune targets

To select the optimal immunotherapy for patients with advanced cancers in general, the target protein needs to be expressed in the tumour at a sufficient level. The level of inflammation in the TME is also important.[36] With regard to the latter, a number of immune signatures have been proposed as well as an analysis of mutation frequency.[37]

We show here that FNA sampling can provide information about the expression levels of a number of well-known immune checkpoint targets, as well as proteins in the TME associated with the development of resistance to pembrolizumab in melanoma.[38] In such cases, repeated sampling is important to follow the tumour development longitudinally. Hence, it is most likely of significant importance to

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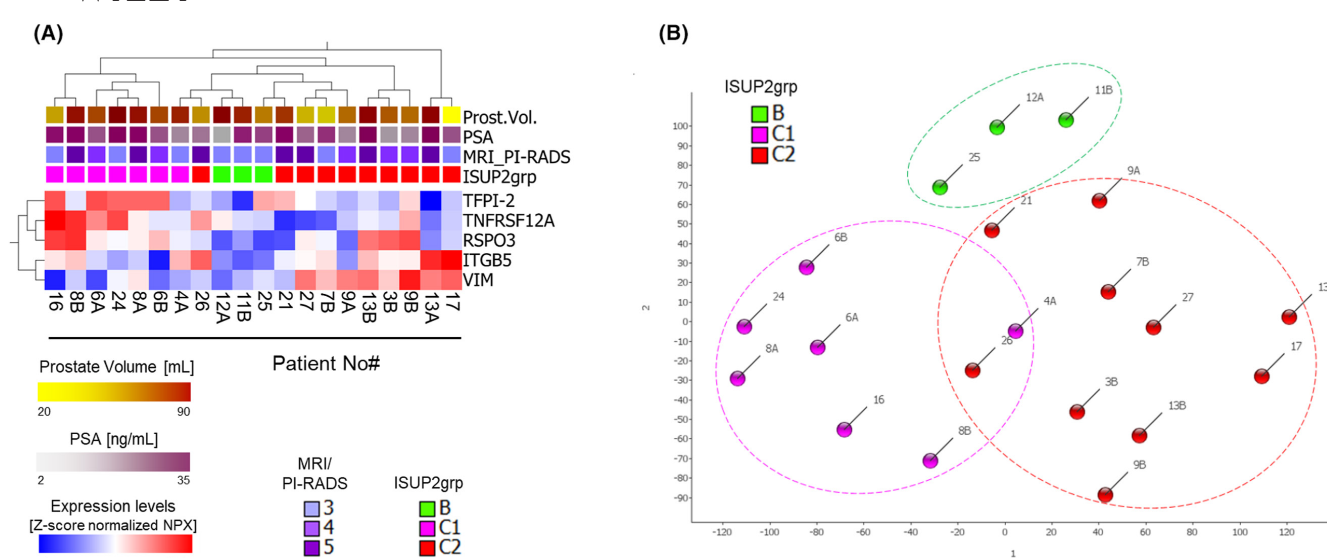

FIGURE 2 Example of one machine learning model is illustrated: Extreme Gradient Boosting (XGBoost) classifier vs the CNB classifications B, C1 and C2 (figure legend "ISUP2grp") (from Table 1). (A) Heatmap showing a five-protein signature with hierarchical clusters. No association with prostate volume, prostate-specific antigen (PSA) or Prostate Imaging Reporting and Data System (PI-RADS) data was observed. (B). A t-distributed stochastic neighbour embedding (t-SNE) plot representing the XGBoost classifier vs ISUP2 group, showing the separation of groups in two dimensions. ITGB5, integrin beta-5; Prost. Vol., prostate volume; RSPO3, R-spondin-3; TFPI-2, tissue factor pathway inhibitor 2; TNFRSF12A, tumour necrosis factor receptor superfamily member 12A; VIM, vimentin.

use minimally invasive sampling that can be performed via FNA. In our analyses we found that multiple immunotherapy targets can be assessed quantitatively in FNA samples from PC including PD-L1, TNFRSF6B/HVEM, TNFRSF9/CD137/4-1BB, and CD73 (Figure S6). In addition, proteins associated with the development of resistance to pembrolizumab, eg CCL3, CCL4, CD4, GZMA, and VEGF, which may be included for prediction of resistance,[38] were also possible to detect.

4 | DISCUSSION

In this pilot study, we combined fusion-imaging guided, minimally invasive FNA sampling of the prostate with multiplex protein profiling (PEA). Consecutive samples from 23 patents were collected; however, samples from seven patients were excluded after cytology quality control due to blood contamination. This contamination was caused by technical issues that can be solved in future follow-up studies and the success rate will most likely improve. Out of the 166 proteins from the Oncology II and Immuno-Oncology PEA panels, 151 proteins were successfully profiled and subjected to advanced data analysis and prediction modelling.

In general, the performance of the models was improved when a subset of proteins was used for the final modelling. Single prediction models were not accurate enough to enable 100% correct prediction of the ISUP grade group of a sampled tumour. The main challenges with our dataset were the limited cohort, ie, the curse of dimensionality (few samples vs many variables) and insufficient degrees of freedom to estimate a full model. The consequence may be unstable predictors and sometimes poor cross-validation performance of the fitted models. Nevertheless, our results suggest that there may be important BM signatures obtained by FNA and subsequent PEA profiling that can predict disease severity and correlate with the ISUP grade group. Given these conditions, and to increase the probability for nominating the best possible BM candidates, we applied an ML ensemble strategy and statistical boosting to identify potential BM signatures related to the ISUP grade group. This statistical approach is not new but has to our knowledge not been applied previously to explore BMs in PC cytology materials.[39,40]

A surprisingly small number ($n = 12$) of recurring BM candidates were identified and some of the most frequently nominated proteins were TFPI-2, ITGB5, VIM, and TGFβ1. Literature and STRING database analysis show that these proteins are functionally associated with extracellular matrix remodelling and invasiveness. Thus, these proteins may also be associated with metastasis and survival. Data from the Protein Atlas (https://www.proteinatlas.org/) provide some support for this hypothesis; eg, PC patients with high levels of TFPI-2 showed slightly better 10-year survival rates compared to those with low levels of TFPI-2. Although high levels of ITGB5 and VIM were unfavourable in PC and renal cancer, respectively, and associated with high ISUP grade groups in our studies, these proteins were not associated with survival in PC in the Protein Atlas. In our analyses members of the tumour necrosis factor receptor superfamily, FADD, TNFRSF12A, and TNFRSF21, showed low levels of expression in benign cases and a trend for elevated levels in PC with high ISUP grade groups. In the Protein Atlas, high expression levels of FADD and TNFRSF12A were associated with unfavourable prognosis in head and neck cancers but were not significantly altered in PC in relation to survival. However, expression differences related to various types of samples analysed, different sample handling, other types of antibodies, and detection methods used may complicate direct comparison of data from the

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Protein Atlas and the results from our study, but could be regarded as suggestions of proteins for future validations. Nevertheless, we identified a core of BM candidates by adding functional network analysis (by STRING database integrated analysis), a powerful tool that has been only sparsely used in PC BM research.[41]

Immunotherapy is a rapidly emerging and promising treatment regime for patients suffering from advanced cancers, including PC. In this study, we profiled four proteins representing immune targets (eg, PD-L1), and four proteins associated with immunotherapy resistance (eg, GZMA). Although these proteins did not show correlation with ISUP grade groups, it was interesting to note that many of these proteins could be assessed above the LOD in most cases. Therefore, our data suggest that FNA samples analysed by PEA may be used to identify BM signatures associated with immunotherapy efficacy and resistance in PC.

Many of the nominated BM candidates described in this pilot study seem highly relevant to PC, even when considered one BM at a time. However, the strength of our study is to show how a potentially predictive *signature* can be identified in a minute amount of material from minimally invasive FNA sampling. Based on our experience, we suggest that an ensemble strategy for data analysis combined with functional network analysis may well be used for pilot studies when a limited cohort is available, for feasibility evaluation or to explore the potential of a BM project at a relatively early stage and within a restricted budget. The potential for molecular diagnostics and protein profiling in FNA samples has developed rapidly in recent years, and most likely it is only a matter of time before the technology is fully valid even as support for diagnosis and treatment prediction in PC.[42]

In conclusion, our pilot study represents a "proof of concept" and shows that multiplex profiling of potential diagnostic and predictive BMs is feasible via minimally invasive FNA sampling.

AUTHOR CONTRIBUTIONS

Conceptualization (HJ, GA, BF), data curation (SL, MH, RCA, BF, PR), formal analysis (BF, SJ, RCA, AD, SJ), funding acquisition (RL, MH, SL, KV), investigation (MH, SL, AD, GA, BF, SJ), methodology (SL, BF, SJ), project administration (PR, BF, SL), resources (MH, SL, AD, RL), software (SJ), supervision (MH, GA), validation (GA, AD), visualisation (BF, AD), writing—original draft (BF, RL, SL), and writing—review and editing (PR, KV, AD, SL, BF, RL, SJ).

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CONFLICT OF INTEREST STATEMENT

The authors declare there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data not included in the article or Supporting Information are available from the corresponding author (bo.franzen@ki.se) or Pontus Röbeck (pontus.robeck@akademiska.se) upon reasonable request.

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