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Detection of candidate biomarkers of prostate cancer progression in serum: a depletion-free 3D LC/MS quantitative proteomics pilot study

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Background: Prostate cancer (PCa) is the most common male cancer in the United Kingdom and we aimed to identify clinically relevant biomarkers corresponding to stage progression of the disease.

Methods: We used enhanced proteomic profiling of PCa progression using iTRAQ 3D LC mass spectrometry on high-quality serum samples to identify biomarkers of PCa.

Results: We identified > 1000 proteins. Following specific inclusion/exclusion criteria we targeted seven proteins of which two were validated by ELISA and six potentially interacted forming an 'interactome' with only a single protein linking each marker. This network also includes accepted cancer markers, such as TNF, STAT3, NF- κ B and IL6.

Conclusions: Our linked and interrelated biomarker network highlights the potential utility of six of our seven markers as a panel for diagnosing PCa and, critically, in determining the stage of the disease. Our validation analysis of the MS-identified proteins found that SAA alongside KLK3 may improve categorisation of PCa than by KLK3 alone, and that TSR1, although not significant in this model, might also be a clinically relevant biomarker.

The most common male cancer in the United Kingdom is prostate cancer (PCa), with 47 300 diagnoses in 2013 (CRUK, 2014a) and 10 837 deaths in 2012 (CRUK, 2014b) from the disease. At disease presentation, ~16% of men in the United States will have locally

advanced or metastatic disease, despite PSA screening, and of the remainder, 30–40% will still suffer biochemical recurrence regardless of radical prostatectomy (Brawley, 2012). Once PCa has metastasised, life expectancy is generally < 5 years. Conversely,

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patients presenting with organ-confined disease have a minimal risk of death within 15 years (Brawley, 2012). The US screening programme is thought to have led to >1 million additional men being diagnosed and treated for PCa between 1986 and 2005. However, a worrying observation is that for every 1 death that is averted 20 men are 'overdiagnosed'. Overdiagnosis is a disturbing problem because of globally acknowledged treatment-associated side effects (Welch and Albertsen, 2009).

In this context it becomes essential to discover modes for improving diagnosis and planning surgical interventions. Novel candidate biomarkers offer potential clinical utility in the more accurate identification of patients with an increased risk of aggressive PCa before invasive treatments.

Proteomic profiling utilising isobaric stable isotope labelling and ultra-performance liquid chromatography linked with high-resolution tandem mass spectrometry (LC-MS) offers extended linear dynamic range in proteome coverage (Zeidan and Townsend, 2008; Zeidan *et al*, 2009a, b; Al-Ruwaili *et al*, 2010) with high analytical precision (Garbis *et al*, 2008, 2011).

Such methodological features are particularly important when it comes to the analysis of serum samples whose protein content spans a wide dynamic range of >12 orders of magnitude with the carrier protein albumin accounting for ~55% of total protein content by mass (Anderson and Anderson, 2002; Boylan *et al*, 2010; Garbis *et al*, 2011; Rehman *et al*, 2012; Tonack *et al*, 2013). Such high abundance of albumin masks or sequesters the presence of lower abundance proteins. Many serum proteomic methods utilise depletion strategies to remove the high abundance proteins (primarily albumin and immunoglobulins) to simplify the analysis of the proteome, but this results in the concurrent removal of many other lower abundance, potentially valuable, proteins (Yocum *et al*, 2005).

Building on the success of previous methods (Garbis *et al*, 2008; Bouchal *et al*, 2009), we developed a quantitative version of a whole serum analysis approach to investigate gender-mediated factors affecting the obesogenic state in humans (Al-Daghri *et al*, 2014). The aim of our current study was to apply this approach to identify serum biomarkers of PCa progression. Our study hypothesis is that the methodological attributes of the iTRAQ 3D LC-MS protocol exhibits sufficient selectivity, specificity and sensitivity to reveal novel and clinically relevant biomarkers that can stage PCa progression.

MATERIALS AND METHODS

Discovery samples. For the mass spectrometry (MS) discovery phase, we used serum from a panel of patients recruited (using informed consent) through the University of Surrey (Professor Pandha SUN study, REC reference 08/H1306/115) categorised as follows: (1) PCa null, <1 ng ml⁻¹ PSA (20 samples in this category); (2) putative benign disease, 4.7–12 ng ml⁻¹ PSA, including benign prostatic hyperplasia, prostatitis, prostatic intraepithelial neoplasia, inflammation and atrophy (15 samples in this category); (3) T1–T2 stage prostate cancer, 3.9–4.8 ng ml⁻¹ PSA (20 samples in this category); and (4) T3–T4 stage PCa (some with metastatic disease), 6.7–17.65 ng ml⁻¹ PSA (20 samples in this category). Serum was collected in red-topped serum activator tubes (BD Biosciences, Oxford, UK), inverted five times and left at room temperature for 30 min before centrifugation at 3000 r.p.m. for 10 min. All samples were centrifuged within 2 h of collection. After centrifugation, the top clear fraction (serum) was removed and aliquoted into cryovials (1 ml per vial) before being stored at –80 °C.

Validation samples. To validate biomarkers by ELISA, we used a separate, independent cohort of samples collected through the University of Manchester (Professor Noel Clarke, Northern Prostate Cancer Collaborative (ProMPT), MREC/01/4/061). These

samples were categorised as follows: (1) PCa null (20 samples); (2) patients with BPH (20 samples); (3) T1–T2 stage PCa, 0.7–31 ng ml⁻¹ PSA (20 samples); and (4) T3–T4 stage PCa (some with metastatic disease), 0.5–1400 ng ml⁻¹ PSA (20 samples). Blood was collected in Gold-topped BD Vacutainer SST II Plus plastic serum tube (BD Biosciences 367955), inverted five times and left at room temperature for a minimum of 30 min (up to 2 h) before centrifugation at 1000 g for 10 min. Serum was removed and aliquoted before storing at –80 °C.

LC-MS proteomics. All aspects of the LC-MS proteomics method used for this study have been reported by the authors (Al-Daghri *et al*, 2014). The offline HILIC peptide separation has also been reported by the authors (Garbis *et al*, 2011; Delehouze *et al*, 2014; Bouchal *et al*, 2015). The discovery experiment was executed once. However, technical replicates of each group were performed using the same samples. The samples were pooled twice and labelled differently to provide these technical repeats (Figure 1A). Each pooled serum category was analysed in parallel under the same offline tryptic peptide LC-MS conditions. This gave us a high degree of analytical precision and the ability to more reliably determine a smaller degree of differential analysis not feasible with label-free methods. The biological and technical reproducibility of the study method has been reported by the authors (Al-Daghri *et al*, 2014). Specific method details may be found in the Supplementary Methods section. The mass spectrometric proteomics data have been deposited to the ProteomeXchange Consortium (Vizcaino *et al*, 2014) via the PRIDE partner repository (Wang *et al*, 2012; Vizcaino *et al*, 2014; Vizcaino *et al*, 2013) with the data set identifier PXD004575.

Our protein selection process is depicted in Supplementary Figure S1. For each group studied by MS, there were two technical replicates, labelled with a different iTRAQ label, resulting in four ratios for each comparison (i.e., 115/113, 115/114, 116/113 and 116/114 for BPH/control). Because of the variability observed between replicates, a measure, termed the 'regulation score' (Equation (1)) was used to summarise both the magnitude and consistency of differential abundance across multiple derived log₂(ratios). For instance, when the mean is high and the s.d. is low, the resulting regulation score is high. The top 40 most consistently regulated, significant ($P < 0.05$) proteins were derived from the regulation score values for the three conditions. This shortlist was used for the selection of validation markers (Figure 2C).

$$\frac{\bar{x}}{(s.d.+1)} \quad (1)$$

Equation (1) shows the calculation of the regulation score.

From the shortlist we selected proteins that differentiated one disease group from the other two, or had a step-wise increase or decrease with progression, and, critically, had commercially available validation reagents (Table 1). Because of lack of commercial availability, we were unable to study the markers that seemed to be specific to early-stage PCa.

ELISA validation. The ELISAs were obtained from Antibodies Online and My Biosource, details are shown in Supplementary Table S1. The ELISAs were performed according to the manufacturer's protocols. More detail can be found in the Supplementary Methods section.

Literature and network analysis. In addition to the ELISA validation of our chosen MS-identified biomarkers, a literature and network analysis of interacting proteins with the seven markers was performed in collaboration with Biorelate (www.biorelate.com). Details can be found in the Supplementary Methods section.

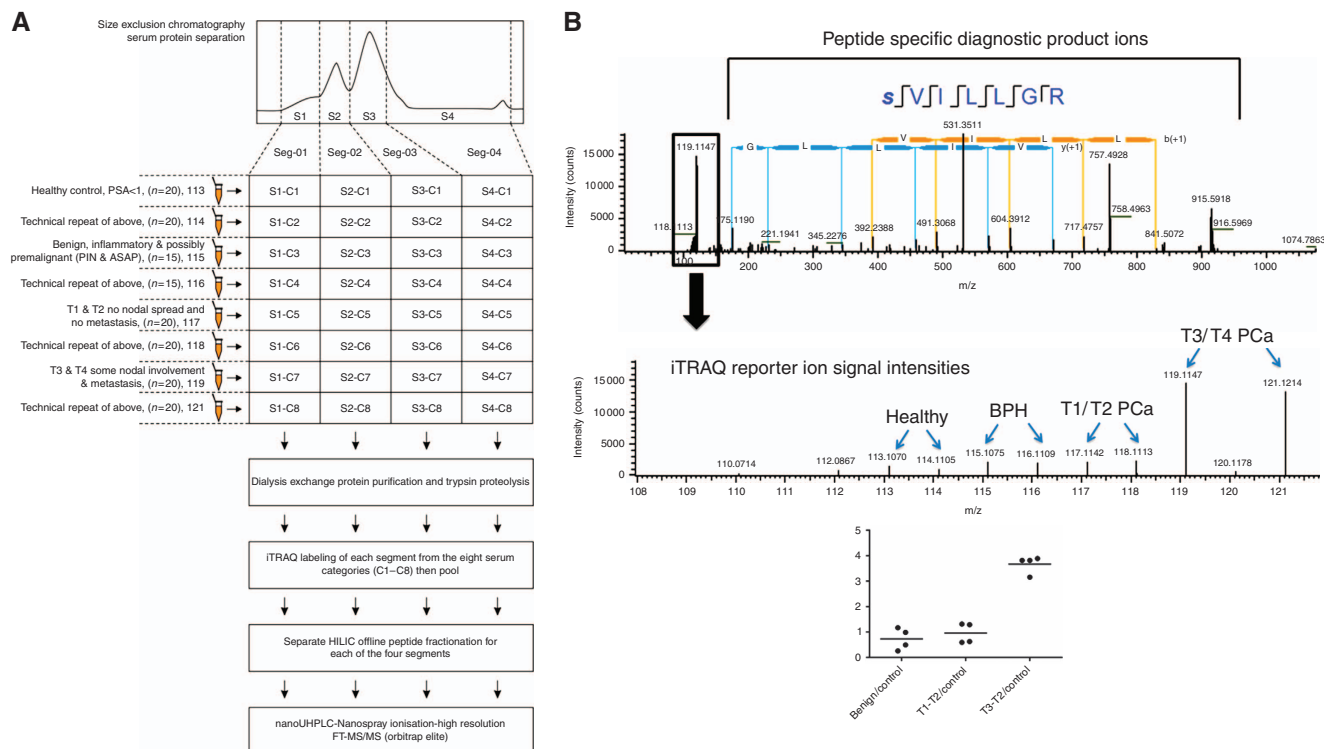


Figure 1. Mass Spectrometry Methodology. (A) Illustration of the multiplex quantitative serum proteomics method used for the discovery findings. TR = technical repeat, iTRAQ labels are in bold. The method utilises multidimensional liquid chromatography, stable isotope labelling of surrogate tryptic peptides and ultra-high resolution/precision tandem mass spectrometry using the state-of-the-art FT-Orbitrap Elite platform. (B) Annotated high-resolution (FTMS) product ion mass spectrum of the tryptic peptide SVLLGR, uniquely traceable to PSA with an expanded view of the low-mass region (and a dot plot) showing the observed iTRAQ reporter ion intensities, demonstrating the highest abundance of the PSA proteotypic peptide occurring for the T3–T4 PCa clinical cohorts.

RESULTS

Discovery MS. Our LC-MS proteomics method (Figure 1A) enabled us to identify a total of 1034 proteins (Supplementary Table S2). Our raw data have been uploaded to the PRIDE database (Accession: PXD004575). As a proof of principle, our method allowed the non-targeted relative quantitative analysis of the low-abundant KLK3 (PSA) protein without the need for mainstream immunodepletion strategies that may have otherwise depleted it (Figure 1B). Our KLK3 finding demonstrates its well-documented limitation to discriminate BPH from early-stage PCa at the serum level, but does demonstrate its utility as a recurrence marker because of the high levels seen in later-stage disease.

As a means to assess the absolute abundance range of our quantified proteome we compared the total number of peptide-spectrum matches (PSMs) for each protein across all four segments with published and estimated concentration data from PeptideAtlas (Farrah *et al*, 2011). We found a linear relationship between our MS-based average counts and the absolute concentrations for >350 proteins, suggesting that approximate absolute abundances for previously unidentified proteins can be predicted by the PSM counts (Supplementary Figure S2).

To identify functional associations between the differentially expressed proteins we created a protein–protein interaction network using the Genes2FANs (Dannenfeler *et al*, 2012) tool (Supplementary Figure S3A) that confirmed that a set of regulated proteins is functionally related although not always through direct interactions. Gene Ontology analysis (Chen *et al*, 2013) showed significant enrichment of extracellular vesicular exosome proteins (Supplementary Figure S3B), suggesting a potential secretion route of the differentially expressed proteins into the blood stream.

A total of 1034 serum proteins were identified with excellent reproducibility between technical replicates (Figure 2A). From this total, we shortlisted 40 for further study based on the regulation score mentioned previously. The distribution of regulation scores for the markers is displayed in Figure 2B and the top 40 in Figure 2C. Of these, seven were selected for validation by ELISA (Supplementary Table S3) based on their ability to differentiate one group (control/benign/T1–T2 PCa/T3–T4 PCa) from another (according to the MS data) and the availability of commercial reagents. These seven markers are summarised in Table 1.

Literature and network analysis. Using all PubMed abstracts and all PubMed Central (PMC) open-access full-text articles, we performed a comprehensive literature analysis of the seven markers to assess their previous relevance with respect to PCa and as biomarkers (Table 2). Only VWA5B2 had not been studied in the context of PCa previously, with just three publications found in total. Research was limited in PCa for both SGCd and TSR1. Of note, Love *et al* (2009) demonstrated SGCd to have a 14-fold increased level of extracellular expression in BPH RNA compared with PCa RNA, whereas Savas *et al* (2010) identified single-nucleotide polymorphisms (SNPs) associated with SGCd and selenium resistance – a dietary trace element shown to protect against various cancers including PCa (Platz and Helzlsouer, 2001; Meillet *et al*, 2004; Neill and Fleshner, 2006). No evidence was found that supported TSR1 as a PCa biomarker. The remaining four biomarkers CST3, SRC, SAA1 and KLK3, were found to have been intensively studied in PCa biology, with each having ≥40 PCa biomarker-associated publications. In keeping with our MS data, CST3 has been shown to be downregulated in PCa (Jiborn *et al*, 2006; Wegiel *et al*, 2009). SAA1 has been identified as a marker for distinguishing PCa patients with bone lesions

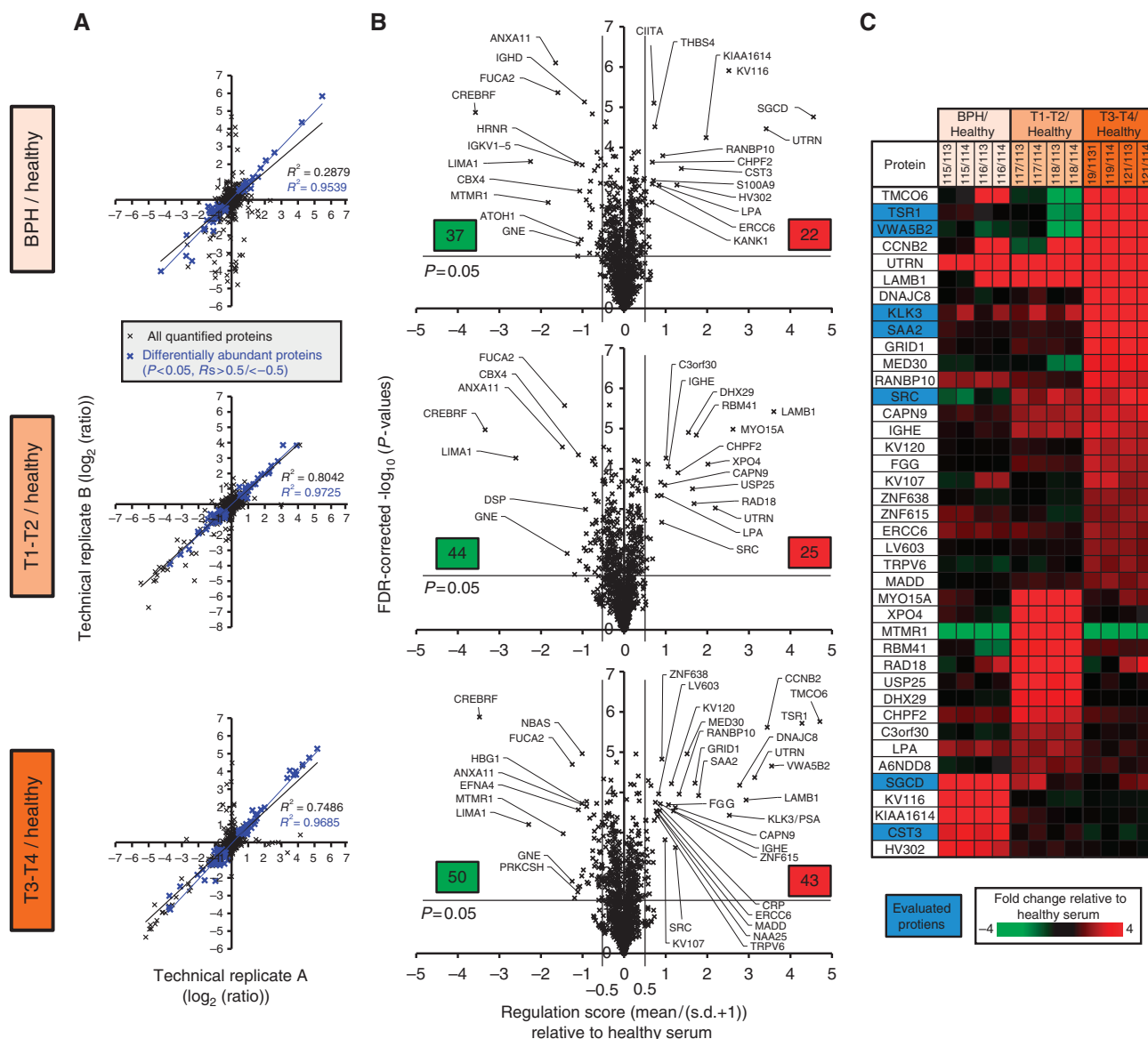


Figure 2. Summary of the reproducible differential serum protein abundance observed in BPH, T1-T2 and T3-T4 relative to healthy serum. (A) The reproducibility between technical replicates for all proteins, highlighting those considered differentially abundant (blue), relative to healthy serum (labels 113 and 114). (B) Volcano plots highlighting significantly, differentially abundant proteins plotting regulation scores (Rs) and $-\log_{10}$ (P-values) of the four ratios derived from the technical/biological replicates for BPH, T1-T2 and T3-T4 relative to healthy donor serum. A total of 72 and 82 proteins demonstrated significant differential abundance ($R_s > 0.5$ or $R_s < -0.5$, $P < 0.05$) in at least one of the three conditions, respectively, totalling 151 distinct differentially abundant proteins. (C) The top 40 significantly ($P < 0.05$) overabundant proteins, sorted by regulation score, across the BPH, T1-T2 and T3-T4 samples relative to healthy serum. Highlighted proteins are the seven selected for further validation by ELISA.

(Le *et al*, 2005). SRC has been shown, as it has with many other cancers, to be upregulated in PCa, with a large resource of research available. The existing use of KLK3 as a gold standard marker for PCa is clearly reflected in the 4908 associated PCa biomarker publications found.

Using each biomarker and their associated publications, we then curated any documented interactions (both direct and indirect) with other proteins, supplementing these with any additional interactions stored in the STRING database. As a result, interactions were discerned for all markers except for VWA5B2. These were then combined to form a network of interactions between proteins linking each of the six markers (Figure 3). Interestingly, these form a coherent graph with SRC, SAA1 and KLK3 contributing the most connecting proteins between the markers. An analysis, using DAVID (Huang *et al*, 2009), of the

enriched Gene Ontology (GO) terms associated with the six markers and their connecting interactants revealed positive regulation of biosynthetic process ($P = 5.82E-18$), positive regulation of cellular biosynthetic process ($P = 8.64E-17$) and positive regulation of multicellular organismal process ($P = 2.81E-16$) as the most enriched terms (Table 2). Other significantly enriched GO terms that may be indicative of this group's role in PCa include regulation of cytokine production ($P = 1.78E-13$) and regulation of cell migration ($P = 2.55E-12$).

Biomarker validation by ELISA. The ELISAs, as a clinically accepted diagnostics method, were performed on an independent cohort of patients and results compared with the discovery MS data (Figure 4A). Kruskal-Wallis analysis of the ELISA data demonstrated that SAA and KLK3 (PSA) were significantly

Table 1. Summary data of the seven proteins identified via mass spectrometry that were shortlisted for ELISA validation

Protein	log ₂ benign/control				log ₂ T1–T2/control				log ₂ T3–T4/control			
Delta-sarcoglycan	5.329	5.457	5.643	5.837	1.159	1.385	0.232	0.377	–0.021	0.104	0.699	0.846
Pre-rRNA-processing protein TSR1 homologue	0.334	0.452	0	0.078	–0.058	0.014	–1.11	–1.012	4.555	4.676	4.642	4.77
Kalikrein 3	0.498	1.174	0.261	0.988	0.633	1.32	0.596	1.295	3.204	3.892	3.161	3.818
von Willebrand factor A domain- containing protein 5B2	–0.312	–0.026	–0.682	–0.435	–0.253	–0.012	–1.836	–1.569	3.991	4.28	4.096	4.393
Serum amyloid A protein	0.471	0.288	0.181	0.209	0.379	0.449	0.325	0.327	2.046	1.992	2.309	1.965
Proto-oncogene tyrosine- protein kinase Src	–0.633	–0.879	–0.151	–0.437	1.118	0.827	1.366	1.1	1.506	1.263	2.044	1.809
Cystatin-C	1.476	1.749	1.461	1.785	0.172	0.456	0.097	0.394	–0.345	–0.06	–0.405	–0.15

Abbreviation: ELISA = enzyme-linked immunosorbent assay. These were selected based on their ability to differentiate one patient group from another (for example, delta-sarcoglycan (SGCd) showed marked overexpression in benign prostatic hyperplasia (BPH) compared with control, but T1–T2 and T3–T4 were similar to the control) or their stepwise increase over the course of the disease (serum amyloid A (SAA)) and the availability of commercial reagents.

Table 2. Literature-informatic analysis for PCa marker proteins

Marker	Total publications	PCa publications	PCa publications enrichment <i>P</i> -value	PCa biomarker publications	PCa biomarker publications enrichment <i>P</i> -value
SGCD	323	3	0.60	2	0.90
TSR1	112	2	0.29	0	1.00
VWA5B2	3	0	1.00	0	1.00
CST3	4431	60	5.85E – 3	50	1.00
SRC	7805	618	5.28E – 342	305	1.19E – 1201
SAA1	3767	58	4.21E – 4	48	1.00
KLK3	44 017	19 295	1.75E – 25 391	4908	1.92E – 3105

Abbreviation: PCa = prostate cancer. Publications were assigned to each protein if they were mentioned in the text. Prostate cancer (PCa) publications are those marker publications filtered that also mention PCa-related terms. The PCa biomarker publications are those PCa publications further filtered for mentions of biomarker-related terms. Enrichment *P*-values were calculated using Fisher's exact test (see Materials and Methods).

differentially expressed across the groups ($P < 0.001$). Pairwise Mann–Whitney *U* analysis showed significant SAA increases in levels in benign and T1–T2 PCa ($P = 0.037$), benign and control ($P = 0.001$), T3–T4 and control ($P < 0.001$) and T1–T2 and T3–T4 ($P = 0.002$). The KLK3 ELISA concentration was consistent with the MS data, with T3–T4 being significantly different to the control ($P < 0.001$), benign ($P < 0.001$) and T1–T2 ($P = 0.010$) groups. T1–T2 was also found to be significantly different to the control group ($P = 0.009$).

TSR1 was found not to be significant by Kruskal–Wallis analysis, but differences were found by pairwise Mann–Whitney *U* with levels increasing in benign vs control, T1–T2 vs control and T3–T4 vs control, although only the T1–T2 vs control was significant ($P = 0.013$). TSR1 was identified as a T3–T4 stage PCa marker according to MS, yet the ELISA data suggest it to be a marker of ‘cellular change’ as it was significantly increased in benign and the two PCa groups compared with the control group, but not differentially expressed between the three disease groups.

Using ELISA, SGCd, SRC, CST3 and VWA5B2 did not show any significant differences in abundance across the disease groups. The ELISA validation was technically difficult as the data imply that the levels of the target proteins largely fall below the detection limits of such assays.

To provide further insight into the utility of these markers, we performed binary logistic regression to produce predictive models that were then analysed by ROC curves (Figure 4B). This analysis showed that KLK3 had an AUC of 0.679 significantly different from the null hypothesis of AUC = 0.5 ($P = 0.006$). Both SAA-1 and TSR1 had AUC values of 0.602 and 0.613, respectively.

Alone these markers were not considered significantly different from the null hypotheses. However, using TSR1 in combination with KLK3 gave an AUC value of 0.727, improving on the predictability of KLK3 alone that is significantly different from the null hypothesis ($P < 0.0005$).

DISCUSSION

The iTRAQ 3D-LC-MS analysis of pooled serum samples yielded many putative targets for validation. Of note, KLK3 (PSA) was identified, and its abundance across the groups was in keeping with current literature and clinical experience. High levels were observed in late-stage PCa but fairly similar levels were found in the control and benign disease groups, with a slight increase in the early-stage PCa group (not significant). There are few other MS studies that have managed to identify KLK3 in serum samples from PCa patients, probably because of the use of immunodepletion strategies used in those studies (Adam *et al*, 2002; Rehman *et al*, 2012). To overcome this, studies have been utilising immunoprecipitation (IP) MS to ‘extract’ PSA for MS analysis. Utilising stable isotope labelling-multiple reaction monitoring MS (SIL/MRM-MS), it has been possible for one group to simultaneously measure multiple biomarkers including various PSA forms (Chen *et al*, 2015).

We chose several potential biomarkers identified by iTRAQ 3D-LC-MS analysis for further analysis in an attempt to find biomarkers that together might allow the improved prediction of

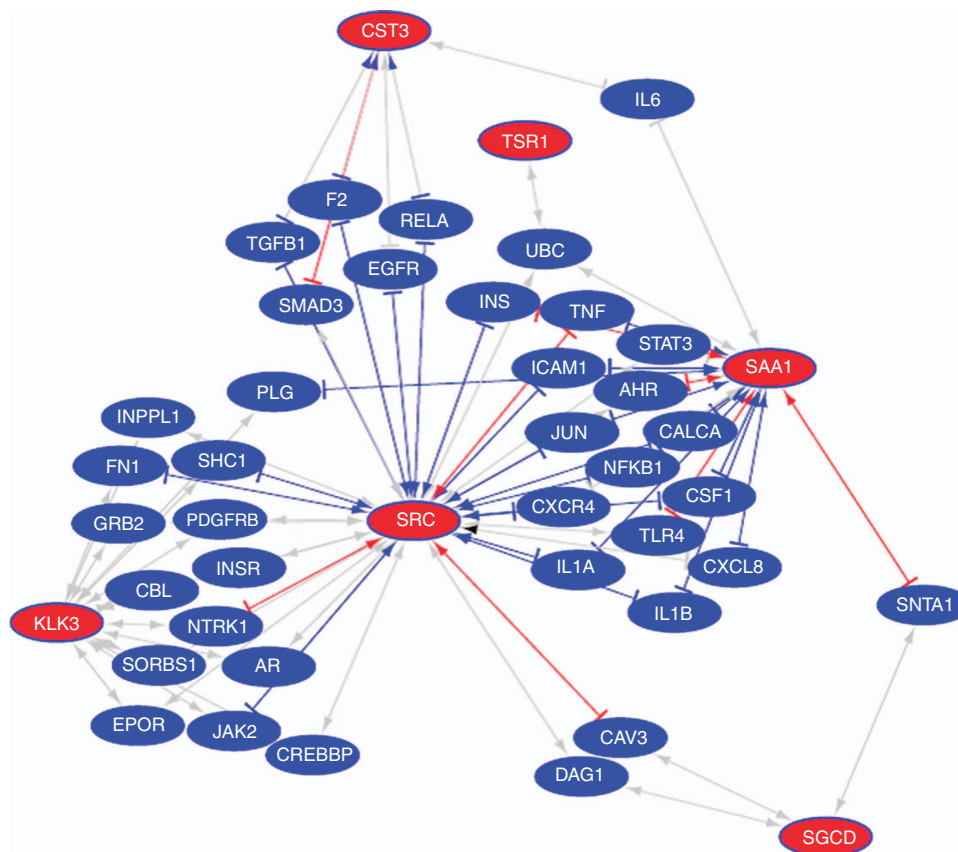


Figure 3. Network of markers excluding VWA5B2 (for which there were no discernible interactions) and proteins that have been shown to interact (directly or indirectly) with at least two of these, as identified from the publications associated with each marker and from the STRING database.

PCa stage. These ELISAs were used to investigate the abundance of these proteins in individual patient samples. Ideally, the panel would include proteins that showed different patterns of abundance between groups to allow additional predictability. The panel thus included proteins that should increase abundance in a single patient group as well as proteins that showed progressive changes across patient groups. Availability of ELISAs for the early-stage PCa markers was limited. An ELISA kit for USP24 was obtained, but was unable to detect the marker in serum (data not shown) and hence this was excluded from further analysis. It was noted that the Cancer Genome Atlas Research Network (2015) identified an amplification of a region that includes XPO4 (13q12.11), one of the markers identified in our MS discovery phase as a putative marker of early-stage disease.

Both SAA and KLK3 were found to be significantly differentiated by Kruskal–Wallis analysis of the ELISA data with very significant agreement between the two diagnostic methods. It is unsurprising that there was a lack of further validated markers as the stability of serum proteins can be poor (Gislefoss *et al*, 2009). For example, studies of KLK3 (PSA) stability advise caution in any analyses done on serum KLK3 after 2 years of storage at -70°C (Woodrum and York, 1998). As MS, by its very nature, studies proteins by looking at peptide signatures of proteins, it is less hindered by this degradation than ELISA for whole proteins would be.

Our literature analysis was useful in providing an improved rigorous understanding of PCa with respect to each associated marker. We found that although TSR1 has not been strongly associated with PCa previously, it has been suggested to play a putative role in the quality control of 18S rRNA precursor production (Tafforeau *et al*, 2013). Taking this alongside the importance of ribosome biogenesis in cancer (van Sluis and

McStay, 2014), it is perhaps unsurprising that a molecule involved in this process has implicated in PCa. In addition, work on tissue has shown TSR1 RNA expression in prostate tissue, although its highest levels of expression are found in the testis (Ardlie *et al*, 2015).

SRC is a non-receptor protein tyrosine kinase that has a number of roles in cell signalling (Wheeler *et al*, 2009). These interactions are thought to lead to several functions such as proliferation, growth differentiation, motility, migration, angiogenesis and survival. Hence, it has been implicated in several cancers including PCa as it underpins many of the hallmarks of cancer as described by Hanahan and Weinberg (2011). Previous studies utilising the SRC inhibitor dasatinib in PCa cell lines suggests that SRC may be a mediator of cell growth and migration (Nam *et al*, 2005). The PCa clinical trials with dasatinib (SRC family kinase inhibitor) have been promising, with a reduction in bone resorption in over half of the patients with progressive metastatic prostate cancer (Wheeler *et al*, 2009), where bone is the prime metastatic site for PCa. Interestingly, the GTEx project database listed prostate as the highest SRC RNA expressing tissue (Ardlie *et al*, 2015).

CST3 has been shown to be downregulated in PCa and is thought to have a role in invasion through the MAPK/ERK and androgen receptor pathways (Wegiel *et al*, 2009). A role for CST3 in neuroendocrine differentiation in PCa has also been suggested (Jiborn *et al*, 2006). Here, CST3 was downregulated in non-neuroendocrine tumour tissue and that this downregulation correlated with increasing Gleason Grade. In PCa neuroendocrine tumours (a highly aggressive subtype), however, the abundance increased with Gleason grade (Jiborn *et al*, 2006). CST3 belongs to a family of cysteine protease inhibitors that prevent proteolysis of, for example, the extracellular matrix and basement membrane. Downregulation of these inhibitors (and dysregulation of the

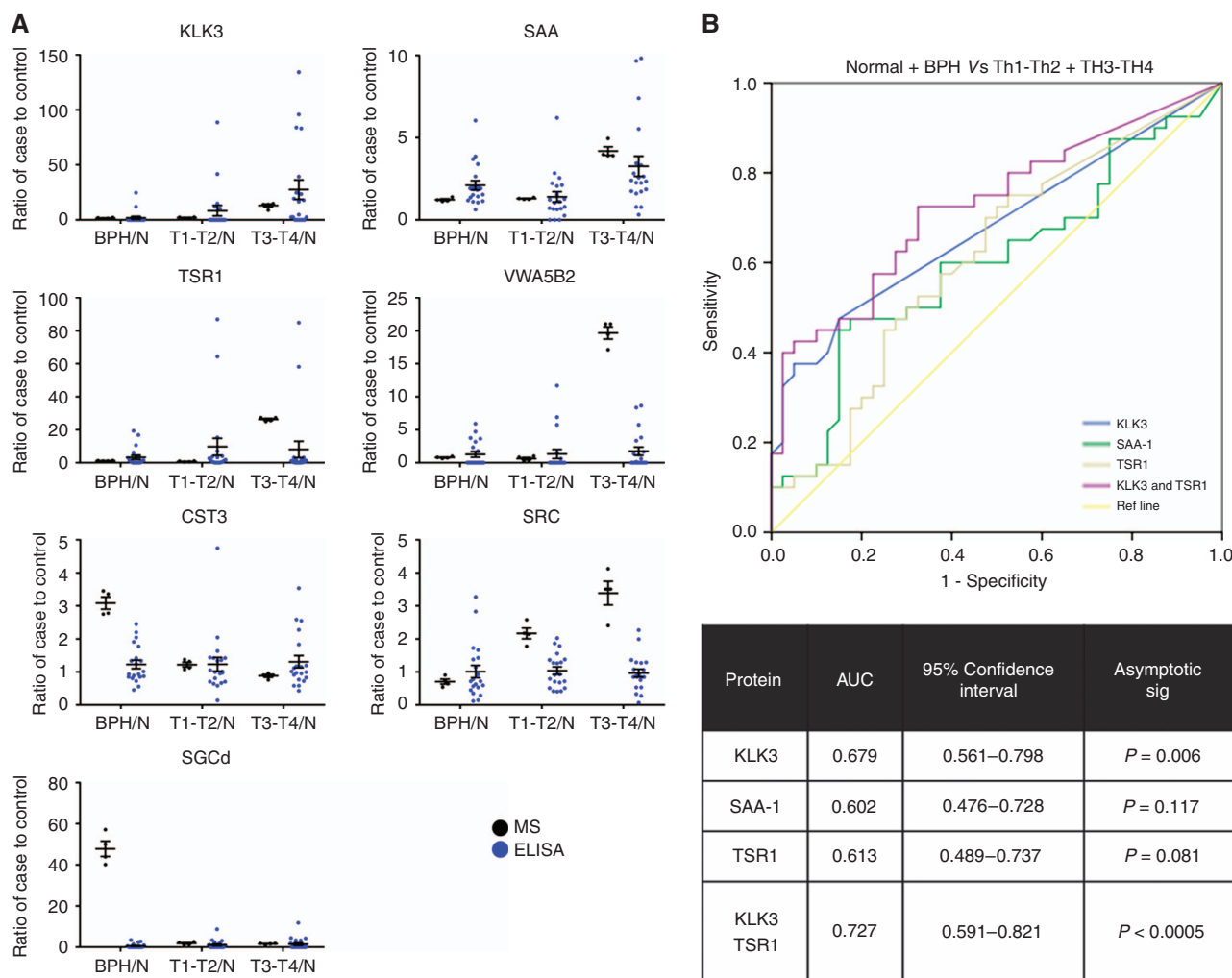


Figure 4. Biomarker Panel Validation. (A) The MS (black dots) and ELISA (blue dots) data for each marker detected. Dots represent ratios of the individual value for each case in the groups (BPH, T1–T2 and T3–T4) to the mean of the normal group. The abundance of each marker was calculated from a 5PL curve of the intensity values from the ELISAs. (B) The ROC curve analysis of individual markers and binary logistic regression model containing KLK3 and TSR1.

proteolytic/anti-proteolytic homeostasis) are associated with malignant progression and shorter mean patient survival (Jiborn *et al*, 2006). Imbalance in these molecules can occur in response to inflammatory diseases that could potentially account for its upregulation in the benign disease group that includes conditions such as prostatitis. CST3 RNA seems to be expressed fairly ubiquitously with higher expression seen in the brain, although expression has been observed in prostate tissue (Ardlie *et al*, 2015), and particularly in PCa (Uhlén *et al*, 2015).

SAA was identified from MS as being more abundant in late-stage disease that was supported by ELISA data. Le *et al* (2005) identified SAA as a marker in PCa patients showing increased levels in serum to be indicative of the presence of bone metastasis. SAA is an acute-phase protein associated with inflammation, and hence it is unlikely to be PCa specific but, in conjunction with other PCa biomarkers, could be a useful addition to a panel of (companion) biomarkers.

A limitation to the iTRAQ 3D LC-MS analysis used for our study was the use of pooled specimens for each clinical cohort. Essential to the pooled clinical cohorts was the implementation of our well-defined inclusion and exclusion criteria that minimised confounding factors. Ideally, the proteomic analysis of individual, non-pooled specimens would have allowed the assessment of heterogeneity between individual samples. The lack of validation of

some of our candidate markers could in part be related to the heterogeneity of PCa itself and the variability between the two cohorts. Prostate cancer is renowned for its clinical heterogeneity in terms of treatment response, speed of growth and overall prognosis, but it is also an incredibly complex disease at the molecular level (Boyd *et al*, 2012). This molecular heterogeneity may account for the difficulty of identifying commonalities with pooled samples, and also for the low validation rate seen between our discovery and validation cohorts that were taken from distinct geographical UK locations.

The inability of ELISA to validate some of the MS identified biomarkers may also be attributed to fundamental differences between the LC-MS approach and the ELISA technique. ELISAs rely upon an intact interaction between an epitope and antigen and are thus dependent on both the integrity of analyte and quality of the antibody. Conversely, MS is not limited by these factors and, in fact, is reliant upon the detection and identification of peptide fragments and hence is less hampered by epitope degradation. The gold standard in verifying the absolute quantitative accuracy of our proposed biomarkers is the use of targeted LC-MS approaches using such tandem mass spectrometry techniques as multiple reaction monitoring (MRM), parallel reaction monitoring (PRM) or selected reaction monitoring (SRM). To increase their sensitivity the LC-MS technique can be combined with affinity capture and purification of target

proteins or their surrogate peptides, as reported in the literature (Boja and Rodriguez, 2012). However, such LC-MS-based approaches require considerable method development and were beyond the scope of this proof-of-concept biomarker discovery study. We chose the ELISA assay as a low-cost alternative that is in wide commercial use for protein measurements.

Despite difficulties with validating potential biomarkers and a relatively small sample size, we identified SAA and TSR1 as biomarkers that could potentially add to the predictability of KLK3 and successfully validated these via ELISA. When analysed using ROC curves, TSR1 in particular was able to add to the predictability of KLK3, increasing the AUC from 0.679 to 0.737. SAA did little to increase the ability of KLK3 to distinguish between cancer and noncancer, but pairwise Mann–Whitney *U* analysis suggested it may have a role in distinguishing different stages of cancer and should not be dismissed as a potentially useful biomarker in a future biomarker panel.

In conclusion, as a proof-of-principle study, our serum proteomics discovery pipeline allows the discovery of novel serological markers of PCa progression of potential clinical utility. Our analysis has identified two potential biomarkers, SAA and TSR1, that could be combined with KLK3 to improve its predictive capability of disease progression. These proposed biomarkers warrant validation across hundreds of samples in a blinded randomised control setting. Such a validation process must also include well-curated serum specimens derived from diverse populations with well-defined patient information (BMI, family history, pharmacological status, etc.). The validation of the proposed biomarker panel constitutes a future perspective and is beyond the scope of this proof-of-concept study.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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