Original Article

New serum biomarkers for prostate cancer diagnosis

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ABSTRACT

Background: Prostate-specific antigen (PSA) is currently used as a biomarker for diagnosis and management of prostate cancer (CaP). However, PSA typically lacks the sensitivity and specificity desired of a diagnostic marker. **Objective:** The goal of this study was to identify an additional biomarker or a panel of biomarkers that is more sensitive and specific than PSA in differentiating benign versus malignant prostate disease and/or localized CaP versus metastatic CaP. **Methods:** Concurrent measurements of circulating interleukin-8 (IL-8), Tumor necrosis factor-α (TNF-α) and soluble tumor necrosis factor-α receptors 1 (sTNFR1) were obtained from four groups of men: (1) Controls (2) with elevated prostate-specific antigen with a negative prostate biopsy (elPSA_negBx) (3) with clinically localized CaP and (4) with castration resistant prostate cancer. **Results:** TNF-α Area under the receiver operating characteristic curve (AUC = 0.93) and sTNFR1 (AUC = 0.97) were strong predictors of elPSA_negBx (vs. CaP). The best predictor of elPSA_negBx vs CaP was sTNFR1 and IL-8 combined (AUC = 0.997). The strongest single predictors of localized versus metastatic CaP were TNF-α (AUC = 0.992) and PSA (AUC = 0.963) levels. **Conclusions:** The specificity and sensitivity of a PSA-based CaP diagnosis can be significantly enhanced by concurrent serum measurements of IL-8, TNF-α and sTNFR1. In view of the concerns about the ability of PSA to distinguish clinically relevant CaP from indolent disease, assessment of these biomarkers in the larger cohort is warranted.

Key words: Castration resistant prostate cancer, interleukin-8, tumor necrosis factor-α, prostate cancer, prostate specific antigen, serum biomarker

INTRODUCTION

According to American Cancer Society, an estimated 238,590 new cases of prostate cancer (CaP) will be diagnosed and 29,720 men will die of CaP in 2013.[1] There is a great deal of controversy regarding the widespread use of Prostate-specific antigen (PSA) testing for the diagnosis of CaP.[2] The adoption of PSA testing has been credited with the significant decline in the proportion of men diagnosed with metastatic disease and the overall reduction in CaP mortality over the last two decades.[2,3] However, PSA testing has been criticized for lacking the specificity to adequately differentiate between men with and without CaP. Furthermore, many men diagnosed with CaP have a normal PSA.[4] Conversely, elevated PSA levels have been found in other diseases including breast cancer,[5] renal cell carcinoma,[6] ovarian cancer[7] and adrenal neoplasm.[6] The widespread use of PSA testing is reported to have resulted in unnecessary prostate biopsies and the over diagnosis and treatment of indolent CaP.[8-10] According to some opponents, PSA testing does not improve CaP survival and may be harmful to men (physically and psychologically)[9] and to society (increasing the cost of health-care without a survival benefit).[11,12] Despite several strategies to enhance the specificity of PSA testing (e.g. PSA-density, PSA-velocity, age adjusted PSA ranges and free to total PSA ratios),[13,14] PSA testing remains a controversial tool for the early detection of CaP. At present, no commercially available biomarker(s) have been identified to differentiate between men with and without CaP or to differentiate high risk CaP from indolent CaP better than the PSA test. Clearly, much benefit would be derived from a serological test that more accurately identifies early stage CaPs or

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correctly distinguishes between men with non-cancerous and cancerous conditions or differentiates men with high risk CaP from those with indolent disease better than the PSA test. In order to improve the accuracy of CaP diagnosis and to better inform CaP treatment decision making, other serum biomarkers should be identified and validated.

Progression of CaP is accompanied by modulation of several key regulatory molecules, including vascular endothelial growth factor, interleukin-8 (IL-8), basic fibroblast growth factor (FGF), transforming growth factor beta, urokinase plasminogen activator, tumor necrosis factor-α (TNF-α) and IGF-1 and their receptors.[15,16] These changes may take the form of up- or down-regulation of growth factors or their receptors as well as changes in paracrine or autocrine mediation of growth. Several reports in the literature document the relevance of serum IL-8 and TNF-α measurements in the diagnosis of CaP.[17] In general, these studies evaluated individual biomarkers and where unable to determine if the biomarkers could be used to enhance the sensitivity and specificity of PSA. In earlier studies several cytokines and growth factors were measured in the sera of all four groups of test subjects included in this study; including transforming growth factor beta, IL-8, TNF-α, soluble tumor necrosis factor-α receptor 1 (sTNFR1), ICAM-1, IFN-γ, FGF, MMP-9. Based upon our objective three biomarkers were selected for further evaluation. This report considers concurrent serum measurements of IL-8, TNF-α and its sTNFR1 in normal healthy individuals (controls), men with elevated prostate-specific antigen with a negative prostate biopsy (elPSA_negBx), patients diagnosed with localized CaP and patients with castration resistant prostate cancer (CRPC). If CaP patients have altered serum concentrations of IL-8, TNF-α and sTNFR1, then the accuracy of PSA-based diagnostic tests may be improved by incorporating these cytokines. The objective of this study is to determine whether IL-8, TNF-α and sTNFR1 individually or in combination can (a) distinguish between men with and without CaP, (b) distinguish between localized CaP from CRPC.

MATERIALS AND METHODS

Participant privacy protection

Samples and patient data were provided from four sources with approved institutional review board protocols: (1) The Roswell Park Cancer Institute's (RPCI) Data Bank Bio-Repository (DBBR). (2) RPCI Screen Clinic and Urology Clinic; (3) participating urologists in the Western New York community; and (4) The Cancer and Leukemia Group B (CALGB); NCI protocol # CALGB - 150 201. All donor blood samples were de-identified to ensure participant confidentiality.

Patient samples

Serum cytokine measurements were obtained from four comparison groups:

1. Controls: Serum samples from 46 healthy males with no prior history of cancer at the time of collection were obtained from the DBBR, RPCI. Two participants were African American and 44 were Caucasian. Median age of this group was 47 years (range: 37-60)
2. elPSA_negBx: Serum samples from men (n = 50) with confirmed PSA > 4 ng/dl who received a negative trans-rectal ultrasound 12 core biopsy of the prostate. The participants were recruited by the Urology clinic at RPCI and by the participating Urologists in Buffalo, NY community. Six participants were African American and 44 were Caucasian. Median age was 69 years (range: 55-81)
3. Localized CaP: Serum samples from 49 patients with localized CaP were provided by the RPCI-DBBR. Serum samples were obtained prior to any therapy. Median age of this group was 64 years (range: 46-85). 40 participants were Caucasian and nine were African American. Median Gleason score was 6 (range: 6-9)
4. CRPC: Serum samples from 109 CRPC patients were provided by the CALGB; NCI protocol #CALGB - 150 201. The sample was restricted to include subjects with (1) histologically documented adenocarcinoma of the prostate, (2) metastatic disease with tumor progression after hormonal therapy[18] and (3) at least 4 weeks of androgen deprivation therapy. For patients with measurable disease, progression was defined as a greater than 25% increase in the sum of the products of the perpendicular diameters of all measurable lesions. For patients with "bone only" disease, a PSA greater than 5 ng/ml, which had risen from baseline on at least two successive occasions at least 4 weeks apart was required. All CRPC patients had metastatic disease demonstrated on imaging at some point during their history, but not necessarily at the time of enrollment. Patients were excluded if they had received prior chemotherapy, immunotherapy, experimental therapy or prior treatment with ketoconazole, aminoglutethimide or corticosteroids if they had a CALGB performance status (PS) of more than 2. Because of potential interactions with ketoconazole, no ongoing or concurrent use of terfenadine, astemizole or cisapride was allowed. The median age was 72.3 years (range: 49-88). Among this group 82% were Caucasian, 16% were African-American and 2% were of another race. Nearly, 89% participants had Gleason score in the range of 5-10.

Each participant was classified as control, elPSA_negBx, localized CaP or CRPC by the participating physicians using established criterion. The participating physicians also determined if any individual, because of other medical

reason(s), may not be the suitable candidate to participate in this study. Venous blood was drawn into vaccutainer tubes by trained technicians in respective clinics. The blood was allowed to clot at room temperature for 1 h; spun at 2000 rpm for 15 min. Different aliquots (200 µl) of each serum sample were frozen at −70°C until assayed. Each aliquot was thawed once and discarded after use.

Monitoring of biomarkers

TNF-α, sTNFR1 and IL-8 measurements were made on each serum sample using highly sensitive commercially available enzyme-linked immunosorbent assay (ELISA) kits. Ultra-sensitive human TNF-α ELISA kit (Cat # KHC 3013) was purchased from BioSource International, Inc.; Camarillo, CA. It has the sensitivity range of 0.5-32 pg/ml. sTNFR1 ELISA kit (Cat #DY-225) was purchased from R and D Systems, Minneapolis, MN. It has the sensitivity range of 10 to1000 pg/ml. Human IL-8 ELISA kit (Cat # 555244) was purchased from B.D. Biosciences, San Diego, CA. It has the sensitivity range of 5-100 pg/ml. PSA levels were measured in all serum samples by ELISA as described before.[19] Commercially available standards were used in all cases. All instructions from manufacturers were strictly followed and inter and intravariations in assays were within the recommended limits. Standard curve and concentrations of each marker were calculated using the KC Junior (Bio-Tek, Inc) software.

Statistical analysis

The analyte expression measures were summarized using descriptive statistics (proportions, median, inter-quartile range [IQR]) within participant disease categories. Kruskal-Wallis tests were used for a global assessment of possible differences in the distributions of the analyte measures across the four disease categories. Following the statistically significant global test, six Wilcoxon Rank Sum Tests were used to test pairwise differences between groups for each analyte. Associations between the expression measurements were assessed conditionally (within disease categories) using Spearman Correlation coefficients. Within the CRPC patients, Wilcoxon Rank Sum Tests were carried out to assess analyte expression differences across dichotomized Gleason score and PS categories.

To assess the ability of the measured analytes to distinguish between benign versus malignant, patients with localized CaP and CRPC groups were combined into a single "malignant" group and compared with the elPSA_negBx patients. The predicted probability of benign (vs. malignant) disease was modeled as a function of each log-transformed analyte using univariable and multivariable logistic regression methods. Multivariable models included more than one analyte, but did not adjust for other baseline characteristics. The modeling methods were also used to assess the ability of these markers to separate localized CaP and CRPC patients. The receiver operating characteristic (ROC) curve provides a visual indication of the predictive accuracy of the model, plotting Sensitivity (or true positive fraction) as a function of 1-specificity (or false positive fraction) at different marker cut-off values. The probability of concordance between the predicted probability and observed disease state is a useful measure of discriminative accuracy. This measure is equivalent to the area under the receiver operating characteristic curve (AUC). The AUC values range from 0.5 (indicating no discriminating ability) to 1.0 (indicating perfect discrimination). For this analysis, AUC values greater than 0.8 were considered as useful in predicting outcomes for individual patients.

All *P* values are two-sided and values ≤0.05 were considered to be statistically significant. 95% confidence intervals (CI) describe the plausible range of values for the associated (true, unknown) parameter in the population. All analyses were performed using SAS version 9.2 (SAS, Cary NC).

RESULTS

Patient demographic and disease characteristics are summarized in Table 1. This study sample included 254 participants (46 controls, 50 elPSA_negBx, 49 localized CaP and 109 CRPC). In all 4 groups, a majority of the men were White. With a median age 47 years, men in the control group tended to be younger than men in the other groups (elPSA_negBx, age 69 years; localized CaP, age 64 years and CRPC, age 72 years). The median PSA levels differed significantly between the control group (1.39 ng/ml, elPSA_negBx (3.80 ng/ml), localized CaP (2.40 ng/ml) and CRPC (16.40 ng/ml). The median PSA of the study groups were significantly higher than the control group ($P < 0.05$). In addition, pairwise comparisons of PSA demonstrated each group was significantly different from each other (all tests $P < 0.001$). The median IL-8 levels differed significantly ($P < 0.001$) between the control group (4.00 pg/ml), elPSA_negBx (8.13 pg/ml), localized CaP (16.90 pg/ml) and CRPC patients (43.50 pg/ml). The median TNF-α levels differed significantly ($P < 0.05$) with among the control group (5.12 pg/ml), elPSA_negBx (1.15 pg/ml), localized CaP (2.20 pg/ml). However, the median TNF-α, level was not significantly different between the control patients (5.12 pg/ml) and CRPC patients (5.50 pg/ml). The median sTNFR1 levels were significantly different between the control patients (670.37 pg/ml) and CRPC patients (1790.20 pg/ml) ($P < 0.05$). However, the median sTNFR1 levels were not significantly different among the control group (670.37 pg/ml), elPSA_negBx (585.0 pg/ml) and localized CaP (978.40 pg/ml) groups. Significant differences

for the median sTNFR1 levels were noted between the elPSA_negBx, the localized CaP and CRPC patients as well as between the localized CaP and CRPC patients.

The ability of single analytes to distinguish between elPSA_negBx and malignant cancer samples is illustrated in Figure 1. TNF-α (AUC = 0.93) and sTNFR1 (AUC = 0.97) were strong predictors of elPSA_negBx vs malignant PCa. Comparing the ROC curves in Figure 1, the sensitivity/specificity profiles of TNF-α and sTNFR1 are independently uniformly superior to those of PSA. To determine the clinical usefulness of the biomarkers, the AUC results (with 95% CIs) for additive combinations of these biomarkers to distinguish between elPSA_negBx and localized cancer are shown in Table 2. The best predictor of elPSA_negBx versus localized cancer was sTNFR1, TNF-α, PSA and IL-8 combined (AUC = 0.991; 95% CI from 0.979 to 1.00). It is important to note that sTNFR1 and IL-8 (AUC 0.988; 95% CI from 0.974 to1.00) and sTNFR1, TNF-α and IL-8 combined (AUC = 0.988; 95% CI from 0.974to 1.00) were extremely good predictors. It can be argued that the addition of more analytes to the combination of sTNFR1 and IL-8 may not improve the biostatical accuracy significantly.

The ability of single analytes to distinguish between the localized CaP and CRPC patients is summarized in Figure 2. The strongest single predictors of localized versus metastatic CaP were TNF-α (AUC = 0.992) and PSA (AUC = 0.963) levels. Similar results for additive combinations of analytes are shown in Table 3. Adding sTNFR1, PSA or IL-8 to a model containing TNF-α resulted in minimal improvements in accuracy.

In order to quantify the impact of Gleason score on analyte activity, exploratory analyses were performed on CRPC patients to study the impact of baseline characteristics on the cytokine expression measurements. The analyte values of patients with Gleason score of 8-10 did not differ from those with Gleason score of <7 (data not shown). Similarly, patients with PS of zero did not differ from those with PS of 1 to 3 in their cytokine values, with the exception of PSA (P = 0.034) (data not shown).

DISCUSSION

Within our study sample, sTNFR1 and TNF-α independently and sTNFR1 and IL-8 in combination were significantly better than PSA at differentiating men PCa with from those

Table 1: Patient demographic and disease characteristics of the study groups

Patient demographics and disease parameters	Controls (*n*=46)	elPSA_negBx (*n*=50)	Localized CaP (*n*=49)	CRPC (*n*=109)
Age in years, median (IQR)	47 (37-57.2)	69 (61-73)	64 (60-70)	72 (66-78)
Race, n (%)				
Caucasian	44 (96)	44 (88)	40 (82)	89 (82)
African-American	2 (4)	6 (12)	9 (18)	17 (16)
Other			0	3 (2)
Years since diagnosis				
Median (IQR)			0.2 (0.1-0.4)	4.5 (2.0-6.9)
Gleason score, n (%)				
2-4			0	9 (8)
5-7			41 (84)	56 (51)
8-10			7 (14)	41 (38)
Unknown			1 (2)	3 (3)
Performance status, n (%)				
0	N/A	N/A	N/A	61 (56)
1				36 (33)
2				9 (8)
3				1 (1)
Disease measurability, n (%)				
Measurable	N/A	N/A	N/A	40 (37)
Evaluable				65 (60)
Unknown				4 (3)
Metastases, n (%)				
Any	0	0	0	102 (94)
Visceral				8 (7)
Bone				87 (80)
Median PSA (ng/ml) IQR (25^{th}, 75^{th})	1.39 (1.10, 1.96)	3.80 (2.70, 5.48)	2.40 (2.20, 3.10)	16.40 (8.20, 30.00)
Median IL-8 (pg/ml) IQR (25^{th}, 75^{th})	4.00 (3.33, 4.78)	8.127 (6.86, 9.88)	16.90 (11.60, 31.40)	43.50 (15.00, 110.70)
Median TNF-α (pg/ml) IQR (25^{th}, 75^{th})	5.12 (4.52, 5.83)	1.15 (0.83, 1.74)	2.20 (1.58, 2.62)	5.50 (4.60, 6.50)
Median sTNFR1(pg/ml) IQR (25^{th}, 75^{th})	670.37 (612.50, 777.89)	585.00 (464.00, 710.00)	978.40 (761.90, 1186.00)	1790.20 (1431.20, 2609.20)

CRPC: Castration resistant prostate cancer, elPSA_negBx: Elevated prostate-specific antigen with a negative prostate biopsy, IQR: Inter-quartile range, PSA: Prostate-specific antigen, IL: Interleukin, TNF: Tumor necrosis factor-α, CaP: Prostate cancer

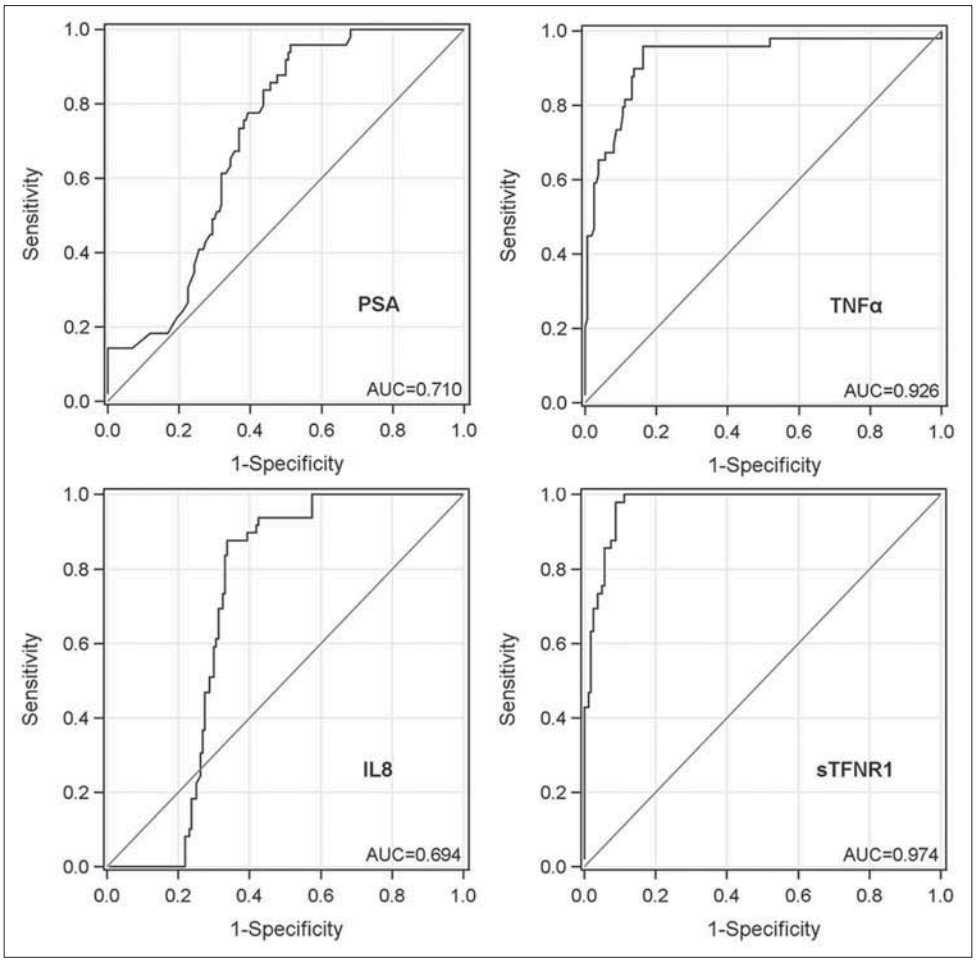

Figure 1: Modeling the probability of distinguishing elevated prostate-specific antigen with a negative prostate biopsy (elPSA_negBx) subjects from prostate cancer (CaP). This measure is equivalent to the area under the receiver operating characteristic curve (AUC). The strongest predictor of not having CaP is log (sTNFR1) with AUC = 0.97, followed closely by log (TNF-α) with AUC = 0.93 (sTNFR1: Soluble tumor necrosis factor-α receptors 1, TNF- α: Tumor necrosis factor-α)

Table 2: AUC (95% CI) estimates for individual biomarkers and their combinations to distinguish between localized CaP and elPSA_negBx

Markers	AUC (95% CI)
PSA	0.707 (0.599-0.815)
IL-8	0.807 (0.701-0.912)
TNF-α	0.822 (0.739-0.905)
sTNFR1	0.906 (0.850-0.962)
TNF-α and sTNFR1	0.912 (0.859-0.966)
TNF-α and PSA	0.836 (0.755-0.917)
TNF-α and IL-8	0.853 (0.775-0.932)
sTNFR1 and PSA	0.926 (0.876-0.975)
sTNFR1 and IL-8	0.988 (0.974-1.000)
PSA and IL-8	0.796 (0.695-0.896)
TNF-α and sTNFR1 and PSA	0.931 (0.885-0.978)
TNF-α and sTNFR1 and IL-8	0.988 (0.974-1.000)
TNF-α and PSA and IL-8	0.867 (0.793-0.942)
sTNFR1 and PSA and IL-8	0.989 (0.976-1.000)
TNF-α and sTNFR1 and PSA and IL-8	0.991 (0.979-1.000)

AUC: Area under the receiver operating characteristic curve, ROC: Receiver operating characteristic, CI: Confidence interval, CaP: Prostate cancer, PSA: Prostate-specific antigen, IL: Interleukin, TNF: Tumor necrosis factor-α, sTNFR1: Soluble tumor necrosis factor-α receptors 1, elPSA_negBx: Elevated prostate-specific antigen with a negative prostate biopsy

without CaP. The reported controversy regarding routine PSA testing is well-known. Those who support PSA testing as a routine CaP early detection tool report that the PSA test is responsible (in part) for a 39% reduction in CaP mortality from 1990 to 2007[2,3,20] as well as a decrease in the proportion of men diagnosed with distant (advanced) CaP.[2,3,20] Those who oppose the use of the PSA test as a routine CaP early detection tool state that the widespread use of PSA testing has resulted in 1.3 million additional men diagnosed and 1.0 million men treated for CaP, possibly unnecessarily.[3,20] In addition, the PSA test has been reported to be of limited value in differentiating benign prostate disease from CaP and this has resulted in 700,000 unnecessary prostate biopsies annually in the United States.[3,20] Whether one supports or opposes the widespread use of PSA testing, most would agree that it is not the optimal test and better test(s) should be developed. The findings of this pilot study support that sTNFR1 and TNF-α are worthy of additional evaluation in a larger sample of men to determine whether it has utility to society as a CaP detection tool.

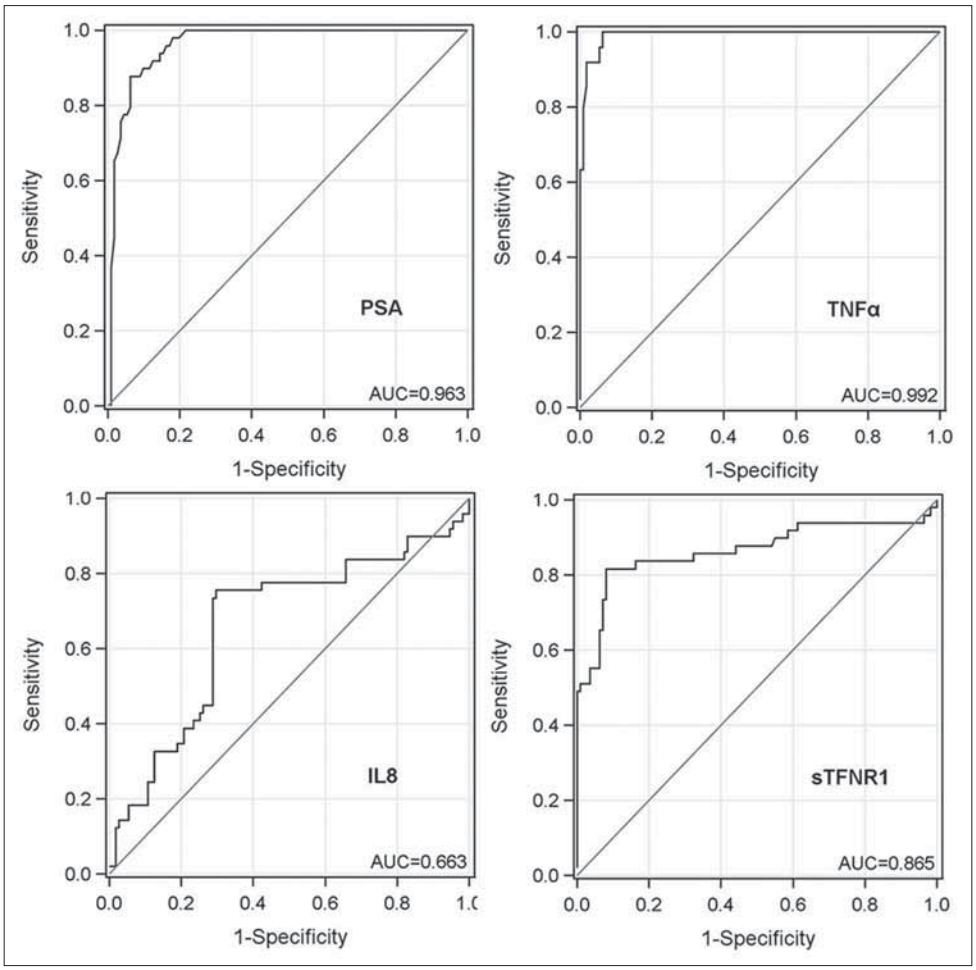

Figure 2: Modeling the probability of local disease versus metastatic CaP. This measure is equivalent to the area under the receiver operating characteristic curve (AUC). Tumor necrosis factor-α and prostate-specific antigen were both very strong predictors of local versus metastatic disease. Also, soluble tumor necrosis factor-α receptor 1 was a reasonably good predictor

The findings of this study support sTNFR1 and TNF-α independently and sTNFR1 and IL-8 in combination are highly significantly predictive in differentiating men with CaP from those without, compared with PSA alone. Although our findings must be validated in a larger sample men in a prospective manner, the preliminary outcomes are compelling as a possible tool to address the reported short comings of PSA testing alone,[2,21] PSA in combination with other tools (for example free PSA)[13,22,23] and CaP risk calculators[24] with regards to their utility in reducing the number of unnecessary prostate biopsies performed annually.

The PSA test has also been criticized for the inability to differentiate men with high risk CaP from those with indolent CaPs and thus resulting in the overtreatment of what has been estimated 1 million men as of 2007.[3,20] Epstein *et al*.[25] reported that 16% of prostatectomy specimens of clinically localized men contains indolent cancers (cancers that will not significantly progress during the lifetime of the individual). This is contrasted to the reports that more than 50% of cancers originally thought to be clinically localized to the prostate were locally

Table 3: AUC (95% CI) estimates for individual biomarkers and their combinations to distinguish between localized versus metastatic CaP

Markers	AUC (95%)
PSA	0.963 (0.937-0.990)
IL-8	0.663 (0.566-0.761)
TNF-α	0.992 (0.983-1.000)
sTNFR1	0.865 (0.788-0.942)
TNF-α and sTNFR1	0.993 (0.985-1.000)
TNF-α and PSA	0.999 (0.998-1.000)
TNF-α and IL-8	0.992 (0.983-1.000)
sTNFR1 and PSA	0.971 (0.950-0.993)
sTNFR1 and IL-8	0.870 (0.801-0.938)
PSA and IL-8	0.964 (0.938-0.990)
TNF-α and sTNFR1 and PSA	0.999 (0.998-1.000)
TNF-α and sTNFR1 and IL-8	0.994 (0.985-1.000)
TNF-α and PSA and IL-8	0.999 (0.998-1.000)
sTNFR1 and PSA and IL-8	0.975 (0.957-0.994)
TNF-α and sTNFR1 and PSA and IL-8	0.999 (0.998-1.000)

AUC: Area under the receiver operating characteristic curve, ROC: Receiver operating characteristic, CI: Confidence interval, CaP: Prostate cancer, PSA: Prostate-specific antigen, IL: Interleukin, TNF: Tumor necrosis factor-α, sTNFR1: Soluble tumor necrosis factor-α receptors 1

advanced at the time of treatment.[26-28] The PSA test alone has not been reported to accurately predict the presence of CaP, its aggressiveness or the risk of post-treatment recurrence.[2,20,22,29,30] In order to better assist men with their CaP treatment decision-making and better determine those who would best benefit from active surveillance those with high risk CaP and therefore should receive definitive treatment. The findings of this study suggest that TNF-α alone or in combination with PSA significantly distinguished men with CRPC from those with localized CaP, compared with PSA test alone. Although CRPC may be considered the extreme of aggressive types of CaP (those who failed androgen deprivation therapy), TNF-α alone or in combination with PSA still performed better than PSA test alone. These results suggest that the utility of TNF-α as a tool for distinguishing between local and metastatic CaP warrants further research.

The findings of this study are interesting, thought provoking and contribute to the literature; however, these findings should be evaluated in the context of the following limitations. First, the majority of the sample population was White. CaP disproportionately impacts Black men.[31] Before any biomarker is used for routine early detection or monitoring of men with CaP, it should be studied in a large, racial diverse sample of men. Second, study was performed as a cohort study over time, which would have allowed for comparative biomarker samples over time and continued followed-up participants. This study was designed as a pilot study to determine the utility of the sTNFR1, TNF-α and IL-8 as CaP biomarkers; therefore, at the onset of the study there was not enough evidence to support the funding of a large prospective study cohort of men at risk of CaP to determine the utility sTNFR1, TNF-α and IL-8 as CaP biomarkers.

CONCLUSION

The scientific exploration for the ideal or at least a better biomarker(s) for the early detection of CaP, to inform CaP treatment decision making and monitoring men with CaP is needed. This pilot study reports some encouraging results regarding the combination sTNFR1 and TNF-α and IL 8 as such biomarkers and they are worthy of further study to confirm that utility as biomarkers for the CaP early detection and treatment decision-making and possibly as a monitoring tool for men with CaP (e.g., men on active surveillance or post CaP treatment).

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