Plasma Sarcosine Measured by Gas Chromatography-Mass Spectrometry Distinguishes Prostatic Intraepithelial Neoplasia and Prostate Cancer from Benign Prostate Hyperplasia

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ABSTRACT

Objective: Sarcosine was postulated in 2009 as a biomarker for prostate cancer (PCa). Here, we assess plasma sarcosine as a biomarker that is complementary to prostate-specific antigen (PSA).

Methods: Plasma sarcosine was measured using gas chromatography-mass spectrometry (GC-MS) in adults classified as noncancerous controls (with benign prostate hyperplasia [BPH], n = 36), with prostatic intraepithelial neoplasia (PIN, n = 16), or with PCa (n = 27). Diagnostic accuracy was assessed using receiver operating characteristic curve analysis.

Results: Plasma sarcosine levels were higher in the PCa (2.0 μM [1.3–3.3 μM], P <.01) and the PIN (1.9 μM [1.2–6.5 μM], P <.001) groups than in the BPH (0.9 μM [0.6–1.4 μM]) group. Plasma sarcosine had "good" and "very good" discriminative capability to detect PIN (area under the curve [AUC], 0.734) and PCa (AUC, 0.833) versus BPH, respectively. The use of PSA and sarcosine together improved the overall diagnostic accuracy to detect PIN and PCa versus BPH.

Conclusion: Plasma sarcosine measured by GC-MS had "good" and "very good" classification performance for distinguishing PIN and PCa, respectively, relative to noncancerous patients diagnosed with BPH.

Keywords: prostate cancer, sarcosine, prostate-specific antigen, prostatic intraepithelial neoplasia, mass spectrometry, biomarkers

Prostate cancer (PCa) is the second most frequent cancer and the fifth leading cause of cancer death in men.[1] PCa is classified according to the extension of the tumor, the clinical or histopathological stage, and the histopathological grade (Gleason score).[2] The most commonly used tests for screening are the prostate-specific antigen (PSA) test and the digital rectal examination.[2] The incorporation of the PSA test in screening allows for an early diagnosis of asymptomatic individuals with the disease.[2] However, there are issues with the validity of this marker.[3] For example, false-positive

Abbreviations:

PCa, prostate cancer; PSA, prostate-specific antigen; GC-MS, gas chromatography-mass spectrometry; BPH, benign prostate hyperplasia; PIN, prostatic intraepithelial neoplasia; AUC, area under the curve; GNMT, glycine-N-methyltransferase; MCF, methyl chloroformate; DTT, D,L-dithiothreitol; PBS, phosphate-buffered saline; ROC, receiver operating characteristic; TP, true positive; TN, true negative; PPV, positive predictive value; NPV, negative predictive value; ANCOVA, analysis of covariance.

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and false-negative cases have occurred in patients with tumors with highly undifferentiated histological grade with normal PSA.[3] The discovery of new biomarkers and validation of candidate markers are needed to complement the PSA test and improve diagnosis in clinical practice.[4]

The amino acid sarcosine, also known as *N*-methylglycine (CH_3NHCH_2COOH), is an intermediary metabolite in glycine metabolism.[5] High concentrations of sarcosine (ie, sarcosinemia disease) occur in rare genetic abnormalities,[6] but in healthy individuals, sarcosine concentrations are expected to be low or negligible.[7] In 2009, Sreekumar et al. postulated elevated sarcosine concentrations as abnormal in metastatic PCa based on a large metabolomic characterization, with sarcosine a potential biomarker to identify PCa.[8] The diagnostic features of this marker have been studied at different stages of PCa progression, having been measured in urine, serum, plasma, and affected tissues.[8–14] However, there are contradictions in the evidence. The role of sarcosine in carcinogenesis has not been fully understood, and there is no consensus on the use of this marker.[7] Metabolically, the formation of sarcosine is catalyzed by glycine-*N*-methyltransferase (GNMT). The GNMT gene has been reported to be overexpressed in patients suffering from PCa, and this overexpression has been also associated with an increased risk of the disease.[15,16] Although there has been an interest in studying sarcosine and PCa, there are still unanswered questions and controversies.[8,14,17–21] Most of the studies have been focused on comparing patients with a high severity of PCa versus healthy controls, but they do not take into consideration intermediate stages of PCa, such as prostatic intraepithelial neoplasia (PIN).[8,19–22] Here, we assess the diagnostic performance of plasma sarcosine as a complementary biomarker to the PSA in individuals diagnosed with PIN and with PCa versus individuals diagnosed with benign prostate hyperplasia (BPH) as confirmed by biopsy.

Materials and Methods

Study Design and Recruitment

A nonexperimental comparison-group design comprising 3 groups was performed. Recruitment was done from May 2017 to September 2017. Patients were recruited at the Research Institute of Urology and Reproductive Health, Sechenov University. Individuals were classified as noncancerous control (BPH), PIN, or PCa based on biopsy results, as follows: the control group comprised individuals diagnosed with BPH, defined as the absence of caverns, poorly differentiated cells, or areas with bad differentiation; the PIN group showed some areas of poorly differentiated cells with several caverns; and the PCa group showed multiple areas of badly differentiated cells with multiple caverns.[23,24]

Ethical Considerations

This research was approved by the Ethics Committee at the I.M. Sechenov First Moscow State Medical University, Moscow, Russia (Document #05-17). Written signed informed consent was obtained from each volunteer before entry into the study. The study was performed in conformity with the ethical principles for medical research involving humans as stated in the Declaration of Helsinki.

Prostate-Specific Antigen, Biopsy, and Gleason Score

Total PSA levels were measured by a chemiluminescent immunoassay method[25] (Department of Uronephrology, Sechenov University). The histological material from biopsy specimens was used for grading according to the Gleason classification system.[26] Patients with PCa were grouped according to Gleason scores of <7 or ≥7.

Specimen Collection

Venous blood specimens (5 ml) were collected in heparin-treated tubes in the morning after an overnight fast of at least 8 hours. Immediately after blood collection, specimens were centrifuged at 5000 rpm for 10 minutes at 4°C to obtain plasma and were stored at −80°C until laboratory analysis.

Plasma Sarcosine Determination

Plasma sarcosine was analyzed as a methyl chloroformate (MCF) derivative according to the procedures published by Windelberg et al.[27] and Midttun et al.[28], with modifications. Plasma (100 µL) was mixed with 1 µL of 1 mM 4-chloro-L-phenylalanine solution (internal standard) and with 25 µl of 500 mM D,L-dithiothreitol (DTT), followed by incubation at room temperature for 20 min. Next, the specimen was deproteinized by adding 450 µl of ethanol.

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Three hundred eighty microliters of the supernatant obtained after centrifugation (5 min at 16,900 relative centrifugal force) was transferred into an empty Eppendorf tube and mixed with 300 µl of water, 50 µl of pyridine, and 250 µl of 20% (v/v) MCF in toluene. Mixing was achieved by repeated pipetting. After incubation at room temperature for 10 min to obtain phase separation, 600 µl of the aqueous phase was replaced by water (500 µl), and the specimen was mixed again. After centrifugation, 100 µl of toluene layer was placed in an autosampler vial, and 1 µl was used for gas chromatography-mass spectrometry (GC-MS) analysis.

GC-MS Analysis

GC-MS analysis was performed using an Agilent 6890 (Santa Clara, CA) equipped with a 30-m-long, 0.25-mm-inner-diameter Rtx5Sil-MS column (Restek Corporation, Bellefonte, PA). Specimens were injected in the splitless mode; the oven temperature was ramped up from 75°C to 85°C at 45°C/min, with a hold for 1 min, and then increased at a rate of 45°C/min to 125°C, followed by a further increase at 60°C/min to 260°C. Helium was used as a carrier gas at a constant flow rate of 1 mL/min. The inlet temperature was 270°C. Detection was done using an Agilent 5850 single quadrupole mass spectrometer (St. Joseph, MI) with a 280ºC transfer line temperature and electron ionization at 70 eV. The scanning mass range was 50 Da to 750 Da.

Chemicals and Reagents

Amino acid standards, DTT, phosphate-buffered saline (PBS), and 4-chloro-L-phenylalanine were purchased from Sigma-Aldrich. Purified water was obtained from the EMD Millipore Milli-Q reference ultrapure water purification system, and ethanol was from J.T. Baker, pyridine was from Biochem Chemopharma, methyl chloroformate was from Fluka, and toluene was from ACROS Organic (Fisher Scientific).

Calibration and Quality Controls

Stock solutions were prepared in PBS solution and stored in brown glass vials at –80°C. For calibration and quality control specimens, appropriate volumes of working solutions were added to PBS buffer (blank specimen) and prepared according to specimen preparation procedures.

Diagnostic Accuracy Assessment

The diagnostic accuracy of plasma PSA and plasma sarcosine was assessed considering the PIN and the PCa groups as the target conditions and the noncancerous control group (BPH) as the reference standard. The rationale to choose this control group as a reference standard was based on choosing an available group of patients with a nononcological alteration of the prostate gland tissues. Youden's index ([sensitivity + specificity] – 1) was used to identify the most appropriate cut points for both markers. This index was obtained from receiver operating characteristic (ROC) curve analysis. The area under the curve (AUC) values obtained from ROC curve analysis were classified for diagnostic accuracy as "test not useful," "bad," "sufficient," "good," "very good," or "excellent" if the AUC was <0.5, 0.5 to 0.6, 0.6 to 0.7, 0.7 to 0.8, 0.8 to 0.9, or 0.9 to 1.0, respectively.[29] Sensitivity, specificity, predictive values, and diagnostic accuracy were used as methods to assess diagnostic accuracy. Sensitivity expresses the proportion of true-positive (TP) patients with the disease divided by the total number of patients diagnosed with the disease. Specificity was expressed as the proportion of true-negative (TN) patients without the disease divided by the total number of patients without diagnosis of the disease. The probability of having or not having PCa was estimated by calculation of the positive and negative predictive values (PPV and NPV, respectively), as follows: PPV equals the number of TP patients divided by the total number of individuals with positive results, and NPV equals the number of TN patients divided by the total number of individuals with negative results. Overall diagnostic accuracy was expressed as the proportion of correctly classified individuals (TP + TN) versus all participants (TP + TN + false positive + false negative).

Statistical Analyses

The distribution of the variables was checked with the Shapiro-Wilk test. The Kruskal-Wallis test was used to compare differences across groups with Dunn's correction for multiple comparisons for crude analyses. Plasma PSA and sarcosine levels were log transformed for analysis of covariance (ANCOVA). Age and prostate volume were considered as covariates based on the well-known influence of these parameters as risk factors for cancer.[30,31] Sidak post hoc correction was used for comparison between groups. For subgroup analyses, the Mann-Whitney U test and

Table 1. General Characteristics

Characteristics	Data by group[a]			*P* Value
	Control	PIN	PCa	
n	38	16	27	–
Age, median (range), y	64 (52–80)	66 (54–75)	67 (53–80)	NS
Prostate volume, median (25^{th} %ile–75^{th} %ile), ml	67.5 (55.5–75.0)[a]	56.6 (46.5–56.6)[a,b]	45.9 (30.0–58.0)[b]	<.001
Gleason score, n (%)				
<7			17 (63)	–
≥7			10 (37)	

PIN, prostatic intraepithelial neoplasia; PCa, prostate cancer; PSA, prostate-specific antigen; NS, not significant. Dash indicated that no statistical test were performed.
[a]*Kruskal-Wallis ANOVA. Different superscript letters denote significant differences across groups.*

ANCOVAs were used to compare the groups with a Gleason score of <7 versus ≥7 in the PCa group. Statistical analyses were performed with STATISTICA 8.0, SPSS Statistics 17.0, and plots were improved with Adobe Illustrator 14.0.

Results

General Characteristics

Based on the biopsy results, the 81 participants were subdivided as noncancerous control (BPH) (n = 38), PIN (n = 16), and PCa (n = 27). The range of age was 52 years to 80 years old, and there were no significant differences in the median ages between the groups. The prostate volumes presented medians of 67.5 ml, 56.6 ml, and 45.9 ml across groups, respectively. Prostate volumes were higher (*P* <.001) in the control group than in the PCa group. Gleason scores available in the PCa group indicative of tumor grade showed that 37% presented a score of ≥7 (**Table 1**).

Comparisons of Plasma PSA and Plasma Sarcosine across Groups

PSA levels were higher in the PCa (24.0 ng/mL [6.1–50.4 ng/mL]) than in the control (4.2 ng/mL [2.2–6.6 ng/mL], *P* <.001) and the PIN (7.7 ng/mL [4.3–10.1 ng/mL], *P* <.01) groups (Median [25^{th}–75^{th} percentile]). PSA levels were not significantly different between the control and PIN groups (**Figure 1A**). Plasma sarcosine concentrations were higher in both the PCa (2.0 µM [1.3–3.3 µM], *P* <.01) and PIN (1.9 µM [1.2–6.5 µM], *P* <.001) groups than in the control (0.9 µM [0.6–1.4 µM]) group. Plasma sarcosine levels were not significantly different between the PIN and PCa groups (**Figure 1B**). These comparisons were performed after adjustment for age and prostate volume as covariates.

Diagnostic Accuracy Assessment to Differentiate PIN versus Noncancerous Control

ROC curve analyses to assess the diagnostic validity of plasma PSA and plasma sarcosine as differential markers for the presence of PIN versus BPH showed "good" discriminative capability for both plasma PSA (AUC, 0.720) and plasma sarcosine (AUC, 0.734) (**Figure 1A** and **1B**). Plasma PSA presented 69% sensitivity and 64% specificity, and plasma sarcosine presented 75% sensitivity and 72% specificity. Using plasma PSA and plasma sarcosine combined improved the overall diagnostic performance. The diagnostic accuracy for both tests used together was 83%, versus 70% and 77% for PSA and sarcosine when used as single markers, respectively (**Table 2**).

Diagnostic Accuracy Assessment to Differentiate PCa versus Noncancerous Control

ROC curve analyses to assess the diagnostic validity of plasma PSA and plasma sarcosine as differential markers for the presence of oncological (PCa) versus nononcological prostate gland enlargements (BPH) showed "very good" discriminative capability for both plasma PSA (AUC, 0.854) and plasma sarcosine (AUC, 0.833) (**Figure 1C** and **1D**). Plasma PSA presented 93% sensitivity but only 64% specificity. Plasma sarcosine had 89% sensitivity and 72% specificity. Using plasma PSA and plasma sarcosine combined improved the overall diagnostic performance. The diagnostic accuracy for the two tests used together was 87%, versus 76% and 79% for PSA and sarcosine when used as single markers, respectively (**Table 3**).

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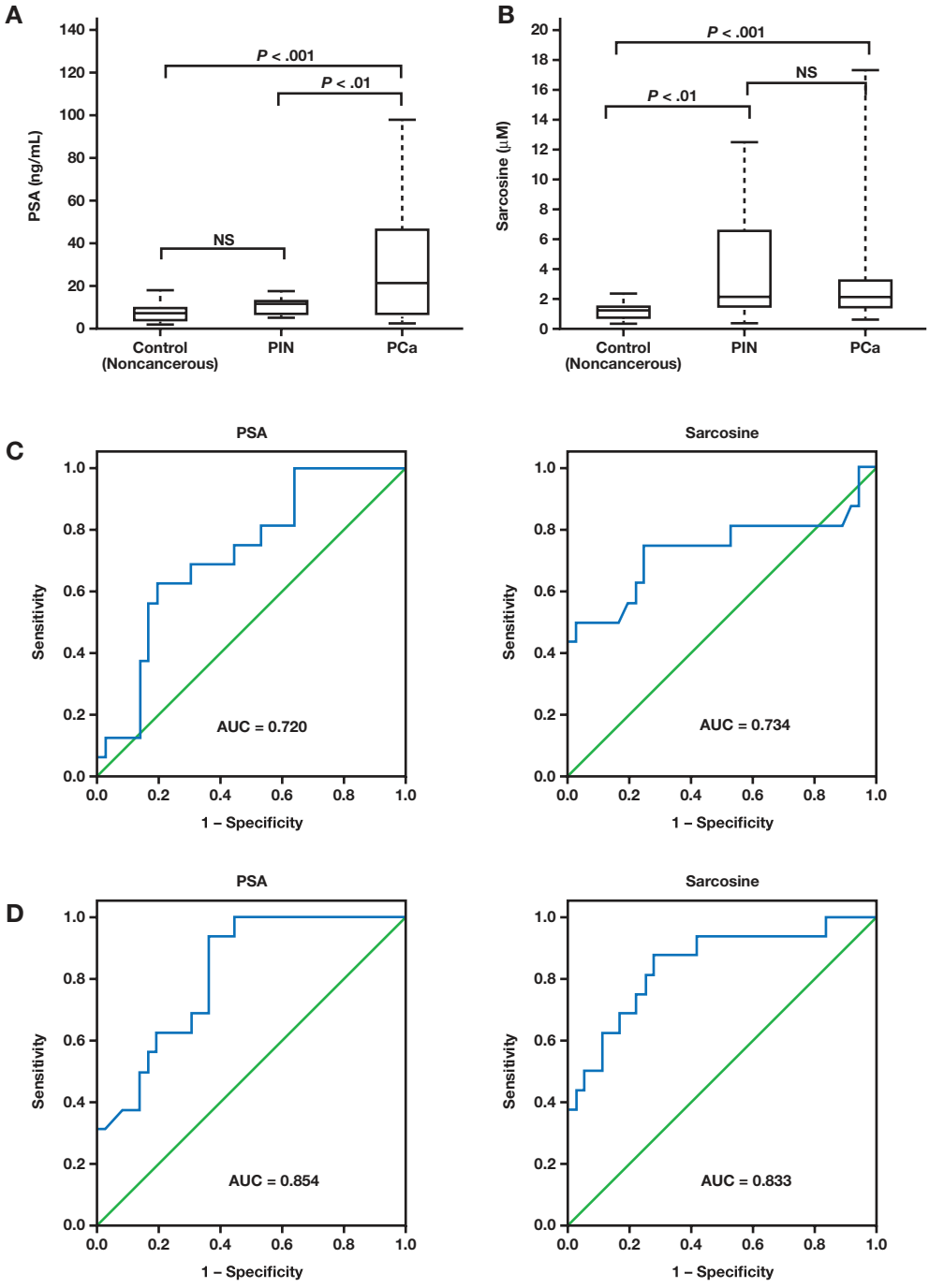

Figure 1

Diagnostic performance of total PSA and plasma sarcosine in noncancerous control, PIN and PCa groups. Panels **A** and **B** correspond to box plots across groups. Panel **C** corresponds to ROC curves for PSA and plasma sarcosine to differentiate noncancerous control versus PIN. Panel **D** corresponds to ROC curves for PSA and plasma sarcosine to differentiate noncancerous control versus PCa. PIN, prostatic intraepithelial neoplasia; PCa, prostate cancer; PSA, prostate-specific antigen; ROC, Receiver Operating Characteristic.

Table 2. Diagnostic Accuracy Assessment to Differentiate PIN versus Noncancerous Control

Parameter	Classification of PIN versus noncancerous control, %					
	PSA		Sarcosine		PSA and sarcosine	
	Estimate	95% CI	Estimate	95% CI	Estimate	95% CI
Sensitivity	68.8	41.3–88.9	75.0	47.6–92.7	50.0	24.7–75.4
Specificity	63.9	46.2–79.2	72.2	54.8–85.8	91.7	77.5–98.3
PPV	45.8	32.9–59.4	54.6	39.8–68.6	72.7	44.8–89.8
NPV	82.1	68.1–90.8	86.7	73.1–94.0	80.5	71.5–87.2
Diagnostic accuracy	70.0	59.0–84.4	76.9	63.2–87.5	82.7	69.7–91.8

PIN, prostatic intraepithelial neoplasia; PSA, prostate-specific antigen; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.

Table 3. Diagnostic Accuracy Assessment to Differentiate PCa versus Noncancerous Control

Parameter	Classification of PCa versus noncancerous control, %					
	PSA		Sarcosine		PSA and sarcosine	
	Estimate	95% CI	Estimate	95% CI	Estimate	95% CI
Sensitivity	92.6	75.7–99.1	88.9	70.8–97.7	81.5	61.9–93.7
Specificity	63.9	46.2–79.2	72.2	54.8–85.8	91.7	77.5–98.3
PPV	65.8	55.1–75.1	70.6	58.2–80.5	88.0	71.0–95.7
NPV	92.0	74.8–97.8	89.7	74.5–96.3	86.8	74.8–93.6
Diagnostic accuracy	76.2	63.8–86.0	79.4	67.3–88.5	87.3	76.5–94.4

PCa, prostate cancer; PSA, prostate-specific antigen; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.

Comparison of PSA and Plasma Sarcosine According to Gleason Score in the PCa Group

Plasma PSA concentrations were significantly (P <.001) different in the group of patients with a Gleason score of <7 (n = 17) versus ≥7 (n = 10). Plasma sarcosine concentrations were not significantly different between the 2 groups (data not shown). Crude and adjusted comparisons had similar statistical differences.

Discussion

In our study, based on predefined groups with diagnoses confirmed by biopsy, we found that plasma sarcosine had "good" and "very good" discriminative capability to contrast the noncancerous control (BPH) group versus the PIN group (AUC, 0.833) and versus the PCa group (AUC, 0.833). Using PSA and sarcosine together improved the overall diagnostic accuracy to detect both PIN and PCa compared to using these as single biomarkers.

Sreekumar et al found that metastatic specimens had increased levels of sarcosine in 79% of the analyzed tissue specimens.[8] Importantly, in their study, none of the noncancerous tissues had detectable levels of sarcosine.[8] Sreekumar et al found significantly higher sarcosine urine levels in biopsy-positive PCa patients than in the biopsy-negative controls.[8] Lucarelli et al found that serum sarcosine concentrations were higher in a group of patients with PCa than in patients with no evidence of malignancy.[9] Serum sarcosine showed a higher predictive value (AUC, 0.668) than did total PSA (AUC, 0.535) in patients with a PSA of <4 ng/mL.[9] In our study, contrasting PCa versus the control group, the AUCs for plasma PSA and plasma sarcosine were 0.854 and 0.833, respectively, regardless of PSA concentration. McDunn et al found elevations in Gleason grade 8 tumors versus benign tissues.[10] In our study, we performed subgroup analyses comparing different grades of Gleason in spite of being restricted to small specimen sizes. Plasma sarcosine did not differentiate individuals with PCa having a score of ≥7 versus <7 compared to plasma PSA.

In our study, plasma PSA had a high capacity to detect true-positive patients with PCa (high sensitivity, 92.6%); however, the test presented a low probability of detecting

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true-negative individuals (low specificity, 63.9%). In clinical practice, this low specificity can increase referrals of patients to unnecessary invasive procedures, such as biopsies, for further diagnostic confirmation. In clinical practice, based on our study population, if both markers are abnormal, there will be a high chance of having PCa, improving early detection of the disease. On the other hand, if any of the 2 markers are not abnormal, there will be a low chance of having the disease (high specificity, 91.7%). Interestingly, plasma sarcosine has 1 more feature. If plasma PSA is not abnormal, indicative of the absence of oncological alterations, but plasma sarcosine is abnormal, there will be a chance of detecting PIN as an early development of PCa. Classification of PCa versus BPH using plasma PSA alone and plasma sarcosine alone presented higher NPVs than PPVs. Using plasma PSA and plasma sarcosine together presented good PPVs and NPVs, with both values above 80%. However, it is important to note that these calculations are affected by the prevalence of having PCa versus the control group found in our study population. This prevalence was 31%. In populations where it is expected to find a higher prevalence, the PPV should increase while the NPV should decrease, and vice versa.[29]

Overall diagnostic accuracy to detect PIN or PCa versus BPH was highly impacted by using the 2 markers together. This overall parameter has to be interpreted with caution because it is influenced by prevalence, and the rest of the parameters should be weighted to make an overall interpretation. In our study, using plasma PSA and plasma sarcosine together presented an improvement in overall diagnostic accuracy accompanied by good diagnostic performance for the rest of the parameters.

The present study measured plasma sarcosine in order to contribute to the current knowledge regarding the use of sarcosine as a biomarker in the diagnosis of PCa. The study design was based on 3 well-discriminated groups with diagnosis made using biopsy specimens. A more informative study would have a narrower age range and collect more information, such as patients' race, family history, genetic profiling, and risk factors causing or predisposing them to develop PIN or PCa. We acknowledge that inference of causality is limited due to comparing groups in an experimental design without follow-up, such as a case-control study, a prospective cohort, or a randomized controlled trial. Statistically, it is possible that there was not enough power to detect significant differences due to limitations with the specimen sizes, especially when making comparisons after stratifying 2 groups with different Gleason scores in the PCa group. We did not include a control group with an absence of prostate gland enlargement. However, regardless of enlargement, our noncancerous control group comprised individuals diagnosed with BPH. They had a confirmed absence of oncological alterations by biopsy. More robust studies are needed to confirm the value of plasma sarcosine concentration as a biomarker and to establish the diagnostic impact of this marker on the therapies and the survival of the patients.

Conclusion

Plasma sarcosine concentrations measured by GC-MS analysis had "good" and "very good" classification performance for distinguishing PIN and PCa, respectively, relative to noncancerous patients, suggesting that it may be a promising complementary biomarker when used together with PSA. Further research is needed to better assess if plasma sarcosine differentiates patients diagnosed with PCa with different Gleason scores. **LM**

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Additional contributions

P.A.M. participated in specimen collection, conducted biochemical analyses, performed statistical analyses, interpreted biological and clinical information, and provided input to the manuscript. A.B. conceptualized the study,

advised on statistical analyses, interpreted biological and clinical information, and wrote the manuscript. N.M. conducted biochemical analyses, interpreted biological information, and provided input to the manuscript. M.F. conceptualized the study and provided input on the clinical implications of this research. E.V.L., Y.V.S., Y.V.L., V.Y.M., N.V.P., D.V.E., and A.V.L. were part of patient recruitment, specimen collection, medical history, and clinical procedures. S.A.A. conceived the main study and has final responsibility for all parts of this research.

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