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TITLE: Magnetic Resonance Spectroscopy (MRS) of Prostatic Fluids for Early Detection of Prostate Cancer

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In this study, the metabolic profiles of expressed prostatic secretions (EPS) from 52 men with prostate cancer (PCa) and from 26 healthy controls were analyzed using quantitative proton nuclear magnetic resonance spectroscopy (1H-NMRS). The metabolites quantified included citrate, spermine, myo-inositol, lactate, alanine, phosphocholine, glutamine, acetate, and hydroxybutyrate. Logistic regression (LR) was used to model the risk of PCa based on metabolite concentrations while adjusting for age. The LR models indicated that the absolute concentrations of citrate, myo-inositol, and spermine were highly predictive of PCa and inversely related to the risk of PCa. The areas under the receiver operating characteristic curves (AUROC) for citrate, myo-inositol and spermine were 0.89, 0.87, and 0.79, respectively. At 90% sensitivity, these metabolites had specificities of 74%, 51% and 34%, respectively. The LR analysis indicated that absolute levels of these three metabolites were independent of age. The results indicate that citrate, myo-inositol and spermine are potentially important markers of PCa in human EPS. Further, the absolute concentration of these metabolites in EPS appears to be independent of age, increasing the potential utility of these markers due to elimination of age as a confounding variable.
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THE METABOLITES CITRATE, MYO-INOSITOL, AND SPERMINE ARE POTENTIAL AGE-INDEPENDENT MARKERS OF PROSTATE CANCER IN HUMAN EXPRESSED PROSTATIC SECRETIONS.

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Running Title: Citrate Validation in Prostatic Cancer

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ABSTRACT

Purpose: Due to specific physiological functions, prostatic tissues and fluids have unique metabolic profiles. In this study, proton nuclear magnetic resonance spectroscopy ($^{1}$H-NMRS) is used to validate potential metabolic markers of prostate cancer (PCa) in human expressed prostatic secretions (EPS).

Methods: Metabolic profiles of EPS from 52 men with PCa and from 26 healthy controls were analyzed using quantitative $^{1}$H-NMRS. The metabolites quantified included citrate, spermine, myo-inositol, lactate, alanine, phosphocholine, glutamine, acetate, and hydroxybutyrate. Logistic regression (LR) was used to model the risk of PCa based on metabolite concentrations while adjusting for age.

Results: The average age of the EPS donors with PCa was 58.0±7.0 years and 52.2±12.1 for the healthy donors. The median Gleason score for the men with PCa was 7 (range 5-9). The LR models indicated that the absolute concentrations of citrate, myo-inositol, and spermine were highly predictive of PCa and inversely related to the risk of PCa. The areas under the receiver operating characteristic curves (AUROC) for citrate, myo-inositol and spermine were 0.89, 0.87, and 0.79, respectively. At 90% sensitivity, these metabolites had specificities of 74%, 51% and 34%, respectively. The LR analysis indicated that absolute levels of these three metabolites were independent of age.

Conclusions: The results indicate that citrate, myo-inositol and spermine are potentially important markers of PCa in human EPS. Further, the absolute concentration of these metabolites in EPS appears to be independent of age, increasing the potential utility of these markers due to elimination of age as a confounding variable.
INTRODUCTION

Prostate cancer (PCa) is the leading non-cutaneous cancer diagnosed in men in the United States.\textsuperscript{1} The American Cancer Society estimates that over 230,000 men will be diagnosed with PCa and almost 30,000 will die of this disease in 2006.\textsuperscript{1} Although controversies over prostate cancer screening persist, the early detection of prostate cancer is likely to result in an intervention at an earlier stage of disease and, thus, an increased likelihood of treatment success.\textsuperscript{2-4} Early detection of prostate cancer is complicated by a lack of definitive markers for the disease. Although serum prostate specific antigen (PSA) and its derivatives are widely considered to be the best PCa markers currently available, their utility is hampered by limited sensitivity and specificity.\textsuperscript{2-5} The sensitivity of the PSA test can be improved to a limited degree by adjusting PSA cutoffs downward from the current 4.0 ng/mL standard. Most proponents of this approach have suggested new PSA cutoffs of around 2.5 ng/mL. Unfortunately, increasing the sensitivity of PSA in this way results in a concomitant reduction in an already low specificity. As a result, a substantial number of false positives occur, often leading to invasive and unnecessary biopsies of the prostate.

The limited specificity of PSA is related – at least in part - to it’s strong positive association with age.\textsuperscript{6,7} Benign prostatic hyperplasia, which is also highly associated with age, contributes to increased PSA levels and can also result in false positive PSA tests.\textsuperscript{8} Therefore, identifying PCa markers that are less correlated with age and that maintain sufficient sensitivity for detecting the disease could greatly benefit men at risk of developing PCa.

Because the prostate gland has physiologic and metabolic functions unique from the rest of the body, it is especially suited for metabolic profile analysis for the identification of potential PCa biomarkers. For example, the normal prostate gland accumulates remarkably high levels of the metabolite citrate relative to other tissues.\textsuperscript{9} This is achieved by the ability to accumulate high cellular levels of zinc in secretory epithelial cells that inhibit citrate oxidation.\textsuperscript{9} The presence of
prostate cancer appears to reduce the amount of citrate in prostatic tissues, suggesting that lowered citrate levels may be a marker of prostate cancer.\textsuperscript{9-11} Zinc and the polyamines myo-inositol and spermine are also found in higher concentrations in the prostate gland and their levels appear to be affected by prostatic disease processes.\textsuperscript{12,13}

Both \textit{in vivo} and \textit{in vitro} magnetic resonance spectroscopy of prostatic tissues have shown promise in identifying metabolic profiles indicative of prostate cancer.\textsuperscript{10,11,14-18} The aim of the current study is to identify potential metabolic markers of PCa within human expressed prostatic secretions (EPS) utilizing quantitative high-resolution proton nuclear magnetic resonance spectroscopy (\textsuperscript{1}H-NMRS). The absolute concentrations of metabolites specific to EPS were analyzed with relation to the risk of prostate cancer and patient age.
METHODS

Patient Population and Sample Collection. The 78 EPS samples utilized in this study were obtained from the biorepositories of two institutions and were collected under IRB approved protocols. All of the EPS donors signed informed consent forms agreeing to donate their EPS and agreeing that the samples would be used in prostate cancer research. All samples were obtained via transrectal prostate massage and were spun down at 13,000 g at 4°C for 5 minutes to remove cell debris immediately upon collection. The supernatant was stored at -80°C until needed for analysis. At the beginning of study, selected samples were initially analyzed for metabolite stability during freezing procedure and storage. Of the 78 samples analyzed, 52 were from men with prostate cancer and 26 were from healthy (non-PCa) controls. Demographic data on EPS donors are given in Table 1.

Sample Preparation and Quantitative ¹H-NMRS analysis. Frozen samples were slowly thawed at 4°C. Depending on the initial volume, 5 to 20 µL of EPS were analyzed. Deuterium oxide (D₂O, 20 to 35 µL), containing trimethylsilyl propionic-2,2,3,3,4 acid (TMSP) as an external standard, was added to each EPS sample resulting in final sample volume of 40 µL. The samples were centrifuged at 4,000 g at 4°C for 5 min. The supernatants were transferred into Bruker 1-mm glass capillaries (Bruker Biospin, Fremont, CA) using 1-mL syringes with thin epidural needles. The glass capillaries were sealed and inserted into the magnet using a 1-mm NMR spinner.

All ¹H-NMRS analyses were performed by NMR scientists (NJS, DJK) who were "blinded" to the clinical background of the EPS samples. All EPS samples were analyzed using a Bruker DRX 500 MHz high-resolution NMR spectrometer (Bruker Biospin, Fremont, CA). The sample temperature was held constant at 5°C inside of the magnet using a Bruker temperature regulator. Bruker 1-mm TXI micro-probe for ultra-small volume samples was used for all experiments. Deuterium lock signal was held for D₂O added to the sample. All ¹H-NMR spectra
were obtained using XWIN-NMR 3.5 or TopSpin software (Bruker Biospin, Fremont, CA). For metabolite quantification, a standard Bruker proton water pre-saturation pulse program “zgpr” was used to suppress water residue signal. The resonance frequency for proton channel was 500 MHz with 80 total acquisitions. A pulse delay of 12.8 seconds was applied between acquisitions for fully relaxed $^1$H-NMR spectra (calculated as $5^*T1$). The total acquisition time was 20 minutes, 45 seconds. TMSP was used as an external reference for metabolite quantification and chemical shift (0 ppm). The absolute concentration of TMSP in deuterium oxide was verified for each experiment set using 20 mM amino acid solution (citrate, alanine, myo-inositol, glutamine) as a quantification standard. Endogenous EPS metabolites were identified from 2D-NMR spectra (H,H-COSY and H,C-HSQC) based on the results from our chemical-shift database. After performing Fourier transformation (with line broadening LB=0.1 Hz) and making phase and baseline corrections, each identified $^1$H peak was integrated using the Bruker 1D WIN-NMR version 4.0 program. The absolute concentrations of single metabolites were then referred to the TMSP integral and calculated according to the equation:

$$C_x = \frac{I_x \times N_x \times C}{I \times 9} \times V \times V_{eps}$$  (1)

where $C_x$ = metabolite concentration

$I_x$ = integral of metabolite $^1$H peak

$N_x$ = number of protons in metabolite $^1$H peak (from CH, CH$_2$, CH$_3$, etc.)

$C$ = TMSP concentration calculated for each experiment set

$I$ = integral of TMSP $^1$H peak at 0 ppm (:9 since TMSP has 9 protons)

$V$ = total volume of the sample (40 µL)

$V_{eps}$ = volume of EPS sample (5 to 20 µL)
Since in $^1$H-NMR spectroscopy, single metabolites can produce multiple peaks, the final concentration for each metabolite was calculated as an average of its $^1$H-NMR detectable peaks. The absolute concentrations of metabolites were reported as µmol per mL of EPS.

**Metabolite Stability Analysis.** To assess any possible metabolic degradation occurring during freezing, -80 °C storage, or thawing procedures, 6 freshly collected EPS samples were analyzed at three different time points post-collection. Each of the 6 fresh EPS samples was divided into three aliquots (10 µL each). The first aliquot was analyzed by $^1$H-NMRS immediately after sample collection (day 0), the second aliquot was analyzed after 1 week of -80 °C storage (week 1), and the third after 1 month of -80 °C storage (week 4).

**Statistical Analysis.** The statistical modeling and analysis group (RHJ, EJG, CO) took no part in the $^1$H-NMRS analysis and were the only staff members who had access to the clinical and outcome data.

The primary statistical method utilized was logistic regression using SAS (2004) PROC GENMOD. The analysis was carried out for each metabolite with the binary response being cancer or no cancer. The log of the metabolite concentration was the independent variable, and age was included in the model to control for the age difference between the group with cancer and the group without cancer.

SAS PROC MIXED was used to analyze the differences between metabolite concentrations due to freezing and thawing in the metabolite stability analysis. The response variable for each metabolite was the log of the concentration of the metabolite. The independent variables were an intercept and a 0, 1 indicator variable for time 1 and a 0, 1 indicator variable for time 2. A random subject effect was included in the model since there are repeated measures on each subject, allowing each subject to have a different mean level. The hypothesis to be tested is whether the two coefficients of the indicator variable are zero, which would indicate that there is no effect from freezing the samples.
RESULTS

The characteristics of the men who provided EPS samples are summarized in Table 1. Biopsy Gleason sums and serum PSA levels were only available for the men with cancer. The difference in mean ages between the men with cancer and the healthy controls was statistically significant ($p = 0.04$).

Table 2 provides a summary of the LR modeling results and includes p-values for coefficients of log of the metabolite concentrations. A small p-value indicates that the log of the metabolite concentrations are predictive of PCa. The p-values for age indicate the effect of controlling for age. A large p-value for age such as those for citrate, myo-inositol and spermine indicate that controlling for age for these variables is not necessary. The logistic regressions for these three variables were then refitted without age in the model. The deviance over the degrees of freedom, also included in Table 2, provides a measure of how well the given LR model was fitted. Values near 1 indicate that the models fit well. A value of the deviance over the degrees of freedom greater than one suggests under-fitting. Based upon the results of the LR modeling, the log of the absolute concentrations of citrate, myo-inositol, and spermine were the most predictive of prostate cancer, while not being dependant on age. Figure 1A shows absolute concentrations of these three metabolites calculated from $^1$H-NMRS of EPS of healthy and PCa subjects (Figure 1B). At 90% sensitivity, these metabolites had specificities of 74%, 51% and 34%, respectively. The concentration cut-points (cut-offs) that corresponded to the 90% sensitivity for each metabolite were 324.0 µmol/mL for citrate, 21.0 µmol/mL for myo-inositol, and 75.0 µmol/mL for spermine. The areas under the receiver operating characteristic curves (AUROC) for citrate, myo-inositol and spermine were 0.89, 0.87, and 0.79, respectively. Figure 2 provides the ROC curves for these three metabolites. Figure 3 illustrates the relationships between the log of the concentrations of citrate, myo-inositol and spermine, and the risk of prostate cancer.
Table 3 summarizes the results of the metabolite stability analysis on six samples. For citrate and spermine various $^1$H-NMR peaks were independently statistically analyzed to confirm the results. The concentration of alanine was below the $^1$H-NMRS lower limit of quantification in one of the EPS samples. Therefore, five measurements of alanine from the six samples analyzed were available for comparison. The p-values greater than 0.05 indicate that no statistically significant changes in metabolite concentrations occurred during the various freeze-thaw cycles for a given metabolite. Thus, only glutamine and alanine underwent significant changes in concentrations (degradation) between freeze-thaw cycles. No metabolic degradation was observed for citrate, myo-inositol, and spermine, as well as for hydroxybutyrate, lactate, phosphocholine or valine in fresh versus frozen samples.
DISCUSSION
In the present study, we report the absolute concentrations of citrate, spermine and myo-inositol as age-independent markers for PCa in expressed prostatic secretions. The median concentrations of citrate (114 μmol/mL in PCa versus 349 in healthy subjects), spermine (57 versus 27 μmol/mL) and myo-inositol (7 vs 21μmol/mL) were significantly decreased in PCa subjects as calculated from $^1$H-NMRS.

High-resolution $^1$H-MRS of bio-fluids and tissues coupled with appropriate statistic methods offer a novel and robust approach for identifying individual metabolites or combinations of metabolites that may serve as cancer markers. This spectroscopic analysis allows simultaneous detection (and, in our laboratory, quantification) of hundreds of low molecular weight (max. 20 kDa) species within a biological matrix at concentrations above 100 μM. This results in the generation of an endogenous metabolic profile that has the potential to reveal characteristics indicative of disease status. This new NMR-based metabolomic approach is intended to be complementary to genetic and proteomic analyses that have gained increasing importance in biomedical research. Metabolomics is defined as the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification.\textsuperscript{20,21}

$^1$H-MRS has rapidly evolved to be a method with increasingly broad applications in tumor diagnosis as well as for evaluating the efficacy of anti-cancer therapy based on metabolic alterations during oncogenesis.\textsuperscript{21-25} For example, total choline and choline metabolism intermediates became an establish NMR marker (both \textit{in vivo} and \textit{ex vivo}) for malignant breast tumors. \textsuperscript{22, 23} In prostate cancer, \textit{ex vivo} as well as \textit{in vivo} MRI/MRS studies from Dr. Kurhanewicz’ group at the University of California, San Francisco (UCSF) have demonstrated a linear correlation between the magnitude of the decrease of citrate and the elevation of choline with pathologic Gleason score.\textsuperscript{10,16,17} The limitations of previous \textit{ex vivo} approaches include the
relatively invasive nature of the biopsy sampling.\textsuperscript{26} For \textit{in vivo} citrate \textsuperscript{1}H-MRSI, the requirement of special \textit{in vivo} equipment (endorectal coils and appropriate software) and a well-developed MRS/MRI protocol, which are not available in most single institutions, potentially limits its clinical utility.

On the other hand, \textit{ex vivo} \textsuperscript{1}H-MRS analysis of body fluids is a feasible method for more institutions while still providing specific reliable markers of human cancer. The analysis of expressed prostatic secretions (EPS) offers an advantage over the analysis of prostate biopsy tissue because EPS collection is relatively less invasive.

A previous report has suggested that the ratio of citrate/spermine measured by \textsuperscript{1}H-MRS is lower in EPS from men with prostate cancer.\textsuperscript{11} However, the study was limited in that it included only low numbers of patients, provided only relative levels for the markers of interest, and suffered from the absence of an appropriate statistical interpretation of the data due to the low number of available samples.

Recently improved high-resolution \textsuperscript{1}H-MRS methods available at our institution have allowed us to determine the absolute concentrations of markers of interest in \textit{ex vivo} EPS samples. Perhaps the most important and novel finding of the current study is that the absolute concentrations of citrate, myo-inositol and spermine are predictive of prostate cancer while being independent of age. It has been previously reported that prostatic cells are zinc-accumulating citrate-producing cells because of their highly specific secretory ability.\textsuperscript{9} Their malignant transformation is characterized by metabolic transformation to citrate-oxidizing cancer cells that lost the ability to accumulate zinc. Polyamine spermine is also specific for prostate tissue and has been proposed as an endogenous inhibitor of prostate cancer growth.\textsuperscript{27} On the other hand, the volume and osmoregulator myo-inositol is expressed in a variety of tissues, and its decrease was observed in breast tumors\textsuperscript{21} (but has not been previously reported for malignant prostate gland). While previous studies have indicated that ratios of citrate to other
metabolites (relative concentrations) are potentially predictive of prostate cancer,\textsuperscript{10,11} the current study, to the best of our knowledge, is the first to indicate that these three metabolites are independent of age. This has important implications because age is a strong confounder in the association between PSA – the leading prostate cancer marker currently in use - and prostate cancer risk. Moreover, benign prostatic hyperplasia (BPH), which is also strongly associated with age, can raise PSA levels and further muddy the waters in terms of the early detection of prostate cancer. Thus, age-independent markers of PCa have the potential to improve early detection and diagnostics while reducing false-positives and their associated harms.

While these results are promising, caution is warranted. The primary limitation of the current study is the small number of samples analyzed (n=65) in relation to the number of metabolites measured (n=9). Ratios of samples-to-variables such as these have the potential to lead to false associations being made between variables due to chance. Another potential limitation of this study is the possibility that the LR models over-fit the data used to develop the models. Over-fitting is a phenomenon where a model describes the available data well, but is not able to generalize effectively with new data. The deviance/DF measures provided in Table 2 suggest that over-fitting may have occurred in the LR modeling of citrate and myo-inositol, but not in spermine. A prospective validation of these models, which is underway, will be necessary to confirm the current results. Finally, the use of EPS as a source for PCa markers may be impractical due to the need for an extended transrectal prostatic massage to collect the sample. Although the procedure does not appear to cause significant short or long-term harm, it does result in some patient discomfort and it is not clear how well it would be tolerated as a routine test.

Possible future directions for this research include investigating whether citrate, myo-inositol and spermine can be as effectively measured in urine collected after a digital rectal exam rather than EPS collected using an extended prostatic massage. Larger multi-institutional studies on
EPS and urine metabolic profile will also help to validate the identified metabolic markers according to the Gleason score and/or benign hyperplasia as well as to assess the efficacy of novel anti-cancer therapies.

In conclusion, the absolute concentrations of the metabolites citrate, myo-inositol and spermine in EPS are potential age-independent markers of prostate cancer. A prospective validation, currently underway, is necessary to confirm these promising results.
ACKNOWLEDGMENTS

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REFERENCES


**TABLES**

**Table 1:** Demographic data on EPS donors and metabolite concentrations in EPS from control (healthy) and PCa subjects calculated from high-resolution $^1$H-NMRS

<table>
<thead>
<tr>
<th></th>
<th>All Subjects</th>
<th>With Cancer</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N</strong></td>
<td>78</td>
<td>52</td>
<td>26</td>
</tr>
<tr>
<td><strong>Age in years – mean (SD)</strong></td>
<td>56.1 (±9.4)</td>
<td>58.0 (± 7.0)</td>
<td>52.2 (± 12.1)</td>
</tr>
<tr>
<td><strong>Biopsy Gleason Sum</strong></td>
<td>na</td>
<td>7 (5 – 9)</td>
<td>na</td>
</tr>
<tr>
<td><strong>PSA in ng/ml</strong></td>
<td>na</td>
<td>5.4 (2.0 – 28.0)</td>
<td>na</td>
</tr>
<tr>
<td>[Citrate] in μmol/ml</td>
<td>240.1 (14.3 – 764.5)</td>
<td>137.2 (14.3 – 444.4)</td>
<td>353.2 (125.9 – 764.5)</td>
</tr>
<tr>
<td>[Spermine] in μmol/ml</td>
<td>43.8 (2.1 – 168.2)</td>
<td>30.6 (2.1 – 133.3)</td>
<td>58.0 (18.9 – 168.2)</td>
</tr>
<tr>
<td>[Myo-Inositol] in μmol/ml</td>
<td>13.6 (.81 – 41.9)</td>
<td>7.2 (.81 – 26.6)</td>
<td>21.2 (7.7 – 41.9)</td>
</tr>
<tr>
<td>[Lactate] in μmol/ml</td>
<td>1.1 (.00* - 13.2)</td>
<td>1.0 (.14 – 13.2)</td>
<td>1.1 (.00* - 7.9)</td>
</tr>
<tr>
<td>[Alanine] in μmol/ml</td>
<td>.47 (.00* – 5.8)</td>
<td>.38 (.01 – 5.4)</td>
<td>.70 (.00* - 5.8)</td>
</tr>
<tr>
<td>[Posphocholine] in μmol/ml</td>
<td>.28 (.01 – 4.6)</td>
<td>.07 (.01 – 4.2)</td>
<td>.42 (.02 – 4.6)</td>
</tr>
<tr>
<td>[Glutamine] in μmol/ml</td>
<td>1.1 (.00* - 9.6)</td>
<td>1.1 (.01 – 9.3)</td>
<td>2.0 (.00* - 9.6)</td>
</tr>
<tr>
<td>[Acetate] in μmol/ml</td>
<td>.00* (.00* – 2.9)</td>
<td>.09 (.00* – 2.9)</td>
<td>.00* (.00* - 00*)</td>
</tr>
<tr>
<td>[Valine, Leucine] in μmol/ml</td>
<td>13.1 (.86 – 37.9)</td>
<td>9.9 (.86 – 37.9)</td>
<td>16.6 (7.7 – 37.7)</td>
</tr>
<tr>
<td>[Hydroxybutyrate] μmol/ml</td>
<td>.60 (.00* - 7.9)</td>
<td>.40 (.01 – 5.4)</td>
<td>.78 (.00* - 7.9)</td>
</tr>
</tbody>
</table>

EPS = expressed prostatic secretions; N = number of subjects; SD = standard deviation; PSA = serum prostate specific antigen; [x] = absolute concentration of x; na = not applicable; *= not detectable
**Table 2:** Results of logistic regression (LR) analysis of EPS metabolites in relation to prostate cancer and age.

The p values <0.05 considered to be statistically significant from healthy control subjects (for PCa analysis) and among different age groups (for age analysis)

<table>
<thead>
<tr>
<th><strong>Log Base 10 of:</strong></th>
<th><strong>p-value (PCa)</strong></th>
<th><strong>deviance/DF</strong></th>
<th><strong>p-value (age)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate</td>
<td>0.0005</td>
<td>0.7762</td>
<td>0.9598</td>
</tr>
<tr>
<td>Myo-inositol</td>
<td>0.0006</td>
<td>0.8295</td>
<td>0.7368</td>
</tr>
<tr>
<td>Spermine</td>
<td>0.0038</td>
<td>1.0811</td>
<td>0.2582</td>
</tr>
<tr>
<td>Valine - Leucine</td>
<td>0.0043</td>
<td>1.0983</td>
<td>0.079</td>
</tr>
<tr>
<td>[Citrate/Lactate]</td>
<td>0.0013</td>
<td>0.9977</td>
<td>0.0348</td>
</tr>
<tr>
<td>[Citrate/Spermine]</td>
<td>0.0003</td>
<td>0.9912</td>
<td>0.0325</td>
</tr>
<tr>
<td>Phosphocholine</td>
<td>0.0475</td>
<td>1.2373</td>
<td>0.0236</td>
</tr>
<tr>
<td>Citrate/phosphocholine</td>
<td>0.9586</td>
<td>1.2775</td>
<td>0.0152</td>
</tr>
<tr>
<td>Citrate/inositol</td>
<td>0.0823</td>
<td>1.2266</td>
<td>0.0131</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.0659</td>
<td>1.2075</td>
<td>0.0102</td>
</tr>
<tr>
<td>OH-Butyrate</td>
<td>0.0125</td>
<td>1.1479</td>
<td>0.0093</td>
</tr>
<tr>
<td>Glutamine</td>
<td>0.0258</td>
<td>1.1176</td>
<td>0.0087</td>
</tr>
<tr>
<td>Lactate</td>
<td>0.5308</td>
<td>1.262</td>
<td>0.0082</td>
</tr>
</tbody>
</table>

EPS = expressed prostatic secretions; PCa = prostate cancer; DF = degrees of freedom
Table 3: Metabolic stability analysis on fresh and frozen EPS samples (n=6)

Six freshly collected EPS samples were analyzed by $^1$H-NMRS at three different time points post-collection (fresh, day 0; 1 week after -80 °C storage and 4 weeks after -80 °C storage). The p values <0.05 considered to be statistically significant among three different time points, indicating metabolite degradation due to cold storage.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Chemical Shift</th>
<th>Overall p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myo-Inositol</td>
<td>4.07 ppm</td>
<td>0.2418</td>
</tr>
<tr>
<td>Phosphocholine</td>
<td>3.24 ppm</td>
<td>0.2157</td>
</tr>
<tr>
<td>Spermine1</td>
<td>3.20 ppm</td>
<td>0.4280</td>
</tr>
<tr>
<td>Citrate1</td>
<td>2.72 ppm</td>
<td>0.2763</td>
</tr>
<tr>
<td>Citrate2</td>
<td>2.58 ppm</td>
<td>0.4427</td>
</tr>
<tr>
<td>Glutamine</td>
<td>2.36 ppm</td>
<td><strong>0.0413</strong></td>
</tr>
<tr>
<td>Spermine2</td>
<td>2.11 ppm</td>
<td>0.9197</td>
</tr>
<tr>
<td>Spermine3</td>
<td>1.78 ppm</td>
<td>0.0719</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.45 ppm</td>
<td><strong>0.0261</strong></td>
</tr>
<tr>
<td>Lactate</td>
<td>1.32 ppm</td>
<td>0.4254</td>
</tr>
<tr>
<td>OH-Butyrate</td>
<td>1.19 ppm</td>
<td>0.1165</td>
</tr>
<tr>
<td>Valine - Leucine</td>
<td>1.01 ppm</td>
<td>0.6667</td>
</tr>
</tbody>
</table>

ppm = parts per million
FIGURE LEGENDS

Figure 1: (A) Median concentrations of citrate, myo-inositol and spermine (given as μmol/mL) in human EPS from healthy control subjects and patients with PCa calculated from $^1$H-NMRS. (B) Two representative high-resolution NMR spectra from human EPS from a healthy control subject (left) and a PCa patient (right, x5 magnification).

Figure 2. Receiver operating characteristic (ROC) curves for citrate, myo-inositol, and spermine.

The areas under the receiver operating characteristic curves (AUROC) for citrate, myo-inositol and spermine were 0.89, 0.87, and 0.79, respectively.

Figure 3: Prostate cancer probability curves for citrate, myo-inositol, and spermine.

The relationships are given between the log of the concentrations of citrate, myo-inositol and spermine, and the risk of prostate cancer.
Figure 2:
Figure 3
KEY RESEARCH ACCOMPLISHMENTS: (Bulleted list of key research accomplishments emanating from this research.)

- Confirmed reports in the literature of the value of the relative concentration of Citrate in EPS as a potential marker of prostate cancer.
- Confirmed reports in the literature of the value of the relative concentration of Spermine in EPS as a potential marker of prostate cancer.
- Found that – in a two-variable LR model - citrate/spermine and citrate/lactate were also predictive of prostate cancer, but to a lesser degree than the absolute concentrations of these metabolites.
- Contradicted reports of other researches, which have indicated that phosphocholine concentration is a marker of cancer. We found no correlation between phosphocholine concentrations in EPS and the risk of prostate cancer.
- Validated the use of frozen EPS via a sub-study where we found no significant metabolic degradation for hydroxybutyrate, citrate, myo-inositol, lactate, phosphocholine, spermine, or valine in samples that were frozen and thawed at 1 week and at 1 month as compared to freshly collected EPS. Only the metabolites alanine and glutamine showed significant changes in concentrations, but were not metabolites of interest in this study.
- Utilized a novel method developed by Co-Investigator N. Serkova to measure the absolute concentrations of metabolites (previous studies have only measured relative concentrations of metabolites in EPS).
- Established a quantitative and validated 1H-NMRS protocol to analyze ultra-small volume human body fluids using a 1-mm micro-probe.
- Found that absolute concentrations of citrate, myo-inositol, and spermine are predictive of prostate cancer while being independent of age.
- Found that the concentrations of citrate and spermine are predictive of Gleason sum.

EPS = expressed prostatic secretions
LR = logistic regression
REPORTABLE OUTCOMES:

Peer-Reviewed Abstracts, Presentations, and Manuscripts:

*Nuclear magnetic resonance spectroscopy of expressed prostatic secretions: Metabolite citrate and derivatives are potential markers of prostate cancer.*


Abstract presented (Abstract and Oral Presentation) at the 16th International Prostate Cancer Symposium, Beaver Creek, Colorado, January 2006

*Validation of citrate and derivatives in expressed prostatic secretions to predict prostate cancer: High-resolution $^1$H-NMR study.*


Abstract presented (poster session) at the 97th Annual Meeting of the American Association for Cancer Research, Washington, DC, April 2006.

*High-resolution nuclear magnetic resonance spectroscopy of expressed human prostatic secretions: The metabolites citrate, myo-inositol, and spermine are potential age-independent markers of prostate cancer.*


Abstract presented for Discussed Poster session at the Annual American Urological Association meeting, Atlanta, Georgia, May 2006.

*Nuclear magnetic resonance spectroscopy of expressed prostatic secretions: Metabolite citrate and derivatives are potential markers of prostate cancer: An Update.*


Abstract presented for Poster Session at the Annual American Society of Clinical Oncologists, Atlanta, Georgia, June 2006
NMR Spectroscopic Validation of Metabolic Markers of Prostatic Cancer in Human Expressed Prostatic Secretions


Abstract accepted for oral presentation at the International Society for Magnetic Resonance in Medicine MR in Cancer Workshop, October 2006.

High-resolution nuclear magnetic resonance spectroscopy of expressed human prostatic secretions: The metabolites citrate, myo-inositol, and spermine are potential age-independent markers of prostate cancer.


Full-length manuscript in progress to be submitted to the Journal of Clinical Oncology by 31 October 2006.
CONCLUSIONS: (Summarize the results to include the importance and/or implications of the completed research and when necessary, recommend changes on future work to better address the problem. A "so what section" which evaluates the knowledge as a scientific or medical product shall also be included in the conclusion of the report.)

Important Findings:

Perhaps the most important and novel finding of this study is that the absolute concentrations of citrate, myo-inositol and spermine are predictive of prostate cancer while being independent of age. While previous studies have indicated that ratios of citrate to other metabolites (relative concentrations) are potentially predictive of prostate cancer, the current study, to the best of our knowledge, is the first to indicate that these three metabolites are independent of age. This is important because age is a strong confounder in the association between prostate specific antigen (PSA) – the most widely used marker of prostate cancer - and prostate cancer risk. Moreover, benign prostatic hyperplasia (BPH), which is also strongly associated with age, can raise PSA levels and further muddy the waters in terms of the early detection of prostate cancer. Thus, age-independent markers of PCa have the potential to improve early detection and diagnostics by eliminating this important confounder while reducing false-positives and their associated harms.

Additional Important Findings:

As part of a no-cost extension of the project, we further analyzed our data to determine if the metabolites citrate, myo-inositol and spermine are predictive of Gleason sum. We found that citrate and spermine were indeed predictive of Gleason sum. Myo-inositol did not reach statistical significance in this regard. The prediction equation derived through logistic regression is:

\[
\text{Gleason sum} = 9.43 - 3.32 \log_{10}[\text{citrate}] + 2.79 \log_{10}[\text{spermine}].
\]

The prediction has a standard deviation from the correct Gleason sum of 0.70 when applied to our data set. For example, in a randomly drawn subject, the concentration of citrate = 22.14 and spermine = 5.83. The resulting prediction is 7.10. The true Gleason sum is 7, making this a good prediction.

The implication of these promising results is that the methods described here could potentially be used to better estimate prostate cancer grade before treatment. This could potentially improve treatment decision-making due to a better estimate of tumor aggressiveness. Current biopsy methods alone do not always give an accurate picture in this regard.

Potential Limitations:

While these results are promising, caution is warranted. The primary limitation of the current study is the small number of samples analyzed (65) in relation to the number of metabolites measured (9). Ratios of samples to variables such as these have the potential to lead to false associations being made between variables due to chance. Another potential limitation of this study is the possibility that the logistic regression (LR) models over-fit the data used to develop the models. Over-fitting is a phenomenon where a model describes the available data well, but is not able to generalize effectively with new data. The deviance/DF measures provided in Table 2 suggest that over-fitting may have occurred in the LR modeling of citrate and myo-inositol, but not in spermine. A prospective validation of these models will be necessary to confirm the current results.
In conclusion, the absolute concentrations of the metabolites citrate, myo-inositol and spermine in EPS are potential age-independent markers of prostate cancer. Age-independent markers of prostate cancer would have great potential in improving the accuracy of screening and early detection. A prospective validation, currently underway, is necessary to confirm these promising results.

**Future Directions:**

We plan to seek additional funding to complete a larger, multi-institutional study to further confirm these promising results. We believe the interest in an age-independent marker of prostate cancer would be high in the research and clinical communities. In addition, the ability to accurately determine cancer grade before treatment has the potential to greatly improve the treatment-decision process.