

Identification of Characteristic Metabolic Panels for Different Stages of Prostate Cancer by ^1H NMR-based Metabolomics Analysis

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Abstract

Background: Prostate cancer (PCa) is the second most prevalent cancer in males worldwide, yet detecting PCa and its metastases remains a major challenging task in clinical research setups. The present study aimed to characterize the metabolic changes underlying the PCa progression and investigate the efficacy of related metabolic panels for an accurate PCa assessment.

Methods: This study analyzed the metabolic profiles of serum samples from PCa patients (n = 75), PCa patients with bone metastasis (PCaB, n = 62), and benign prostatic hyperplasia patients (BPH, n = 50) by ¹H nuclear magnetic resonance (NMR)-based metabolomics analysis.

Results: Multivariate analysis revealed that BPH, PCa, and PCaB groups showed distinct metabolic divisions, while univariate statistics integrated with variable importance in the projection (VIP) scores identified a differential metabolite series, which included energy, amino acid, and ketone body metabolism. Additionally, our results also revealed the metabolic panels of discriminant metabolites coupled with the clinical parameters (age and body mass index) for discrimination between PCa and BPH, PCaB and BPH, PCaB and PCa achieved the AUC values of 0.828, 0.917, and 0.872, respectively.

Conclusions: Overall, this study characterizes the potential metabolic alterations involved in the PCa progression and its metastases, and might provide potential metabolic biomarkers for the diagnosis and classification of PCa.

Background

Prostate cancer (PCa) is the second most frequent cancer among men globally, which poses a major detriment to men's health [1]. There were an estimated 1.4 million new cases of prostate cancer, and almost 375,304 cancer deaths occurred in 2020 [2]. Several significant efforts have been focused on the development of sensitive and accurate diagnostic tools for PCa since it is more likely to be cured if it is diagnosed early [3]. Currently, PCa is majorly screened by the prostate-specific antigen (PSA) blood test or a digital rectal examination (DRE) combined with a subsequent ultrasound-guided prostate biopsy (PBs) that confirms the cancerous growth presence [4]. Several limitations still exist in the traditional diagnostic methods like, higher procedural costs, longer examination time, low sensitivity and specificity, which may sometimes even lead to overdiagnosis and overtreatment [5, 6]. Therefore, there is an urgent need to discover novel biomarkers for improving the diagnosis, prognosis and treatment of PCa.

As dysregulated cellular metabolism is a vital hallmark of cancer [7], employing metabolomics analysis in cancer samples could provide critical insights for monitoring the cancer progression [8, 9]. Recently, multiple studies have demonstrated the potential of metabolomics analysis in PCa research. For example, Lima et al. [10] performed a urinary volatile profile of 40 PCa patients and 42 healthy controls *via* gas chromatography-mass spectrometry (GC-MS) and obtained a panel of six volatile biomarkers for PCa diagnosis by analyzing different metabolite expression patterns. Based on the liquid chromatography-mass spectrometry (LC-MS) and GC-MS, Huang et al. [11] suggested that serum N-oleoyl

taurine and sterol metabolites levels were linked to a decreased PCa survival rate. Additionally, a metabolomics analysis integrated with GC-MS and magnetic resonance spectroscopy (MRS) identified acylcarnitines, glycerophospholipids, and arginine as potential diagnostic markers from BPH to PCa by providing crucial insights into the PCa metabolic characteristics [12]. Hence, this concept can be put forth that metabolomics analysis has a strong potential as a precise diagnostic tool for exploring metabolic biomarker's expression patterns and elucidating the potential PCa metabolic mechanisms [13].

Nuclear magnetic resonance (NMR) spectroscopy is a promising noninvasive technique for metabolic profiling analysis due to innate advantages like simple sample preparation, non-destructive analysis, and high reproducibility [14]. In our previous study, ^1H -NMR based metabolomics approach was successfully applied to characterize the significant metabolic differences in tissue and biofluid samples in different PCa stages [15] as well as in hormone-sensitive and castration-resistant PCa cases [16]. The present study characterized the metabolic profiling in serum samples from PCa patients (PCa), PCa patients with bone metastasis (PCaB), and benign prostatic hyperplasia patients (BPH) via a ^1H NMR based metabolomics approach. The aim of this study was (1) to identify metabolic alterations among PCa, PCaB, and BPH, (2) to investigate key metabolic pathways involved in the PCa and PCaB progression, (3) to elucidate the potential biomarker panels for differentiating among BPH, PCa and PCaB subjects.

Methods, Materials And Participants

Study Participants

This study included all participants diagnosed by PSA measurements and pathological examinations based on the PCa National Comprehensive Cancer Network (NCCN) guidelines [17]. A total of 187 participants from Renji Hospital Affiliated to Shanghai Jiaotong University School of Medicine were enrolled, which included 75 BPH, 62 PCa, and 50 PCaB patients. The study protocol was approved by the Ethics Committee of Shanghai Jiao Tong University School of Medicine (IRB number: Renji [2013] 26) along with the informed written consents obtained from all the participants. The important biochemical and clinical subject parameters are listed in Table 1.

Table 1
Participants' clinical characteristics

Parameters	BPH	PCa	PCaB	P value		
				PCa vs BPH	PCaB vs BPH	PCaB vs PCa
N	75	62	50	-	-	-
Age	65.15 ± 7.46	68.53 ± 7.58	73.82 ± 8.17	0.011	< 0.001	<0.001
BMI	24.10 ± 2.56	24.62 ± 2.56	23.38 ± 2.90	0.261	0.155	0.019
PSA	12.64 ± 5.27	18.51 ± 15.92	401.16 ± 124.46	0.949	0.001	0.002
Data were presented as Mean ±SD; BPH, patient with benign prostatic hyperplasia; PCa, prostate cancer; PCaB, prostate cancer with bone metastasis; BMI, body mass index; PSA, prostate-specific antigen.						

Sample Collection and Preparation

On the morning following overnight fasting, the peripheral venous blood samples (5 mL) were collected from the participants, transferred into a centrifuge tube, and kept at room temperature for 30 min, followed by centrifugation at 1,500 g for 15 min. Furthermore, the serum supernatant was collected and stored at -80°C for further analysis. Before undertaking the analysis, serum samples were thawed at 4°C and vortexed for ten seconds, followed by the dilution of serum sample (200 μL) with phosphate buffer (250 μL , 0.2 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH = 7.4) as well as deuterium oxide (D_2O , 50 μL). Thereafter, the mixed serum sample was vortexed for ten seconds and centrifuged at 10,000 g for 15 min at 4°C followed by the supernatant transfer (500 μL) into a 5-mm NMR tube for further metabolomics analysis.

^1H NMR-based Metabolomics Analysis

^1H NMR-based metabolomics analysis was performed on a Bruker AVANCE III 600 MHz NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a five mm Triple Resonance Probe (TXI) probe. The ^1H NMR spectra of serum samples were acquired by using a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence with a fixed receiver-gain value at 37°C for negating the comprehensive effects of lipid or protein macromolecules. The main NMR acquisition parameters, as considered in our previous study [18], were determined as follows: data points = 256 K; relaxation delay = 4 s; spectral width = 12,335.5 Hz; acquisition time = 2.66 s per scan. All CPMG spectra were transferred to TopSpin 3.0 software (Bruker BioSpin, Rheinstetten, Germany) for automatic phase and baseline corrections while the chemical shifts of the serum spectra were directed toward the anomeric signal of α -glucose at 5.23 ppm. The icoshift algorithm based on spectral interval's calibrations was applied to align NMR spectra in

MATLAB (R2012a, The Mathworks Inc., Natick, MA, USA) [19]. Henceforth, the spectral region from 0.0 to 9.0 ppm, excluding the water peak from 4.70 to 5.00 ppm, was subdivided and integrated into binned datasets with sizes of 0.01 ppm and 0.0015 ppm for multivariable and quantitative analyses, respectively. Accordingly, the metabolites were assigned by using Chenomx NMR suite 7.7.2 software (Chenomx Inc., Alberta, Canada) and the Human Metabolome Database (HMDB) [20].

Multivariate Analysis

This study conducted multivariate data analysis by using SIMCA 12.0 software (Umetrics AB, Umea, Sweden) that utilized partial least squares discriminant analysis (PLS-DA) to obtain a metabolic pattern discrimination overview among BPH, PCa and PCaB subjects. Furthermore, the orthogonal partial least squares discriminant analysis (OPLS-DA) model was also employed to distinguish between the two groups and identify the differential metabolites contributing to the discriminations for any two groups. The OPLS-DA model's quality was evaluated by cross-validated analysis of variance (CV-ANOVA), in which R^2 and Q^2 values were calculated as the goodness of fit and goodness of prediction parameters, respectively. The importance of each variable in the OPLS-DA loading was evaluated by corresponding VIP scores, whereas metabolites with a VIP value >1 were selected for further analysis.

Statistical Analysis

The unpaired Student's t-test was employed to evaluate the significance of each metabolite among BPH, PCa, and PCaB in SPSS 22.0 software (IBM Corp, Armonk, NY), whereas the statistically significant difference was considered at $p\text{-value} < 0.05$. Our study selected metabolites with $VIP > 1$ and $p\text{-value} < 0.05$ and considered them as the significant differential metabolites that could discriminate within the groups for an accurate disease assessment. Metabolic pathways were manually drawn by CorelDRAW Graphics Suite (Corel Inc., Ottawa, Canada) based on KEGG pathways (www.genome.jp/kegg/) and Small Molecule Pathway Database (SMPDB, www.smpdb.ca/).

Receiver-Operating Characteristic (ROC) Analysis

Receiver operating characteristic (ROC) curves analysis was used to evaluate the diagnostic potential of variable-identified metabolites among BPH, PCa, and PCaB groups along with the generation of the area under the ROC curve (AUC) value by MedCalc software (MedCalc Software, Ostend, Belgium). The significance level was defined at a $p\text{-value} < 0.05$, and the AUC value < 1 indicated excellent diagnostic accuracy.

Results

Participants' Clinical Characteristics

A total of 187 subjects, including 75 BPH patients, 62 PCa patients, and 50 PCaB patients, were included, while their clinical characteristics are presented in Table 1. In the cohort, the mean age of patients was significantly greater in PCaB, followed by PCa and BPH subjects. Although the body mass index (BMI) of

PCaB had decreased compared to PCa, no significant alteration was observed in PCa and PCaB patients compared to BPH subjects. Furthermore, PCaB patients had distinct higher PSA levels than PCa and BPH groups; however, no difference was observed in PSA levels between BPH and PCa cases.

Metabolic Segregations among BPH, PCa, and PCaB patients

This study comprehensively analyzed the metabolic profiles of serum samples obtained from 75 BPH, 62 PCa, and 50 PCaB patients by a $^1\text{H-NMR}$ based metabolomics approach. Figure 1A illustrates a typical $^1\text{H-NMR}$ spectrum of BPH serum sample containing 25 metabolites that were mainly involved in energy metabolism (citrate, creatine, creatinine, glucose, lactate, and pyruvate), amino acid metabolism (alanine, glutamine, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, threonine, tyrosine, and valine), ketone body metabolism (3-hydroxybutyrate (3-HB), acetone and acetoacetate), lipid metabolism (LDL/VLDL and glycerol) and fatty acid metabolism (acetate and formate).

The PLS-DA, based on the serum metabolome characteristics, was carried out for determining the metabolic pattern changes among BPH, PCa, and PCaB groups. As illustrated in Figure 1B, the PLS-DA score plot revealed a clear metabolic pattern demarcation between BPH, PCa, and PCaB subjects. Subsequently, a supervised OPLS-DA model was utilized for characterizing the discrimination between the two groups as well as the VIP score, which confirmed the differential metabolites. As can be seen from Figure 1C-E, the discriminant capabilities of the PLS-DA models were observed in the following groups: between BPH and PCa ($R^2 = 0.574$, $Q^2 = 0.47$) (Figure 1C), between BPH and PCaB ($R^2 = 0.585$, $Q^2 = 0.522$) (Figure 1D) as well as between PCa and PCaB ($R^2 = 0.594$, $Q^2 = 0.475$) (Figure 1E). The BPH and PCa segregation as displayed by the VIP score of OPLS-DA was mainly attributed to metabolites like 3-HB, alanine, acetone, glutamine, tyrosine, histidine, and formate (Figure 1C). In contrast, significant metabolites like leucine, isoleucine, valine, acetoacetate, pyruvate, creatine, phenylalanine, histidine, and formate played a remarkable role in metabolic differentiation between BPH and PCaB patients (Figure 1D). Additionally, LDL/VLDL, leucine, isoleucine, valine, 3-HB, alanine, acetone, acetoacetate, pyruvate, citrate, and creatine were also associated with PCa and PCaB dissociation in the serum metabolome (Figure 1E).

Quantification of Differential Metabolites among BPH, PCa, and PCaB

Statistical analysis of detected metabolites was obtained for exploring the characteristic metabolic changes among BPH, PCa, and PCaB groups in Table 2. When compared with BPH, PCa had significantly lower levels of histidine, glutamine, acetone, and 3-HB as well as higher serum levels of tyrosine and alanine, whereas PCaB had significantly increased levels of phenylalanine, formate, glucose, and pyruvate along with a remarkable decrease in histidine, creatine, glutamine, acetoacetate, acetate, valine, isoleucine and leucine when compared to BPH cases. Furthermore, in comparison with PCa, PCaB subjects had higher levels of phenylalanine, citrate, pyruvate, and alanine in serum metabolome coupled with lower creatine, lysine, acetone, 3-HB, valine, isoleucine, leucine, and LDL/VLDL levels.

Table 2
Metabolic changes in serum samples from BPH, PCa, and PCaB.

Metabolites	BPH	PCa	PCaB	P value		
				PCa vs BPH	PCaB vs BPH	PCaB vs PCa
3-HB	9.31 ± 5.69	6.18 ± 3.51	8.86 ± 7.11	0.001	0.6520	0.010
Acetate	2.11 ± 0.61	1.99 ± 0.63	1.92 ± 0.99	0.255	0.0107	0.158
Acetoacetate	2.36 ± 1.19	2.14 ± 0.89	1.94 ± 0.92	0.204	0.0221	0.304
Acetone	5.77 ± 4.36	3.21 ± 2.24	5.21 ± 5.21	0.000	0.4529	0.009
Alanine	16.80 ± 2.71	18.57 ± 2.52	17.37 ± 3.45	0.001	0.2625	0.027
Creatine	2.62 ± 0.62	2.63 ± 0.55	2.36 ± 0.70	0.921	0.0189	0.019
Creatinine	1.03 ± 0.32	1.02 ± 0.29	1.00 ± 0.35	0.892	0.2292	0.304
Citrate	2.44 ± 0.56	2.34 ± 0.43	2.56 ± 0.61	0.062	0.1402	0.010
Formate	0.09 ± 0.04	0.11 ± 0.04	0.12 ± 0.04	0.062	0.0009	0.140
Glutamine	18.97 ± 3.20	17.84 ± 2.68	17.70 ± 4.18	0.032	0.0255	0.841
Glycine	8.29 ± 1.87	8.01 ± 1.54	8.41 ± 2.48	0.417	0.7400	0.285
Glycerol	9.60 ± 1.77	9.50 ± 2.58	10.18 ± 2.75	0.815	0.1689	0.125
Histidine	1.20 ± 0.21	1.13 ± 0.18	1.09 ± 0.21	0.032	0.0025	0.338
Isoleucine	2.73 ± 0.47	2.72 ± 0.39	2.55 ± 0.43	0.922	0.0189	0.031
Lactate	100.63 ± 36.14	92.09 ± 29.81	90.63 ± 33.26	0.138	0.0935	0.815
LDL/VLDL	56.61 ± 21.04	61.83 ± 20.69	52.34 ± 23.60	0.258	0.1700	0.018
Leucine	13.53 ± 2.02	13.31 ± 1.64	12.63 ± 2.03	0.478	0.0041	0.034
Lysine	2.46 ± 0.33	2.54 ± 0.46	2.37 ± 0.45	0.253	0.243	0.030
Phenylalanine	0.68 ± 0.26	0.72 ± 0.31	0.84 ± 0.35	0.468	0.005	0.043
Pyruvate	1.65 ± 0.44	1.65 ± 0.38	1.93 ± 0.56	0.973	0.001	0.001
Threonine	1.76 ± 0.31	1.73 ± 0.40	1.70 ± 0.33	0.606	0.292	0.595
Tyrosine	0.94 ± 0.19	1.02 ± 0.15	1.01 ± 0.33	0.044	0.086	0.819

Data were presented as Mean ±SD; BPH, patient with benign prostatic hyperplasia; PCa, prostate cancer; PCaB, prostate cancer with bone metastasis; 3-HB, 3-hydroxybutyrate.

Metabolites	BPH	PCa	PCaB	P value		
				PCa vs BPH	PCaB vs BPH	PCaB vs PCa
Valine	9.37 ± 1.47	9.35 ± 1.32	8.65 ± 1.57	0.916	0.005	0.010
α-Glucose	14.19 ± 3.07	14.89 ± 2.89	15.31 ± 3.46	0.199	0.046	0.464
β-Glucose	12.81 ± 3.10	12.98 ± 3.29	12.97 ± 3.45	0.756	0.780	0.984

Data were presented as Mean ±SD; BPH, patient with benign prostatic hyperplasia; PCa, prostate cancer; PCaB, prostate cancer with bone metastasis; 3-HB, 3-hydroxybutyrate.

Diagnostic Performance of Potential Metabolic Biomarkers

ROC curves analysis, including the AUC value was conducted for evaluating the efficient utilization of the identified significant metabolites for BPH, PCa and PCaB patients screening, based on the metabolites with VIP value > 1 and *p-value* < 0.05. Figure 2 illustrates the diagnostic performance of potential metabolic biomarker panels (AUC value > 0.6, *p-value* < 0.05) as they account for discrimination between BPH and PCa groups due to the exhibition of good response by the five metabolite levels that included 3-HB (AUC = 0.678, sensitivity = 43.55%, specificity = 84%), alanine (AUC = 0.708, sensitivity = 58.06%, specificity = 81.33%), acetone (AUC = 0.687, sensitivity = 74.19%, specificity = 60%), tyrosine (AUC = 0.648, sensitivity = 87.10%, specificity = 38.67%) and histidine (AUC = 0.602, sensitivity = 70.97%, specificity = 46.67%). Moreover, the ROC analysis revealed that these five metabolites in combination had a 0.815 AUC value, along with 75.81% and 72% sensitivity and specificity, respectively. While an integration of these five metabolites with age and BMI might achieved 0.828 AUC value coupled with a sensitivity and specificity of 56.45% and 94.67%, respectively.

The ROC analysis results based on the BPH and PCaB differentiating values (AUC value > 0.6 and *p-value* < 0.05) are shown in Figure 3 which identified eight total of 8 metabolites, that included leucine (AUC = 0.607, sensitivity = 60%, specificity = 62.57%), isoleucine (AUC = 0.614, sensitivity = 54.55%, specificity = 68%), valine (AUC = 0.631, sensitivity = 61.82%, specificity = 61.33%), acetoacetate (AUC = 0.625, sensitivity = 38.18%, specificity = 85.33%), pyruvate (AUC = 0.644, sensitivity = 60%, specificity = 68%), phenylalanine (AUC = 0.675, sensitivity = 62.5%, specificity = 70.83%), histidine (AUC = 0.683, sensitivity = 72.73%, specificity = 58.67%) and formate (AUC = 0.651, sensitivity = 56.36%, specificity = 72%). Moreover, the combination of all these metabolites resulted in a 0.794 AUC value, and the sensitivity and specificity were 65.45% and 85.33%, respectively. Notably, the amalgamation of these eight metabolites with age and BMI was extremely capable of differentiating BPH from PCaB cases, with an AUC value of 0.917, 89.36% sensitivity and 90.67% specificity.

Similarly, the ROC analysis identified top eight metabolites for extricating PCaB diagnosis from PCa as shown in Figure 4, while their combination exhibited a better classification (AUC = 0.828, sensitivity = 78.18%, specificity = 74.19%) than a singular metabolite: LDL/VLDL (AUC = 0.642, sensitivity = 54.55%, specificity = 74.19%), isoleucine (AUC = 0.622, sensitivity = 43.64%, specificity = 85.48%), valine (AUC = 0.626, sensitivity = 49.09%, specificity = 75.81%), 3-HB (AUC = 0.604, sensitivity = 80%, specificity = 41.94%), alanine (AUC = 0.614, sensitivity = 56.36%, specificity = 70.97%), pyruvate (AUC = 0.659, sensitivity = 89.09%, specificity = 38.71%), citrate (AUC = 0.613, sensitivity = 32.73%, specificity = 98.39%) and creatine (AUC = 0.622, sensitivity = 34.55%, specificity = 88.71%). Additionally, the merging of eight metabolites with age and BMI displayed the best predictability with an AUC value, sensitivity and specificity of 0.872, 87.23% and 74.19%, respectively.

Metabolic Pathway Analysis of Differential Metabolites among BPH, PCa, and PCaB

Metabolic pathway analysis was carried out based on the differential metabolite values (p -value < 0.01 and AUC value > 0.6) for exploring pathway-based metabolic features, as shown in Figure 5. Our results suggested that a series of metabolic pathways like energy, amino acid, and ketone body metabolism were implicated in metabolic discrimination among BPH, PCa, and PCaB subjects.

Discussion

Early diagnosis plays a crucial role in the successful treatment for PCa [21]; however, the non-selective use of traditional screening tools for PCa generally leads to overdiagnosis and overtreatment that does not give expected results [22]. Several studies have reported that metabolic disturbances have been associated with increased PCa incidence [23]. Nowadays, metabolomics has emerged as an immensely powerful screening tool in PCa biomarker development due to its inherent advantages, such as noninvasive procedures, high reproducibility, and lower costs [24]. This study examined the metabolic profiles of BPH, PCa, and PCaB serum samples obtained by ¹H NMR-based metabolomics approach and revealed that serum metabolome analysis exhibited clear discriminations among the three subject groups: BPH, PCa, and PCaB. After conducting univariate statistical and VIP analyses, the most important metabolites were selected, and the diagnostic capacity of these metabolites was evaluated by ROC analysis, while the potential biomarker panels were identified in serum samples for segregating BPH, PCa, and PCaB subjects. Accordingly, it was observed that metabolic panels based on serum metabolome levels exhibited superior performance in discriminating the three groups. Especially, the metabolic biomarker panels in the cluster of differential metabolites and clinical characteristics (age and BMI), showing the AUC values > 0.8. Based on this, it was suggested that serum metabolomics analysis shows great potential in becoming an accurate, diagnostic supplemental tool for diagnosing PCa and its metastases. In our study, it was observed that among all identified potential metabolic biomarkers for different PCa stages, a series of metabolic pathways were promptly involved, which included mainly energy, amino acid, and ketone body metabolism.

Energy metabolism, which expedites the uptake and incorporation of glucose into the biomass needed to produce new cells, is critical for maintaining the abnormal growth and intensive proliferation of cancer cells [25]. Furthermore, the glycolytic breakdown of glucose, the main substrate for energy supply, results in pyruvate production that consequently gets oxidized through the tricarboxylic acid (TCA) cycle for ATP production or converted into lactate by anaerobic glycolysis [26]. It was also consistent with the findings of Schwartz et al. in 2017 [27] that according to the Warburg effect, most cancer cells primarily convert glucose to lactate through anaerobic glycolysis for meeting the energy requirements for cellular cell proliferation. However, our study revealed that the pyruvate concentration in serum samples was significantly enhanced in PCaB cases compared to PCa and BPH, which might further imply that PCaB may have a reverse Warburg effect in serum metabolome that still needs validation. Similarly, citrate is not only an important substrate for de novo lipid synthesis but also serves as a key TCA cycle intermediate for ATP production [28, 29]. Costello and his colleagues [30] demonstrated that the malignant prostate cells oxidized citrate for ATP generation for combating increased energy demand. However, the present study revealed that the citrate level of PCaB serum samples was higher than the PCa samples, while the concentration of creatine, an important regulator of energy metabolism, was remarkably increased in the PCaB serum levels when compared to PCa levels. As stated by Rodriguez-Enriquez et al. in 2019 [31], that increased energy metabolites might play a pivotal role in malignant tumor cell growth and proliferation; our findings suggested that the two energy substrates (citrate and creatine) in serum samples were robustly upregulated for supplying the elevated energy demand of cancerous cells while playing a crucial part in tumor progression and metastasis.

Out of the many energy sources, amino acids are precisely involved in biosynthesis as well as are important reserves for supporting the survival and proliferation of cancer cells [32]. A recent study investigated the emerging roles of amino acids in epigenetic regulation and initiating immune responses that were related to tumorigenesis and metastatic pathways [33]. Our study noted that a majority of amino acids were increased in the serum samples when compared from BPH to PCa subjects but gradually decreased when the disease progressed to PCaB. It was suggested that leucine, isoleucine, and valine are members of the branched-chain amino acids (BCAAs) that can be catabolized to TCA cycle intermediates for energy production [34], which was also supported by Teahan et al. [35] that BCAAs are potential biomarkers for PCa aggression by using NMR-based metabolomics. Hence, it was evident that increased levels of serum BCAAs are utilized as an energy source for tumor proliferation and development. In contrast, it was also observed that the serum BCAAs levels were significantly decreasing in PCaB when compared with PCa or BPH patients, thereby suggesting that downregulated BCAAs might be closely related to bone metastasis in PCa progression. Recent evidence indicated that histidine catabolism was associated with PCa progression [15] and was consistent with our findings that the histidine level was markedly decreased in the serum levels of both PCa and PCaB patients as compared to BPH patients. As proposed by Lapek et al. in 2015 [36] that histidine phosphorylation might be greatly associated with metastatic PCa. Hence, this concept could be put forth that the downregulation of PCa and PCaB histidine levels, when compared to BPH patients, might be attributed to the upregulated histidine phosphorylation in PCa and the resultant metastasis development. Several previous studies

have indicated that alanine level was significantly higher in serum and biopsy tissues when progressing from BPH to PCa [37, 38]. In contrast, a decreased alanine level was associated with advanced cancer stage and poor cancer-specific survival [39]. Accordingly, our study denoted that due to alanine's differential nature, it was observed that tumor proliferation was consistent with increased protein synthesis; as a result, the alanine serum level was distinctly elevated from BPH to PCa but decreased from PCa to PCaB subjects. Correspondingly, tyrosine and phenylalanine were two additional metabolites that exhibited increased trends in the serum of PCa and PCaB patients. Several studies have demonstrated that dysregulated tyrosine and phenylalanine metabolism is closely related to PCa progression [40, 41]. Another observation in our study was the presence of higher levels of tyrosine and phenylalanine in PCa and PCaB patients' serum when compared with BPH patients, respectively. It was substantiated by Gomez-Cebrian et al. [42] that phenylalanine hydroxylase (PAH) is the enzyme that metabolizes excess phenylalanine into tyrosine; hence, it is also reported to have a direct association with protein acetylation and energy production [41, 43]. Moreover, our study revealed that a decreased PAH expression might be directly proportional to the enhanced levels of both phenylalanine and tyrosine in PCa and its metastatic progression.

Ketone bodies (3-HB, acetoacetate, and acetone) are alternative mitochondrial energy reserves that can be converted into acetyl-CoA and reutilized as energy substrates [44, 45]. Several studies have stated that ketone body metabolism is critical for tumor biomass expansion [46], as well as the fact that ketogenesis plays an important role in PCa progression [47]. A study by Martinez et al. [48] generated a series of cells and fibroblasts overexpressing the enzyme initiating ketone body production and suggested that the production and reutilization of ketone bodies promote tumor progression and metastasis. A prominent ketone body, 3-HB, has been proved to be specifically associated with metastatic prostate cancer [49]. The current study displayed a significant reduction in the 3-HB level in PCa cases relative to BPH, but a significant elevation was duly observed in PCaB subjects when compared to PCa cases. Collectively, the characteristic changes in the 3-HB level might be applicable for the potential detection of the PCa progressive stages. However, it was observed that the acetoacetate and acetone concentrations were significantly decreased in PCaB as compared to BPH patients, which may be due to the uptake of ketone bodies from the tumor tissues in PCa proliferation and development.

Formate is a member of short-chain fatty acids and can be remodeled back for re-synthesizing serine *via* a one-carbon metabolism pathway [50]. Additionally, formate is an extremely vital component of one-carbon metabolism for tumor proliferation and growth [51, 52]. A study by Meiser et al. [53], revealed that since mice with oxidative cancers have higher circulating formate levels than the healthy controls, thereby proposed that elevated formate overflow is a hallmark of oxidative cancers. Our study results also observed an increased formate level in the PCaB patients' serum when compared to BPH, which was also confirmed by our previous study that depicted a higher formate level in the metastatic PCa tissue when compared to other PCa stages [15]. Thus, our results might imply that upregulated formate metabolism mainly occurred in the tumor tissue and serum samples of the tumor in its metastatic stage.

LDL/VLDL is an essential member of lipoproteins, fundamental to the reverse cholesterol transport pathway and lipid homeostasis [54, 55]. Recent studies have demonstrated that lipoprotein might be considered as a risk factor for PCa [56, 57]. However, several literary insights display contradictory findings regarding the association between lipid metabolism and PCa progression and development. A study by Bull et al. [58], suggested there is a weaker association between higher lipoprotein levels and aggressive PCa risk, which was also substantiated by a meta-analysis of 14 large prospective studies that indicated an absence of association between the blood lipoprotein levels and overall PCa or high-grade PCa risk cases [59]. Our results revealed that PCaB cases had lower circulating LDL/VLDL levels than PCa, thus, suggesting that PCaB may have a disruptive lipid metabolism relative to PCa. Moreover, the lipid metabolism inconsistency along with several metabolic aberrations in PCa development could contribute to the initiation of several more studies focused on the extremely significant role of lipid metabolism in PCa progression.

Conclusion

To conclude, ¹H NMR-based metabolomics analysis of serum metabolic profiles in BPH, PCa, and PCaB patients can be successfully utilized to establish the significant metabolic signatures related to PCa progression and its metastases. The identified metabolic panels might provide crucial insights into the diagnosis and classification of PCa. However, there are several potential limitations in this study: (1) Although the potential metabolic biomarkers were identified in serum samples, this finding still needs validation by using other biofluids and matching tissues. (2) As our study was a prospective, single-center study with smaller sample size, large clinical multi-center cohorts would be required for extracting the clinical potential of our findings. (3) Since ¹H NMR-based metabolomics analysis is generally limited to small metabolites at higher concentrations, multi-omics analysis integrating several useful genes, proteins, and metabolome are recommended for elucidating potential mechanisms underlying the PCa progression and its metastases.

Abbreviations

PCa: prostate cancer; PCaB: PCa patients with bone metastasis; BPH: benign prostatic hyperplasia; NMR: nuclear magnetic resonance; BMI: body mass index; PSA: prostate-specific antigen; 3-HB, 3-hydroxybutyrate.

Declarations

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Authors' contributions

X.Z., B.X. and H.Z. contributed equally to this work. H.Z., B.D. and H.G. contributed to experimental design. B.X., Y.Z. and X.S. contributed to clinical diagnosis and sample collection. X.Z., J.N. and B.L. contributed to NMR metabolomic analysis. X.Z., B.X. and H.Z. contributed to data analysis. B.D. and H.G. contributed to result interpretation and writing. All authors have read and approved the final manuscript.

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Availability of data and materials

The data used and/or analyzed are available from the corresponding author on a reasonable request.

Ethics approval and consent to participate

All procedures carried out in this study were in accordance with the ethical standards of the institutional and national responsible committee on human experimentation and the Helsinki Declaration of 1964 and its later amendments or equivalents. This study was approved by the Ethics Committee of Shanghai Jiao Tong University School of Medicine (IRB number: Renji [2013] 26). Informed consent was obtained from all individual patients included in the study.

Consent for publication

Not applicable.

Competing Interests

The authors have declared that no competing interest exists.

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Figures

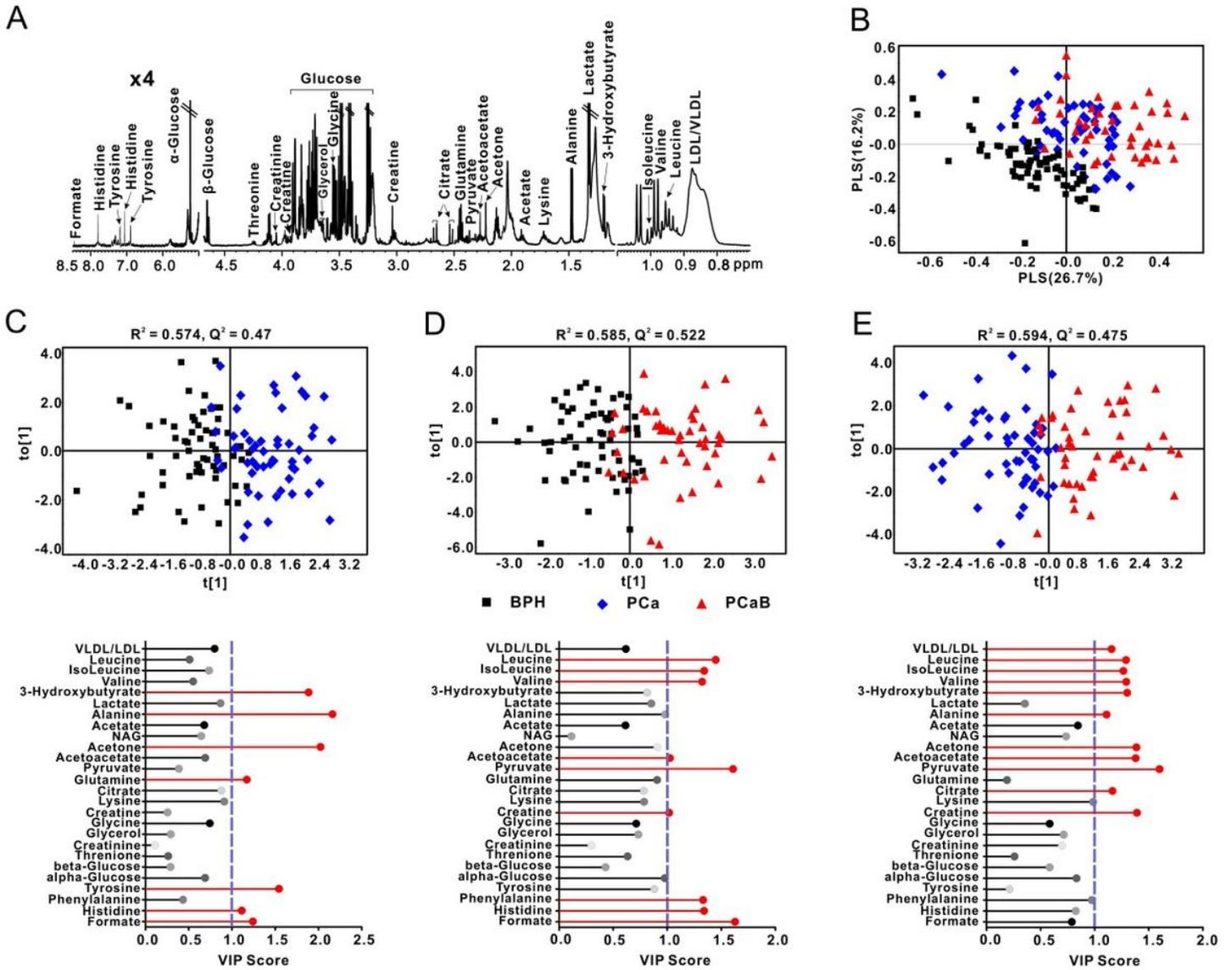


Figure 1

^1H NMR-based metabolomics analysis. (A) Representative 600 MHz ^1H NMR spectra obtained from BPH serum samples; (B) PLS-DA-based metabolic pattern discrimination among BPH, PCa and PCaB; OPLS-DA model-based classification between (C) BPH and PCa, (D) PCaB and BPH, (E) PCaB and PCaB as well as corresponding VIP scores.

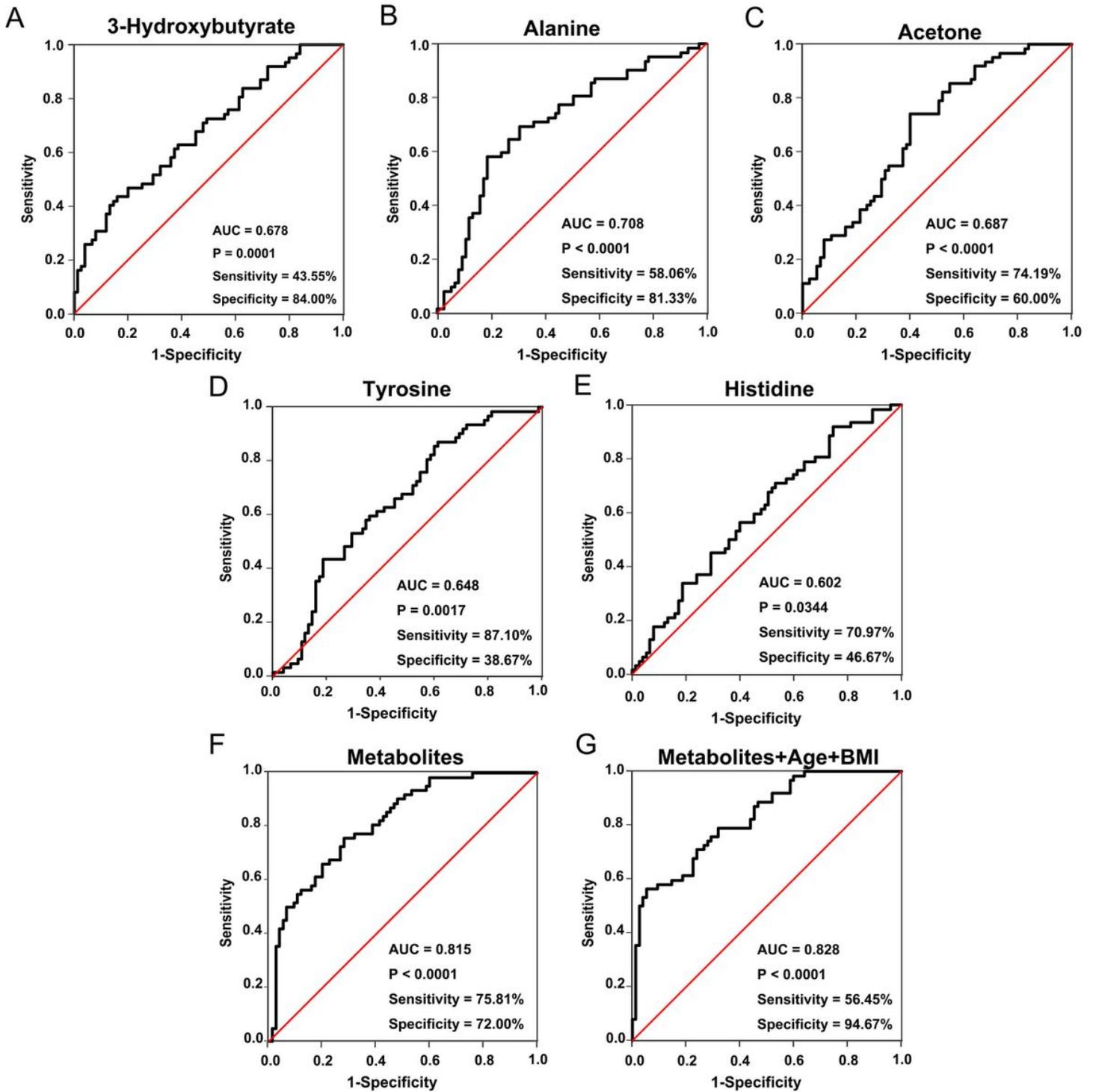


Figure 2

ROC curve analysis of the selected metabolites for discriminating PCa from BPH. The assessment of the diagnostic performance of (A) 3-HB; (B) alanine; (C) acetone; (D) tyrosine; (E) histidine; (F) a combination of five metabolites; (G) a merger of five metabolites with age and BMI.

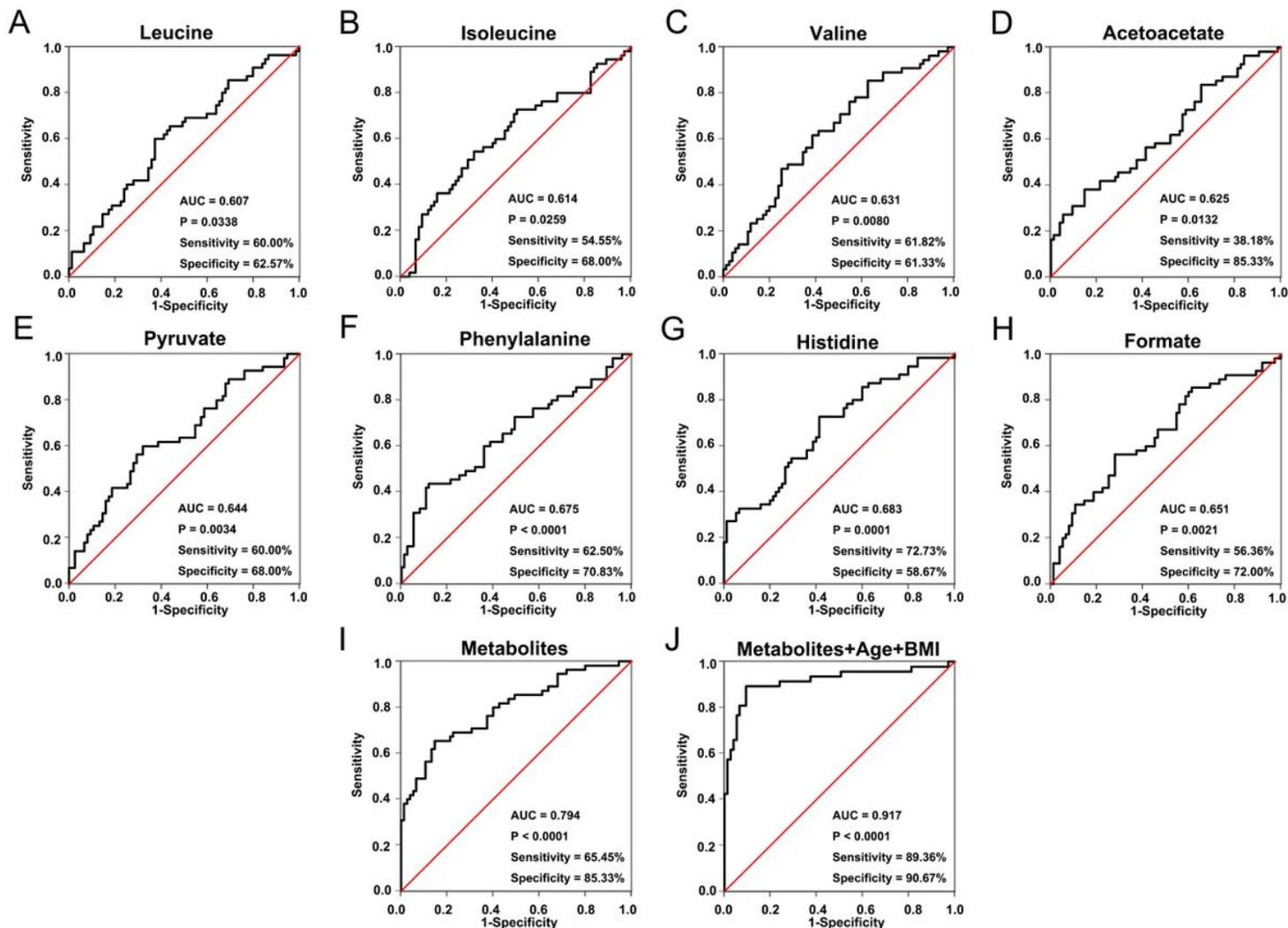


Figure 3

ROC curve analysis of the selected metabolites for differentiating PCaB from BPH. The assessment of the diagnostic performance of (A) leucine; (B) isoleucine; (C) valine; (D) acetoacetate; (E) pyruvate; (F) phenylalanine; (G) histidine; (H) formate; (I) a combination of eight metabolites; (J) a merger of eight metabolites with age and BMI.

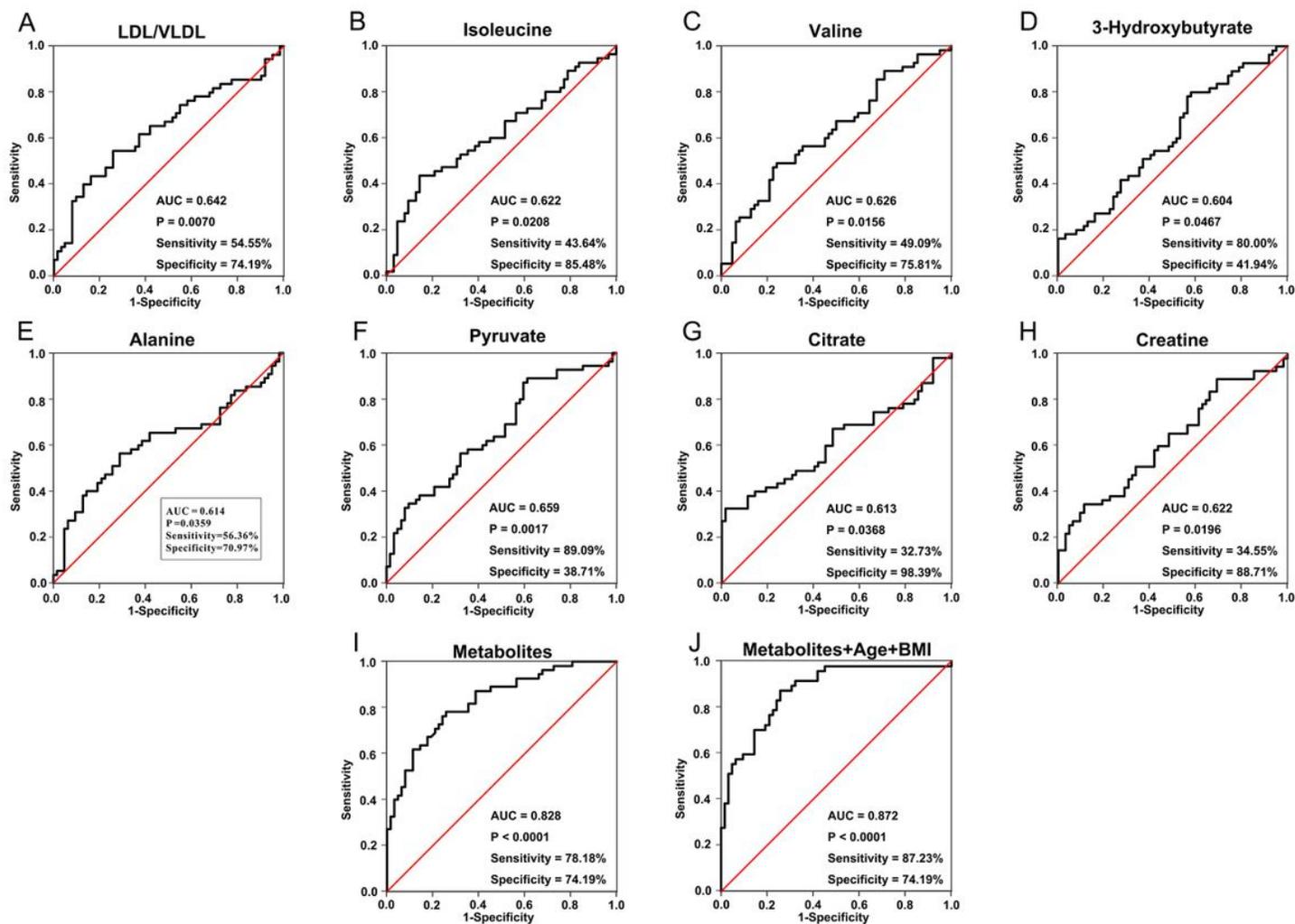


Figure 4

ROC curve analysis of the selected metabolites for discriminating PCaB from PCa. The assessment of the diagnostic performance of (A) LDL/VLDL; (B) isoleucine; (C) valine; (D) 3-HB; (E) alanine; (F) pyruvate; (G) citrate; (H) creatine; (I) a combination of eight metabolites; (J) a merger of eight metabolites with age and BMI.

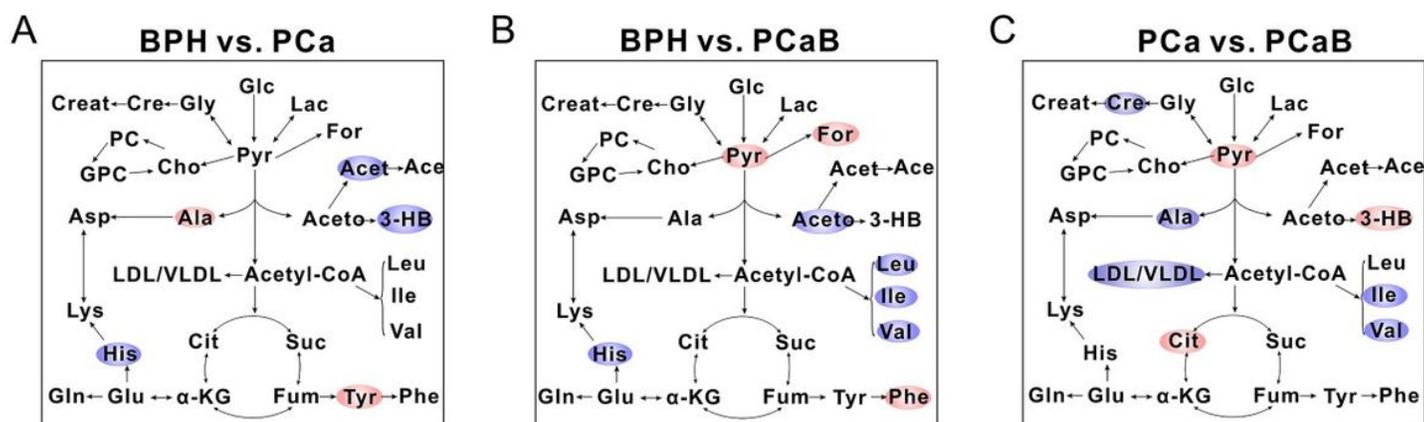


Figure 5

Metabolic pathway analysis. The metabolic pathway was drawn according to the KEGG database and SMPDB database. The red or blue shadings represent the significantly increased or decreased levels of metabolites from BPH to PCa, BPH to PCaB, and PCa to PCaB. Metabolite abbreviation: 3-HB, 3-hydroxybutyrate; Ace, acetate; Aceto, Acetoacetate; Acet, acetone; Ala, alanine; Asp, aspartate; Cho, choline; Cre, creatine; Creat, creatinine; Cit, citrate; For, formate; Fum, fumarate; Glc, glucose; Glu, glutamate; Gln, glutamine; Gly, glycine; GPC, sn-glycero-3-phosphocholine; His, histidine; Ile, isoleucine; Lac, lactate; Leu, leucine; Lys, lysine; PC, phosphocholine; Phe, phenylalanine; Pyr, pyruvate; Suc, succinate; Tyr, tyrosine; Val, valine.