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Integrative Metabolomic Analysis of Serum and Selected Serum Exosomal microRNA in Metastatic Castration-Resistant Prostate Cancer

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Abstract: Metastatic castration-resistant prostate cancer (mCRPC) remains a lethal disease due to the absence of effective therapies. A more comprehensive understanding of molecular events, encompassing the dysregulation of microRNAs (miRs) and metabolic reprogramming, holds the potential to unveil precise mechanisms underlying mCRPC. This study aims to assess the expression of selected serum exosomal miRs (miR-15a, miR-16, miR-19a-3p, miR-21, and miR-141a-3p) alongside serum metabolomic profiling and their correlation in patients with mCRPC and benign prostate hyperplasia (BPH). Blood serum samples from mCRPC patients (n=51) and BPH patients (n=48) underwent metabolome analysis through ^{1}H-NMR spectroscopy. The expression levels of serum exosomal miRs in mCRPC and BPH patients were evaluated using quantitative real-time polymerase chain reaction (qRT–PCR). The ^{1}H-NMR metabolomics analysis revealed significant alterations in lactate, acetate, citrate, 3-hydroxybutyrate, and branched-chain amino acids (BCAAs, including valine, leucine, and isoleucine) in mCRPC patients compared to BPH patients. MiR-15a, miR-16, miR-19a-3p, and miR-21 exhibited a down-regulation of more than twofold in the mCRPC group. Significant correlations were predominantly observed between lactate, citrate, acetate, and miR-15a, miR-16, miR-19a-3p, and miR-21. The importance of integrating metabolome analysis of serum with selected serum exosomal miRs in mCRPC patients has been confirmed, suggesting their potential utility as clinical diagnostic markers.

Keywords: metastatic castration-resistant prostate cancer; microRNA; ^{1}H-NMR metabolomics

1. Introduction

Prostate cancer stands as the second most prevalent cancer globally, exhibiting the highest incidence rates in North and South America, Europe, Australia, and the Caribbean [1]. Despite being commonplace, the existing diagnostic methods for prostate cancer, incorporating digital rectal examination (DRE), serum prostate-specific antigen (PSA) levels, and transrectal ultrasound (TRUS)-guided biopsy, remain unsatisfactory [2]. Radiological imaging techniques, such as multiparametric magnetic resonance imaging (mpMRI), and nuclear medicine methods, notably skeletal scintigraphy and positron emission tomography (PET) utilizing ^{68}Ga prostate-specific membrane antigen (PSMA) ligands, play pivotal roles in diagnosing advanced stages of prostate cancer [3]. There is also an increasing focus on MRI-guided prostate biopsy [4].

Metastatic castration-resistant prostate cancer represents the terminal stage of prostate cancer, marked by the failure of antiandrogen therapy and the metastasis of cancer to distant organs, such as the skeleton [5]. This condition is inevitably linked with a grim prognosis for the patient [6]. Recent advances in the systemic treatment of mCRPC encompass chemotherapy with Docetaxel and Cabazitaxel, immunotherapy, and nuclear medicine therapies such as ^{223}RaCl2 (Xofigo), ^{177}Lu PSMA, and ^{225}Ac PSMA [7].

Cancer cells have long been recognized for their extensive metabolic alterations, and the reprogramming of cellular energy metabolism represents an emerging hallmark of cancer, exemplified by the Warburg effect [8]. In normal prostate epithelial cells, aerobic conditions typically lead to glycolysis instead of oxidative metabolism. These cells utilize glucose and aspartate to synthesize and secrete citrate into the lumen, a crucial component of semen [9,10]. This metabolic profile is a consequence of zinc accumulation in prostate cells, inhibiting the tricarboxylic acid (TCA) cycle enzyme, m-aconitase [11]. The progression of prostate cancer involves a decrease in zinc concentrations, leading to the reactivation of m-aconitase and the initiation of citrate oxidation through the TCA cycle [12]. In contrast to many tissues in the human body, the metabolism of primary prostate cancer cells is characterized by high lipogenesis, lower glycolysis, and dependence on oxidative phosphorylation [13]. Consequently, citrate can be exported to the cytoplasm and converted back into acetyl-CoA for *de novo* synthesis of fatty acids and cholesterol [14]. Characteristic of advanced metastatic stages, prostate cancer cells become highly glycolytic, inhibiting mitochondrial respiration and exhibiting the Warburg effect. Additionally, deregulated anabolism/catabolism of fatty acids and amino acids have been identified as metabolic regulators supporting cancer cell growth [15].

MicroRNAs (miRs) are small non-coding RNA molecules that regulate gene expression and can be dysregulated in various types of diseases, including prostate cancer [16]. Analyzing their expression in prostatic tissues or biological fluids, such as blood, can help identify specific patterns associated with the disease. Additionally, these panels provide prognostic markers, with specific miRs linked to tumor aggressiveness and prognosis, offering insights into disease spread and treatment response. Furthermore, changes in miRs expression during treatment can be monitored for insights into therapy effectiveness and potential therapeutic targets in manipulating miRs for influencing prostate cancer growth or metastasis. The amalgamation of miRs and PSA data exhibited heightened sensitivity and specificity for prostate cancer diagnosis compared to utilizing PSA alone. This integrated diagnostic strategy outperformed the use of PSA in isolation, where sensitivity and specificity are increased [17].

The analysis of a miRs panel in prostate cancer offers additional insights into the molecular aspects of the disease. The presence of malignancy in an organism is evident through altered metabolism, and it is known that many miRs play a critical role in regulating cellular metabolism under normal and pathological conditions. Therefore, the present study aimed to determine the changes in the expression of selected exosomal miRs (miR-15a, miR-16, miR-19a-3p, miR-21, and miR-141a-3p) along with the serum metabolomic profiles between mCRPC and BPH patients, for use as potential non-invasive candidate biomarkers for accurate prostate cancer diagnosis.

2. Results

2.1. Relative changes in blood metabolites

Statistically significant differences were observed in the relative concentrations of blood serum metabolites associated with energy metabolism, including lactate, acetate, citrate, 3-hydroxybutyrate, and essential amino acids BCAAs between mCRPC patients and the BPH group (Table 1). The most notable alterations were evident for citrate (relative difference of 38%) and acetate (relative difference of 24%). Valine and leucine, sharing numerous biochemical pathways, exhibited very similar changes, both decreasing by about 18% in the mCRPC group compared to the BPH group. The alteration in blood serum levels of their third co-metabolite isoleucine was weaker, with a marginally significant decrease of 11% in mCRPC patients against BPH patients. Figure 1 presents boxplots illustrating the significantly altered metabolites in the serum of mCRPC patients and the BPH group.

Table 1. Statistical comparison of relative concentration of blood serum metabolites in mCRPC patients and BPH group. P value derived from Mann-Whitney-U test, percentual change derived from medians.

Metabolite	**Percentage change** mCRPC *vs* BPH group	**P-value**
Lactate	22%	0.04
Citrate	38%	0.003
Valine	-18%	0.02
Leucine	-18%	0.002
Isoleucine	-11%	0.06
3-hydroxybutyrate	-19%	0.03
Acetate	24%	0.00002

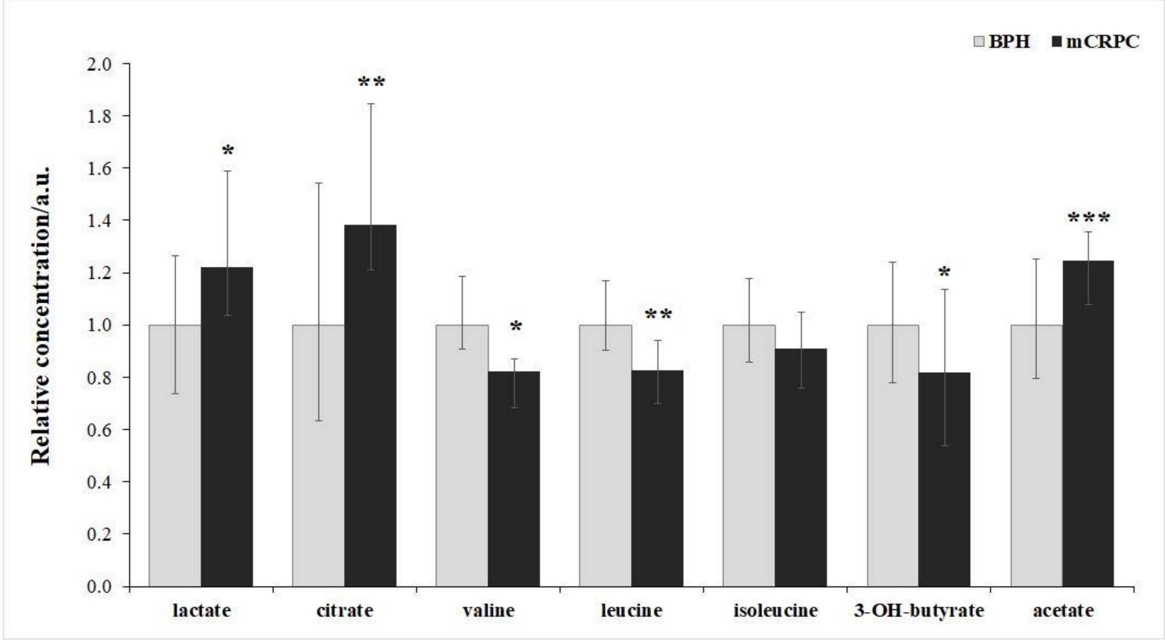

Figure 1. Relative concentrations of metabolites in blood serum in mCRPC patients and BPH patients. Data are presented as medians normalised to median of BPH set to 1, data variance is shown by Q1 and Q3. *p<0.05; **p=0.05-0.01; *** p<0.001.

Comparison of median serum concentrations of metabolites such as alanine, glucose, pyruvate, phenylalanine, tyrosine, glutamine, threonine, lysine, branched-chain keto acids (BCKAs), creatine, creatinine, proline, histidine, succinate, and tryptophan did not reveal significant differences.

2.2. *Exosomal miRNA expression in the serum samples of mCRPC*

Table 2 presents the fold regulation levels of selected exosomal miRs, namely miR-15a, miR-16, miR-19a-3p, miR-21, and miR-141a-3p. The results demonstrate that the expression levels of serum exosomal miR-15a, miR-16, miR-19a-3p, and miR-21 were significantly down-regulated in mCRPC patients compared with BPH patients ($p < 0.05$). Among them, the highest three-fold decrease was observed in the expression level of miR-16 in mCRPC compared to BPH patients. However, the expression level of miR-141a-3p did not exhibit a significant variation within the mCRPC patient group compared to the BPH group ($p > 0.05$).

Table 2. Fold regulation levels of exosomal miRs (miR-15a, miR-16, miR-19a-3p, miR-21, and miR-141a-3p) between mCRPC and BPH patients.

miR	Fold regulation +/-	P-value
miR-15a	-2.00	0.006
miR-16	-3.24	4.33×10^{-6}
miR-19a-3p	-2.78	1.11×10^{-5}
miR-21	-2.39	0.003
miR-141a-3p	+1.23	0.41

+, up regulation (comparing to BPH group); -, down regulation (comparing to BPH group).

Table 3 shows the Pearson correlation coefficient (PCC) for selected serum exosomal miRs (miR-15a, miR-16, miR-19a-3p, miR-21, and miR-141a-3p). The analysis revealed a large number of significant correlations at the $p < 0.05$ level. We found a significant positive correlation between miR-15a, miR-16, miR-19a-3p, and miR-21 levels in patients with mCRPC.

Table 3. Correlation analysis between the serum exosomal miRs.

		miR-15a	miR-16	miR-19a-3p	miR-21	miR-141a-3p
miR-15a	Pearson Corr.	1.00	0.73	0.80	0.81	0.13
	P-value	-	**5.94×10^{-9}**	**6.22×10^{-12}**	**3.99×10^{-9}**	0.38
miR-16	Pearson Corr.	0.73	1.00	0.79	0.38	0.01
	P-value	**5.94×10^{-9}**	-	**9.88×10^{-12}**	**0.01**	0.95
miR-19a-3p	Pearson Corr.	0.80	0.79	1.00	0.72	0.22
	P-value	**6.22×10^{-12}**	**9.88×10^{-12}**	-	**7.44×10^{-9}**	0.13
miR-21	Pearson Corr.	0.81	0.38	0.72	1.00	0.29
	P-value	**3.99×10^{-12}**	**0.01**	**7.44×10^{-9}**	-	**0.04**
miR-141a-3p	Pearson Corr.	0.13	0.01	0.22	0.29	1.00
	P-value	0.38	0.95	0.13	**0.04**	-

2.3. Correlations between miRs and metabolites levels

We investigated the correlation between the expression profile of selected serum exosomal miRs and metabolites in mCRPC patients (Table 4). Lactate showed a significant correlation with miR-15a, miR-16, and miR-19a-3p ($p < 0.05$), respectively. The results demonstrated that citrate and lysine were negatively correlated with serum miR-15a, miR-19a-3p, and miR-21 ($p < 0.05$), respectively. A significant correlation of 3-hydroxybutyrate with miR-15a and miR-21 ($p < 0.05$) was also observed. Furthermore, acetate was positively correlated with miR-19a-3p, miR-21, and miR-141a-3p ($p < 0.05$), respectively. Additionally, glutamine levels were positively correlated with miR-15a and miR-16 ($p < 0.05$). BCAAs did not show a significant correlation with selected exosomal miRs.

Table 4. Correlation analysis of serum exosomal miRs and metabolites.

		miR-15a	miR-16	miR-19a-3p	miR-21	miR-141a-3p
Lactate	Pearson Corr.	0.35	0.31	0.33	0.27	0.01
	P-value	**0.01**	**0.03**	**0.03**	0.06	0.92
Citrate	Pearson Corr.	-0.47	-0.24	-0.35	-0.47	-0.24
	P-value	**9.23×10^{-4}**	0.11	**0.02**	**7.61×10^{-4}**	0.09
Valine	Pearson Corr.	0.05	0.05	0.15	0.14	-0.09
	P-value	0.73	0.071	0.33	0.35	0.55
Leucine	Pearson Corr.	-0.04	-0.02	0.05	0.08	-0.09
	P-value	0.78	0.89	0.72	0.55	0.55
Isoleucine	Pearson Corr.	-0.23	-0.09	-0.07	-0.12	-0.08
	P-value	0.11	0.52	0.62	0.44	0.59
3-hydroxybutyrate	Pearson Corr.	0.28	0.09	0.17	0.33	0.05
	P-value	**0.05**	0.51	0.24	**0.03**	0.72
Acetate	Pearson Corr.	0.16	0.01	0.30	0.46	0.42
	P-value	0.29	0.96	**0.04**	**0.001**	**0.003**

Glutamine	Pearson Corr.	0.28	0.28	0.21	0.19	-0.14
	P-value	**0.05**	**0.05**	0.16	0.21	0.36
Lysine	Pearson Corr.	-0.44	-0.22	-0.29	-0.39	-0.04
	P-value	**0.002**	0.14	**0.05**	**0.006**	0.81

3. Discussion

As of the present date, mCRPC remains an incurable disease, and ongoing research is focused on novel therapeutic agents aimed at maximizing the survival and quality of life for these patients. Consequently, it is crucial to identify critical events in the progression of prostate malignancy through the integration of metabolomic and other omics techniques. NMR spectroscopy has proven to be a suitable methodology for evaluating the metabolic profile, effectively distinguishing between mCRPC patients and those with benign prostatic hyperplasia. Our findings indicate that certain metabolites, such as lactate, acetate, and citrate, are present in higher concentrations in the blood serum of mCRPC patients, while others, including 3-hydroxybutyrate and branched-chain amino acids (BCAAs), are decreased. Furthermore, our investigation reveals that serum exosomal miRs, specifically miR-15a, miR-16, miR-19a-3p, and miR-21, exhibit significant potential as diagnostic markers for mCRPC, especially when combined with lactate, citrate, and acetate.

Normal prostate epithelial cells exhibit an inefficient energy metabolism characterized by the inactivation of the tricarboxylic acid (TCA) cycle, resulting in high citrate production [9]. The neoplastic transformation in prostate cells coincides with the restoration of the TCA cycle and an increased generation of ATP from glucose oxidation [18]. In advanced stages of disease, prostate cancer cells display overexpression of glucose transporters and key glycolytic enzymes [9], leading to increased glucose consumption and lactate release [19]. The elevated lactate produced in hypoxic tumor areas is secreted into the extracellular environment [20], resulting in a high concentration of serum lactate (approximately 40 mM) observed in the serum of various cancer patients compared to the lactate concentration in healthy tissue and serum (1.5 to 3 mM) [21]. We observed elevated serum lactate levels in mCRPC patients compared to the BPH group, which aligns with previously reported findings in prostate cancer tissues [22–24]. We propose that cells in the tumor microenvironment produce lactate, serving as the source of increased blood serum lactate in patients with prostate cancer, and it may contribute to tumor cell invasion, metastasis, and immunosuppression [21].

Previously, it was demonstrated that intracellular citrate concentrations in the normal prostate peripheral zone exceed those in other soft tissues (10,000-15,000 nmols/gram vs. 250-450 nmols/gram). Similarly, citrate concentrations in prostatic ductal fluids are higher than in blood plasma (40-150 mM vs. approximately 0.2 mM [25,26]. Numerous studies employing animal models, cell lines, and tissue extracts have consistently shown reduced citrate levels in prostatic tissue of individuals with prostate adenocarcinoma compared to those with normal prostate peripheral zone and BPH [27–30]. The exact cause of this reduction, whether due to altered citrate production (e.g., low Zn^{2+} levels leading to increased m-aconitase activity) and/or changes in citrate transportation, remains unclear [31].

In our study, we observed significantly higher blood serum citrate levels in mCRPC patients compared to BPH patients. We hypothesize that prostate cancer cells take up this serum citrate through specific transporters expressed in the plasma membrane [32,33], and intracellularly utilize it to support prostate cancer metabolism, proliferation, fatty acid synthesis, and protein acetylation [34]. Similar findings were reported by Buszewska-Forajta et al. (2022)[35], who observed higher serum concentrations of citrate in the prostate cancer group compared to the BPH group, with no significant changes in citrate concentration based on the clinical stage of the tumor. In contrast, Kumar et al. (2016) reported a significant decrease in citrate levels in filtered serum obtained from prostate cancer patients compared to BPH patients[30]. Huang et al. (2017) found a lower risk of T4 prostate cancer in men with higher serum citrate and fumarate concentrations compared to controls, as determined by ultrahigh performance liquid chromatography/mass spectroscopy (LC-MS) and gas chromatography/mass spectroscopy (GC-MS)[36].

Prostate cancer bone metastases represent the final stage of metastasis, associated with aggressive tumor growth and the development of primarily osteoblastic bone disease [37]. Typically, bone metastases in prostate cancer are osteoblastic, involving the deposition of newly formed bone, but they can also manifest as osteolytic, characterized by the destruction of normal bone, or mixed [38]. During bone formation, osteoblasts synthesize citrate, which becomes incorporated into the new bone. Conversely, during bone resorption, citrate is released from the bone into the blood [27]. Hence, we suggest that this constitutes another significant source of citrate in mCRPC patients, contributing to the elevated citrate levels observed in the serum.

Moreover, the process of oncogenesis is linked to alterations in the uptake and metabolism of amino acids. Amino acids serve as the building blocks of proteins and also act as intermediate metabolites fueling various biosynthetic pathways [39]. Branched-chain amino acids (BCAAs), including leucine, isoleucine, and valine, are preferentially taken up by tumors. Being essential amino acids, their plasma levels are contingent on dietary intake and whole-body protein turnover [40,41]. Numerous studies have identified associations between circulating BCAAs levels and various human cancer types [42–44]. The study by Giskeødegård et al. (2015) demonstrated increased serum levels of BCAAs in prostate cancer patients compared to the BPH group [45]. Our findings, indicating significantly lower levels of serum BCAAs in the mCRPC group compared to the BPH group, align with the results of a few studies [46,47]. Similarly, Zhang et al. (2022) also observed significantly decreased serum BCAAs levels in prostate cancer patients with bone metastasis compared to prostate cancer or BPH patients, suggesting that downregulated BCAAs might be closely related to bone metastasis in prostate cancer progression [48]. We hypothesize that the decrease in serum BCAAs levels could result from higher BCAAs uptake through the L-type amino acid transporter LAT1 (SLC7A5), which is highly expressed in prostate tumor tissues [49], and subsequent catabolism of BCAAs for energy production by prostate cancer cells. Furthermore, BCAAs metabolism might be affected by genetic mutations, the tumor microenvironment, food intake, and the individual's health status [50].

Interestingly, we did not observe any differences in the corresponding branched-chain keto acids (BCKAs), such as ketoleucine, ketoisoleucine, and ketovaline, as well as glucose serum levels between the mCRPC and BPH groups. Additionally, we did not notice changes in alanine and glutamine levels, the main amino acids responsible for ammonia detoxification in extrahepatic tissues. Similarly, no differences were detected for other evaluated essential amino acids – phenylalanine, histidine, threonine, and tryptophan. These observations, coupled with the above-discussed facts, support the hypothesis of the selective utilization of branched-chain amino acids (BCAAs) by prostate cancer cells as an energy substrate rather than their accelerated usage in proteosynthesis during the formation of new cells.

Another metabolite that we found altered in the serum of mCRPC patients relative to BPH patients is 3-hydroxybutyrate, a representative ketone body. Ketone body metabolism is dysregulated in various types of cancer, and most tumor cells are unable to use ketone bodies for energy due to abnormalities in mitochondrial structure or function [51,52]. It has been reported that tumor cells can use ketone bodies as precursors for lipid synthesis rather than as energy substrates [53]. The study by Rodrigues et al. (2017) showed that the administration of 3-hydroxybutyrate may accelerate tumor growth [54]. However, there are also many studies presenting the anti-cancer effect of a ketogenic diet, a condition linked with increased ketone bodies levels [55].

Interestingly, Saraon et al. (2013) identified the ketogenic pathway as a novel bioenergetic pathway potentially involved in the progression of prostate cancer from low-grade to high-grade disease, followed by androgen independence[56]. Moreover, increased expression of both ketogenic and ketolytic enzymes (ACAT1, BDH1, HMGCL, and OXCT1) was reported with prostate cancer progression, gradually increasing with tumor grade [56,57]. Huang et al. (2017) showed that serum 3-hydroxybutyrate was associated with an increased risk of fatal prostate cancer in men diagnosed with metastatic disease[36].

Acetate, a short-chain fatty acid, serves as a substrate for the synthesis of acetyl coenzyme A (acetyl-CoA), primarily utilized in the *de novo* synthesis of fatty acids in prostate cancer cells, despite the abundance of circulating fatty acids. This metabolic phenotype is associated with prostate cancer progression and androgen independence [58,59]. Our findings align with previous studies reporting elevated serum acetate levels in various cancer types, including squamous oral carcinoma [60], lung cancer [61], and colon cancer [62]. We surmise that higher serum acetate levels may originate from the diet, with a significant portion generated by the metabolism of intestinal contents by the gut microbiome and/or from endogenous sources [63]. This pool of acetate serves as an alternative carbon source for fatty acid synthesis in cancer cells, potentially supporting the growth or metastasis of prostate tumors.

Numerous miRs have been reported to play significant roles in physiological and pathological processes, including cancer. Exosomes containing miRs, secreted by cancer cells, can be internalized by neighboring or distant recipient cells, facilitating tumor development [16]. The miRs from the miR-15/16 cluster are acknowledged as tumor suppressors, with documented reductions in various cancers, such as chronic lymphocytic lymphoma [64], pituitary adenomas [65], and prostate cancer [66]. Consistent with prior research, we observed a significant downregulation of serum exosomal miR-15a and miR-16 (fold regulation of -2.00 and -3.24, respectively) in mCRPC patients. Jin et al. (2018) proposed that miR-15a/16 inhibit components of the TGF-β signaling pathways in the LNCaP cell line, implying a potential association with prostate cancer progression and metastasis [67]. Specifically, miR-15a and miR-16-1 exert their effects by targeting multiple oncogenes, including BCL2, MCL1, CCND1, and WNT3A. Moreover, the reduced expression of miR-15 and miR-16 in cancer-associated fibroblasts significantly enhances tumor growth and progression [68]. Additionally, miR-15a and miR-16-1 impact fatty acid metabolism, primarily downregulating fatty acid synthase expression in mammary cells [69]. Thus, we speculate that the modulation of *de novo* fatty acid synthesis by miR-15a and miR-16, in conjunction with elevated serum levels of acetate and citrate, could potentially increase lipid synthesis in prostate cancer cells, and promotes tumor invasiveness and metastatic ability.

MiRNA-19a-3p has been identified as a suppressor of invasion and metastasis in prostate cancer by inhibiting SOX4 (SRY-related high-mobility group box 4), a factor involved in the development, differentiation of cells and organs, as well as the initiation and progression of cancer [70]. In our study, a significant downregulation of serum exosomal miR-19a-3p expression was observed in mCRPC patients. Previous research demonstrated that overexpression of miR-19a-3p led to the downregulation of proteins associated with invasion and metastasis in prostate cancer DU145 cells [71]. They reported a significant reduction in miR-19a-3p expression in 121 archived prostate cancer tissues, including 76 non-bone metastatic prostate cancer tissues and 45 bone metastatic prostate cancer tissues. Furthermore, they found that upregulation of miR-19a-3p repressed osteolytic bone lesions. We hypothesize that the downregulation of miR-19a-3p could potentially promote osteolysis and the release of citrate from bone into the bloodstream.

MiR-21 exhibits a dual nature, acting both as an oncogene and a tumor-suppressor [72]. Primarily, miR-21 downregulates PTEN expression, fostering the activation of the PI3K/Akt signaling pathway, thereby propelling cancer progression. Its overexpression impedes apoptosis and plays a crucial role in initiating pro-survival autophagy [73]. MiR-21 significantly contributes to metabolic reprogramming by inducing glycolysis and lactate production, consequently enhancing tumor advancement [74,75]. Moreover, it has been shown that miR-21 plays an essential role in lipid synthesis, fatty acid oxidation, and lipoprotein formation [76,77]. To our knowledge, this is likely the first study reporting the correlation of miR-21 levels with citrate and acetate levels in the serum of mCRPC patients, potentially affecting lipid homeostasis in prostate cancer cells. Moreover, miR-21 stimulates epithelial-mesenchymal transition (EMT) and upregulates the expression of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9), promoting tumor metastasis. MiR-21 is a target of anti-cancer agents like curcumin and curcumol, and its downregulation blocks tumor progression. However, upregulation of miR-21 can result in cancer resistance to chemotherapy and radiotherapy [78]. Variations exist in the findings of these studies, and further investigations are required to determine the suitability of miR-21 as a reliable marker for prostate cancer. In line with our study, Damodaran et al. (2021) revealed significant down-regulation of miR-21 in prostate cancer patients[79]. Conversely, Kim et al. (2021) reported no significant alterations in miR-21 and miR-141 levels in extracellular vesicles of prostate cancer patients compared to the control group [66]. Our investigation showed decreased expression of serum exosomal miR-21 levels in mCRPC patients. Jokovic et al. (2018) compared plasma and exosomal levels of miRs, indicating elevated exosomal miR-21 levels in prostate cancer patients with increased serum PSA values and those with aggressive prostate cancer, while plasma samples did not yield significant results [80]. Consequently, their observations suggest potential prognostic significance for exosomal miR-21 expression levels in prostate cancer.

MiR-141 exhibits distinct expression patterns across various malignancies, influencing tumor behaviors such as EMT, proliferation, migration, metastasis, invasion, and drug resistance, contingent upon cancer type. The study by Xiao et al. (2012) revealed that small heterodimer protein (Shp) is a direct target of miR-141, leading to Shp downregulation [81]. Acting as a corepressor and metabolic regulator, the reduced Shp, induced by miR-141, transcriptionally regulates androgen receptor genes in prostate cells. This underscores miR-141's significance in influencing the androgen receptor signaling pathway, contributing to prostate cancer progression. Sharma and Baruch (2019) identified miR-141 as a promising marker for metastatic prostate cancer [82]. Akalin et al.'s (2022) study suggests potential diagnostic value for miR-141-3p in identifying aggressive prostate cancer [83]. In a meta-analysis by Ye et al. (2020), encompassing seven studies and 414 prostate cancer patients, miR-141 demonstrated diagnostic sensitivity and specificity of 0.70 (95% CI 0.64-0.75) and 0.73 (95% CI 0.64-0.80), respectively[28]. Conversely, other studies, consistent with our findings, reported no significant alteration of miR-141 in prostate cancer tissues or blood extracellular vesicles [39,66].

4. Materials and Methods

4.1. Study population

This study received approval from the Ethical Board of Jessenius Faculty of Medicine, Comenius University, and was conducted in accordance with the Declaration of Helsinki. Prior to obtaining blood samples, informed written consent was obtained from all participants. The study included 51 patients with mCRPC and 48 patients diagnosed with BPH. Three mCRPC patients were excluded from the study due to low measured PSA levels. Men diagnosed with BPH served as the disease control group.

Examinations for mCRPC patients were conducted at the Clinic of Nuclear Medicine at the University Hospital Martin and Jessenius Faculty of Medicine, Comenius University. Skeletal scintigraphy was performed, revealing positive findings indicative of multiple skeletal metastases. Additionally, clinical staging and tumor grading, along with the assessment of PSA levels (Beckman Coulter Access® Hybritech® assay, Beckman Coulter, Fullerton, CA, USA), were carried out. Venous blood samples were collected in collaboration with the Clinic of Nuclear Medicine and the Clinic of Urology at the University Hospital Martin, Slovakia. Table 5 provides information on the age, PSA levels within both studied groups, as well as the staging characterization and histopathological grading of mCRPC patients.

Table 5. Description of the cohort under study.

	mCRPC patients	**BPH patients**	**P-value**
Number	51	48	> 0.05
Age (years, mean ± SD)	73.5 ± 7.65	67.5 ± 6.54	< 0.05
Range	(58 - 85)	(55 - 84)	
PSA (ng/ml, mean ± SD)	103.5 ± 1478.7	3.29 ± 9.77	< 0.05
Range	(2.2 - 9506)	(0.2 – 66.5)	
T Staging	No.		
T2	2		
T3	35	N/A	–
T4	14		
N staging	No.		
N0	40	N/A	–
N1	11		

M staging*	No.	N/A	–
M1a	8		
M1b	51		
M1c	4		
Gleason score	No.	N/A	–
6	1		
7	9		
8	17		
9	20		
10	4		

*one or more staging for each patient. N/A, not applicable.

4.2.1. H-NMR metabolomics

Blood handling for metabolomic measurements. Blood specimens were collected using VACUETTE® serum tubes and promptly subjected to centrifugation within one hour of collection. The centrifugation process at 2000 rpm/4°C/10 min resulting in the separation of serum. The samples were stored at -80°C until use. Serum denaturation was carried out according to Nagana et al. (2015): 600 µl of methanol was added to 300 µl of blood serum, the mixture was vortexed and frozen at -24°C for 30 min. After subsequent centrifugation at 14 000 rpm/room temperature/30 min, 700 µl of supernatant was taken, dried, and stored at -80°C [84]. Before NMR measurement, the dried matter was soluted in 500 µl of deuterated water and 100 µl of stock solution (phosphate buffer 200 mM, pH 7.4), 0.30 mM TSP-d_4 (trimethylsilylpropionic acid-d4) as a chemical shift reference in deuterated water. Finally, the final solution was transferred into a 5 mm NMR tube.

Data acquisition and processing. Nuclear Magnetic Resonance (NMR) data were acquired using a 600 MHz NMR spectrometer, Avance III, from Bruker, equipped with a TCI CryoProbe, operating at a temperature of 310 K. Initial settings, including field shimming, receiver gain, and water suppression frequency, were optimized using an independent sample and then applied consistently across all measurements. After preparation, samples were stored in a Sample Jet automatic machine for a maximum of 2 hours, kept at approximately 5°C. Before measurement, each sample underwent preheating at 310 K for 5 minutes. An exponential noise filter with 0.3 Hz line broadening was applied prior to Fourier transform. All data were subjected to zero-filling. The acquisition order of samples was randomized for the experiments.

Bruker profiling protocols were modified as follows: noesy with presaturation (noesygppr1d): FID size 64k, dummy scans 4, number of scans: 64, spectral width 20.4750 ppm; profiling cpmg (cpmgpr1d, L4 = 126, d20 = 3 ms): number of scans: 256, spectral width 20.4750 ppm. For a subset of 15 randomly chosen samples, 2D spectra were measured: cosy with presaturation (cosygpprqf): FID size 4 k, dummy scans 8, number of scans 16, spectral width 16.0125 ppm. Homonuclear J-resolved (jresgpprqf) utilized a FID size of 8 k, 16 dummy scans, 32 scans. All experiments were conducted with a relaxation delay of 4 s. Across all samples, the half-width of the TSP-d_4 signal was consistently maintained below 1.0 Hz.

Spectra were solved using multiple references, including a human metabolomic database (www.hmda.ca)[85], the Chenomx software (free trial version), an internal metabolite database, and a review of relevant metabolomic literature [84]. Proton chemical shifts were compared with the TSP-d_4 signal assigned a chemical shift of 0.000 ppm. Peak multiplicities were verified through J-resolved spectra, while homonuclear cross-peaks were confirmed using 2D cosy spectra.

All spectra were binned into bins of 0.001 ppm in size. No additional normalization methods were applied to the data since an equal volume of blood serum was used for all measurements. Subsequently, the intensities of selected bins were summed exclusively for spectra subregions where only one metabolite was assigned or minimally affected by other co-metabolites. Metabolites exhibiting weak intense peaks or significant peak overlap were omitted from the analysis. The resulting values were utilized as relative concentrations of metabolites in each sample.

4.3. Exosomal miRs expression

The serum exosomal miR-15a, miR-16, miR-19-3p, miR-21 and miR-141a-3p were selected based on their demonstrated high diagnostic efficacy in previous studies. Total exosomal RNA containing miR was isolated through a spin column-based method (Serum/Plasma ExoRNAse Midi Kit, Qiagen, Hilden, Germany) following the manufacturer's instructions. Before isolation of total RNA, 0.5 ml of serum was initially centrifuged at 3000×g/room temperature/15 min. The RNA purity and quantity were measured using a NanoPhotometer (Implen, GmbH, Germany).

Total exosomal RNA was reverse transcribed into cDNA using an TaqMan MicroRNA Reverse Transkription Kit (Applied Biosystems, Foster City, CA, USA), and TaqMan MicroRNA Assay Kit (Applied Biosystems) containing miR RT primers of selected miRs (miR-15a, miR-16, miR-19a-3p, miR-21 and miR-141a-3p). QRT-PCR was performed using TaqMan Universal PCR Master Mix II, no UNG (Applied Biosystems) and carried out by CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) and processed as described previously [86,87]. The expression levels of exosomal miRs were normalized to miR-1233, identified as the most suitable control for data normalization. Each sample was analyzed in duplicate, and the data were processed using the $2^{-\Delta\Delta Ct}$ method.

4.4. Statistical analysis

^{1}H-NMR metabolomics. The distribution of metabolic data was approached by Shapiro-Wilk test which is suitable to detect non-normality in small sample size (n<50)[88]. The test rejected normality in about half of the data. The null hypothesis of equality of population medians among mCRPC patients and BPH patients by non-parametrical Mann-Whitney-U test. A p-value of 0.05 was used as a threshold to claim significance. Calculations were carried out in OriginPro 2019 (v.9.6.0.172, OriginLab, USA) and Matlab (v. 2015b, Mathworks, USA).

Exosomal miR expression. We employed the Shapiro-Wilk test to assess the normality of the dataset. Subsequently, we utilized the non-parametric Mann-Whitney U test to discern significant differences between groups. Differences were deemed statistically significant at a threshold of $p<0.05$. Visualization of the results was accomplished using GraphPad.

5. Conclusion

In conclusion, this study highlights the potential of metabolomic changes and serum exosomal miR-15a, miR-16, miR-19a-3p, and miR-21 as promising diagnostic markers for mCRPC. A major strength of this study lies in the observed changes, which exhibit high diagnostic efficiency and contribute to a better understanding of prostate cancer pathogenesis. However, the study's limitation is the relatively small sample size, restricting its clinical applicability, and larger studies are warranted in the future. Additionally, the use of BPH patients as the control group may pose a limitation, despite accumulating evidence suggesting important anatomic, pathologic, and genetic links between BPH and prostate cancer, along with their well-established epidemiologic association [89].

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Informed Consent Statement: Informed consent was obtained from all the subjects involved in the study.

Data Availability Statement: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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