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Comparison of the metabolome in urine prior and eight weeks after radical prostatectomy uncovers pathologic and molecular features of prostate cancer

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ABSTRACT

Prostate cancer (PCa) is associated with cellular metabolism alterations leading to changes of the metabolome. So far, studies investigating these alterations mainly focused on comparisons of metabolite profiles of PCa patients and healthy controls. In the present study we compared for the first time metabolite profiles in a significant number of paired urine samples collected before and eight weeks after radical prostatectomy (rPX) in 34 patients with PCa. Our comprehensive non-targeted liquid chromatographic-mass spectrometric metabolomics approach covered > 3000 metabolite ion masses. We annotated 23 metabolites showing significant changes eight weeks after rPX. While the levels of uridine and six acylcarnitines in urine were increased before surgery, lower levels were detected for 16 metabolites, like e.g. citrate, phenyl-lactic acid, choline, myo-inositol, emphasizing a relevant pathophysiological role of these biomarkers and the associated metabolic pathways. These results have important implications for potential use of metabolome analyses for detection of prostate cancer and related pathologic and molecular features.

1. Introduction

Prostate carcinoma (PCa) represents the most common cancer in males older than 70 years and globally accounts for more than one million newly diagnosed patients every year. Worldwide each year 270,000 prostate cancer patients die due to their malignancy. While patients with localized PCa are mostly asymptomatic, patients in locally advanced or metastatic stages may suffer from lower urinary tract symptoms and bone pain. Early detection of PCa and effective therapeutic interventions are major goals to increase the quality of life and extend life span of affected males.

Therapeutic options in localized stage include active surveillance, radical prostatectomy and radiotherapy whereas androgen deprivation therapy and other systemic treatment options represent the standard treatment in advanced stages [1]. Despite the high prevalence and growing knowledge about alterations on the molecular level in PCa tissue through recent years, concepts for targeted therapies tailored to the individual or groups of individuals with similar molecular tumor characteristics are rare. First steps towards tailored treatment for individuals were recently made by the approval of the PARP-inhibitor Olaparib

Abbreviations: FAO, fatty acid oxidation; LC–MS, liquid chromatography-mass spectrometry; PCa, prostate cancer; rPX, radical prostatectomy

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for the treatment of metastatic, castration resistant PCa with alterations in BRCA1/BRCA2 and disease progression after a next-generation hormonal therapy [2]. However, there is still an urgent need to gain deeper insights in the pathophysiological process of PCa development and progression to provide more ideas and targets for the development of novel individualized therapeutic approaches.

It is well known that neoplastic cells are characterized by metabolic alterations compared to benign tissue. Therefore, cellular metabolism and particularly distinct metabolic pathways are among the promising targets for the development of new anti-cancer drugs and treatment strategies. The oncogenic transformation is characterized by changes in metabolism. For instance, in PCa cells unique alterations in energy metabolism, namely fatty acid metabolism, glycolysis, and tricarboxylic acid (TCA) cycle have been reported [3]. As a consequence, one prerequisite to develop new therapeutic approaches is an in-depth understanding of metabolic alterations occurring in cancer cells. The application of high-throughput metabolic analyses further promotes our knowledge on cancer metabolism. Various studies investigated metabolite profiles in blood and tissue of PCa patients whereas studies on alterations in urine samples are rare [4]. The effect of prostatectomy on metabolic profiles of urine samples is unknown. It can be hypothesized, that, if metabolic biomarkers originating from cancer tissue of the prostate can be found in urine, a comparison of the metabolomes in urine before and after removing the prostate (including all cancer tissue) should uncover PCa associated metabolites. The present study represents the first analysis of metabolic profiles of pre- and post-radical prostatectomy (rPX) urine specimens in a significant number of patients with PCa.

In order to detect urinary metabolites potentially associated with the development of PCa, we applied comprehensive, non-targeted metabolomics profiling using liquid chromatography coupled to mass spectrometry (LC–MS). A total of 34 paired pre- and post-prostatectomy urine samples after convalescence were analyzed to evaluate the changes in metabolite profile's in the presence and absence of PCa. We found significant differences in the level of 23 annotated metabolites in PCa patients comparing urines collected before and eight weeks after surgery. To the best of our knowledge, this is the first extensive metabolomics study investigating urines of PCa patients before and after radical prostatectomy, opening perspectives towards metabolites and corresponding metabolic pathways as new biomarkers and drug targets involved in PCa metabolism.

2. Materials and methods

2.1. Chemicals

HPLC-grade solvents (methanol and acetonitrile) and formic acid were purchased from Merck (Darmstadt, Germany) and Fluka (Buchs, Switzerland). Deionized water used in the study was purified from a Milli-Q system (Millipore, Billerica, MA).

Sixteen internal standards were applied including carnitine C2:0-d3, carnitine C3:0-d3, carnitine C4:0-d3, carnitine C6:0-d3, carnitine C10:0-d3, leucine-d3, phenylalanine-d5, tryptophan-d5, cholic acid-2,2,4,4-d4, chenodeoxycholic acid-2,2,4,4-d4, leucine enkephalin, kynurenic acid-d5, 5-hydroxy-L-tryptophan-d3, p-tolyl sulfate-d7, guanosine-13C,15 N2, Pantothenic acid-13C3,15 N. The standards were dissolved in methanol for metabolite extraction. Detailed information of internal standards is given in Table S1 in the Supporting Information.

2.2. Sample collection and pretreatment for metabolomics analysis

The study comprised a collective of 34 patients who underwent radical prostatectomy at the University Hospital Tübingen, Department of Urology, due to organ confined prostate cancer between 06/2017 and 01/2018. The study was conducted according to the declaration of Helsinki of 1964 and its later amendments in accordance with the local ethics committee of the University of Tübingen (No. 214/2017BO2). Informed written consent was obtained from all participants before any study procedure. Paired spontaneous first void urine samples were collected before and 8 weeks after prostatectomy. Characteristics of the study collective are given in Table 1. For metabolite extraction, 180 µL urine was mixed with 4 times volume of methanol containing 16 internal standards, vortexed for 2 min and incubated for 15 min at 4 °C, then centrifuged at 4 °C at 12,000 rpm for 10 min. Aliquots of 240 µL supernatant were lyophilized for metabolomic analysis in positive and negative mode, respectively. 180 µL acetonitrile/water (5:95, v/v) was used to reconstitute the freeze-dried residue. Quality control (QC) samples were prepared by pooling equal aliquots from each sample. QCs were analyzed after every 10 sample injections.

2.3. Nontargeted metabolomics analysis

The metabolite profiling of urine samples was performed with a Waters ACQUITY-UHPLC system (Waters Corp, Milford, USA) coupled to AB SCIEX Triple Q TOF 5600 plus System (AB SCIEX, Framingham, USA) by a modified method reported in detail in a previous study [5].

Table 1
Demographic and histopathological characteristics of the participants.

Characteristics (n = 34)	
Age at baseline/surgery [years]	
Median	68.5
Range	50.0–88.0
Body mass index [kg/m²]	
Median	26.7
Range	22.5–34.0
Smoker status [n]	
Smoker	5
Non-Smoker	26
n.a.	3
Nutrition [n]	
Vegetarian	1
Non-Vegetarian	23
n.a.	9
Prostate specific antigen [ng/mL]	
Median	6.8
Range	2.8–41.8
Diabetes [n]	
Yes	2
No	32
Histopathological features	
Prostate Weight [g]	
Median	48.5
Range	19.0–89.0
T-Status [n]	
T2a	5
T2b	1
T2c	20
T3a	1
T3b	7
N-Status [n]	
N0	32
N1	2
Gleason-score	
6	1
7a	11
7b	10
8	4
9	7
10	1
Risk-Stratification (D'Amico) [n]	
Intermediate-risk	20
High-risk	14

The separation was performed on a 2.1 × 100 mm ACQUITYTM 1.8 μm HSS T3 column (Waters, Ireland). The mobile phases were (A) water with 0.1 % formic acid and (B) acetonitrile with 0.1 % formic acid. The flow rate was 0.35 mL/min and the total run time was 26 min. The gradient elution started with 5 % B for 1 min, then increased linearly to 50 % B at 18 min and increased to 100 % B within 0.5 min, kept for 4 min, followed by returning to 5 % B in 0.5 min and equilibrating for 3 min before next injection. Column temperature was set at 40 °C. The injection volume was 5 μL. For positive and negative mode analyses the same chromatographic conditions were applied.

The ion spray voltage of mass spectrometry was set at 5500 V in positive mode and −4500 V in negative mode. The interface heater temperature was 500 °C. Ion source gas 1, ion source gas 2 and curtain gas were set at 50 Psi, 50 Psi and 35 Psi, respectively. The scan range was set at *m/z* 80–1200.

2.4. Data preprocessing

Peak extraction and alignment of raw data were performed using MarkerView software (AB SCIEX, Framingham, U.S.A.) applying the following parameters: mass tolerance 20 ppm, retention time tolerance 0.3 min, noise threshold 1000, maximum number of peaks 3000. Subsequently the modified 80 % rule was applied to remove missing values [6]. Furthermore, the intensities of all retained features were calibrated using isotope-labeled internal standards (IS). The different IS of the panel should a) be evenly distributed within the total retention time window of the analysis, b) must not interfere with the detection of metabolites, c) be chemical diverse to cover as many chemical properties of metabolites as possible, d) IS quantification must be robust and highly reproducible. IS showing the lowest coefficient of variation in or close to the retention time window of a metabolite of interest are chosen as the most suitable one for the corresponding metabolite [7]. All metabolite levels were adjusted to urinary creatinine levels. The annotation of metabolites was performed according to our published strategy either by confirmation with the corresponding standard or according to exact *m/z*, MS/MS fragments and retention time [5].

2.5. Statistical analysis

MATLAB (Mathworks Inc., Natick, USA) was employed to perform paired nonparametric tests to identify the features with significant difference ($p < 0.05$). Multi Experiment Viewer software (Version 4.7.4) and Microsoft Excel (Microsoft Corporation, Redmond, USA) were used to construct the heat maps to visualize the metabolite concentrations in different groups. Volcano plot was generated using R package ggrepel (version 0.9.1) to visualize significantly different metabolites. Metabolic pathway analysis was conducted using MetaboAnalyst 5.0 to discover disease-associated pathways [8].

3. Results and discussion

3.1. Characteristics of the prostate carcinoma patients

We aimed to identify significant metabolic changes in spontaneous first-void urine samples of PCa patients before and 8 weeks after radical prostatectomy (rPX). The study population comprised 34 male individuals suffering from localized PCa. According to D'Amico 20 of them were categorized in the intermediate-risk and 14 in the high-risk group. The median age of the patients was 68.5 years (Range 50.0–88.0) and the median PSA level was 6.8 ng/mL (Range 2.8–41.8) (Gleason score: < 8 ($n = 22$) and ≥ 8 ($n = 12$)). The population also included patients suffering from metabolic disease such as overweight, represented by a median body mass index of ≥26.7 and a fraction of patients with diabetes ($n = 5$ [15 %]). No patient of the study cohort received prior to the surgery any prostate specific medication, such as 5-α-reductase-inhibitors or α-blockers. Only one patient received androgen deprivation therapy prior to his salvage prostatectomy. No changes in medication for concomitant diseases has been reported during the observation period. Patients' characteristics and comorbidities are shown in Table 1.

After radical prostatectomy patients regularly undergo in-patient rehabilitation for several weeks with partly tremendous changes in daily life and nutrition supply. To exclude after rPX interfering effects on the studied urine metabolome caused by the well-known effects of nutrition changes [9] and inflammation [10] these samples were collected after a time interval of two months when patients had returned to their domestic environment and their usual habits as before surgery. Only one patient declared vegetarian nutrition habits. Despite the described effects of diabetes and nutrition habits on urine metabolome, we found no significant inter-cohort differences regarding metabolic diseases and nutrition habits in the change of urine metabolome prior to and after radical prostatectomy which may be due to the persistence of the underlying disease [11].

3.2. Identification of significantly altered metabolites in urine collected before and eight weeks after radical prostatectomy (rPX)

Several studies have identified metabolic biomarkers in PCa in blood and urine specimens, which were exclusively elucidated in comparisons of PCa patients vs. benign or healthy controls (see references in Table S2). Each study defined significantly altered metabolic biomarkers predicting PCa development, diagnostic as well as progression. However, the proposed biomarkers showed tremendous interstudy heterogeneity with poor reproducibility. Only very few studies performed validation of their proposed biomarkers in an independent dataset. To the best of our knowledge no study has investigated metabolic changes in a significant number of PCa patients during the course of disease after local therapy with a curative intent. In our study paired spontaneous first-void urine samples were analyzed by non-targeted LC–MS in both positive and negative ionization mode. After peak alignment and application of the "modified 80 % rule" to remove missing values [12] normalization and calibration of all metabolite ion features was performed using creatinine concentrations [13] and appropriate internal standards (Table S1). In total 1699 and 1396 metabolite ion features were covered in positive and negative ion mode, respectively. Analytical robustness and precision of the metabolomics approach is demonstrated in Fig. S1 in the supplement.

In a first step we aimed to achieve a comprehensive overview of possible alterations in the metabolite profiles between urines collected pre and post radical prostatectomy (rPX). We performed a paired Wilcoxon signed-rank tests to identify metabolites that significantly differed between pre- and post rPX samples ($p < 0.05$). Fig. 1 provides an overview of differences in the metabolite profiles of urines before and 8 weeks after prostatectomy.

Based on the detected alterations of distinct metabolite ion masses in the heatmap between pre- and post-rPX we performed ion-feature analysis of the detected metabolites. Information-dependent acquisition (IDA)-auto MS2 mode in high resolution LC–MS was applied for the identification of metabolites. Finally, we annotated 119 metabolites (listed in supplemental Table S3) applying our previously published strategy for metabolite identification [5]. The identity of 11 metabolites was confirmed by a comparison with the corresponding standard compound, and the other metabolites were annotated by exact mass, retention time, and specific MS/MS fragmentation patterns. A complete list of all 119 identified metabolites is provided in supplemental Table S3. A representative example of a high-resolution LC–MS/MS-based metabolite identification is given in Fig. S2 showing the identification of indoleacetic acid by retention time, MS1 and MS2 in comparison to the corresponding standard compound.

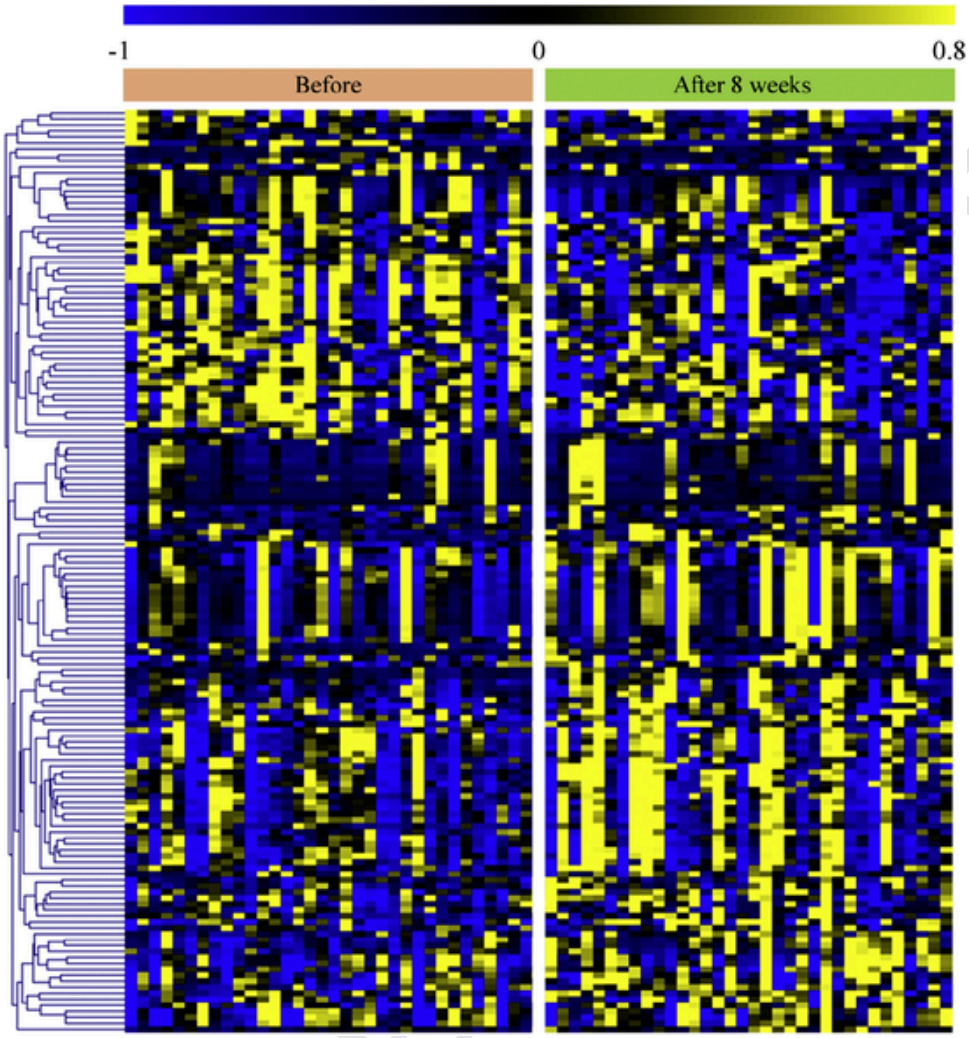

Fig. 1. Heatmap of metabolite ion features showing significant difference in urine before and eight weeks after radical prostatectomy based on paired Wilcoxon signed-rank tests ($p < 0.05$). Exemplarily metabolite ion features detected in negative ion mode are shown. Yellow indicates a higher and blue a lower level in urine in comparison to the average level (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Significant differences in the urine levels after rPX were detected for 23 annotated metabolites illustrated by a volcano plot in Fig. 2. Subsequently, pathway analysis was performed using these biomarkers, which revealed glutamate metabolism, Warburg effect, citric acid cycle and phospholipid biosynthesis (Fig. S3), i.e. pathways highly relevant for the neoplastic metabolism of cancer. Additional detailed information to these metabolites, including pre vs post fold alterations, p-values, and, if existing, citations of PCa references for several of the detected biomarkers, which underlines the reliability of our findings, are given in supplementary Table S2. Significantly increased levels in the urine before prostatectomy was detected for six acylcarnitines (C2:0-, C3:0-, C5:0-, C8:0-OH-, C10:0-OH- and C11:1-carnitine) as well as uridine (Figs. 2 and 3; Table S2). Carnitines and their derivatives, which can be synthesized endogenously or taken up from the diet, play an essential role in the transport of activated long-chain fatty acids from cytosol to mitochondrial for fatty acid oxidation (FAO) [14]. Furthermore, acylcarnitines are generally accepted markers reflecting the activity of beta-oxidation [15]. Our findings achieved in paired samples from the same individuals emphasize prior findings of identified alterations of acylcarnitines in PCa patients compared to healthy control or benign prostatic hyperplasia [16,17]. Notably, in our previous study investigating PCa tissues, carnitine and hydroxylated short-chain acylcarnitine were increased, while medium-chain and long-chain acylcarnitines were decreased (except carnitine C18:0) in PCa tissue compared with adjacent benign tissues [18]. Recently, a case-control study conducted on preoperative plasma from PCa patients and healthy controls demonstrated that medium-chain acylcarnitines (C6-C12) were positively associated with the risk of PCa progression while long-chain acylcarnitines (C14–16) were inversely associated with local progression and bone progression [19]. Despite starting from different sample species, it is remarkable that our study underlines the published results thereby stressing the hypothesis of significantly abnormal acylcarnitine metabolism in PCa. With regard to the significantly altered acylcarnitine metabolism in PCa, the carnitine shuttle system as a pivotal regulator could be a potential drug target for the prevention and treatment of PCa. Inhibition of CPT1 and lipid synthesis/lipolysis decreased viability of androgen-dependent prostate cell lines (LNCaP and VCaP) and patient-derived benign and PCa cells [20]. Furthermore, Valentino et al. reported that microRNAs greatly contribute to deregulation of FAO via carnitine system modulation in PCa cells, and forced expression of miRNAs (has-miR-124-3p, has-miR-129-5p and has-miR-378) affecting proliferation, migration and invasion of tumor cells regardless of their hormone sensitivity, suggesting the carnitine system as a primary regulator of adaptive metabolic reprogramming in PCa cells [21].

Citric acid, glycochenodeoxycholate sulfate (GCDCS), 7-methylguanine, *N*-acetyl-glucosamine-6-phosphate, phenylacetyl-

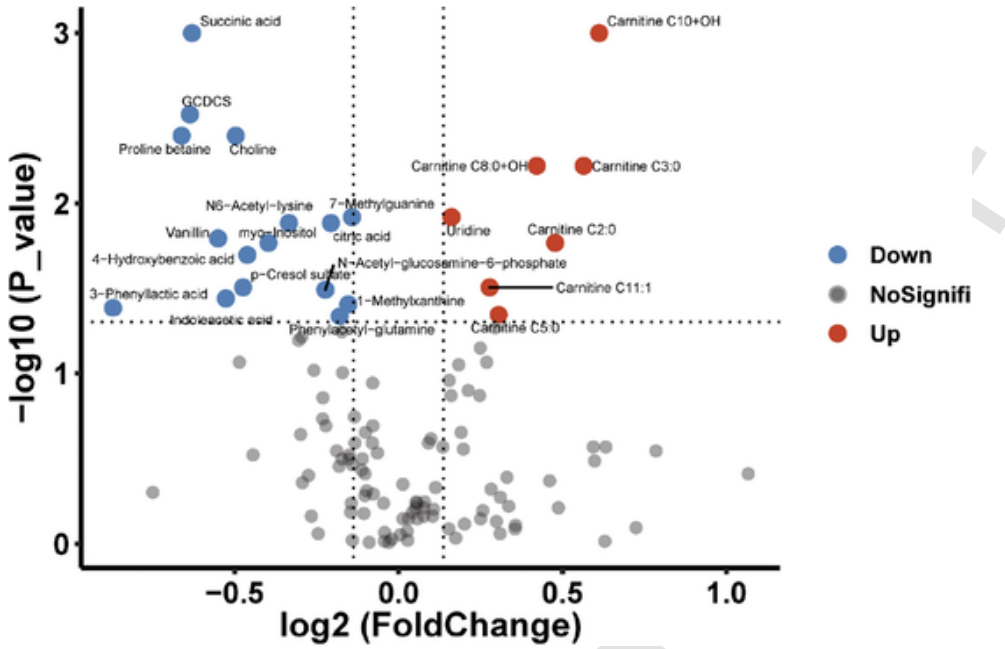

Fig. 2. Volcano plot of 23 annotated metabolites showing significant changes in urine levels 8 weeks after radical prostatectomy.

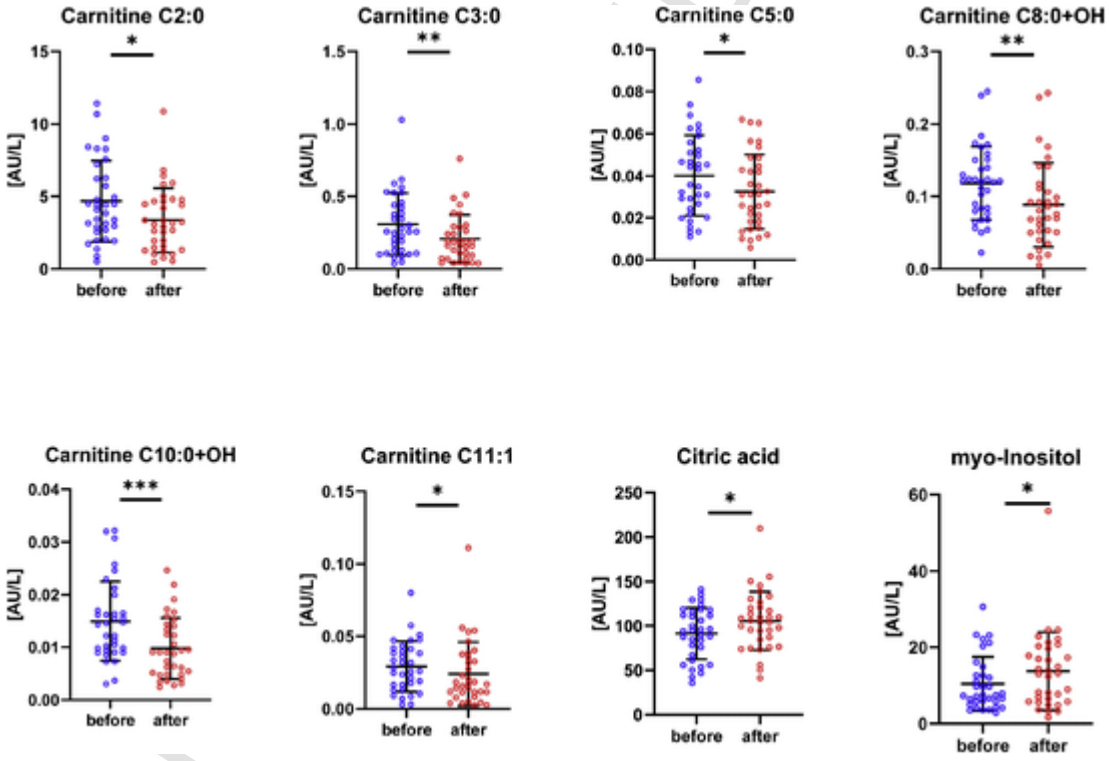

Fig. 3. Comparison of the levels of selected metabolites in urine before and 8 weeks after radical prostatectomy (rPX). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. AU = arbitrary units.

glutamine, 4-hydroxybenzoic acid, p-cresol sulfate, N6-acetyl-lysine, proline betaine, indoleacetic acid, choline, succinic acid, 3-phenyllactic acid, 1-methylxanthine, myo-inositol, and vanillin showed lower levels before rPX (Table S2). Fig. 3 provides details on the individual levels of some selected metabolites. The relative level of urinary citric acid in PCa patients, found to be lower before prostatectomy, is in line with previous reports describing citric acid metabolism playing a critical role in prostate tumor growth and aggressiveness [22]. A comparison of prostate tissues of normal and benign (benign prostatic hyperplasia, BPH) subjects revealed that PCa is characterized by a significant decrease of citric acid level, which makes it a unique and potent biomarker for PCa [23]. A study based on nude mice suggested that urinary citric acid determination with europium–oxytetracycline complex as biosensor could be applied for the detection of PCa and estimation of PCa stages [24]. Kline et al. demonstrated that citric acid determinations in unprocessed semen and expressed prostatic secretions (EPS) via NMR showed better diagnostic performance than prostate specific antigen (PSA) testing [25]. Another investigation showed that citric acid as

well as myo-inositol in human EPS are potential age-independent markers for PCa [26], which was further consolidated by the observation in our study that the urines collected from 8 weeks after surgery show a slight but significant increase in citric acid and myo-inositol level as shown in Fig. 3. Moreover, Dittrich et al. revealed by high-resolution magic angle spinning proton magnetic resonance spectroscopy (HRMAS[1]HMRS) that low citric acid level in unit volume tissue of histologically benign epithelial glands reflect rapidly elevating PSA values, which is an indicator of fast-growing PCa [27]. Regarding the detected central role of energy metabolism, namely altered citric acid levels and acyl-carnitines, further studies may provide a deeper understanding in PCa bioenergetic shift from glycolysis to fatty acid oxidation aiming to provide acetyl-CoA for the TCA cycle. Perspectives of these investigations could be on the one hand the identification of novel therapeutic targets, and on the other hand insights of the relevance of dietary factors in this pathological process of PCa.

Additionally, we detected changes in the urine levels of other metabolites originating from various different metabolic pathways after radical prostatectomy (Table S2). Several of these metabolites exhibit anticancer activity in *in vitro* experiments. For example phenyl lactic acid, showing the lower levels before prostatectomy (Table S2), has been reported to reduce the proliferative capacity of prostate carcinoma cell lines PC3 and DU145 [28] and induce cell cycle arrest in LNCaP, androgen-independent LNCaP, and PC3 cells, mediated by an increase of p27 [29]. Proline betaine, also known as stachydrine, was reported to lead to a significant inhibition of expression of chemokine receptors in PC-3 and LNcaP cells, affecting the invasive capacity of PCa cells [30]. 4-hydroxybenzoic acid was identified as HDAC6-specific inhibitor leading to decreased cancer cell viability and proliferation [31]. Other metabolites showing decreased levels before rPX were glycochenodeoxycholate sulfate (GCDCS), indoleacetic acid, choline, p-cresol sulfate, and phenylacetyl-glutamine (Fig. 2 and Table S2). This is in accordance with metabolic patterns reported in prior studies containing plasma and tissue specimens of PCa patients and healthy controls and underlines the relevance of the results of the present study particularly considering that in our study no healthy control-group was implemented. With respect to risk stratification of PCa, Gleason-score, T-Stage or PSA-level no clear association to the mentioned metabolites could be detected. There have been no specific metabolite pattern indicating dedifferentiation of PCa.

Although the composition of compounds especially in urine is discussed to be affected by several external conditions such as diet or daytime of acquisition, our data are well in accordance with findings in tissue and plasma, as well as mechanistic *in vitro* investigations in PCa cell line models, where the above-mentioned alterations especially in FAO and citric cycle were also mentioned. Pre-analytical effects on the metabolome during 24 h transportation of urine at room temperature from home to the laboratory can be excluded based on the data of a recent report of Wang and colleagues [32].

However, aside the detection of potent PCa biomarkers our study has several limitations. From the analytical point of view, we applied a scan range starting at m/z 80 up to m/z 1200, consequently small metabolites like glycine were not covered. Additionally, polyamines, which are important compounds in the scenario of prostate cancer [33], are not covered by common reversed-phase chromatographic metabolomics approaches, as we applied in this study. Furthermore from the biomedical point of view, alterations caused by delayed wound healing 8 weeks after surgery cannot completely be excluded, but seem to be very unlikely for the following reasons: a) wound healing is generally terminated after 4–6 weeks [34]; b) based on our practical clinical experience in the time courses of more than 250 prostatectomies per year, and c) based on the fact that almost all detected biomarkers show a more or less close association to cancer metabolism. Longitudinal falsifications due to pharmaceutical products are possible, but can also nearly be excluded in our study cohort since in the observation period no changes in daily medication of each individual were reported. Finally, we are aware that the findings of our study are only a starting point and further detailed diagnostic and functional investigations are needed to validate and confirm these findings, particularly to exclude that the detected biomarkers reflect non-cancer prostate tissue. However, as already mentioned above, the pathway analysis as well as the majority of all metabolite biomarkers are meaningful compounds of distinct metabolism of tumor cells.

4. Conclusions

Taken together, our study elucidated in urine eight weeks after radical prostatectomy metabolite biomarkers of various metabolic pathways altered in prostate malignancy, including significantly altered bioenergetic pattern shifting from glycolysis towards fatty acid oxidation in PCa, thereby emphasizing fatty acid metabolism as a potential drug target for PCa treatment. The data provide insights in metabolic pathomechanisms of PCa for possible future therapeutic purposes and suggests biomarkers as potential diagnostic tools.

Author contributions

Conceptualization, T.T., R.L., G.X., S.W.; methodology, T.T., J.H., Mi.Ho., Q.W., X.J., M.M., X.L.; software, M.M., Q.W., J.H.; resources, T.T., A.S., A.P., H.U.H., G.X.; original draft preparation S.W., Q.W., R.L.; review and editing, T.T., A.S., J.H., X.Z., Q.W., X.L., A.P., H.U.H., R.L., G.X. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2021.114288.

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