# Exploration of urological biomarkers by urine metabolome NMR-analysis in an Asian patient cohort of prostate cancer

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# Contents

1. Introduction ..... 4
1.1. Prostate Cancer (PC) ..... 4
1.1.1. Epidemiology ..... 4
1.1.2. PC classification and grading ..... 5
1.2. PC Biomarkers ..... 7
1.3. PC prevalence is not equal in different populations ..... 8
1.4. Aims of the study ..... 8
1.5. Material and Methods ..... 8
1.5.1. Patients and sampling ..... 8
1.5.2. Urine analysis using NMR technology ..... 9
2. Publications ..... 14
2.1. Yang B et al. (2017) J Zhejiang Univ Sci B;18(11):921-33 ..... 14
2.2. Neuhaus J and Yang B (2018) Diagnostics; 8(4),68 ..... 28
2.3. Yang et al. (2021) Diagnostics;11(2):149 ..... 48
3. Summary ..... 67
3.1 Introduction ..... 67
3.2 Material and Methods ..... 67
3.3 Results ..... 68
3.3.1 Identification of important metabolites and the metabolic changes ..... 68
3.3.2 Acquisition of the most prominent metabolites,
Correlation analysis and ROC analysis ..... 68
3.3.3 Subgroup analysis ..... 69
3.3.4 Analysis of the metabolite interaction networks
and corresponding pathways ..... 69
3.4 Discussion ..... 69
4. References ..... 71
5. Attachments (Supplemental Materials) ..... 75

6. Description of the scientific share of the applicant
in the included publications ……..……..... 75

7. Declaration (Selbstständigkeiterklärung) ..... 78

8. Curriculum vitae ..... 79

9. Scientific Publications and Awards ..... 81

9.1. Publications ..... 81

9.2. Published abstracts ..... 81

9.3. Awards ..... 81

10. Acknowledgements ..... 82

# 1. Introduction

## 1.1. Prostate Cancer (PC)

### 1.1.1. Epidemiology

Prostate cancer (PC) is one of the major threats to men's health worldwide (Siegel et al., 2016; Brawley, 2012; Jahn et al., 2015; Center et al., 2012). In the United States PC was estimated to make up roughly 20% of the new cancer cases in men in 2016. Deaths from PC are expected to account for 8% of cancer associated deaths (Siegel et al., 2016). Epidemiological data from China are still rare and incomplete but were recently supplemented by high-quality data provided by the National Central Cancer Registry of China (NCCR) (Chen et al., 2016).

The incidence rate of prostate cancer in China increased from 1998 to 2008 by a factor of 3, from 35.2/100,000 to 110.0/100,000 and the average annual growth rate was as high as 12.07% reaching 60,300 cases in 2015 (Zhu et al., 2015; Coffey, 2001; Baade et al., 2013; Chen et al., 2016). While incidence rates in rural areas remained stable between 2006 and 2009, there was an increase in urban areas, especially documented in Hong Kong and Shanghai. The rapid rise of the incidence rate may be in part related to the aging of the population but there seems to be a strong link to Western-style diet (Lin et al., 2015).

A comparison of the incidences of prostate cancer in 2015 showed that although the total number of patients in the United States has reached 3.66 times that of China, the estimated death tolls in the two countries are almost similar (Table 1) (Siegel et al., 2016; Ervik et al., 2016; Chen et al., 2016).

**Table 1.**

**Estimated new cases and deaths from prostate cancer in the United States and China.**

| Country / Region | New cases | Deaths | Ratio |
|---|---|---|---|
| US | 220800 | 27540 | 0.12 |
| EU* | 400364 | 92328 | 0.23 |
| China | 60300 | 26600 | 0.44 |

Note that while the number of patients in the US was almost 4 times that in China, the death tolls are almost equal, indicating a much higher mortality from PC in China, reflected in the new cases / deaths ratio. * WHO – region.

Interestingly, the numbers in the European Union (EU, WHO region) are in between which might reflect more regional variations in living conditions and diet. However, further investigations are required to come to valid conclusions. Effectivity of PC treatment and cancer recurrence heavily depend on early detection and proper risk stratification (Moller et al., 2015; Schroder et al., 2012; Klotz et al., 2015; Moyer, 2012).

In the US, the proportion of localized prostate cancer accounts for more than 80% of all cases, which is also one of the major reasons the mortality/morbidity rate in the US is much lower than that in Asian countries, and continues to decrease (Moller et al., 2015; Jemal et al., 2015; DeSantis et al., 2014). Therefore, early detection and diagnosis is the most effective way by which to improve the survival rate, and development of new biomarkers and/or reasonable combination of current diagnostic methods is a hot spot in the field of prostate cancer research (Felgueiras et al., 2014).

Among countries that have implemented prostate cancer screening strategies, five-year survival rates have improved rapidly in Japan, with an average annual increase of about 11.7% and a 5-year survival rate of 93%, while in China, the annual increase was only 3.7% and the 5-year survival rate was 69.2%(Yao et al., 2021).

In 2018, there were 1.3 million new cases of prostate cancer worldwide, and its morbidity and mortality ranked second and fifth among male malignancies, respectively.

However, to date no serum or urine biomarker or biomarker panel meets the requirements for highly sensitive and specific detection of PC and differentiation between indolent and significant PC. We here explore the prospects of metabolomics to improve prostate cancer detection, patient stratification and treatment monitoring.

### 1.1.2. PC classification and grading

The prostate gland is a walnut-sized gland located between the bladder neck and the external urethral sphincter. There are four main zones in the prostate gland: the peripheral zone (posteriorly), the fibromuscular zone (anteriorly), the central zone (centrally) and the transitional zone (surrounding the urethra). The anatomy of the prostate gland is shown in Fig. 1 (Adapted from: Bhavsar et al., 2014).

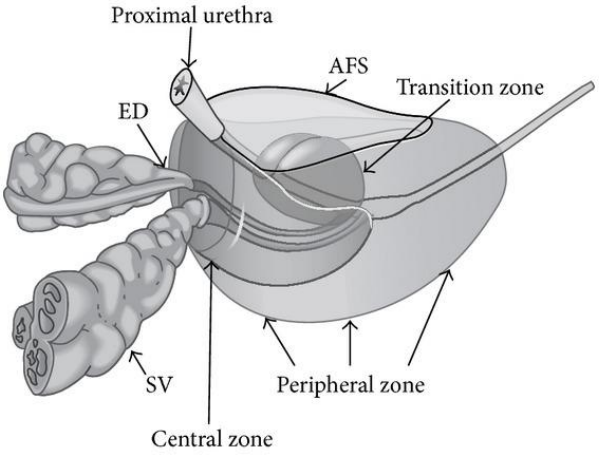


**Fig. 1.** Zonal anatomy of the prostate gland. ED: ejaculatory ducts; SV: seminal vesicles; AFS: anterior fibromuscular stroma.

Prostate cancer does not occur uniformly throughout the prostate. Although cancers of the prostate often are multifocal, from 80% to 85% arise from the peripheral zone, 10% to 15% arise from the transition zone, and 5% to 10% arise from the central zone (Buyyounouski et al., 2017).

The biopsy Gleason grading system is the most important prognostic marker for prostate cancer. The higher the Gleason score, the higher the malignant degree of prostate cancer. The TNM staging system proposed by AJCC is a widely used independent index that can reflect the progression and prognosis of prostate cancer. Table 2 shows the definitions for clinical and pathological T, N, and M classifications (Buyyounouski et al., 2017).

**Table 2.** Definitions of the American Joint Committee on Cancer TNM Criteria

| CATEGORY | CRITERIA |
|---|---|
| **Clinical (cT)** | |
| **T category** | |
| **TX** | Primary tumor cannot be assessed |
| **T0** | No evidence of primary tumor |
| **T1** | Clinically inapparent tumor that is not palpable |
| **T1a** | Tumor incidental histologic finding in 5% or less of tissue resected |
| **T1b** | Tumor incidental histologic finding in more than 5% of tissue resected |
| **T1c** | Tumor identified by needle biopsy found in one or both sides, but not palpable |
| **T2** | Tumor is palpable and confined within prostate |
| **T2a** | Tumor involves one-half of one side or less |
| **T2b** | Tumor involves more than one-half of one side but not both sides |
| **T2c** | Tumor involves both sides |
| **T3** | Extraprostatic tumor that is not fixed or does not invade adjacent structures |
| **T3a** | Extraprostatic extension (unilateral or bilateral) |
| **T3b** | Tumor invades seminal vesicle(s) |
| **T4** | Tumor is fixed or invades adjacent structures other than seminal vesicles, such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall |
| **Pathologic (pT)** | |
| **T category** | |
| **T2** | Organ confined |
| **T3** | Extraprostatic extension |
| **T3a** | Extraprostatic extension (unilateral or bilateral) or microscopic invasion of bladder neck |
| **T3b** | Tumor invades seminal vesicle(s) |
| **T4** | Tumor is fixed or invades adjacent structures other than seminal vesicles, such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall |
| **N category** | |
| **NX** | Regional lymph nodes were not assessed |
| **N0** | No positive regional lymph nodes |
| **N1** | Metastases in regional lymph node(s) |
| **M category** | **M criteria** |
| **M0** | No distant metastasis |
| **M1** | Distant metastasis |
| **M1a** | Nonregional lymph node(s) |
| **M1b** | Bone(s) |
| **M1c** | Other site(s) with or without bone disease |

Radical prostatectomy (RP) has become the most effective method for the treatment of localized prostate cancer and some high-risk prostate cancer. RP is used when the cancer is believed to be confined to the prostate gland. During the procedure, the prostate gland and some tissue around the gland, including the seminal vesicles, are removed. Transurethral resection of the prostate, or TURP, which also involves removal of part of the prostate gland, is an approach performed through the penis with an endoscope (small, flexible tube with a light and a lens on the end). This procedure doesn't cure prostate cancer but can remove the obstruction while the doctors plan for definitive treatment. Laparoscopic surgery, done manually or by robot, is another method of removal of the prostate gland.

Shortcomings in comprehensive medical check-ups in low- and middle-income countries lead to delayed detection of PC and are causative of high numbers of advanced PC cases at first diagnosis. The performance of available biomarkers is still insufficient and limited applicability, including logistical and financial burdens, impedes comprehensive implementation into health care systems. There is broad agreement on the need of new biomarkers to improve (i) early detection of PC, (ii) risk stratification, (iii) prognosis, and (iv) treatment monitoring.

### 1.2. PC Biomarkers

Serum prostate specific antigen (PSA) level and digital rectal examination (DRE) constitute the major screening tests for prostate cancer (PC) diagnosis, while the transrectal ultrasound-guided prostate biopsy provides the final confirmation of cancer presence (Velonas et al., 2013). PSA level has been extensively used as a biomarker to detect PC. Nevertheless, due to prostate physiology, PSA testing results in a large frequency of false positives leading to numerous men each year undergoing unnecessary prostate biopsy procedures (Vickers et al., 2008; Link et al., 2004; McDunn et al., 2013; Roberts et al., 2011; Djavan et al., 2000). Hence, a non-invasive, cost-effective, efficient, and reasonably accurate test for early identification of PC is urgently needed. Compared with serum, urine is easier to obtain and handle, needs less sample preparation, and has higher amounts of metabolites and lower protein content (Rigau et al., 2013; Wilkosz et al., 2011; Zhang et al., 2013). Therefore, in attempt to solve this diagnostic dilemma, many previous studies have focused on urinary metabolomic profile, to identify the predictive biomarkers for PC (Chistiakov et al., 2018). Yang and colleagues conducted a study searching for urine metabolite biomarkers for the detection of PC. They found twenty differentially expressed urine metabolites in a cohort of 50 prostate cancer patients compared to non-cancerous individuals (Yang et al., 2021; Gordetsky et al., 2016; Nam et al., 2018; Di Meo et al., 2017). The combination of solely three metabolites, representing alterations in Glycine, Serine, and Threonine metabolism (KEGG database pathway), was able to identify PC

patients with 77% accuracy at 80% sensitivity and 64% specificity. Furthermore, those metabolites could separate significant PC (Gleason score ≥ 7) from indolent PC (GS 6), which confirms urine metabolomics as a promising diagnostic tool in PC. However, to date, no single urine biomarker/biomarker panel meets the requirements for highly sensitive, and specific detection of PC. Therefore, the search for PC-specific biomarkers still is an active area of research.

### 1.3. PC prevalence is not equal in different populations

There is a racial difference in incidence rate and interpatient heterogeneity of prostate cancer. By contrast, Asian men have lower disease prevalence compared with Asian-American or American PC cohorts. Despite lower PC incidence, the Asian populations have a higher prevalence of advanced disease, probably due to the lack of availability of more sensitive diagnostic tools (Ateeq et al., 2016). Therefore, it's necessary to define the urine metabolome in an Asian population.

### 1.4. Aims of the study

- Exploration of novel biomarkers for the detection of PC in an Asian cohort.
- Are urinary metabolomics suitable to develop new PC biomarkers?
- What are the advantages of urine biomarkers?
- How to identify novel biomarkers in the urine and to investigate the possible functions and roles of potential biomarkers in PC?

### 1.5. Material and Methods

#### 1.5.1. Patients and sampling

Urine samples were collected from PC patients from January 2017 to December 2018 from Sir Run Run Shaw Hospital, HangZhou and Zhoupu Hospital, Shanghai, China. Clinical diagnosis of individuals was performed according to serum PSA, DRE, biopsy results/pathological results after operation and Gleason score. A total of 50 patients with prostate cancer were included in this study. The control group consisted of 50 non-cancerous men, who were without evidence of PC, based on PSA levels, negative findings in imagological examination and DRE. Clinical and demographics characteristics of the individuals are shown in Table 3.

**Table 3.** Characteristics of the individuals

| | Control group (n=50) | | PC group (n=50) | | |
|---|---|---|---|---|---|
| **Characteristics** | **mean (SD)** | **Group size** | **mean (SD)** | **Group size** | **P-value** |
| Age (years) | 63.30 (9.61) | 50 | 70.00 (8.98) | 50 | < 0.0001 |
| Prostate volume (mL) | 26.24 (8.77) | 24 | 39.77(19.00) | 50 | 0.0169 |
| PSA (⩽ 10ng/mL) | 1.56 (0.89) | 50 | 6.69 (1.96) | 14 | |
| PSA (10.1 - 20ng/mL) | NA | 0 | 14.01 (2.08) | 14 | |
| PSA (> 20ng/mL) | NA | 0 | 89.82 (86.28) | 22 | |
| GS (pre) 6 | NA | NA | NA | 13 | |
| GS (pre) ⩾ 7 | NA | NA | NA | 34 | |
| GS (post) 6 | NA | NA | NA | 6 | |
| GS (post) ⩾ 7 | NA | NA | NA | 35 | |
| **Treatment:** | | | | 50 | |
| Radical operation | | | | 41 | |
| Seed implantation | | | | 5 | |
| Endocrine | | | | 2 | |
| Chemotherapy | | | | 1 | |
| TURP | | | | 1 | |

GS = Gleason Score; GS (pre) = GS of biopsy; 41 patients have accepted radical operation and got the post-operation GS (GS (post)); SD = standard deviation; prostate volume was calculated as volume: Vml = (length×width×height) × π/6. TURP = Transurethral resection of the prostate.; NA=not applicable; PC=prostate cancer

Patient-recruitment and sampling procedures were performed in accordance with the Declaration of Helsinki and applicable local regulatory requirements and laws. All patients provided written informed consent. Ethical approvals were obtained from the local ethics committees of the Sir Run Run Shaw Hospital affiliated to Zhejiang University (Ethical review approval number: 20190725-290) and Shanghai University of Medicine & Health Sciences (Ethical review approval number: HMMEP-2016-017).

### 1.5.2. Urine analysis using NMR technology

Nuclear magnetic resonance (NMR) spectroscopy, liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) are the most common techniques used in the study of cancer metabolomics and biomarker development. NMR technology stands out for the rapid detection and excellent reproducibility at high resolution, acceptable sensitivity (mMol range) and quantitative accuracy. NMR spectroscopy is a well-established non-destructive analytical method

based on quantum physical effects of atomic nuclei. It makes use of atomic nuclear spins being aligned when placed into a strong magnetic field and moving out of alignment by absorbance of isotope specific radiofrequencies. The tiny differences in realignment of the atomic spins with the magnetic field is then detected. Due to interference with nearby nuclei and electrons, information on the molecular makeup and structure of a probe can be deduced. While many nuclei can be detected, most used isotopes are $^{1}H$ and $^{13}C$. High field NMR instruments (≥ 600 MHz) are needed to provide sufficient sensitivity and spectral resolution. Since sensitivity for $^{1}H$ (protium) is highest, one-dimensional $^{1}H$ analyses are preferred in most studies. NMR spectroscopy allows the direct identification, quantification and structural analysis of small organic molecules, nucleic acids, proteins and carbohydrates. Since most measured signals may come from aqueous solvents deuterium ($^{2}H$) is often substituted. With a spin of 1 it does not show up in proton ($^{1}H$, spin 1/2) NMR. NMR signals are calibrated to known peaks, e.g. tetramethylsilane (TMS) for $^{1}H$-NMR. The analysis includes identification of molecules by their specific chemical shift fingerprints and quantification may be done by comparison to peaks of pure standards in validation experiments. The application of NMR spectroscopy to biomarker detection involves extended multivariate statistical analyses, e.g. principle component analysis (PCA) or partial least square-discriminant analysis (PLS-DA).

In addition, the simplicity of sample preparation, low volume requirement (typically a few hundred µl), non-destructive measurement and finally cost efficiency led to rapid acceptance of NMR in noninvasive diagnostics (Motta et al., 2012; Soininen et al., 2015; Ibrahim et al., 2013; Nagana Gowda and Raftery, 2015). The most outstanding point is that hundreds of metabolites can be analyzed in just one NMR measurement (Duarte et al., 2014). One characteristic of cancer cells is the switch from aerobic oxygen-consuming energy production to glycolytic metabolism, known as the Warburg effect (Warburg, 1956). Changes in glycolytic metabolites and related amino acids are amongst the most promising for cancer detection: high lactate levels indicating enhanced anaerobic energy metabolism; enhanced serine and glycine levels as result of de novo synthesis of serine via a side branch of glycolysis in highly proliferative cancer cells (Yang and Vousden, 2016). Serine is crucial for the growth and survival of many cancer cells and is closely related to the folate cycle as a donor of one-carbon units. Therefore, the enzymes involved in serine de novo synthesis, i.e. phosphoglycerate dehydrogenase (PHGDH), phosphoserine aminotransferase 1 (PSAT1) and phosphoserine phosphatase (PSPH), may be good targets for therapeutic intervention (Yang and Vousden, 2016).

To significantly outperform current PC biomarkers and to overcome the shortcomings of PSA screening, NMR-metabolomics must overcome several challenges. While liquid biopsy, especially urine, is well accepted by patients, standardization is a major issue for quality management concerning sample preparation, measurement, data

processing and statistical analysis. NMR data are highly comparable between different institutions which is a great advantage and a prerequisite for comprehensive worldwide application (Ward et al., 2010).

New tools are under development for multivariate statistics of huge data volumes acquired by NMR and automatic classification of discriminatory from non-discriminatory metabolites (Motegi et al., 2015; Zou et al., 2016).

The workflow for implementation of urine NMR-metabolomics into routine PC diagnostics and treatment monitoring is shown in Fig. 2.

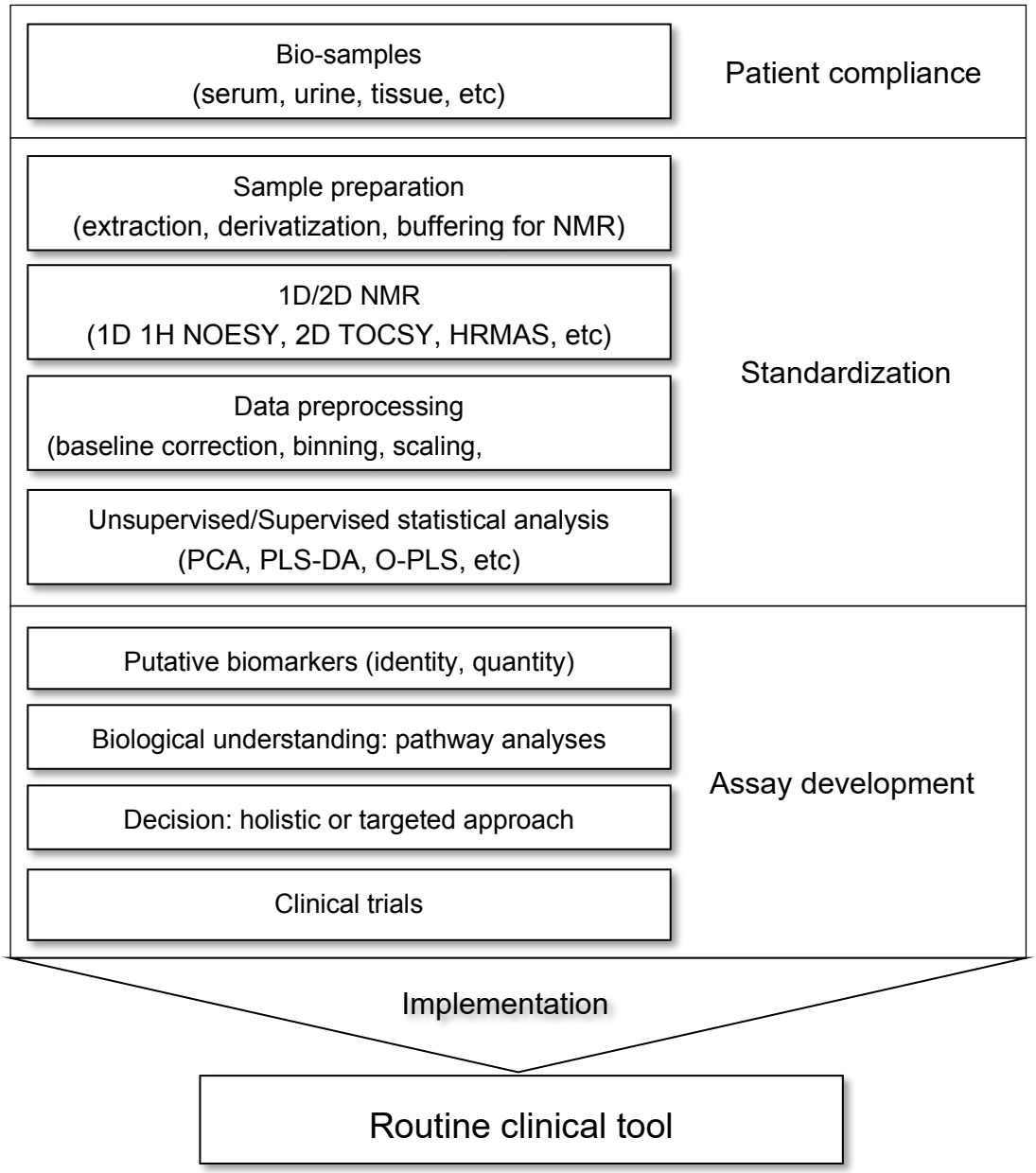

**Fig. 2.** NMR-based metabolomics workflow. Left hand: developmental steps; right hand: challenges. Patient compliance will be best when using urine as a noninvasive source. Standardization is the major challenge: development of a standard platform for comparability across laboratories and time. The major decision in assay development is between the holistic approach (as often preferred in MS/MS analyses or next generation sequencing (NGS)) and targeted metabolite quantification. Clinical trials in different countries with different socio-economic and genetic backgrounds are required for final adjustment of the assay. PCA: principal component analysis; PLS-DA: partial least square-discriminant analysis; OPLS-DA: orthogonal projection to latent structure discriminant analysis

NMR-analytics have been used for biomarker detection in humans in several tumor entities others than prostate cancer, for example: non-small-cell lung cancer (Doskocz et al., 2015), oral squamous cell carcinoma (Gupta et al., 2015), gastric cancer (Jung et al., 2014), myeloma (Lodi et al., 2013), pancreatic ductal adeno carcinoma (Davis et al., 2013), lung cancer (Carrola et al., 2011) and bladder cancer (Bansal et al., 2013). For additional information see Table 2 in Yang et al. 2007).

Squamous cell carcinoma is a good example of the high performance of $^{1}$H-NMR in conjunction with PCA and OPLS-DA analyses. Serum metabolomics successfully separated patients with oral leukoplakia (OLK, n=100) and oral squamous cell carcinoma (OSCC, n=100), from healthy controls (HC) by the $^{1}$H-NMR technique (Gupta et al., 2015). OSCC and HC were accurately separated with high area under the curve (AUC = 0.97) of the receiver operating characteristics (ROC) according to the expression differences of four biomarkers, namely glutamine, propionic acid ester, acetone, and choline, while the comparative analysis of glutamine, acetone, ethyl acetate and choline can accurately distinguish OLK and OSCC (AUC = 0.96). The two groups were separated with almost ideal sensitivity and specificity (sensitivity 92.7%, specificity 93.8%; Gupta et al., 2015).

These considerations resulted in a multicenter study demonstrating the feasibility of $^{1}$H-NMR urine metabolomics for the detection of novel biomarkers in an Asian cohort (Yang et al. 2021). Figure 3 summarizes the approach.

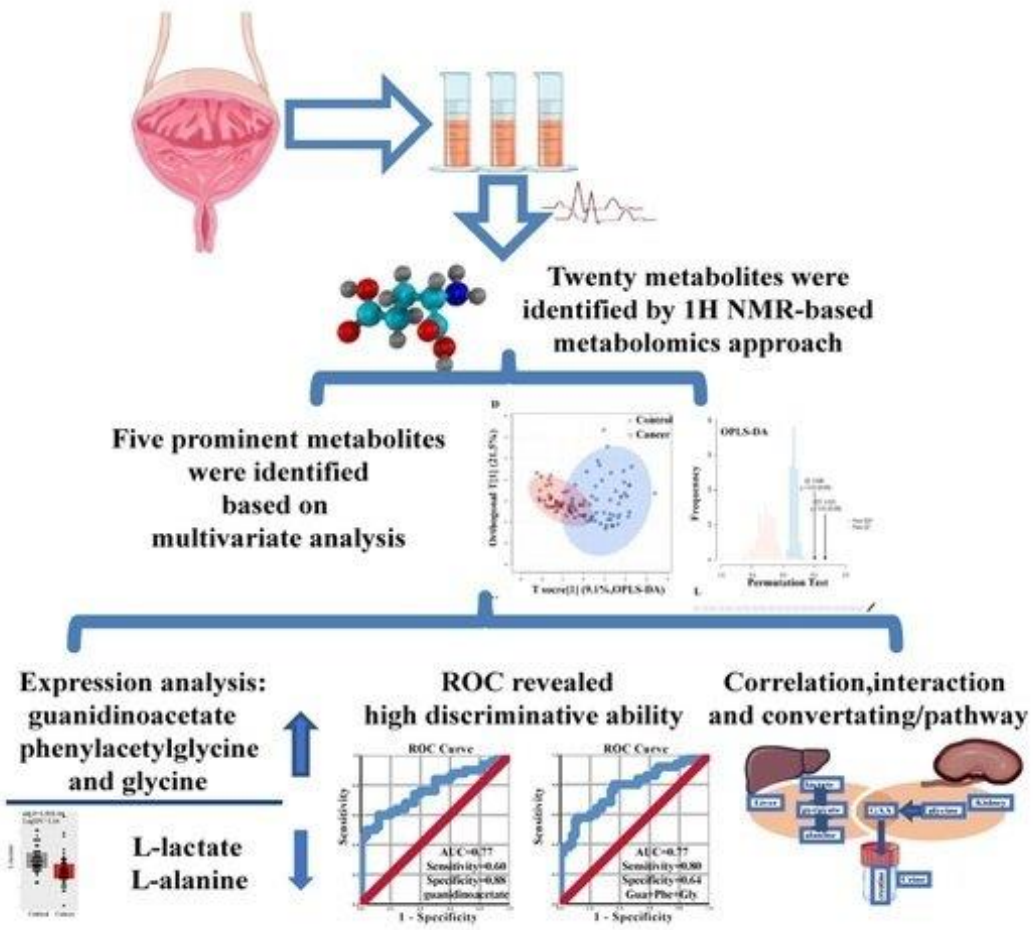


Figure 3. Detection of PC-related metabolites in the urine. $^{1}$H-NMR was used to search for metabolite biomarkers in an Asian cohort. The multicenter study was performed in two big Chinese cities (Shanghai and Hangzhou) and the results were published in 2021 (see publication 2.3.: Yang et al. 2021).

This cumulative doctoral thesis consists of three related publications.

1) Yang, B.; Liao, G.Q.; Wen, X.F.; Chen, W.H.; Cheng, S.; Stolzenburg, J.U.; Ganzer, R.; Neuhaus, J. Nuclear magnetic resonance spectroscopy as a new approach for improvement of early diagnosis and risk stratification of prostate cancer. *J Zhejiang Univ Sci B* **2017**;*18(11):*921-33. doi:10.1631/jzus.B1600441
2) Neuhaus, J.; Yang, B. Liquid Biopsy Potential Biomarkers in Prostate Cancer. *Diagnostics (Basel)* **2018**;*8(4):68.* doi:10.3390/diagnostics8040068
3) Yang, B.; Zhang, C.; Cheng, S.; Li, G.; Griebel, J.; Neuhaus, J. Novel Metabolic Signatures of Prostate Cancer Revealed by $^{1}$H-NMR Metabolomics of Urine. *Diagnostics* **2021**;*11(2):*149. doi:10.3390/diagnostics11020149

## 2. Publications

**2.1.** Yang, B.; Liao, G.Q.; Wen, X.F.; Chen, W.H.; Cheng, S.; Stolzenburg, J.U.; Ganzer, R.; Neuhaus, J. Nuclear magnetic resonance spectroscopy as a new approach for improvement of early diagnosis and risk stratification of prostate cancer. *J Zhejiang Univ Sci B* **2017**;*18(11):*921-33, doi:10.1631/jzus.B1600441

Impact Factor (2017): 2.074.

In this review we summarized the literature up to 2017 on $^{1}$H-NMR metabolomics in cancer biomarker research and evaluated the potential application to PC. We found that $^{1}$H-NMR urine metabolomics is a promising tool for the development of new biomarker panels for early diagnosis and risk stratification of PC.

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***Review:***

# Nuclear magnetic resonance spectroscopy as a new approach for improvement of early diagnosis and risk stratification of prostate cancer*

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**Abstract:** Prostate cancer (PCa) is the second most common male cancer worldwide and the fifth leading cause of death from cancer in men. Early detection and risk stratification is the most effective way to improve the survival of PCa patients. Current PCa biomarkers lack sufficient sensitivity and specificity to cancer. Metabolite biomarkers are evolving as a new diagnostic tool. This review is aimed to evaluate the potential of metabolite biomarkers for early detection, risk assessment, and monitoring of PCa. Of the 154 identified publications, 27 and 38 were original papers on urine and serum metabolomics, respectively. Nuclear magnetic resonance (NMR) is a promising method for measuring concentrations of metabolites in complex samples with good reproducibility, high sensitivity, and simple sample processing. Especially urine-based NMR metabolomics has the potential to be a cost-efficient method for the early detection of PCa, risk stratification, and monitoring treatment efficacy.

**Key words:** Prostate cancer; Metabolomics; Nuclear magnetic resonance (NMR); Biomarker
http://dx.doi.org/10.1631/jzus.B1600441 **CLC number:** R737.25

## 1 Introduction

Prostate cancer (PCa) is one of the major threats to men's health worldwide (Brawley, 2012; Center *et al.*, 2012; Jahn *et al.*, 2015; Siegel *et al.*, 2016). In the United States, PCa was estimated to make up roughly 20% of the new cancer cases in men in 2016. Deaths from PCa are expected to account for 8% of cancer-associated deaths (Siegel *et al.*, 2016). Epidemiological data from China are still rare and incomplete but were recently supplemented by high-quality data provided by the National Central Cancer Registry of China (NCCR) (Chen *et al.*, 2016).

The incidence rate of PCa in China from 1998 to 2008 increased by a factor of 3, from 35.2/100000 to 110.0/100000, and the average annual growth rate was as high as 12.6% reaching 60300 cases in 2015 (Coffey, 2001; Baade *et al.*, 2013; Zhu *et al.*, 2015; Chen *et al.*, 2016). While incidence rates in rural areas remained stable between 2006 and 2009, there was an

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increase in urban areas, especially documented in Hong Kong and Shanghai. The rapid rise of the incidence rate may be in part related to the aging of the population but there seems to be a strong link to Western-style diet (Lin *et al.*, 2015).

A comparison of the incidences of PCa in 2015 showed that although the total number of patients in the United States has reached 3.66 times that of China, the estimated death tolls in the two countries are almost similar (Table 1) (Chen *et al.*, 2016; Ervik *et al.*, 2016; Siegel *et al.*, 2016). Interestingly, the numbers in the European Union (EU, World Health Organization (WHO) region) are in between, which might reflect more regional variations in living conditions and diet. However, further investigations are required to come to valid conclusions.

**Table 1 Comparison of the estimated new cases and deaths from prostate cancer in the United States, the EU (WHO region), and China, 2015**

| Country/ region | Estimated new cases | Estimated deaths | Ratio |
|---|---|---|---|
| US | 220800 | 27540 | 0.12 |
| EU | 400364 | 92328 | 0.23 |
| China | 60300 | 26600 | 0.44 |

Data are from the studies of Chen *et al*. (2016), Ervik *et al*. (2016), and Siegel *et al*. (2016)

Effectivity of PCa treatment and cancer recurrence heavily depend on early detection and proper risk stratification (Moyer, 2012; Schroder *et al.*, 2012; Klotz *et al.*, 2015; Moller *et al.*, 2015). In the United States, the proportion of localized PCa accounts for more than 80% of all cases, which is also one of the major reasons why the mortality/morbidity rate in the United States is much lower than that in Asian countries, and continues to decrease (DeSantis *et al.*, 2014; Jemal *et al.*, 2015; Moller *et al.*, 2015). Therefore, early detection and diagnosis is the most effective way to improve the survival rate, and development of new biomarkers and/or reasonable combination of current diagnostic methods are hot spots in the field of PCa research (Felgueiras *et al.*, 2014).

However, to date no serum or urine biomarker or biomarker panel meets the requirements for highly sensitive and specific detection of PCa and differentiation between indolent and significant PCa. We here explore the prospects of metabolomics to improve PCa detection, patient stratification, and treatment monitoring.

## 2 Metabolomics—a window into tumor pathology

### 2.1 Metabolomics in cancer diagnostics

Metabolites, typically less than 1000 Da, represent the end products of complex metabolic pathways. The metabolome closely reflects any changes in those pathways and therefore provides a reasonable basis for clinical diagnosis. Specific changes in the metabolome are thought to reflect pathological states of patients (Dunn *et al.*, 2013).

Depending on the grade of degeneration, tumor cells show alterations of basic biochemical processes. Therefore, defining the metabolic signature of malignancies and precursor cells is the current hot spot in cancer metabolic research.

Several cancer entities have been analyzed, aiming to better understand the pathological alterations in metabolic pathways and to uncover new diagnostic biomarkers.

#### 2.1.1 Colon cancer

Chen *et al.* (2014) found altered glycolytic enzyme activity in the transcriptome of stem cell-like $CD133^+$ colon cancer initiating cells (CCICs) compared to $CD133^-$ colon cancer cells. Those alterations in metabolic enzyme expression correlated with metabolite production through the tricarboxylic acid (TCA) cycle and cysteine/methionine metabolism pathways. This suggests that the metabolic signature can be used as a starting point to determine the potential biological markers and the colorectal cancer therapeutic target.

#### 2.1.2 Thyroid cancer

Clinical studies showed that there was a significant difference in the endogenous metabolism between patients with papillary thyroid cancer, benign thyroid tumors and healthy people. Compared to healthy people, in the serum samples of papillary thyroid cancer patients the valine, leucine, isoleucine, lactate, alanine, glutamine, and glycine levels were increased, while lipids, choline, and tyrosine levels were reduced. Interestingly, glycine levels were not different between benign thyroid lesions and healthy controls. Therefore, glycine could be a useful biomarker for early tumor detection (Zhao *et al.*, 2015).

2.1.3 Ovarian cancer

Jiang *et al*. (2015) found that the clinical staging of ovarian cancer patients was significantly correlated with the urine metabolites analyzed by ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS).

2.1.4 Renal cancer

In their research on the potential risk of the development of chronic liver disease to hepatocellular carcinoma, Trovato *et al.* (2015) proposed that in the future the periodic monitoring of specific metabolic markers for the urine of patients with chronic liver disease will make it possible to achieve clinical application as an effective method for cancer prevention or early diagnosis.

2.1.5 Bladder cancer

Various studies using mass spectrometric (MS) methods reported promising results for bladder cancer (BCa) detection and separation of invasive BCa from non-invasive BCa (Issaq *et al.*, 2008; Huang *et al.*, 2011; Pasikanti *et al.*, 2013; Chan *et al.*, 2015; Zhou *et al.*, 2016). However, cohort sizes were small in most studies and the effects of renal cancer and bladder inflammation were the major problem in BCa detection (Van *et al.*, 2011). In conclusion, MS-based metabolomics can improve BCa detection and risk stratification but the financial burden may hinder comprehensive routine implementation.

2.1.6 Prostate cancer

To date only a few studies have used nuclear magnetic resonance (NMR) to define metabolomic signatures of PCa for diagnosis and risk assessment (Kumar *et al.*, 2016a), while most studies used liquid chromatography-mass spectrometry (LC-MS) or capillary electrophoresis-mass spectrometry (CE-MS) (Roberts *et al.*, 2011; McDunn *et al.*, 2013; Thapar and Titus, 2014; Struck-Lewicka *et al.*, 2015).

### 2.2 Nuclear magnetic resonance spectroscopy in the study of cancer and biomarker development

NMR, LC-MS, and gas chromatography-mass spectrometry (GC-MS) are the most common techniques used in metabolomics. NMR technology stands out for the rapid detection and excellent reproducibility at high resolution, acceptable sensitivity (mmol), and quantitative accuracy.

NMR spectroscopy is a well-established non-destructive analytical method based on quantum physical effects of atomic nuclei. It makes use of atomic nuclear spins being aligned when placed into a strong magnetic field and moving out of alignment by absorbance of isotope specific radio-frequencies. The tiny difference in realignment of the atomic spins with the magnetic field is then detected. Due to interference with nearby nuclei and electrons, information on the molecular makeup and structure of a probe can be deduced. While many nuclei can be detected, most commonly used isotopes are $^{1}H$ and $^{13}C$. High-field NMR instruments (≥600 MHz) are needed to provide sufficient sensitivity and spectral resolution. Since sensitivity for $^{1}H$ is highest, one-dimensional $^{1}H$ analyses are preferred in most studies. NMR spectroscopy allows the direct identification, quantification, and structural analysis of small organic molecules, nucleic acids, proteins, and carbohydrates. Since most measured signals may come from aqueous solvents, deuterium ($^{2}H$) is often substituted. With a spin of 1, it does not show up in proton ($^{1}H$, spin 1/2) NMR. NMR signals are calibrated to known peaks, e.g. tetramethylsilane (TMS) for $^{1}H$-NMR. The analysis includes identification of molecules by their specific chemical shift fingerprints and quantification may be done by comparison to peaks of pure standards in validation experiments. The application of NMR spectroscopy to biomarker detection involves extended multivariate statistical analyses, e.g. principal component analysis (PCA) or partial least square-discriminant analysis (PLS-DA).

In addition, the simplicity of sample preparation, low volume requirement (typically a few hundred microliters), non-destructive measurement, and last but not least cost efficiency led to rapid acceptance of NMR in noninvasive diagnostics (Motta *et al.*, 2012; Ibrahim *et al.*, 2013; Nagana Gowda and Raftery, 2015; Soininen *et al.*, 2015). The most outstanding point is that hundreds of metabolites can be analyzed in just one NMR measurement (Duarte *et al.*, 2014). One characteristic of cancer cells is the switch from aerobic oxygen-consuming energy production to glycolytic metabolism, known as the Warburg effect (Warburg, 1956). Changes in glycolytic metabolites and related amino acids are amongst the most promising for cancer detection: high lactate levels indicating enhanced anaerobic energy metabolism; enhanced

serine and glycine levels as result of de novo synthesis of serine via a side branch of glycolysis in highly proliferative cancer cells (Yang and Vousden, 2016). Serine is crucial for the growth and survival of many cancer cells and is closely related to the folate cycle as a donor of one-carbon units. Therefore, the enzymes involved in serine de novo synthesis, i.e. phosphoglycerate dehydrogenase (PHGDH), phosphoserine aminotransferase 1 (PSAT1), and phosphoserine phosphatase (PSPH), may be good targets for therapeutic intervention (Yang and Vousden, 2016).

To significantly outperform current PCa biomarkers and to overcome the shortcomings of prostate specific antigen (PSA) screening, NMR-metabolomics must overcome several challenges. While liquid biopsy, especially urine, is well accepted by patients, standardization is a major issue for quality management concerning sample preparation, measurement, data processing, and statistical analysis. NMR data are highly comparable between different institutions, which is a great advantage and a prerequisite for comprehensive worldwide application (Ward *et al.*, 2010). New tools are under development for multivariate statistics of huge data volumes acquired by NMR and automatic classification of discriminatory from non-discriminatory metabolites (Motegi *et al.*, 2015; Zou *et al.*, 2016).

The workflow for implementation of urine NMR metabolomics into routine PCa diagnostics and treatment monitoring is shown in Fig. 1.

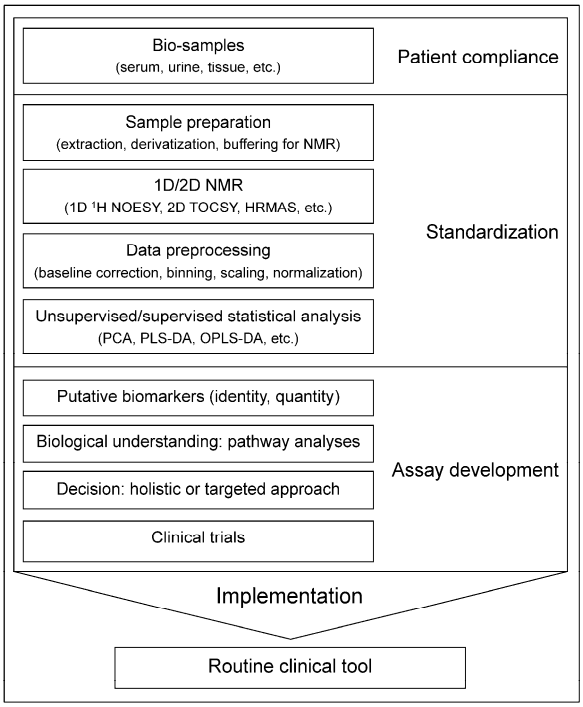


**Fig. 1 NMR-based metabolomics workflow**
Left hand: developmental steps; right hand: challenges. Patient compliance will be best when using urine as a non-invasive source. Standardization is the major challenge: development of a standard platform for comparability across laboratories and time. The major decision in assay development is between the holistic approach (as often preferred in MS/MS analyses or next generation sequencing (NGS)) and targeted metabolite quantification. Clinical trials in different countries with different socio-economic and genetic backgrounds are required for final adjustment of the assay. PCA: principal component analysis; PLS-DA: partial least square-discriminant analysis; OPLS-DA: orthogonal projection to latent structure discriminant analysis

NMR analytics have been used for biomarker detection in humans in several tumor entities other than PCa (Table 2): non-small-cell lung cancer (Doskocz *et al.*, 2015), oral squamous cell carcinoma (OSCC) (Gupta *et al.*, 2015), gastric cancer (Jung *et al.*, 2014), myeloma (Lodi *et al.*, 2013), pancreatic ductal adenocarcinoma (Davis *et al.*, 2013), lung cancer (Carrola *et al.*, 2011), and BCa (Bansal *et al.*, 2013).

### 2.2.1 Squamous cell carcinoma

Serum metabolomics successfully separated patients with oral leukoplakia (OLK; $n$=100) and OSCC ($n$=100) from healthy controls by the $^1$H-NMR technique (Gupta *et al.*, 2015). OSCC and healthy control were accurately separated with high area under the curve (AUC; receiver operating characteristic (ROC): 0.97) according to the expression differences of four biomarkers, namely glutamine, propionic acid ester, acetone, and choline, while the comparative analyses of glutamine, acetone, ethyl acetate, and choline can accurately distinguish OLK and OSCC (ROC: 0.96). The two groups were separated with almost ideal sensitivity and specificity (Gupta *et al.*, 2015).

### 2.2.2 Gastric cancer

In a study on gastric cancer, Chan *et al*. (2016) analyzed 77 metabolites by $^1$H-NMR in urine samples and found a clear gastric cancer specific metabolite profile. LASSO regularized logistic regression (LASSO-LR) identified three discriminatory metabolites: 2-hydroxyisobutyrate, 3-indoxylsulfate, and alanine, which clearly separated gastric cancer from healthy controls (AUC: 0.95). However, benign gastric disease showed large overlap with gastric

cancer, illustrating the major problem of current metabolomic biomarker studies, patient pre-selection (Chan *et al.*, 2016).

2.2.3 Lung cancer

Carrola *et al.* (2011) reported on urine analysis for lung cancer patients using $^{1}$H-NMR combined with PCA, PLS-DA, and orthogonal partial minimum variance discriminant analysis. The results showed that the development process of lung cancer was closely related to potential biological markers, including hippurate, gourd trigonelline, β-hydroxyisovaleric acid, α-hydroxyisobutyric acid, *N*-aceglutamide, and creatine anhydride.

**Table 2 Urine NMR metabolomics in biomarker research**

| Publication year | Cancer entity | Topic | Method | Findings | Reference |
|---|---|---|---|---|---|
| 2015 | Non-small-cell lung cancer | Assessment of toxic kidney's injury | J-RES NMR; focused on amino acids and organic acids (lactid acid and pyruvic acid) profiles | Increase of alanine, leucine, isoleucine, and valine concentrations after the application of cisplatin | Doskocz *et al.*, 2015 |
| 2015 | Oral squamous cell carcinoma | Early stage detection of oral cancer (OC) and oral leukoplakia (OLK) | $^{1}$H-NMR; PCA; OPLS-DA | Accurate separation of OC by glutamine, propionate, acetone, and choline (AUC 0.97; sensitivity 92.7%, specificity 93.8%) | Gupta *et al.*, 2015 |
| 2014 | Gastric cancer | Development of non-invasive screening method | $^{1}$H-NMR; pre- and post-surgery urine; PCA; OPLS-DA | GC predicted with high accuracy (AUC 0.9731); 86% of sensitivity and 92% of specificity; 17 urinary metabolites identified as potential biomarkers | Jung *et al.*, 2014 |
| 2013 | Myeloma | Prediction of risk of disease state | $^{1}$H-NMR; paired blood and urine samples; PCA; PSL-DA | Carnitine and acetylcarnitine potential biomarkers; diagnosis and relapse; pathways may be involved in pathology | Lodi *et al.*, 2013 |
| 2013 | Gastric adenocarcinoma; mouse model | Tumor biomarkers; monitoring of treatment effect (adriamycin) | $^{1}$H-NMR; PCA; PSL-DA | Significantly altered metabolites in tumor model: trimethylamine oxide, hippurate, taurine, 3-indoxylsulfate, trigonelline citrate, trimethylamine, and 2-oxoglutarate | Kim *et al.*, 2013 |
| 2013 | Pancreatic ductal adenocarcinoma | Tumor biomarkers; screening | $^{1}$H-NMR; targeted profiling; PCA; OPLS-DA | Sixty-six metabolites quantified; AUROC (0.988); 21 "key" metabolites | Davis *et al.*, 2013 |
| 2012 | Bladder cancer; canine model | Tumor detection and grading | Spontaneous canine transitional cell carcinoma; $^{1}$H-NMR | Statistical model (PLS-DA) based on 6 metabolites: urea, choline, methylguanidine, citrate, acetone, β-hydroxybutyrate; TCC detection: AUC (0.85), sensitivity (86%), specificity (78%) | Zhang *et al.*, 2012 |
| 2011 | Lung cancer | Tumor detection | $^{1}$H-NMR; PLS-DA; OPLS-DA; Monte Carlo Cross Validation | AUC (0.935); sensitivity (93%); specificity (94%) | Carrola *et al.*, 2011 |
| 2010 | Bladder cancer | Tumor detection and grading | 400 MHz $^{1}$H-NMR; OPLS-DA | Citrate lowered, hippurate elevated in BCa; taurine exclusively detected in BCa; no discrimination of tumor grades | Srivastava *et al.*, 2010 |
| 2010 | Gastric cancer; mouse model | Toxico-metabolomics | $^{1}$H-NMR; PLS-DA; OPLS-DA | Altered in gastric cancer: trimethylamine oxide (TMAO), 3-indoxylsulfate, hippurate, citrate levels, and 3-indoxylsulfate | Kim *et al.*, 2010 |

To be continued

Table 2

| Publication year | Cancer entity | Topic | Method | Findings | Reference |
|---|---|---|---|---|---|
| 2015 | None | New multivariate statistical approach to NMR data analysis | $^{1}$H-NMR spectral dataset analysis of known standard mixtures | Cluster-aided MCR-ALS | Motegi *et al.*, 2015 |
| 2015 | None; hypertensive disorders of pregnancy | Screening for prediction of preeclampsia | Prospective study; $^{1}$H-NMR analysis of urine and serum | Prediction of preeclampsia from urine metabolomic profiles (51.3% sensitivity); hippurate most important metabolite | Austdal *et al.*, 2015 |
| 2015 | None | Monitoring changes of metabolites in urine during diurnal rhythm | $^{1}$H-NMR | Thirty-two metabolites identified; diurnal rhythm (wake-sleep) has significant influence on urine metabolites | Giskeodegard *et al.*, 2015 |
| 2014 | None | Technological optimization | $^{1}$H-NMR | Optimized workflow | Dona *et al.*, 2014 |
| 2014 | None; acute pancreatitis | Phenotyping | $^{1}$H-NMR | Pancreatitis: high levels of urinary ketone bodies, glucose, plasma choline, and lipid, and relatively low levels of urinary hippurate, creatine, and plasma-branched chain amino acids; able to distinguish between cholelithiasis and colonic inflammation | Villaseñor *et al.*, 2014 |
| 2012 | None | Metabolic phenotyping; effects of cadmium exposure | $^{1}$H-NMR; PLS-DA; OPLS-DA | Six metabolites associated with cadmium exposure: citrate, 3-hydroxyisovalerate, 4-deoxyerythronic acid, dimethylglycine, creatinine, and creatine | Ellis *et al.*, 2012 |
| 2011 | None | Diet effects (cruciferous vegetables) on urine metabolomics; cancerogens | $^{1}$H-NMR; J-RES NMR; PLS-DA; OPLS-DA | Four single peaks identified as *S*-methyl-L-cysteine sulfoxide (SMCSO) and 3 other peaks related to SMCSO can serve as biomarkers for putatively cancerogenic cruciferous vegetable diet | Edmands *et al.*, 2011 |
| 2010 | None; autism | Phenotyping; autistic subjects; their siblings, unrelated healthy subjects | $^{1}$H-NMR; J-RES NMR; PLS-DA; OPLS-DA | Creatine, creatinine, glycine, hippurate, NMNA, NMND, PAG, 4-cresol sulfate, succinate, and taurine; indication of differences in microbiota | Yap *et al.*, 2010a |
| 2010 | None; heart disease; stroke | Population metabolic phenotyping: northern vs. southern Chinese population | $^{1}$H-NMR; J-RES NMR; PLS-DA; OPLS-DA; 24 h urine samples | Higher in the north: dimethylglycine, alanine, lactate, branched-chain amino acids (isoleucine, leucine, valine), *N*-acetyls of glycoprotein fragments (including uromodulin), *N*-acetyl neuraminic acid, pentanoic/heptanoic acid, and methylguanidine; higher in the south: hippurate, 4-cresyl sulfate, phenylacetylglutamine, 2-hydroxyisobutyrate, succinate, creatine, scyllo-inositol, proline betaine, and trans-aconitate | Yap *et al.*, 2010b |

PubMed search (access: Aug. 26, 2016) revealed 154 publications of which 29 review articles were identified. Twenty-seven and 38 were original research papers filtered by the additional keywords "urine" and "serum", respectively. Those 65 papers were evaluated and relevant papers were summarized. J-RES: J-resolved; NMR: nuclear magnetic resonance; PCA: principal component analysis; OPLS-DA: orthogonal projection to latent structure discriminant analysis; PSL-DA: partial least-square discriminant analysis; AUC: area under the curve; AUROC: area under receiver operating characteristic curve; TCC: transitional cell carcinoma; MCR-ALS: multivariate curve resolution-alternating least squares; NMNA: *N*-methylnicotinic acid; NMND: *N*-methylnicotinamide; PAG: phenylacetylglycine

#### 2.2.4 Bladder cancer

Only two NMR studies are reported in the literature. One study on serum $^{1}$H-NMR revealed good separation of low- and high-grade BCa from healthy controls (AUC 0.95, sensitivity 96% and specificity 94%) (Bansal *et al.*, 2013). A panel of six metabolites (dimethylamine (DMA), malonate, lactate, glutamine, histidine, and valine) was derived from the orthogonal projection to latent structure discriminant analysis (OPLS-DA) model. Low- and high-grade BCa could be best separated (AUC 0.97) by using a panel of three metabolites (DMA, malonate, and lactate) (Bansal *et al.*, 2013). Another $^{1}$H-NMR study showed altered levels of citrate, DMA, taurine, phenylalanine, and hippurate in BCa compared to healthy controls in urine (Srivastava *et al.*, 2010). However, the authors were not able to differentiate between carcinoma in situ (CIS) and stage Ta or T1 tumors.

#### 2.2.5 Prostate cancer

Kline *et al.* (2006) reported that citrate measured by high-field $^{1}$H-NMR in seminal fluid outperformed PSA in detection of PCa in PCa patients when compared to healthy controls. Recently, Kumar *et al*. (2016b) showed that panels of metabolites from serum can separate benign prostatic hyperplasia (BPH)+ PCa from healthy controls and PCa from BPH. Interestingly, [$^{68}$Ga] citrate was recently successfully used as a radiotracer in positron emission tomography for imaging of PCa in a small study group of castration-resistant prostate cancer (CRPC) ($n$=8), demonstrating the diagnostic potential of newly detected metabolites (Behr *et al.*, 2016).

Giskeødegård *et al.* (2015a; 2015b) analyzed blood plasma and serum samples from 29 PCa patients and 21 controls with BPH by a combination of magnetic resonance spectroscopy, MS, and GC. They could separate PCa from BPH patients with good sensitivity (81.5%) and specificity (75.2%), demonstrating that fatty acids (acyl carnitines), choline (glycerophospholipids), and amino acids (arginine) can be used as metabolic markers for the diagnostic differentiation between PCa and BPH.

Clinical studies have demonstrated that using the NMR metabolic proteomics method to describe the metabolic signature of potential cancer patients and identifying cancer-associated characteristics of early biological markers can help early diagnosis of PCa, while also providing a reference indicator for prognosis and therapeutic effect evaluation (Bertini *et al.*, 2012; Smolinska *et al.*, 2012; Beger, 2013; Emwas *et al.*, 2013; James and Parkinson, 2015).

### 2.3 What is the best sample for NMR cancer metabolomics?

#### 2.3.1 Serum

Serum is the most versatile body fluid and can be used for quantitative NMR metabolic analysis of many different malignancies (Bertini *et al.*, 2012; Zhang *et al.*, 2013; Wang *et al.*, 2013; Armitage and Barbas, 2014; Kumar *et al.*, 2015; Jobard *et al.*, 2015). However, the technical challenges are higher in serum than in urine, since serum has the prospect of possible interference of high abundance metabolites with low abundance target metabolites requiring special fractionation procedures (Ferreiro-Vera *et al.*, 2012).

#### 2.3.2 Seminal fluid

In the case of PCa, seminal fluid has been appreciated as a direct reflection of prostate consistence by the use of ejaculate or expressed prostatic secretions in PCa biomarker research mostly based on proteomics (Drake *et al.*, 2010; Kim *et al.*, 2012; Neuhaus *et al.*, 2013; Principe *et al.*, 2013; Trock, 2014).

However, it is difficult to establish the ejaculate analysis as a routine clinical test due to critical acceptance by the patients. Thus, in consideration of patient compliance, clinical work flow, and technical feasibility, urine is the most promising body fluid for NMR metabolic studies.

#### 2.3.3 Urine

Urine is outstanding in reflecting the health of a patient, since being composed of renal draining urine contains a wealth of biomarkers derived from all organs. Therefore, urine analyses can detect diseases as different as inflammatory bowel disease (Stephens *et al.*, 2013) and Alzheimer's disease (Fukuhara *et al.*, 2013). PCA3 (prostate cancer gene 3) in urine is the only Food and Drug Administration (FDA)-approved urinary biomarker of clinical PCa, while the fusion gene *TMPRSS2 ERG*, α-formyl coenzyme A racemic enzyme (*AMACR*), single nucleotide polymorphism (*SNP*), and others have also been reported and confirmed to have correlations with PCa. Therefore, they

could be rated as potential PCa biomarkers in urine and may find their way into clinical diagnosis and assessment of therapeutic efficacy (Prensner *et al.*, 2012; Salagierski and Schalken, 2012; Salami *et al.*, 2013; Shipitsin *et al.*, 2014; Wei *et al.*, 2014; Bansal *et al.*, 2015; Frantzi *et al.*, 2015). Recently, prostate born exosomes in urine—called prostasomes—have been used as a promising source of biomarkers (Ronquist and Brody, 1985; Duijvesz *et al.*, 2011; Zijlstra and Stoorvogel, 2016). Surprisingly, only one original study on urine metabolomics in the PCa context is available (Öman *et al.*, 2014). As the ultimate product, the metabolite is more stable than DNA and proteins (Patel and Ahmed, 2015). Further standardization of sampling, analysis, and statistical methods will improve the reliability and outcome of NMR-based urine metabolomics (Emwas *et al.*, 2016).

In summary, individual metabolic signatures are likely to develop into valuable tools for the non-invasive detection of diseases, which are characterized by massively altered local or systemic metabolism. NMR technology has evolved into a precise, generally applicable and, at least in high throughput, cost-efficient method (Sokolenko *et al.*, 2013; Mathé *et al.*, 2014). The idea of using a complex mixture of small urine compounds to assay for PCa is the basis of metabolomics in its holistic approach. Interestingly, even organic compounds evaporated from urine can be used for PCa discrimination from BPH by using unspecific multisensor ion mobility spectrometry, supporting the notion of disease-specific urine metabolite composition (Roine *et al.*, 2014). We performed a PubMed search focusing on urine NMR metabolomics and summarized the most relevant papers in Table 2.

## 3 Concluding remarks

Metabolomics, recognized as a new diagnostic technology, has made rapid progress following genomics, proteomics, and transcriptome studies. In a short time span of 20 years, it has achieved broad development and application prospects in the fields of disease diagnosis, research, and development of new drugs, drug mechanisms of action research, and food science. To date NMR-based metabolomics of PCa has focused on serum in a small number of studies. However, the results are encouraging and especially urine metabolomics has the potential to create a new path for the clinical diagnosis and risk stratification of PCa.

In its holistic approach, unlabeled NMR will be able to detect small changes in urine metabolite composition, reflecting disease-specific alterations in cancer-bearing organs or body metabolism. While even at this early stage NMR technology will be able to be used in clinical laboratories, the definition of disease-specific biomarker panels will increase the acceptance and might open new horizons for the development of novel and easy to use detection equipment. There is good support for the notion that NMR technology will be a cost-efficient tool to be also used in PCa risk assessment, prognosis and monitoring of PCa progression and treatment efficacy. Because of its feasibility in large patient cohorts, NMR-based urine analysis bears the promise of uncovering population-specific metabolic peculiarities in Western and Asian populations, thereby helping to understand the differences in PCa prevalence in those countries.

Given the special Chinese situation with growing PCa detection rates and increasing morbidity of the detected PCa, early non-invasive NMR urine diagnostics could completely change the situation of PCa in China, saving tens of thousands of lives each year, resulting in fundamental changes of the diagnosis and treatment of PCa. In contrast, risk assessment, monitoring, and treatment control will be the most promising avenues in Western countries.

### Compliance with ethics guidelines

Bo YANG, Guo-qiang LIAO, Xiao-fei WEN, Wei-hua CHEN, Sheng CHENG, Jens-Uwe STOLZENBURG, Roman GANZER, and Jochen NEUHAUS declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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## 中文概要

**题　目：核磁共振波谱作为提高前列腺癌早期诊断和危险度分级的新方法**

**概　要：**前列腺癌（PCA）是全球第二个最常见的男性癌症，同时也是男性癌症死亡的第五大原因。早期发现和危险度分级是提高前列腺癌患者生存率最有效的方法。目前前列腺癌的生物标志物缺乏足够的敏感性和特异性，而代谢产物作为生物标志物可以作为一种新的提高早期诊断的工具。我们检索了 154 篇出版物，其中 27 篇和 38 篇是分别关于尿液和血清代谢组学分析的原研论文，提示了核磁共振波谱分析是一种很有前景的检测方法，可用于测量复杂的样本中代谢物的浓度，具有良好的重现性、高灵敏度和样本处理的便捷性。特别是基于核磁共振的代谢组学检测尿液已成为检测前列腺癌的早期潜在的危险度分级和监测治疗效果的有效的方法。

**关键词：**前列腺癌；代谢组学；核磁共振（NMR）；生物标志物

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In this paper, we evaluated the use of liquid biopsies as a source of prostate cancer biomarkers. We focused on biomarkers able to discriminate between indolent PC (Gleason score (GS) < 7) from high-grade significant PC with a GS ≥ 7. While the available biomarkers still lack performance in risk stratification of naïve patients, some biomarkers with high negative predictive values may help to reduce unnecessary biopsies. Our study supports the view of the urgent need for novel urine (non-invasive) biomarkers.

diagnostics

MDPI

*Viewpoint*

# Liquid Biopsy Potential Biomarkers in Prostate Cancer

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**Abstract:** Prostate cancer (PCa) is the second most common cancer in men worldwide with an incidence of 14.8% and a mortality of 6.6%. Shortcomings in comprehensive medical check-ups in low- and middle-income countries lead to delayed detection of PCa and are causative of high numbers of advanced PCa cases at first diagnosis. The performance of available biomarkers is still insufficient and limited applicability, including logistical and financial burdens, impedes comprehensive implementation into health care systems. There is broad agreement on the need of new biomarkers to improve (i) early detection of PCa, (ii) risk stratification, (iii) prognosis, and (iv) treatment monitoring. This review focuses on liquid biopsy tests distinguishing high-grade significant (Gleason score (GS) $\geq$ 7) from low-grade indolent PCa. Available biomarkers still lack performance in risk stratification of biopsy naïve patients. However, biomarkers with highly negative predictive values may help to reduce unnecessary biopsies. Risk calculators using integrative scoring systems clearly improve decision-making for invasive prostate biopsy. Emerging biomarkers have the potential to substitute PSA and improve the overall performance of risk calculators. Until then, PSA should be used and may be replaced whenever enough evidence has accumulated for better performance of a new biomarker.

**Keywords:** prostate cancer (PCa); biomarkers; liquid biopsy; diagnosis high-grade PCa; risk stratification; risk calculators

## 1. Introduction

Prostate cancer (PCa) is the second most common cancer in men worldwide, with an incidence of 14.8% (estimated at 1.1 million in 2012) and a mortality of still 6.6% (an estimated 307,000 deaths in 2012), which is at 5th rank among cancers. The five-year prevalence of 148.6/100,000 is the highest seen in all cancers in adult men [1].

There is a significant variation of incidence, with the highest rates seen in Western and Northern Europe, Australia/New Zealand, and Northern America and the lowest rates in Asian countries, which may be partly due to the comprehensive use of prostate-specific antigen (PSA) testing and close monitoring of the male population in more developed regions. As recently reported in China, the poor coverage of PSA monitoring seems to be the major cause of advanced PCa at first diagnosis, translating into a high mortality of 44% in China compared to 23% in the EU and 14% in the USA [2–4].

In this review we will evaluate the performance of available and emerging biomarkers in risk stratification, focusing on liquid biopsies and distinguishing high-risk Gleason score (GS) $\geq$ 7 tumours from indolent, low-risk tumours, especially in biopsy-naïve patients.

## 2. Current PCa Diagnostics

At present, the recommendations of the American Urological Association (AUA) and the European Association of Urology (EAU) guidelines for the clinical examination methods of prostate cancer include digital rectal examination (DRE), rectal ultrasound (TRUS) examination, and prostate biopsy [5–7].

Biopsy is still the only standard in preoperative diagnosis. This invasive operation causes obvious physical pain to patients and the detection rate was closely related to the number of puncture points and prostate volume, and is likely to cause haematuria, urinary retention or infection, sepsis, and other serious complications leading to distress. At the same time, DRE and TRUS have disadvantages such as poor accuracy and are affected by the skill and experience of the operator and other subjective factors. For instance, 24% of 126 patients with suspected DRE had no PCa on biopsies in a prospective study by Leyten and co-workers [8].

In addition, all three, DRE, TRUS and biopsy, are inevitably faced with an important issue, namely low sensitivity to early cancer, after which the patient has missed the most ideal treatment time [9–11].

As proposed by Dr TA Stamey in the early 1980s, PSA diagnostics rapidly developed into comprehensive screening programs in the USA and worldwide [12]. PSA population screening in 6260 Americans showed a significant decrease of high- and intermediate-risk patients from 68.9% to 52.3% and an increase of low-risk disease from 31.2% to 47.7% between 1989 and 2002 [13]. PSA screening caused prostate cancer mortality to fall by 21%, demonstrated in the 13-year follow-up of the European Randomised Study of Screening for Prostate Cancer (ERSPC), indicating that early diagnosis and treatment of prostate cancer were indeed significantly improved by the popularity of serum PSA screening [14–16].

However, in the past decade more and more evidence accumulated challenging the benefit of extensive PCa screening programs. Comprehensive PSA screening, in particular, may lead to significant overdiagnosis and overtreatment [17–19]. Overdiagnosis and unnecessary prostate biopsies were calculated to occur in 23–42% in PSA screening programs [20]. Thus, there are considerable concerns about the predictive value of PSA levels, PSA density, and even first biopsy in the group of patients eligible for active surveillance following the recommendations of the EAU [21].

One major problem of PSA screening is the different cutoff values used. The threshold of 4.0 ng/mL (commonly used with the Tandem-R, Hybritech test) has to be revised based on the findings of 15.2% PCa in men with PSA $\leq$ 4 ng/mL in a cohort of 2950 men (aged 62–91 years) by Thompson and co-workers [22]. A considerable 14.9% of those were diagnosed with high-grade (GS $\geq$ 7) tumours. Even at very low PSA levels of $\leq$0.5 ng/mL, they found 6.6% PCa, of which 12.5% were high-grade tumours [22]. These findings clearly illustrate the diagnostic shortcomings of PSA.

Age-adjusted PSA cutoffs were tested to improve performance. While age-adjusted PSA cutoffs for total serum PSA (tPSA) and complexed serum PSA (cPSA) were superior to a fixed cutoff in a cohort of 3597 men who underwent routine biopsy, they could not improve the PCa detection rate of approximately 39% within the range of 2.0 ng/mL–20.0 ng/mL [23].

In light of those diagnostic restrictions, there is still an ongoing dispute on PSA testing worldwide. In the USA, PSA screening was evaluated by the U.S. Preventive Services Task Force (USPSTF), leading to amendment of the recommendations on PSA screening in 2008 [24]. The American Urological Association (AUA) stated that PSA screening should apply only to men aged 55 to 69 years and suggested that routine examination be performed once every two years or longer [7,18,25].

However, this diagnostic practice may significantly increase the risk of missing PCa in men <55 years [26] and might increase mortality, which has to be analysed after longer follow-up [27,28]. Nevertheless, recent data analysis shows a continuous increase in the morbidity of biopsies in conjunction with reduction of total biopsies since the 2008 USPSTF recommendations [29]. Amongst the many attempts to solve this diagnostic dilemma, only a few biomarkers are established in clinical practice.

In summary, to date no single serum or urine biomarker or biomarker panel meets the requirements for highly sensitive and specific detection of PCa and differentiation between indolent

and significant PCa. Imaging technologies have been greatly improved but still are not sufficiently validated or standardized. Some of the diagnostic tools are already established, such as PSA and its derivatives; others are under critical evaluation and some are exploring the potency of the latest high-end analytics to improve (i) early detection of PCa, (ii) risk stratification, (iii) disease prognosis, and (iv) treatment monitoring.

## 3. Current PCa Biomarker Tests for Discrimination-Significant and Indolent PCa

The need for better PCa diagnostics has led to a huge number of new strategies for meaningful combinations of established and innovative approaches to open up new biomarker resources. Table 1 gives an overview of commercially available biomarker tests, using liquid biopsy for the detection of high-grade (≥GS 7) PCa and assisting the physician with identifying patients for prostate biopsy and those eligible for active surveillance (for detailed information and more specialized tests, see [30]). Table 1 also demonstrates that PSA cutoff values vary between studies. Age-adjusted use of PSA cutoff values could significantly improve the sensitivity of PSA testing [23].

### *3.1. Prostate-Specific Antigen (PSA)*

PSA, alone or in combination with free/total PSA (f/t PSA) ratio, formerly thought to be of value for distinguishing PCa from BPH, shows only limited sensitivity and insufficient specificity (Table 1; recent meta-analysis by Huang et al. [31]).

Longitudinal PSA screening to determine PSA velocity has been initially described to distinguish PCa from BPH in men aged >60 years at an average rate of change (ng/mL per year) of ≥0.75 with 90% specificity compared to 60% single PSA value ≥4 ng/mL [32]. However, in the following studies these promising results could not be confirmed [31].

**Table 1.** PCa biomarker tests for prediction of high-grade PCa (GS $\geq$ 7).

| Biomarker(s) | Source | Commercial Product | Predict | Avoid Biopsies | Sens. | Spec. | AUC | PPV | NPV | Targeted Patients | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| PSA | blood (serum) | **Tandem-R®** monoclonal immunoradiometric assay (Hybritech Inc., San Diego, CA, USA) | PCa on first biopsy | n.a. | 79% at PSA $\geq$ 4 ng/mL | 59% at PSA $\geq$ 4 ng/mL | 0.64 | 40% | 89% | age > 50 years PSA $\geq$ 4 ng/mL | [33] |
| PSA | blood (serum) | **Tandem-R®** (Hybritech) | PCa (vs. BPH/Controls) | n.a. | 78% at PSA $\geq$ 4 ng/mL | 60% (PCa vs. BPH); 94% (PCa vs. Control at PSA $\geq$ 4 ng/mL | n.r. | n.r. | n.r. | age > 60 years | [32] |
| PSA velocity (0.75 ng/mL/year) | blood (serum) | **Tandem-R®** (Hybritech) | PCa (vs. BPH/Controls) | n.a. | 72% at PSA $\geq$ 4 ng/mL | 90% (PCa vs. BPH); 100% PCa vs. Control: at PSA $\geq$ 4 ng/mL | n.r. | n.r. | n.r. | age >60 years | [32] |
| PSA | blood (serum) | **Access Hybritech®** | Risk of GS $\geq$ 7 | n.a. | 90% at PSA $\geq$4.3 ng/mL | 9% at PSA $\geq$ 4.4 ng/mL | 0.55 | n.r. | n.r. | age $\geq$ 50 years PSA 4–10 ng/mL, neg. DRE | [34] |
| fPSA/tPSA | blood (serum) | n.r. | PCa (vs. BPH) | n.a. | 70% (pooled data) | 58% (pooled data) | 0.76 (pooled data) | 41% | 86% (1) | PSA 4.0–10.0 ng/mL | meta-analysis [31] |
| PHI (p2PSA/fPSA $\times$ $\sqrt{}$tPSA) | blood (post-DRE serum) | **PHI**, prostate health index Beckman Coulter, Atlanta, GA, USA) | Risk of GS $\geq$ 7 | n.r. | 90% (pooled data) | 17% (pooled data) | 0.67 (pooled data) | n.r. | n.r. | age $\geq$ 50 years PSA 4–10 ng/mL, neg. DRE | meta-analysis [35] |
| p2PSA/fPSA (%p2PSA) | | **PHI**, prostate health index Beckman Coulter, Atlanta, GA, USA) | Risk of GS $\geq$ 7 | n.r. | 96% (pooled data) | 9% (pooled data) | 0.54 (pooled data) | n.r. | n.r. | age $\geq$ 50 years PSA 4–10 ng/mL, neg. DRE | meta-analysis [35] |
| PHI (p2PSA/ fPSA $\times$ $\sqrt{}$tPSA) | blood (post-DRE serum) | **PHI**, prostate health index Beckman Coulter, Atlanta, GA, USA) | Risk of GS $\geq$ 7 | 30.1% | 90% (cutoff 29.8) | 30% (cutoff 29.8) | 0.71 | n.r. | n.r. | age $\geq$ 50 years PSA 4–10 ng/mL, neg. DRE | [34] |
| intact PSA, free PSA, total PSA, kallikrein-related peptidase 2 (hK2) | blood (post-DRE serum) | **4KScore®** Test (OPKO Lab, Nashville, TN, USA) | Risk of GS $\geq$ 7 | 43% | n.r. | n.r. | 0.82 | n.r. | n.r. | PSA $\geq$ 3 ng/mL; | [36] |
| expression of 8 auto-antibodies against: CSNK2A2, cestrosomal protein 164 kDa, NK3 homeobox 1, aurora kinase interacting protein 1,5′-UTR BMI1, ARF6, chromosome 3′-UTR region Ropporin/RhoEGF, desmocollin 3 | blood (serum) | **Apifiny®** (Armune Bioscience, Kalamazoo, MI, USA) | Risk of GS $\geq$ 7 | n.r. | 60% at PSA > 4 ng/mL [37] | 69% at PSA > 4 ng/mL [37] | 0.69 at PSA > 4 ng/mL [37] | 30% [37] | 89% [37] | PSA $\geq$ 2.5 ng/mL, initial biopsy | [37,38] |

**Table 1.** *Cont.*

| Biomarker(s) | Source | Commercial Product | Predict | Avoid Biopsies | Sens. | Spec. | AUC | PPV | NPV | Targeted Patients | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| prostate cancer gene 3 (PCA3) + PSA mRNA ratio | post-DRE urine | **Progensa**™ (Gen-Probe Inc., San Diego, CA, USA) | PCa | n.r. | 58% [39]<br>78% [40]<br>76% [41] | 72% [39]<br>57% [40]<br>52% [41] | 0.68 [39]<br>n.r. [40]<br>0.80 [41] | n.r. [39]<br>34% [40]<br>n.r. [41] | n.r. [39]<br>90% [40]<br>88% [41] | age $\geq$ 50 years neg. prior biopsy, repeat biopsy | [39–41] |
| exosomes (EV) + (SOC: prostate-specific antigen level, age, race, family history); gene expression (targets revealed): SPDEF, ERG and PCA3 | urine | **ExoDx**® Prostate IntelliScore urine exosome assay (Exosome Diagnostics, Inc., Waltham, MA, USA) | Risk of GS $\geq$ 7 | n.r. | 92% | 34% | 0.73 | 36% | 91% | PSA 2–20 ng/mL, initial biopsy | [42] |
| serum PSA + urine PCA3 mRNA + urine TMPRSS2:ERG mRNA | blood (serum); post-DRE urine | **Progensa**™ (Hologic, Bedford, MA, USA); **MiPS** test; University of Michigan (MLabs) | Risk of GS $\geq$ 7 | 35–47% | n.r. | n.r. | 0.77 (PSA + T2:ERG + PCA3 | n.r. | n.r. | elevated PSA (initial biopsy), prior negative biopsy (repeat biopsy) | [43] |
| HOXC6 mRNA + DLX1 mRNA + serum PSA + PSA density + DRE status + age + family history | post-DRE urine | **SelectMDx** (MDx Health, Irvine, CA, USA) | Risk of GS $\geq$ 7 | 42% of total; 53% of unne-cessary biopsies | 91% (HOXC6 + DLX1) | 36% (HOXC6 + DLX1) | 0.76 (HOXC6 + DLX1); 0.90 + clin. Para-meters | 28% | 98% | PSA > 4 ng/mL; negative index biopsy | [44] |
| STHLM3 risk-based model: PSA, fPSA, iPSA, hK2, β-microseminoprotein (MSMB), macrophage inhibitory cytokine 1 (MIC1), genetic polymorphisms [232 SNPs], age, family history, previous prostate biopsy, DRE, prostate volume | blood | various | Risk of GS $\geq$ 7 | 32% biopsies (GS $\geq$ 7); 44% benign biopsies | n.r. | n.r. | n.r. | n.r. | n.r. | PSA $\geq$ 3 ng/mL; age 50–69 years; highly selected patients; validation in standard populations needed | [45] |

Abbreviations: sensitivity (sens.); specificity (spec.); receiver-operation-characteristics (ROC) area under the curve (AUC); positive predictive value (PPV); negative predictive value (NPV); benign prostate hyperplasia (BPH); Gleason score (GS); free PSA (fPSA); total PSA (tPSA); not applicable (n.a.); not reported (n.r.). [(1)] calculated by authors from Table 1 in Huang et al., 2018 [31].

### *3.2. Prostate Health Index and Derivates*

The Prostate Health Index (PHI Beckman Coulter, Atlanta, GA, USA) was FDA-approved in 2012. The PHI score combines total, free, and [–2] proPSA in one test and a score is calculated indicating the probability of PCa positive biopsy (phi-score = [−2] proPSA/fPSA) × $\sqrt{}$tPSA) [46].

In an early meta-analysis, AUCs of %[–2] proPSA and PHI were comparable in patients with PSA values of 2–10 ng/mL, reaching between 0.76 and 0.78 for prediction of PCa-positive biopsies [47]. As recently analysed by White and co-workers, PHI alone impacts the decision-making of physicians and resulted in a significant reduction in biopsies of 40% [48]. However, the PHI score was not very good at predicting high-grade PCa and as such did not help with clinical decision-making in a large study using pT3 stage and/or GS $\geq$ 7 as outcome measures [49] (see also Table 1 [35]).

### *3.3. 4KScore® Test*

Promising data have also been reported for the 4KScore® Test (OPKO Lab, Nashville, TN, USA) using 4-Kallikrein markers in blood serum after DRE. In a recent study including 496 participants with PSA $\geq$ 3.0 ng/mL, the accuracy of predicting PCa GS $\geq$ 7 was AUC 0.738 in the standard model (PSA + age) and AUC 0.820 in the advanced model integrating 4KScore® Test ($p < 0.001$). In a model with 6% cutoff, the risk calculated by age and 4KScore® would avoid 43% of biopsies, detect 119 of 133 (89.5%) GS $\geq$ 7 high-risk tumours, and delay diagnosis of 14 (10.5%) of the significant tumours [36].

### *3.4. Progensa™ (Gen-Probe Inc., San Diego, CA, USA)*

The prostate cancer gene 3 (PCA3) test detects long non-coding RNA (lncRNA), which has been shown to be associated with PCa. The Progensa™ PCA3-score calculates the ratio of PCA3 and PSA mRNA of exosomes isolated from post-DRE urine and has been proposed for the identification of patients eligible for active surveillance [50]. It was FDA-approved in 2012 and several studies reported variable performance (sensitivity: 58–78%; specificity: 57–72%) for detection of PCa (Table 1) [39–41]. The NPV was reported to be 88% and 90%, respectively, which was regarded as helpful for biopsy decision-making [40,41]. However, as pointed out by Vickers and co-workers, PCA3 was approved by the FDA only to add the decision of repeat biopsy. In biopsy-naïve patients, there is a high risk of missing high-grade PCa with low levels of PCA3 [51].

### *3.5. Further Non-Commercial and Integrative Tests*

Several other tests are already available for risk evaluation in patients with elevated PSA, usually at a threshold of $\geq$4 ng/mL (Table 1). While their positive predictive value is low (28–36%), those tests showed highly negative predictive values (88–98%), which make them valuable for clinical decision-making on invasive biopsy diagnostics. Tomlins et al. reported avoidance of 35–47% of biopsies using the MiPS test (University of Michigan, MLabs) [43]. Van Neste calculated that 42% of total biopsies and 53% of unnecessary biopsies can be avoided by combining the SelectMDx (MDx Health, Irvine, CA, USA) measuring HOXC6 mRNA and DLX1 mRNA in post-DRE urine with serum PSA, PSA density, DRE status, age, and family history [44].

As summarized in Table 1, more or less critical restrictions regarding the target patients apply to most biomarker test available. Only a few are useful in a broad clinical setting, as required for screening or routine check-up examinations, i.e., for application in biopsy-naïve patients. Especially for the detection of critical high-risk patients ("risk of Gleason score $\geq$ 7") who need to undergo prostate biopsy, the specificity of the available tests is poor. For detailed information, the reader is referred to the literature given in Table 1.

## 4. Do We Need More Biomarkers, or Do We Need a New, Consistent Concept?

In view of the huge number of different biomarkers available and new approaches, one has to ask whether there is a realistic chance that these advanced methods will finally provide a set of biomarkers

able to meet all requirements in PCa detection, stratification, and monitoring. Most probably, we will need a combination of specialized biomarkers with good performance within their restricted fields. The primary goal should be to reduce the need for invasive prostate biopsies to improve the benefit-harm balance.

An ideal biomarker concept should support low-invasive, organ-saving treatments if possible; radical surgery if necessary at the earliest time point to avoid PCa progression to metastatic and androgen-insensitive disease [52].

## 5. Emerging Biomarkers for Detection of Significant PCa

### *5.1. Polypeptides*

Seminal plasma is a body fluid directly related to the prostate. Therefore, PCa specific analytes are expected to be available at higher concentrations and better accessible than in urine or blood. An in-depth proteome analysis of expressed prostatic secretions (EPS) was conducted in 2010 by Drake and co-workers to provide a resource for the development of biomarkers [53]. In a small cohort of patients with advanced ($n$ = 8) or organ-confined ($n$ = 8) prostate cancer, a total of 624 unique proteins were identified in EPS by mass spectrometry [54]. Fourteen candidates with 133 differently expressed proteins were further analysed for suitability as biomarkers, including PSA and PAP, which were significantly elevated in organ-confined PCa. The authors concluded that EPS-urines are a promising source for new PCa biomarkers [54].

In a multicentre, open-label case/control study our group analysed 125 patients for PCa-specific polypeptides in seminal plasma from fresh ejaculate donation after physiological liquefaction. The idea was to create stable conditions reflecting the enzymatic activity of pathological protease network in PCa, and to analyse the resulting protein fragments, $\leq$20 kDa polypeptides by capillary-electrophoresis mass spectroscopy (CE-MS). We were able to define a panel of 11 polypeptides from seminal plasma-based CE-MS analysis with 80% sensitivity at 82% specificity in discriminating patients with GS 7 and organ-confined (<pT3a) or advanced disease ($\geq$pT3a) [55].

Proteomic signatures of polypeptides have also been used to detect PCa in the urine of biopsy-naïve patients without known PCa or suspect DRE [56]. The biomarker panel of 12 polypeptides detected PCa with 89% sensitivity and 51% specificity (AUC 0.70). By inclusion of age and fPSA, the performance was augmented to 91% sensitivity and 69% specificity [56]. Unfortunately, the data were not analysed for prediction in high-risk patients.

### *5.2. Metabolites*

Metabolites were shown to closely reflect aggressiveness of PCa [57]. A prospective study including 1122 cases tested the performance of sarcosine to predict the risk of prostate cancer. This study revealed an association of serum sarcosine levels normalized to alanine with low-grade (non-aggressive) PCa but no association with aggressive PCa [58], and a recent study showed that sarcosine is not indicative of PCa in urine [59].

Post-DRE urine pellet is used as a source for metabolites to predict high-grade PCa (GS $\geq$ 7) in the Polarix® test (Metabolon Inc., Morrisville, NC, USA). In a retrospective study, McDunn and co-workers identified metabolites associated with the aggressiveness of a tumour and constructed a panel of four metabolites (5,6-dihydrouracil, choline phosphate, glycerol, and methylpalmitate) predicting the probability of organ-confined PCa with an accuracy of AUC = 0.62. Using a panel of three metabolites (7-a-hydroxy-3-oxo-4-cholestenoate, pregnen-diol disulfate, and mannosyl tryptophan), they were also able to improve the prediction of progression-free survival to AUC = 0.64 [57]. While these results are promising, the performance of urine metabolites is still not satisfying. However, the study provides the basis for further development of metabolite biomarkers.

Most interestingly, PCa-specific metabolites have been found in urine exosomes, implying potential use as a new biomarker source to address PCa pathogenesis and progression. Out of 248 metabolites identified, 76 were differentially expressed in PCa and BPH [60].

Metabolomics is a hot topic in current biomarker research. However, so far, even large studies did not successfully identify meaningful metabolites [61].

### *5.3. MicroRNA (miRNA)*

MiRNAs have been acknowledged to be important for gene regulation in normal and pathological conditions. Based on tissue analyses, dozens of miRNAs have been shown to be dysregulated in PCa (see [62,63]).

In a comprehensive screening study using radical prostatectomy samples of 34 patients, 34 miRNA were significantly upregulated in the tumour epithelium compared to normal epithelium [64]. The authors also compared GS 6 PCa with high-grade GS $\geq$ 8 PCa tissue. They found 18 differentially expressed miRNAs ($p < 0.005$): 11 were up- and seven were downregulated (Table 2).

Schaefer et al. reported five upregulated and 10 downregulated miRNAs during miRNA microarray analysis of 76 radical prostatectomy specimens comparing matched tumour and adjacent normal tissues [65]. The expression of five miRNAs correlated with Gleason score, and upregulated miR-96 predicted biochemical recurrence (Table 2).

**Table 2.** Micro RNAs in prostate cancer diagnosis.

| **Reference** | **Song et al. 2018 [62]** | | **Schaefer et al. 2010 [65]** | | **Walter et al. 2013 [64]** | |
|---|---|---|---|---|---|---|
| **Type** | **Meta-Analysis of 104 Studies** | | **Original Article** | | **Original Article** | |
| **Samples** | **Tissue, Blood, Urine** | | **RPE Frozen Tissue (76 PCa, 79 PCa)** | | **FFPE RPE Tissue (37 PCa)** | |
| **Method(s)** | **Various** | | **miRNA Microarray; 470 miRNAs** | | **PCR Array Profiling** | |
| **Measure** | **Expression in PCa** | | **Expression in PCa** | | **Expr. in GS $\geq$ 8 vs. GS 6** | |
| | miR-1 ↓ | a | miR-16 ↓ | | miR-9 ↑ | i |
| | miR-18a ↑ | a | miR-31 ↓ | j | miR-27 ↓ | i |
| | **miR-21 ↑** | **c,l** | miR-96 ↑ | e,g,j | **miR-30c ↑** | **h,l** |
| | miR-23b ↓ | a | miR-125b ↓ | k | miR-34 ↑ | i |
| | miR-27b ↓ | a | miR-145 ↓ | | miR-92 ↓ | i |
| | miR-30c ↓ | a,c | miR-149 ↓ | e | miR-96 ↓ | i |
| | miR-31 ↑ | b | miR-181b ↓ | | miR-122 ↑ | h,i |
| | miR-34a ↑ | a | miR-182 ↑ | e | miR-125a ↑ | h |
| | miR-99b ↓ | a | miR-182 * ↑ | | miR-125 ↓ | i |
| | miR-106b ↑ | a | miR-183 ↑ | f | miR-126 ↓ | i |
| | miR-129 ↓ | c | miR-184 ↓ | | miR-138 ↑ | i |
| | miR-139-5p ↓ | a | miR-205 ↓ | e,f,j,k | miR-144 ↑ | i |
| | **miR-141 ↑** | **a,l** | miR-221 ↓ | | miR-146b-5p ↑ | h |
| | miR-145 ↓ | c | miR-222 ↓ | k | miR-148 ↓ | i,m |
| | miR-152 ↓ | a | **miR-375 ↑** | **e,l,m** | miR-181a ↑ | h |
| | miR-182 ↑ | a | | | miR-181c ↑ | h |
| | miR-183 ↑ | a | | | miR-184 ↑ | h,i |
| | miR-187 ↓ | a | | | miR-193 ↑ | i |
| | miR-200a ↑ | a | | | miR-193b ↑ | h |
| | miR-200b ↑ | a | | | miR-198 ↑ | i |
| | miR-204 ↓ | a | | | miR-214 ↑ | h |
| | miR-205 ↓ | a | | | miR-215 ↑ | i |
| | miR-224 ↓ | a | | | miR-222 ↓ | i |
| | miR-301a ↑ | a | | | miR-335 ↑ | h,i |
| | **miR-375 ↑** | **a,d,l,m** | | | miR-373 ↑ | i |
| | miR-452 ↓ | a | | | | |
| | miR-505 ↓ | a | | | | |
| | let-7c ↓ | a,b,c | | | | |

FFPE = formalin-fixed paraffin-embedded; TURP = transurethral resection of the prostate; RPE = radical prostatectomy; a = differentiate PCa from BPH/HC; b = differentiate advanced metastatic from local/primary PCa; c = prediction of poor recurrence free survival; d = worse overall survival; e = AUC of 0.88 combining 5 miRNAs; f = AUC of 0.88 combining two miRNAs; g = can predict biochemical recurrence; h = $p < 0.005$ in PCa vs. normal epithelium; i = differentiate GS $\geq$ 8 from GS 6; j = correlation with Gleason score; k = correlation with tumour stage; l = of diagnostic value in serum; m = of diagnostic value in urine; * = indicates reverse miRNA sequence; ↑ = upregulated; ↓ = downregulated.

However, Stephan et al.'s study of miR-183 (upregulated) and miR-205 (downregulated) failed to detect high-grade PCa in patients with and without PCa (38 each group) using urine sediment, while PCA3 was able to separate those patient groups [66]. In a recent meta-analysis, Song et al. identified from an extensive literature survey 10 upregulated and 14 downregulated miRNAs with potential for separating PCa from BPH or normal controls (Table 2). Furthermore, high expression of miR-32 and let-7c differentiated local from metastatic PCa. The authors also found that the expression profiles of urine, blood serum, and tissue differed considerably [62].

Circulating miRNAs were isolated from various body fluids, including blood plasma and serum being protected against ribonuclease degradation by inclusion in lipid compartments, extracellular vesicles of 40–5000 nm in diameter [67].

In serum, miR-141 levels can distinguish PCa from healthy controls with an AUC of 0.907 with 60% sensitivity at 100% specificity in a cohort of 25 PCa and 25 age-matched healthy control individuals [68]. In a recent study Porzycki et al. found that the combination of miR-141, miR-21, and miR-375 could distinguish PCa (mean PSA of 21.3 ng/mL) from healthy controls with an AUC of 0.864 and a sensitivity of 93% at 63% specificity. However, the group sizes were quite small (20 PCa vs. eight healthy controls), requiring further validation of the findings in larger cohorts [69].

Recently, Tinay et al. found a significant upregulation of miR-9-3p, miR-330-3p-3p, and miR-345-5p in PCa patients ($n$ = 25) compared to healthy controls ($n$ = 20). MiR-345-5p was further analysed due to its direct targeting of CDKN1A encoding the cyclin-dependent kinase inhibitor p21 [70]. Interestingly, the overlap between the miRNAs in serum with tissue miRNAs is limited to miR-21, miR-141, and miR-375 (boldface and labelled "l" in Table 2).

In urine, PCa-specific miRNAs patterns can be detected in exosomes by next-generation sequencing (NGS) and RT-qPCR. For example, miR-196a-5p and miR-501-3p levels were significantly downregulated in a preliminary study of 28 PCa (GS $\geq$ 7) vs. 19 healthy controls [71]. In a larger study, including 215 PCa patients, 23 BPH patients, and 62 asymptomatic control individuals, Stuopelyte et al. found 100 out of 754 miRNAs scanned deregulated in PCa. MiR-148a and miR-375 were the most abundant miRNAs in urine and showed high sensitivity and specificity (85.31% and 65.22%, respectively), with an AUC of 0.79 in differentiation between PCa ($n$ = 72) and BPH ($n$ = 23). In combination with serum PSA, the AUC was 0.85, with 84.29% sensitivity at 76.19% specificity. Within the grey zone PSA levels of 4–10 ng/mL AUC increased to 0.90 with 83.87% sensitivity at 81.82% sensitivity [72]. Overlapping miRNAs were in boldface and labelled "m" in Table 2.

None of the current miRNA approaches provide high PPV for the detection of high-grade PCa. The largest NPV of 0.939 has been reported to predict the absence of high-grade PCa compared to BPH for a 14-miRNA panel: miR-24, -26b, -30c, -93, -100, -103, -106a, -107, -130b, -223, -146a, -451, -874, and let-7a [73].

The high number of miRNAs found to be dysregulated in PCa and the ability of subsets to either detect PCa, differentiate high-grade from low-grade PCa, or predict recurrence-free/overall survival encourages further attempts to define miRNA biomarker panels. Most interestingly, the overlap between tissue and liquid biopsies is rather limited. This problem has to be investigated more deeply.

### *5.4. Gene Expression of PCa-Related Genes in Exosomes*

Exosomes can be used to measure gene expression of PCa-related genes (among others: SPDEF, ERG and PCA3). Combined into a score (ExoDx Prostate IntelliScore urine exosome assay (Exosome Diagnostics, Inc., Waltham, MA, USA)) with standard of care parameters: prostate-specific antigen level, age, race, and family history [42], this score was able to predict high-risk PCa, defined as GS $\geq$ 7, with a sensitivity of 92%. However, specificity was low (34%) resulting in a positive predictive value (PPV) of only 36%. In contrast, the negative predicted value (NPV) was high (91%), thereby unnecessary biopsies could have been avoided in 27%, missing only 5% of patients with high-risk PCa

(GS 4 + 3) [42], indicating a considerable clinical value of tests providing high NPV, even if the PPV is low (Table 1).

### *5.5. Long Non-Coding RNA (lncRNA)*

Non-coding RNA makes up the vast majority of our genetic information. Only <3% of the human DNA comprises protein-coding gene sequences. LncRNAs are key regulators of the genome and their involvement in several disease states, especially cancer has recently emerged [74–76]. Thus, lncRNAs are regarded as promising biomarkers as well as therapeutic targets in urologic cancers [77,78]. Several biomarker tests including lncRNA (PCA3) are already on the market (Table 1).

Recently investigated promising lncRNAs include: TINCR [79], FR0348383 [80], SChLAP1 [81], and MALAT1 [82] (Table 3). LncRNA biomarker research is rapidly expanding and more lncRNA biomarkers are likely to emerge in the near future [83,84].

**Table 3.** Long non-coding RNAs (lncRNA) potential biomarkers.

| Name | Function | Diagnostic Value | Reference |
|---|---|---|---|
| PCA3 ↑ (prostate cancer associated 3) | increase of cell proliferation, migration and invasion; inhibition of apoptosis; [85] | predict risk of GS > 7 | [86] |
| TINCR ↓ (Terminal differentiation induced non-coding RNA) | growth inhibition via TRIP13 suppression [79] | not determined | [79] |
| FR0348383 ↑ | unknown | predict PCa-positive biopsy; avoid 52% unnecessary biopsies without missing high-grade PCa | [80] |
| SChLAP1 ↑ (SWI/SNF complex antagonist associated with prostate cancer 1) | increase of cell proliferation, metastasis via downregulation of miRNA-198 and activation of MAPK1 pathway [87] | predict high-risk, lethal PCa; biochemical recurrence after RPE | [81] |
| MALAT1 ↑ (metastasis-associated lung adenocarcinoma transcript 1) | interacts with EZH2, promoting proliferation and invasion [88] | predict PCa-positive biopsy; discriminate between PCa and BPH, PCa and HC | [82] |

RPE = radical prostatectomy; HC = healthy controls; ↑ = upregulated; ↓ = downregulated.

### *5.6. Circulating Tumour Cells (CTC)*

At the moment circulating tumour cells (CTC) are not used for early detection of high-grade PCa because they are rarely detected in localized PCa [89–92]. CTCs are being investigated for use as a prognostic biomarker of mCRPC and to predict treatment efficacy [93–96]. However, the first long-term follow-up studies have questioned the prognostic value of preoperative CTCs for the prediction of early biochemical recurrence. Meyer et al. detected CTCs in only 11% (17/152) of patients before radical prostatectomy. The CTC counts did not correlate with PSA levels, disease status, or biochemical recurrence [97]. Recently, Murray et al. concluded that the biological characteristics of circulating prostate cells (CPCs) may be more important than the number of circulating cells. They found that patients with CD82-negative CPCs had a worse prognosis in a study of 285 men at a follow-up of 10 years. CD82 is a tumour suppressor and the expression on CPCs may indicate high metastatic potential [98].

## 6. Integrative Scoring Systems/Risk Calculators

The overall goal of all biomarkers is the improvement of the prediction of the individual risk of the patient. To this end, standard null hypothesis significance testing (NHST) methods are not conducive, since they do not report quantitative percent individual risk evaluation. Bayesian data analysis can overcome this weakness and provide direct access to meaningful risk evaluation. Risk calculators such as the online Prostate Cancer Prevention Trial Risk Calculator (PCPTRC) developed in 2006 predicting the likelihood of detecting no versus low-grade (GS < 7) versus high-grade (GS $\geq$ 7) in a biopsy for an individual patient can be continuously adjusted on the basis of newly available epidemiologic

data [99–101]. New biomarkers replacing lower-performing ones may be included when available, thus continuously improving the performance of those calculators. Several complex scoring models for risk assessment of PCa GS $\geq$ 7 have been developed, including population adaption: the Stockholm model 3 (STHLM3) [45], the Rotterdam Prostate Cancer Risk Calculator (RPCRC) [102], the Indonesian prostate cancer risk calculator (IPCRC) [103], a Chinese (Hong Kong) adaptation of the ERSPC risk calculator [104], the Huashan risk calculator [105], and the Chinese Prostate Cancer Consortium Risk Calculator (CPCC-RC) [106].

## 7. Conclusions

In this review we focused on the liquid biopsy biomarkers currently in use and emerging for distinguishing patients with low, insignificant PCa from patients with high-risk PCa with a Gleason score $\geq$ 7. Biomarker development faces some common challenges that may limit the usability of biomarkers in clinical routine. The accuracy of transrectal ultrasound (TRUS)-guided needle biopsy is limited by a false negative rate of 23% [107]. Serial biopsies can improve the detection rate of organ-confined PCa from 77% at first biopsy to 99% at fourth biopsy [107]. However, since this initial study in 2002 the number of biopsy cores to be obtained increased from quadrant biopsy (four cores) to sextant biopsies (six cores) and, recently, to a standard of 10–12 cores, as recommended by the guidelines of the EAU. Nevertheless, detection rates are still in the range of 35% [36]. This results in significant uncertainty when using systematic biopsy as a reference standard. In addition, there is a significant upgrading of tumour grade of up to 56.7%, as demonstrated by studies comparing biopsy and final Gleason score after radical prostatectomy [21,108,109]. This causes another serious problem in defining the reference standard in biomarker studies, because whole-gland histopathological evaluation is only available after tumour radical prostatectomy and in rare cases of prostate enucleation due to large volume BPH. Furthermore, in healthy control groups neither biopsy material nor whole-gland tissue is available, reducing the determination of "tumour-free" status to clinical observation and exclusion of other biomarkers (in practice, mostly suspicious PSA levels). Another challenge is the population bias, e.g., shown in metabolomic studies. Special care has to be taken in conception, sampling, and sample processing to account for ethnic and lifestyle differences [110,111]. Population-based adjustment of biomarker panels and cutoffs is required, e.g., for Asian and Western countries [112–115]. All biomarkers have to compete against PSA and most outperform PSA in certain patient groups. While PSA assays are standardized, comparable, and easy to handle, with a low logistical burden, many of the novel biomarkers make higher demands on clinical staff, organization, laboratory equipment, and data analysis (Figure 1).

In addition, the superiority to PSA has to be validated in large prospective studies, which usually takes at least five years. There are already good data for the biomarkers established in the market (Table 1), but only a few of the novel biomarkers can provide clinical data. Furthermore, distinct restrictions apply to the tests ("targeted patients" in Table 1), which need to be taken into account when comparing the performance of different biomarkers. Currently, histopathological evaluation of needle biopsies is the gold standard and the basis of treatment decisions. Biomarkers should be able to predict the initial biopsy outcome in respect of high-grade disease, i.e., they should have a highly positive predictive value. At present, none of the available biomarkers and tests alone can achieve this goal.

Therefore, because of the low application threshold, PSA monitoring is indispensable at the moment, and should be integrated into routine health examinations of men aged $\geq$45 years, as recommended in the latest 2018 German S3-guidelines for Prostate Cancer [116]. In case of suspect PSA findings, additional biomarkers should be used to further characterize disease state and safe stratification of patients into treatment groups. Risk calculators should be used for transparent decision-making and to improve the inclusion of the patient.

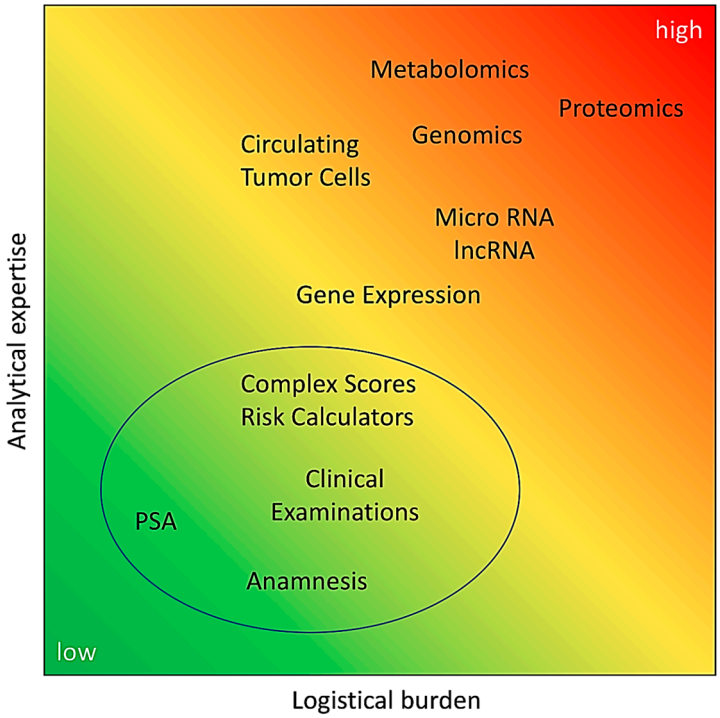


**Figure 1.** Feasibility of liquid biomarker-based diagnostics. The financial burden is coded by colour (green = low, yellow = medium, red = high); at present, advanced analytical methods come with higher high technical requirements and the need for very high analytical expertise; generally, gene expression, genomics, proteomics, and metabolomics require specialized analysis laboratories. In most cases, e.g., for untargeted analyses, standards have not been defined yet; this accelerates the threshold for comprehensive establishment in the clinical routine. For instance, proteomic analyses are still expensive, require high analytical expertise, and are not comprehensively available. On the other hand, proteomic analyses are fairly good and standardized. Comparing metabolic analyses, they require higher expertise than the more standardized proteomics, but are less expensive. The logistical burden grows with the complexity of the clinical and analytical requirements; circle indicates currently well established methods of PCa diagnostics.

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This multicenter diagnostic (prospective) study urine samples of 50 PC patients and 50 healthy controls were analyzed by $^{1}$H-NMR and a metabolite profile was created using 20 differentially expressed metabolites. Further analysis using PCA and OPLS-DA showed an acceptable discrimination of PC patients from HC using the combination of guanidino-acetate, Phenylacetylglycine, and glycine (AUC = 0.77; sensitivity = 80%, specificity = 64%). In addition, we identified potentially involved pathways by bioinformatics analysis. The KEGG “Glycine, Serine, and Threonine metabolism” pathway seemed to be associated with PC. To the best of our knowledge, this is the first study identifying guanidinoacetate, and phenylacetylglycine as potential novel biomarkers in PC.

diagnostics

MDPI

*Article*

# Novel Metabolic Signatures of Prostate Cancer Revealed by $^{1}$H-NMR Metabolomics of Urine

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**Abstract:** Prostate cancer (PC) is one of the most common male cancers worldwide. Until now, there is no consensus about using urinary metabolomic profiling as novel biomarkers to identify PC. In this study, urine samples from 50 PC patients and 50 non-cancerous individuals (control group) were collected. Based on $^{1}$H nuclear magnetic resonance ($^{1}$H-NMR) analysis, 20 metabolites were identified. Subsequently, principal component analysis (PCA), partial least squares-differential analysis (PLS-DA) and ortho-PLS-DA (OPLS-DA) were applied to find metabolites to distinguish PC from the control group. Furthermore, Wilcoxon test was used to find significant differences between the two groups in metabolite urine levels. Guanidinoacetate, phenylacetylglycine, and glycine were significantly increased in PC, while L-lactate and L-alanine were significantly decreased. The receiver operating characteristics (ROC) analysis revealed that the combination of guanidinoacetate, phenylacetylglycine, and glycine was able to accurately differentiate 77% of the PC patients with sensitivity = 80% and a specificity = 64%. In addition, those three metabolites showed significant differences in patients stratified for Gleason score 6 and Gleason score $\geq$7, indicating potential use to detect significant prostate cancer. Pathway enrichment analysis using the KEGG (Kyoto Encyclopedia of Genes and Genomes) and the SMPDB (The Small Molecule Pathway Database) revealed potential involvement of KEGG "Glycine, Serine, and Threonine metabolism" in PC. The present study highlights that guanidinoacetate, phenylacetylglycine, and glycine are potential candidate biomarkers of PC. To the best knowledge of the authors, this is the first study identifying guanidinoacetate, and phenylacetylglycine as potential novel biomarkers in PC.

**Keywords:** prostate cancer; urine metabolomics; $^{1}$H-Nuclear Magnetic Resonance; metabolite biomarkers



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## 1. Introduction

Prostate cancer is one of the most commonly cancers and the leading cause of cancer-related deaths in men worldwide [1]. Serum prostate specific antigen (PSA) level and digital rectal examination (DRE) constitute the major screening tests for prostate cancer (PC) diagnosis, while the transrectal ultrasound-guided prostate biopsy provides the final confirmation of cancer presence [2]. PSA level has been extensively used as a biomarker to detect PC. Nevertheless, due to prostate physiology, PSA testing results in a large frequency of false positives leading to numerous men each year undergoing unnecessary prostate biopsy procedures [3–7]. Hence, a non-invasive, cost-effective, efficient, and reasonably accurate test for early identification of PC is urgently needed.

Compared with serum, urine is easier to obtain and handle, needs less sample preparation, and has higher amounts of metabolites and lower protein content [8–10]. Therefore, in attempt to solve this diagnostic dilemma, many previous studies have focused on urinary metabolomic profile, to identify the predictive biomarkers for PC [11–14]. However, to date, no single urine biomarker/biomarker panel meets the requirements for highly sensitive, and specific detection of PC. Therefore, biomarker discovery in relation to PC continues to be an active area of research.

Nuclear magnetic resonance (NMR) spectroscopy is a powerful analytical approach for both identification and quantification of analytes with superior advantages, such as good reproducibility and simple sample processing. In the last decade, NMR has been applied toward identifying metabolic alterations in PC that may provide clinically useful biomarkers [15–19]. $^{1}$H-NMR spectroscopy followed by multivariate analysis is a systems biological approach that has been used to identify essential changes in metabolism. Therefore, metabolomics profiling offers a robust methodology for understanding the biochemical process of diseases.

Our current study aimed to identify novel biomarkers in the urine and to investigate the possible function and role of potential biomarkers in PC. Based on $^{1}$H-NMR, we identified 20 metabolites from urine samples. All spectra were analyzed by multivariate statistical analysis to extract the vital variables. Moreover, to evaluate the discrimination ability of the variables for diagnosis of PC. Additionally, metabolomics analysis cannot provide direct information about the active pathways related to the diseases. Furthermore, the regulation of the reactions and metabolic programs still need to be addressed [20]. Figure 1 summarizes the study design and workflow.

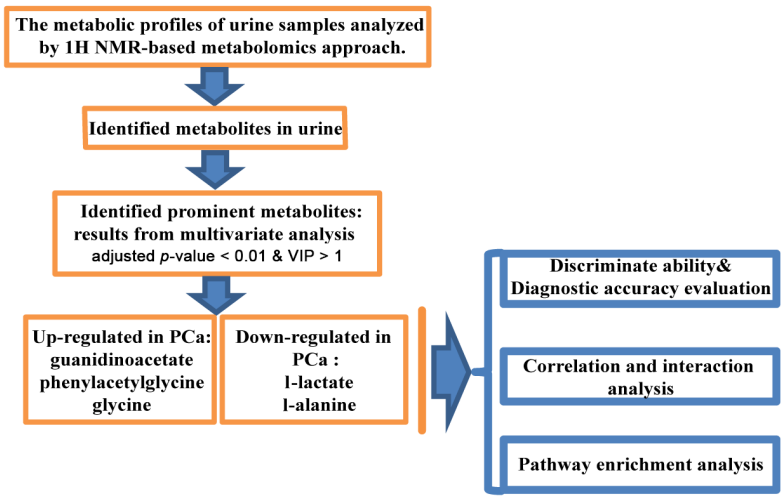


**Figure 1.** Study design. The workflow of the analysis steps.

## 2. Materials and Methods

### *2.1. Clinical Samples Selection and Ethics Statement*

Urine samples were collected from PC patients from January 2017 to December 2018 from Sir Run Run Shaw Hospital, HangZhou and Zhoupu Hospital, Shanghai, China. Clinical diagnosis of individuals was performed according to serum PSA, DRE, biopsy results/pathological results after operation and Gleason score. A total of 50 patients with prostate cancer were included in this study. The control group consisted of 50 non-cancerous men, who were without evidence of PC, based on PSA levels, negative findings in imagological examination and DRE. Clinical and demographics characteristics of the individuals are shown in Table 1.

**Table 1.** Characteristics of the individuals.

| Characteristics | Control Group ($n$ = 50) | | PC Group ($n$ = 50) | | Significance |
|---|---|---|---|---|---|
| | Mean (SD) | Group Size | Mean (SD) | Group Size | $p$-Value |
| Age (years) | 63.30 (9.61) | 50 | 70.00 (8.98) | 50 | <0.0001 |
| Prostate volume (mL) | 26.24 (8.77) | 24 | 39.77(19.00) | 50 | 0.0169 |
| PSA ($\leq$ 10 ng/mL) | 1.56 (0.89) | 50 | 6.69 (1.96) | 14 | |
| PSA (10.1–20 ng/mL) | NA | 0 | 14.01 (2.08) | 14 | |
| PSA (> 20 ng/mL) | NA | 0 | 89.82 (86.28) | 22 | |
| GS (pre) 6 | NA | NA | NA | 13 | |
| GS (pre) $\geq$7 | NA | NA | NA | 34 | |
| GS (post) 6 | NA | NA | NA | 6 | |
| GS (post) $\geq$7 | NA | NA | NA | 35 | |
| Treatment: | | | | 50 | |
| Radical operation | | | | 41 | |
| Seed implantation | | | | 5 | |
| Endocrine | | | | 2 | |
| Chemotherapy | | | | 1 | |
| TURP | | | | 1 | |

GS = Gleason Score; GS (pre) = GS of biopsy; 41 patients have accepted radical operation and got the post-operation GS (GS (post)); SD = standard deviation; prostate volume was calculated as volume: volume (mL) = (length $\times$ width $\times$ height) $\times$ $\pi/6$. TURP = Transurethral resection of the prostate; NA = not applicable; PC = prostate cancer.

Patients recruitment and sampling procedures were performed in accordance with the Declaration of Helsinki and applicable local regulatory requirements and laws. All patients provided written informed consent. Ethical approvals were obtained from the local ethics committees of the Sir Run Run Shaw Hospital affiliated to Zhejiang University (Ethical review approval number: 20190725-290) and Shanghai University of Medicine & Health Sciences (Ethical review approval number: HMMEP-2016-017).

### *2.2. Sample Preparation and $^1$H-NMR Based Metabolomics Analysis*

Midstream urine samples of all PC patients and controls were taken in the morning during standard clinical routine procedure. The samples were frozen within 1 h after collection and stored at $-80$ °C. At the time of $^1$H-NMR analysis, urine samples were thawed in an ice-water bath. Where not otherwise stated, chemicals were from Sigma-Aldrich Trading Co., Ltd., Shanghai, China. Two hundred µL of phosphate buffered saline (PBS) solution (0.1ml $Na_2HPO_4$ and 0.1ml $NaH_2PO_4$; 10% $D_2O$ and 0.03% TSP (trimethylsilylpropionic acid-$d_4$ sodium salt; pH 7.4) was added and the samples were centrifuged at 13,000 rpm for 20 min. After this, 550 µL of the supernatants were transferred to a 5-mm NMR tube for analysis. $^1$H-NMR spectral acquisition was performed using a Bruker Avance III NMR spectrometer equipped with 600 MHz magnets Ultrashield Plus (spectrometer frequency: 600.13 MHz; Bruker BioSpin Corporation, Billerica, MA. USA). All $^1$H-NMR experiments were performed at 25 °C.

All spectra were phase and baseline corrected, and chemical shifts were adjusted with reference to TSP signal using MestRenova 6.2 software (Mestrelab Research S.L., Santiago de Compostela, Spain). The spectra were binned into 0.02 ppm buckets between 0.52 and 9.30 ppm, and the region between $\delta$ 4.32 and 6.10 ppm, including the water ($\delta$ 4.32 and 5.26 ppm), and urea signal ($\delta$ 5.58 and 6.10 ppm) regions, was excluded from the analysis to avoid interference arising from differences in water suppression and variability from the urea signal.

### *2.3. Data Modelling and Statistical Analysis*

Before data analysis, we checked the data integrity. All missing values, zeros, and negative values were replaced by the 1/5 of the minimum positive value of each variable [21,22]. In addition, after the replacement, we compared the two data sets: before replacement and after replacement. We made sure that all the necessary information has been collected,

and that there was no significant difference between the two data sets (Table S1) and subgroups (cancer group and control group) (Tables S2 and S3). The normalization of the spectra was performed by R statistical package 4.0.2 (http://www.r-project.org) based on geometric mean, and generalized log transformation was performed to make features more comparable (Figure S1 Supplementary Materials; Figure 2).

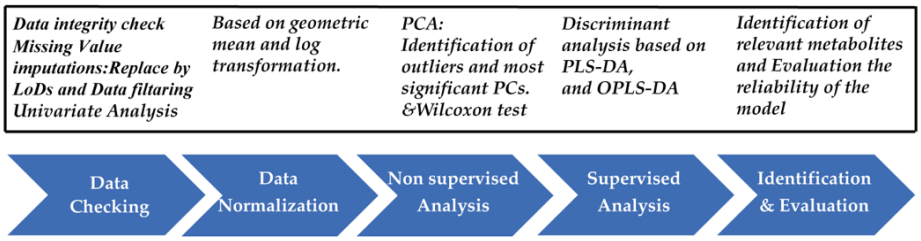


**Figure 2.** General scheme of the data modelling and statistical analysis procedures (sample size, $n$ = 100; variables size, $n$ = 20).

### *2.4. Identification of Relevant Metabolites*

For identification of relevant metabolites, we used several statistical approaches resulting in the definition of a subset of metabolites identified by at least two methods. The Multivariate statistical analysis was carried out using R packages "MetaboAnalyst" [21–23], "ropls" [24], "mixOmics" [25]. Principal component analysis (PCA) as a non-supervised statistical method, we used to uncover the outliers and the directions that best explain the variance in the dataset. Partial Least Squares discriminant analysis (PLS-DA), and Orthogonal Partial Least Squares discriminant analysis (OPLS-DA) were used to reduce the number of metabolites in high-dimensional data to produce robust and easy-to-interpret models, and to identify spectral features that drive group separation. Subsequently, based on R, Wilcoxon rank sum test was performed to find the difference between the cancer group and control group. The difference was considered significant at a Bonferroni-adjusted $p$-value < 0.05.

The variable importance in projection (VIP), and corresponding loading/contribution value in each model was used to identify the variables responsible for distinguishing. Furthermore, a permutation test with 100 permutations was employed to validate the performance of PLS-DA models and OPLS-DA models. For quality criterion we chose in PCA model, $R^2X > 0.4$; in PLS-DA or OPLS-DA, $R^2Y$ (goodness of fit parameter) and $Q^2$ (predictive ability parameter) > 0.5 [26,27].

### *2.5. Acquisition of the Pathways and Biological Processes Corresponding to Metabolites*

To explore the significance of a specific metabolite for prostate cancer, we used public databases to identify associated pathways. We focused on the most prominent metabolites defined by several criteria: (i) the metabolite was at least recommended in two different models (PCA, PLS-DA, or OPLS-DA); (ii) Wilcoxon test adjusted $p$-value < 0.01; (iii) VIP-values of the OPLS-DA >1.

Furthermore, the R package "MetaboAnalyst" [21–23] was performed analyze the contribution of the metabolites in depth. To implement a knowledge-based network of metabolite-metabolite interactions we used the Search Tool for Interactions of Chemicals (STITCH) database [28]. We also performed a Metabolite Sets Enrichment Analysis (MSEA), including pathway enrichment analysis based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) and the Small Molecule Pathway Database (SMPDB) [29,30]. A hypergeometric test was used to evaluate whether a particular metabolite set is represented, and the metabolite set contains at least more than 2 metabolites in the given compound list. Additionally, one-tailed $p$-values were provided after adjusting for multiple testing. A $p$-value < 0.05 was considered statistically significant.

### *2.6. Statistics*

All statistical analyses were performed using SPSS software (version 26; IBM Corp., Armonk, NY, USA) or R statistical package 4.0.2 (http://www.r-project.org). Univariate analysis was performed using ANOVA, *t*-test, Wilcoxon test, hypergeometric test and permutation test. Bonferroni was used to adjust *p*-values. The correlation analyses were performed by Pearson's test. Multivariate analyses were also performed using the PCA, PLS-DA, and OPLS-DA model. Subsequently, we used binary regression and a linear fitting model to do receiver operating characteristic (ROC) curve analysis to evaluate the performance of the metabolite or metabolite panel for the prediction of PC. *p*-values < 0.05 or adjusted *p*-values < 0.05 were considered statistically significant.

## 3. Results

### *3.1. Metabolites in Urine Samples of PC*

NMR offers the opportunity of quantifying metabolites directly from $^{1}$H-NMR metabolite profiles through analyzing the chemical shift, coupling constant, and shapes of peaks from NMR experiments, and to identify the metabolites based on existing public databases and literature reports [31–36]. Typical $^{1}$H-NMR spectra were derived from urine samples of the PC group and the Control group; interesting metabolites were identified (labeled as digits from 1 to 30 in Figure 3).

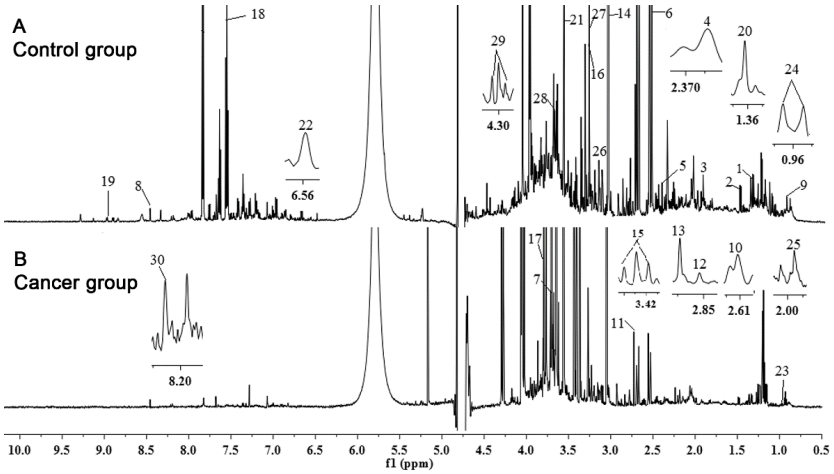


**Figure 3.** Representative 600 MHz $^{1}$H-NMR spectrum and assignment of identified metabolites (digits from 1 to 30) in two representative urine samples. Signals were analyzed from δ 0.52 to 9.30 ppm, excluding water and urea regions (δ 4.32–6.10 ppm). (**A**) Control group; (**B**) Cancer group; f1 (ppm) = chemical shift to TSP.

The region at 0.0–3.10 ppm shows aliphatic compounds including prominent signals from organic acids and amino acids, such as L-alanine, citric acid, pyruvate, succinate, and L-lactate; the region at 5.5–9.0 ppm shows aromatic compounds, such as hippurate and also formate, deeply downshifted due to the adjacent carboxy group. Additionally, moieties and chemical shifts of the 30 metabolites were summarized in Table S4. Finally, after removal of metabolites with overlapping signals, we got 20 metabolites which were further analyzed in this study (Table 2). For intensity quantification, the peak areas of these 20 metabolites were integrated using sodium trimethylsilyl propionate (TSP) as standard for further analysis.

**Table 2.** Twenty identified metabolites.

| Key | Metabolites | HMDB ID | Moieties | Chemical Shifts [a] | VIP |
|---|---|---|---|---|---|
| 1 | L-lactate | HMDB0000190 | αCH, $\beta CH_3$ | 1.33 (d,J = 6.6Hz),4.13 (q,J = 4.8Hz) | 1.43 |
| 2 | L-alanine | HMDB0000161 | $\beta CH_3$ | 1.48 (d, J = 7.2Hz) | 1.76 |
| 3 | acetate | HMDB0000042 | $CH_3$ | 1.92 (s) | 1.45 |
| 5 | succinate | HMDB0000254 | $CH_2$ | 2.41 (s) | 0.06 |
| 6 | citrate | HMDB0000094 | half $CH_2$, half $CH_2$ | 2.54 (d,J = 16.2 Hz), 2.70 (d, J = 15.6 Hz) | 0.42 |
| 7 | dimethylglycine | HMDB0000092 | N-$CH_3$, $CH_2$ | 2.92 (s), 3.72 (s) | 1.06 |
| 8 | formate | HMDB0000142 | CH | 8.46 (s) | 0.99 |
| 11 | dimethylamine | HMDB0000087 | $CH_3$ | 2.73 (s) | 0.82 |
| 12 | methylguanidine | HMDB0001522 | $CH_3$ | 2.85 (s) | 0.17 |
| 13 | trimethylamine | HMDB0000906 | $CH_3$ | 2.88 (s) | 0.89 |
| 14 | creatinine | HMDB0000562 | $CH_3$, $CH_2$ | 3.04 (s), 4.06 (s) | 0.45 |
| 15 | taurine | HMDB0000251 | S-$CH_2$, N-$CH_2$ | 3.27 (t), 3.42 (t) | 0.29 |
| 16 | betaine | HMDB0000043 | N($CH_3$)$_3$, $CH_2$ | 3.27 (s), 3.90 (s) | 0.09 |
| 17 | guanidinoacetate | HMDB0000128 | $CH_2$ | 3.80 (s) | 1.94 |
| 18 | hippurate | HMDB0000714 | $CH_2$, CH, CH, CH | 3.97 (d,J = 6Hz), 7.55 (t,J = 7.8Hz), 7.64 (t,J = 7.8Hz), 7.84 (d,J = 7.2Hz) | 0.02 |
| 19 | N-methylnicotinamide | HMDB0003152 | 2-CH, 4-CH, 6-CH, 5-CH, $CH_3$ | 9.29 (s), 8.97 (d,J = 6Hz), 8.91 (dt), 8.19 (m), 4.48 (s) | 0.55 |
| 20 | 2-Hydroxyisobutyrate | HMDB0000729 | $CH_3$ | 1.36 (s) | 0.36 |
| 21 | glycine | HMDB0000123 | $CH_2$ | 3.57 (s) | 1.36 |
| 22 | fumaric acid | HMDB0000134 | CH | 6.56 (s) | 0.32 |
| 28 | Phenylacetylglycine | HMDB0000821 | $CH_2$, CH, CH | 3.68 (s), 7.37 (m), 7.43 (m) | 1.59 |

[a] Signal position in parts per million (ppm) in relation to TPS (set to 0 ppm).

### *3.2. Identification of Important Metabolites and the Metabolic Changes*

PCA, PLS-DA, and OPLS-DA were performed to evaluate the metabolic pattern changes in PC patients compared to non-cancerous controls. PCA could not distinguish the cancer patients from the non-cancerous cases (Figure 4(A1)). The first two principal components (PC) explained 66.2% variables; however, no trends in differences were detected (Figure 4(A2)). Based on the contribution value, we obtained the top seven metabolites, including guanidinoacetate, betaine, phenylacetylglycine, taurine, dimethylglycine, L-alanine, and L-lactate (Figure 4(A3)) (Table S3). The goodness of fit of the PCA model was $R^2X = 0.607$.

Key numbers are related to the metabolite numbering in Figure 1; the variable importance in the projection (VIP) values were obtained from the OPLS-DA model.

If PLS-DA was used as classification model, we found a trend to distinguish cancer from the control (Figure 4(B1)). In this model, the first two principal components explained 55.6% of the variance (Figure 4(B2)). Based on the |loading values| > 0.2, we found 8 significant metabolites: guanidinoacetate, L-alanine, phenylacetylglycine, L-lactate, glycine, acetate, dimethylglycine, and formate (Figure 4(B3)) (Table S3). Furthermore, the PLS-DA performance was assessed by the goodness of fit $R^2Y = 0.628$ and quality assessment statistic $Q^2Y = 0.447$; the outcome indicated good class separation and a moderate predictive ability.

Further improvement in discrimination of the sample groups was achieved by using the OPLS-DA model (Figure 4(C1)). Based on the |loading values| > 0.2, OPLS-DA identified nine critical metabolites: guanidinoacetate, L-alanine, phenylacetylglycine, acetate, l-lactate, glycine, dimethylglycine, formate, and trimethylamine (Figure 4(C3)) (Table S3).

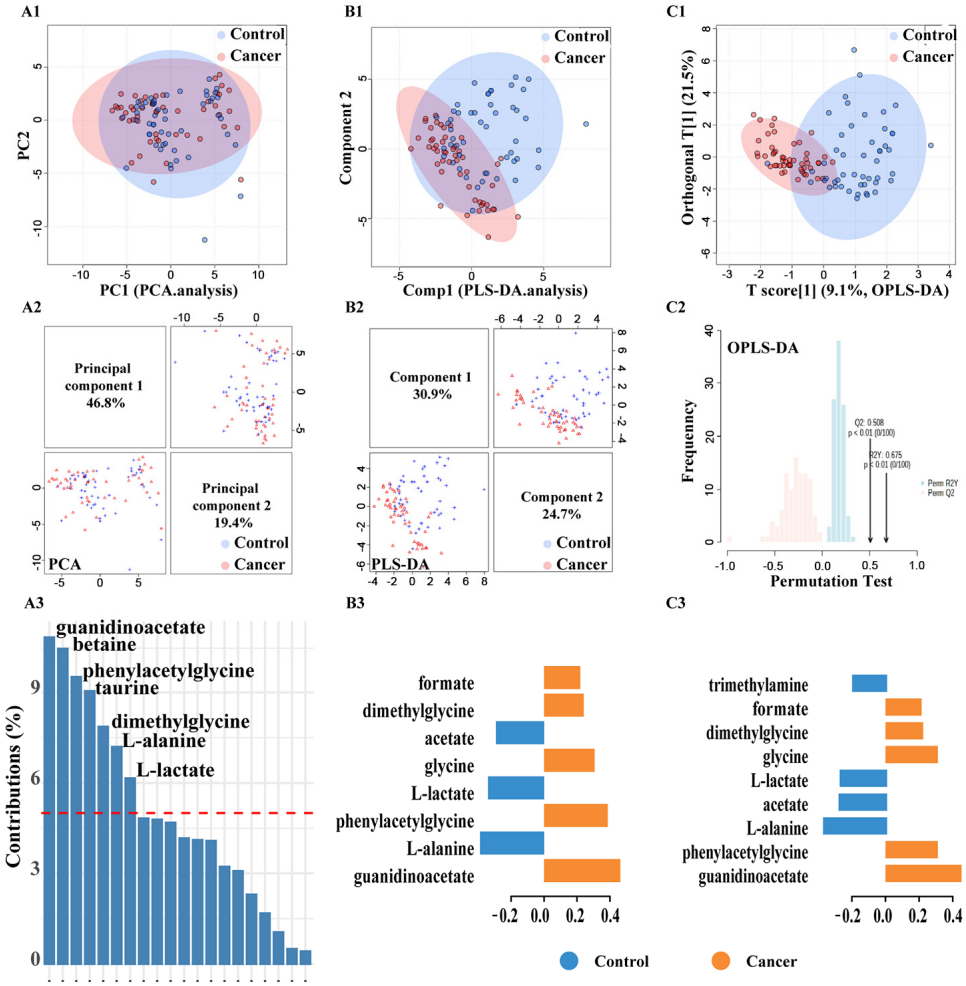


**Figure 4.** Metabolic pattern recognition analysis. Classifying PC from non-cancerous men based on the metabolomic profiles in the urine; (**A1**) PCA based on the first two principal components; (**A2**) sample scatterplot displays the first two components in each data set in PCA; (**A3**) contribution of each feature selected on the first component in PCA; (**B1**) PLS-DA based on the first two components; (**B2**) sample scatterplot display the first two components in each data set in PLS-DA; (**B3**) loading plot weights of each feature selected on the first component of PLS-DA; (**C1**) OPLS-DA based separation of the groups; (**C2**) internal validation of the corresponding OPLS-DA model by permutation analysis (n = 100); fraction of the variance of descriptor class response (Y) (R2Y) = 0.675 (Green bar), *p*-value < 0.01; fraction of the variance predicted (cross-validated)(Q2) = 0.508 (Red bar), *p*-value < 0.01; (**C3**) loading plot weights of each feature selected from OPLS-DA; The color in B3 and C3 indicates the class in which the variable has the maximum level of expression; control = blue; cancer = orange.

As shown in Figure 4, we could not completely discriminate the two groups based on PLS-DA and OPLS-DA scores plot. However, more samples were separated in OPLS-DA in contrast to the PCA method. This OPLS-DA model showed a proper fitting of the data (R2Y = 0.675, *p*-value < 0.01), and exhibit predictive power (Q2 = 0.508, *p*-value < 0.01) (Figure 4(C2)).

The variable importance in the projection (VIP) values of all peaks from OPLS-DA models were taken for selection, and those variables with VIP > 1 [37] were considered as potential biomarker candidates for group discrimination (Table 2). Accordingly, metabolomics revealed prominent alterations in seven metabolites: guanidinoacetate, l-alanine, phenylacetylglycine, acetate, l-lactate, glycine, and dimethylglycine (Table S3). In summary, the $^{1}$H-NMR spectra potentially discriminate the urine samples between PC patients and controls.

For direct comparison of the levels of the 20 metabolites, an integrated strategy combining Wilcoxon analysis was used to identify critical metabolites between the PC and the control group. We compared the urinary metabolomic profiles of the two groups, based on the Bonferroni method of *p*-value adjustment. The analysis revealed a total of eight significant metabolites (adjusted *p*-value < 0.05): guanidinoacetate, l-lactate, l-alanine, phenylacetylglycine, glycine, acetate, formate, and dimethylglycine (Figure 5A–I).

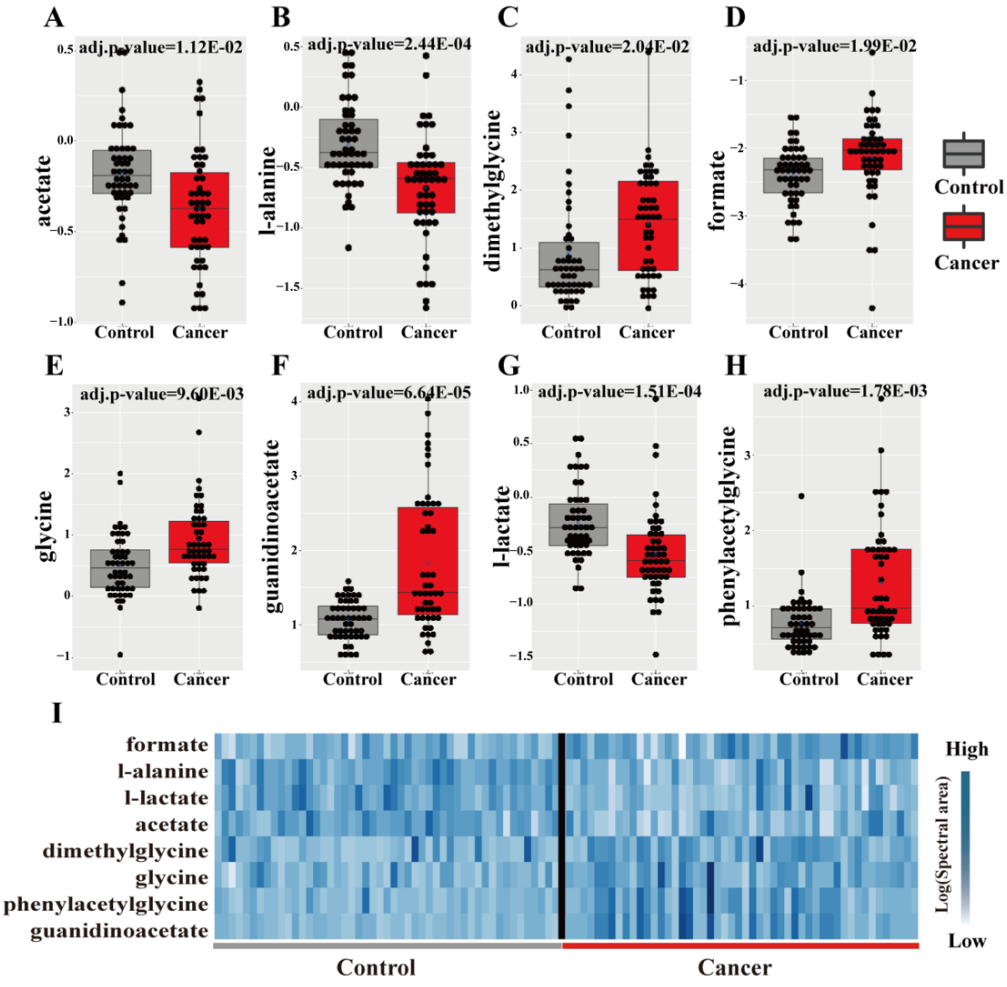


**Figure 5.** Wilcoxon test results and hierarchical clustering of the metabolites. (**A**–**H**) Box plots of levels of significant metabolites based on Wilcoxon test; (**I**) hierarchical clustering of the significant metabolites; the samples on the left of the black bar are non-cancerous samples (control group, $n$ = 50); the samples on the right of the black bar are PC samples ($n$ = 50); values in the heatmap = Log (Spectral area); *p*-values were Bonferroni-adjusted.

### *3.3. Acquisition of the Most Prominent Metabolites, Correlation Analysis, and ROC Analysis*

Regarding the criterion of the most prominent metabolites: (i) the metabolite was at least recommended in two different models, from PCA, PLS-DA and OPLS-DA (Figure 6 (A1)) (Table S5); (ii) Wilcoxon test adjusted *ps* < 0.01 (Figure 6 (A2); Table S5); (iii) the VIP-values of the OPLS-DA >1 (Figure 6 (A2); Table S5). Herein, after the overlapping progression, we focused on the five most prominent metabolites: guanidinoacetate, phenylacetylglycine, glycine, L-lactate and L-alanine (Figure 6 (A3); Table S5). Interestingly, based on the Human Metabolome Database (HMDB) [37], guanidinoacetate and phenylacetylglycine have not been detected in prostate tissue, so far (Table S4). We found a strong positive correlation between guanidinoacetate and phenylacetylglycine (Pearson's correlation coefficient; $r = 0.93$, $p$-value < 0.001), and moderate positive correlations between l-alanine and l-lactate ($r = 0.65$, $p$-value < 0.001), guanidinoacetate and glycine ($r = 0.67$, $p$-value<0.01), and phenylacetylglycine and glycine ($r = 0.64$, $p$-value < 0.001) (Figure 6B).

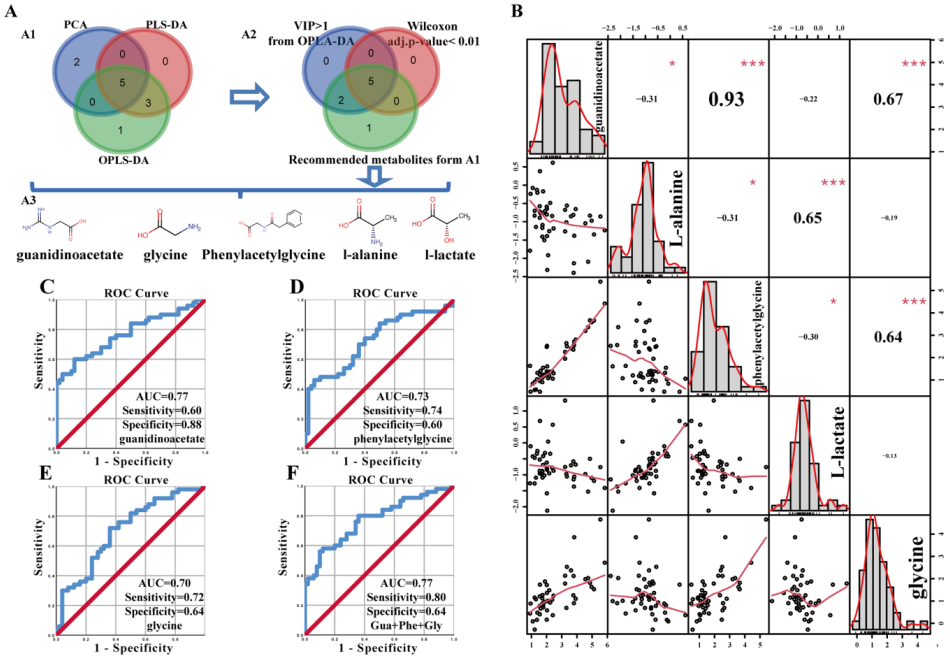


**Figure 6.** Correlations between the five most prominent metabolites and the representative ROC analyses. (**A**) Venn diagram describing the overlapping results from different models. (**A1**) overlapping of the significant metabolites from different models, revealed 8 metabolites at least recommended in two models. Significant metabolites detected by PCA (7 metabolites, marked in blue), PLS-DA (8 metabolites, marked in red), and OPLS-DA (9 metabolites, marked in green); (**A2**) overlapping of the significant metabolites from three different models revealed 5 metabolites, which were common between OPLS-DA (blue circle, VIP > 1, $n = 7$), metabolites with Wilcoxon test adjusted $p$-value < 0.01 (red circle, n = 5), and overlap result was obtained from A1 (green circle, $n = 8$); (**A3**) identification and chemical formula of the 5 significant metabolites. (**B**) Correlation between the 5 metabolites in PC: (i) the histogram of the kernel density estimation and distribution of each variable is shown on the diagonal, (ii) On the bottom of the diagonal: the bivariate scatter plots with a fitted line are displayed, (iii) On the top of the diagonal: the value of the correlation plus the significance level as stars; each significance level is associated to a symbol: $p$-values (0, 0.001, 0.01, 0.05, 1) relate to symbols ("***", "***", "**", "*", " "); the number in the charts is the Pearson's correlation coefficient ($r$); (iv) Numbers at the sides of the charts indicate the range of variable values are depicted as Log(Spectral area). (**C**–**F**) Representative ROC curves showing the diagnostic accuracy (AUC) based on guanidinoacetate (**C**), phenylacetylglycine (**D**), glycine (**E**), and in (**F**) the combination of the three metabolites: guanidinoacetate (Gua), phenylacetylglycine (Phe), and glycine (Gly).

ROC analysis of significant metabolites in multiple t-test revealed for guanidinoacetate an AUC of 0.77 (sensitivity = 60%, specificity = 88%; Figure 6C), phenylacetylglycine an AUC of 0.73 (sensitivity = 74%, specificity = 60%; Figure 6D), and glycine an AUC of 0.70 (sensitivity = 72%, specificity = 64%; Figure 6E). The AUCs of l-alanine and l-lactate were lower than 0.70, respectively (data not shown).

Based on a linear fitting model, various combinations were evaluated for their ability to predict PC. The combination of guanidinoacetate, phenylacetylglycine and glycine identified PC with an AUC = 0.77, sensitivity = 80%, and specificity = 64%. However, while improving the sensitivity from 60% to 80% ($p$-value = 0.03), this combination did not significantly improve the diagnostic probability of PC (Figure 6F). The combination of guanidinoacetate, phenylacetylglycine, glycine, l-alanine, and l-lactate showed less performance (AUC = 0.65, sensitivity = 52%, specificity = 80%; data not shown), as did the combinations of l-alanine and l-lactate and others (AUCs < 0.7 with low specificity and sensitivity; data not shown).

### *3.4. Subgroup Analysis*

To explore the property of the metabolites to separate between different PC stages, we compared the urine levels of the five metabolites L-lactate, L-alanine, glycine, guanidinoacetate, and phenylacetylglycine in different subgroups of PC. Three metabolites: glycine, guanidinoacetate, and phenylacetylglycine showed significant differences between low GS $\leq$ 6 and high GS $\geq$ 7 when using the biopsy GS (GS (pre)) or final post-surgery GS (GS (post)) for stratification (Figures S3 and S4, ANOVA with Bonferroni-adjusted $p$-values, $p < 0.05$).

In addition, we found significant differences in the urine levels of glycine, guanidinoacetate and phenylacetylglycine between PSA-groups (low PSA: $\leq$10 ng/mL and high PSA: >20 ng/mL), while l-lactate and l-alanine were not different (Figure S5). Comparison of TNM or risk groups did not reveal significant differences (data not shown).

### *3.5. Analysis of the Metabolite Interaction Networks and Corresponding Pathways*

The network explorer module is a comprehensive tool to describe potential impacts, and to visualize interactions between metabolites. Network analysis highlights potential functional relationships between a broad set of annotated metabolites. Based on the degree of interaction cut-off value >2, we found another 16 annotated metabolites potentially interacted with the five metabolites defined above, and we also found 53 different interactions among them (Figure 7A).

According to the $p$-values from the pathway enrichment analysis, the pathways containing at least two components of the five prominent metabolites are listed in Figure 7B. Based on KEGG database analysis, "Glycine, serine, and threonine metabolism" and "Aminoacyl-tRNA biosynthesis" were the associated pathways with $p$-value < 0.05. Figure 7C describes the five associated pathways based on SMPDB, such as "Glycine and Serine Metabolism" and "Arginine and Proline Metabolism". Figure 7D Detailed view of the "Glycine, serine, and threonine metabolism" as the most significant pathway.

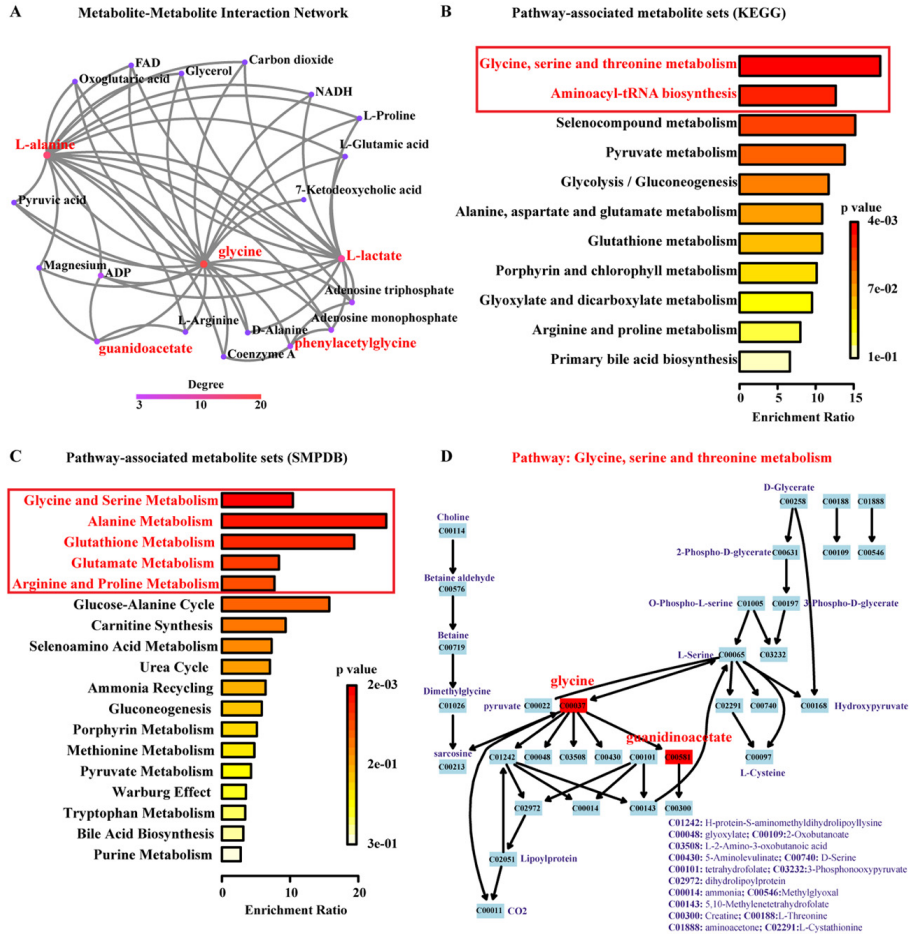


**Figure 7.** Interaction network analysis and pathways associated with the five identified metabolites. (**A**) Interaction map of the annotated metabolites; nodes are color coded for the degree of the metabolite interactions; (**B**) pathways associated with the most prominent metabolites based on KEGG analysis; (**C**) pathways associated with the most prominent metabolites based on SMPDB analysis; (**D**) detailed view of the "Glycine, serine and threonine metabolism" (KEGG map00260) as the most significant pathway according to the KEGG analysis; the numbers in the boxes represent the IDs of annotated metabolites in KEGG database; prominent metabolites as a result of the current analysis are marked in red. Key signaling pathways with *p*-values < 0.05 were marked in boxes with red font in (**B**,**C**).

## 4. Discussion

### *4.1. The Location and Expression of Metabolites in PC*

Notably, urine is a challenging bio-specimen used for biomarker discovery due to its compositional variability [38,39]. Multiple factors can affect the composition and quality of urine liquid biopsy, such as disease state, prescription taken by individuals, diet, gender, and collection time [38,39]. In the present study, multivariate statistical models were used to identify reliable candidate biomarkers of PC. Eventually, we found that guanidinoacetate, phenylacetylglycine, glycine, l-lactate, and l-alanine were the most prominent metabolites.

Lima and colleagues reported that lactate and alanine were frequently altered in PC tissues [40]. Our finding of glycine upregulation is supported by Giskeodegard GF et al. [41], who studied the metabolome in prostate cancer tissue from a Spanish cohort by high reso-

lution magic angle spinning magnetic resonance spectroscopy (HR-MAS). While glycine, L-lactate, and L-alanine have already been shown in literature, to the best knowledge of the authors, the present study for the first time describes guanidinoacetate and phenylacetylglycine as significant metabolites in PC [42].

More evidence for PC-specific metabolic alterations come from metabolomics studies in serum. Kumar et al. found by $^{1}$H-NMR that alanine, pyruvate, glycine, and sarcosine were significantly altered in serum of an Indian cohort of PC patients [43]. These results were supported by Miyagi et al., using high performance liquid chromatography-electrospray ionization mass spectrometry (HPLC-ESE-MS), showing a significant change of alanine, glutamine, valine, tryptophan, arginine and isoleucine, ornithine, and lysine levels associated with PC in a Japanese cohort [44]. However, while Kumar et al. found an upregulation of alanine [43], the alanine levels were downregulated in the study by Miyagi et al. [43]. The discrepancy of two studies showed that the different methods potentially may cause different findings [45].

Extensive literature survey revealed only few studies of urine metabolite levels in PC (Table 3). Only one study reported changes for two of the metabolites identified in our study: glycine and dimethylglycine. However, opposite to our results, those metabolites were downregulated in the study of Pérez-Rambla and colleagues [46]. No urine level data are available for the other metabolites that turned up significantly altered in our PC cohort.

**Table 3.** Metabolites studied in previous studies of Prostate cancer (PC).

| Metabolites | | Samples (Methods) | Reference | Ethnos |
|---|---|---|---|---|
| **Up-Regulated** | **Down-Regulated** | | | |
| BCAA, glutamate; pseudouridine | Glycine [@], dimethylglycine [@], fumarate, 4-imidazole-acetate | Urine (1H-NMR) | Pérez-Rambla et al. [46] | Spanish |
| glycocholic acid, hippurate, chenodeoxycholic acid | 5-Hydroxy-l-tryptophan, taurocholic acid | Urine (FPLC/MS) | Liang, et al. [47] | Chinese (Northern of China) |
| | citrate, Myo-inositol, spermine | EPS (1H-NMR) | Serkova et al. [48] | American |
| sarcosine | | Urine/PT/Plasma (GC-MS) | Sreekumr et al. [49] | American |
| propenoic acid, dihyroxybutanoic acid xylonic acid | pyrimidine, creatinine, purine, glucopyranoside, xylopyranoseand, ribofuranoside | Urine (GC-MS) | Wu et al. [50] | Chinese (Southern of China) |

[@] opposite to the present study; EPS: Human expressed prostatic secretions; BCCA: Branched-chain amino acids; PT: Prostate Tissue; GC-MS: Gas chromatography/mass spectrometry; FPLC/MS: Faster ultrahigh performance liquid chromatography-mass spectrometry; $^{1}$H-NMRS: Proton nuclear magnetic resonance spectroscopy.

The interpretation of these differences is difficult. Different compositions of the PC cohorts in respect to tumor stage may be one reason, as the majority of our samples were from patients with metastasis and high-grade tumors. In addition, the control cohort in the study of Pérez-Rambla et al., were BPH patients, which could possibly explain the different findings [46]. Only 36% (18/50) of our control patients were diagnosed with BPH and the expression levels of glycine and dimethylglycine were not significantly different between BPH and non-BPH patients (Figure S2). Furthermore, the studies listed in Table 3, were done in different populations. Caucasian population samples were from western countries, which not only have a different genetic background but also represent different lifestyle and diet [51]. The study populations of two other studies were from Chinese patients, as in our study, but used different methods. Moreover, the lifestyle and diet of the patients from northern and southern China may not be comparable to the urban population we studied. Therefore, the results might reflect a research method, ethnic peculiarity and/or lifestyle or diet impact [45,51].

### *4.2. Potential Biomarkers of PC*

Over the past 30 years, NMR and MRSI (magnetic resonance spectroscopic imaging) as a non-invasive test, are continuous performed to identify predictive/prognostic

metabolic marker of PC [52]. Furthermore, considerable efforts are ongoing to develop high precision, reliable, safe and non-invasive diagnosis strategies. Kumar proposed a great question: "Metabolomics-Derived Prostate Cancer Biomarkers: Fact or Fiction?" In fact, their findings confirmed that NMR-based serum metabolomics analysis is a promising method for probing PC [43].

Using serum metabolomics, Kumar et al. found that L-alanine, pyruvate, glycine, and sarcosine were able to accurately differentiate 90.2% of cancer cases from healthy persons, with high sensitivity (84.4%) and specificity (92.9%) [43]. Glycine alone showed an AUC of 0.817 [43]. In our $^{1}$H-NMR study, we found, that glycine in urine was up-regulated in PC, and ROC analysis revealed for glycine an AUC of 0.70 (sensitivity = 72%, specificity = 64%), which is comparable to the performance in serum. Furthermore, ROC analysis was also performed to evaluate the various combination; however, the best combination of guanidinoacetate, phenylacetylglycine, and glycine did not significantly improve the discriminant ability (AUC of 0.77, sensitivity = 80%, and specificity = 64%), but significantly improved the sensitivity. In essence, the ROC findings revealed that guanidinoacetate, phenylacetylglycine, and glycine were potential biomarkers.

### *4.3. Metabolite Interactions and Pathways Potentially Involved in PC*

A better understanding of relative correlation and interaction of the potential biomarkers in urine could provide insights into the pathological progression of the disorder. Interestingly, we observed a strong positive correlation between guanidinoacetate and phenylacetylglycine ($r$ = 0.93, $p$-value < 0.001), while only moderate positive correlation between guanidinoacetate and glycine, phenylacetylglycine, and glycine. Furthermore, the comprehensive network showed that the direct and indirect interactions between the prominent metabolites (Figure 7A). Thus, the above results probably indicated that these metabolites are conditioning each other through direct or intermediates interaction.

Glycine is a nonessential amino acid with a central role in protein metabolism and also functions as inhibitory neurotransmitter in the central nervous system [53,54]. Additionally, glycine is involved in the body's production of DNA and in the energy balance [55–57]. Notably, its role in the biosynthesis of purines and in mitochondrial oxidative phosphorylation has been recognized as driver of cancer initiation and proliferation [58,59]. The elevated glycine urine levels in PC support this view and could explain the higher guanidinoacetate levels measured. Guanidinoacetate is a direct metabolite of glycine formed by the glycine aminotransferase. Interestingly, guanidinoacetate is further methylated by the guanidinoacetate N-methyltransferase to creatine, which can be converted to creatinine, which also was elevated in our PC patients as a trend [60]. In addition, Kim et al. found an association of aberrant genes of the "Glycine, serine, and threonine metabolism" pathway with metastasis in PC [61]. Our results also support the notion of altered "Glycine, serine, and threonine metabolism" pathway in PC, and that two of the related metabolites, namely glycine and guanidinoacetate, are potential biomarkers for differentiation of PC from healthy controls.

Previous research described that phenylacetylglycine is working as an acyl glycine [45]. As we known, acyl glycines as classical minor metabolites are one kinds of fatty acids [45,62,63]. Together with phenylacetylglutamine and phenylalanine, phenylacetylglycine is a representative of the phenylalanine/tyrosine metabolism (KEGG: map00360) and showed significant association with T stage in gastric cancer [42,64]. Our study for the first time shows elevated levels of phenylacetylglycine in the urine of prostate cancer patients and thereby further supports the importance of the phenylalanine/tyrosine metabolic pathway in cancer.

### *4.4. The Major Findings of the Present Study*

In summary, the present study identified five prominent metabolites: guanidinoacetate, phenylacetylglycine, glycine, l-lactate, and l-alanine. NMR-derived urinary metabolomics seem sufficiently robust to detect PC. In comparison with previous studies, the most interesting findings were

I. The metabolites guanidinoacetate, phenylacetylglycine, and glycine were significantly upregulated in urine samples of PC. On the contrary, l-alanine and l-lactate were significantly downregulated. Furthermore, the majority of them were positively correlated. Especially strong correlations were seen between guanidinoacetate, phenylacetylglycine and glycine.
II. Guanidinoacetate, phenylacetylglycine, and glycine urine levels were significantly different between PC patients stratified for low GS ($\leq$6) and high GS ($\geq$7).
III. Using the network module, we comprehensively described the potential interaction between the most prominent metabolites. ROC analyses of prominent metabolites revealed a reasonably high diagnostic accuracy of guanidinoacetate, phenylacetylglycine, and glycine.
IV. Pathway enrichment analysis indicated "Glycine, Serine, and Threonine metabolism" as the most importantly altered pathway. Those results provide evidence for the metabolites, and associated pathway potentially playing an essential role in PC.
V. Here, we reported for the first time that guanidinoacetate, and phenylacetylglycine could be promising novel urine biomarkers for PC.

The limitations of our study are (1) the cohort size is small, and we lack an external validation cohort; therefore, our results are at risk of overfitting; (2) as the aim of this study was to evaluate the performance of urine $^{1}$H-NMR metabolomics in an Asian cohort, we did not include Caucasian patients for comparison; (3) due to the small cohort we were not able to analyze PC subgroups, e.g., PSA/Gleason Score/Metastases; and (4) In addition, we focused on the metabolites in urine. Therefore, we cannot estimate the differences of discrimination ability between the blood sample, urine sample and tissue sample at the same time.

Further research will have to validate the urine metabolite biomarker panel in a larger cohort. Comparison to a matched Caucasian cohort could provide interesting insights into ethnical differences, which would have a severe impact on the clinical implementation of urine metabolomics biomarker in different populations.

## 5. Conclusions

Based on the metabolic profiling of urine, the present study showed that PC could be distinguished from non-cancerous individuals by guanidinoacetate, phenylacetylglycine, and glycine. The findings may add to our understanding of the basic mechanisms and progression of PC and indicated that these metabolites are potential candidate markers for PC. Moreover, the present study supported the view that urine metabolomics-derived biomarkers for PC can be a new option for non-invasive PC diagnostics.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/2075-4418/11/2/149/s1, Figure S1: Data normalization, density and intensity before and after data normalization; Figure S2: glycine and dimethylglycine levels. Comparion between normal cases, BPH cases and cancer patients; difference are significant between normal and cancers, but not between BPH and normal cases; ANOVA, Tukey's multiple comparison test, ** $p < 0.01$; **** $p < 0.0001$; Figure S3: subgroup analysis based on biopsy GS (GS (pre)); Figure S4: subgroup analysis based on radical prostatectomy GS (GS (post)); Figure S5: subgroup analysis based on PSA of PCa; stratification following the guidelines of the EAU [65]: PSA $\leq$ 10 ng/mL ($n$ = 14), PSA 10.1–20 ng/mL ($n$ = 14), PSA > 20 ng/mL ($n$ =22); Figure S3 and Figure S5: data presented as box plots with scatter plot, line in box indicates mean, whiskers indicate 95% CI; Table S1: data cleansing of the combined data sets; Table S2: data cleansing of the cancer data set; Table S3: data cleansing of the control data set; Table S4: listing of the thirty metabolites analyzed; Table S5: tissue location and expression of the five most prominent metabolites; Table S6: metabolite detection by different models and variable reduction process; Table S7: Urine levels of the top 8 metabolites.

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## Abbreviations

| | |
|---|---|
| AUC | Area under the receiver operating characteristic (ROC) curve |
| CI | Confidence Interval |
| DRE | Digital rectal examination |
| FC | Fold change |
| GAA | Guanidinoacetate |
| GC/MS | Gas chromatography-mass spectrometry |
| GS | Gleason score |
| HMDB | Human Metabolome Database |
| NMR | Nuclear magnetic resonance |
| OPLS-DA | Orthogonal Partial Least Squares discriminant analysis |
| PBS | Phosphate buffer solution |
| PC | Prostate cancer |
| PCA | Principal component analysis |
| PLS-DA | Partial Least Squares discriminant analysis |
| PSA | Prostate specific antigen |
| ROC | Receiver operating characteristic curve |
| STITCH | Search tool for interactions of chemicals |
| TSP | Trimethylsilylpropionic acid-$d_4$ sodium salt |
| TURP | Transurethral resection of the prostate |
| VIP | Variable importance in projection |

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## 3. Summary

### 3.1 Introduction

Prostate cancer (PC) is one of the most common male cancers worldwide. Until now, there is no consensus about using urinary metabolomic profiling as novel biomarkers to identify PC. Nuclear magnetic resonance (NMR) spectroscopy is a powerful analytical approach for both identification and quantification of analytes with superior advantages, such as good reproducibility and simple sample processing. In the last decade, NMR has been applied toward identifying metabolic alterations in PC that may provide clinically useful biomarkers (Smolinska et al., 2012; Srivastava et al., 2010; Zhang et al., 2012; Bertini et al., 2012; Yang et al., 2017). $^{1}$H-NMR spectroscopy followed by multivariate analysis is a systems biological approach that has been used to identify essential changes in metabolism. Therefore, metabolomics profiling offers a robust methodology for understanding the biochemical process of diseases.

Our current study aimed to identify novel biomarkers in the urine and to investigate the possible function and role of potential biomarkers in PC. Based on $^{1}$H-NMR, we identified 20 metabolites from urine samples. All spectra were analyzed by multivariate statistical analysis to extract the vital variables. Moreover, to evaluate the discrimination ability of the variables for diagnosis of PC. Additionally, metabolomics analysis cannot provide direct information about the active pathways related to the diseases. Furthermore, the regulation of the reactions and metabolic programs still need to be addressed (Cai et al., 2020).

### 3.2 Material and methods

Midstream urine samples of all PC patients (n=50) and non-cancerous individuals (n=50) were taken in the morning during standard clinical routine procedure. The samples were frozen within 1 h after collection and stored at −80 °C. At the time of $^{1}$H-NMR analysis, urine samples were thawed in an ice-water bath. All $^{1}$H-NMR experiments were performed at 25 °C. All spectra were phase and baseline corrected, and chemical shifts were adjusted with reference to TSP signal using MestRenova 6.2 software (Mestrelab Research S.L., Santiago de Compostela, Spain).

Before data analysis, we checked the data integrity. All missing values, zeros and negative values were replaced by the 1/5 of the minimum positive value of each variable (Chong et al., 2019; Xia et al., 2016). In addition, after the replacement, we compared the two data sets: before replacement and after replacement. We made sure that all the necessary information had been collected, and that there was no significant difference between the two data sets and subgroups.

For identification of relevant metabolites, we used several statistical approaches resulting in the definition of a subset of metabolites identified by at least two methods. The Multivariate statistical analysis was carried out using R packages “MetaboAnalyst”

(Chong et al., 2019; Xia et al., 2016; Pang et al., 2020), ”ropls” (Thevenot et al., 2016),”mixOmics” (Rohart et al., 2017).

To explore the significance of a specific metabolite for prostate cancer, we used public databases to identify associated pathways. We focused on the most prominent metabolites defined by several criteria: (i) the metabolite was at least recommended in two different models (PCA, PLS-DA or OPLS-DA); (ii) Wilcoxon test adj. p-value < 0.01; (iii) VIP-values of the OPLS-DA >1.

All statistical analyses were performed using SPSS software (version 26; IBM Corp., USA) or R statistical package 4.0.2 (http://www.r-project.org). Univariate analysis was performed using ANOVA, t-test, Wilcoxon test, hypergeometric test and permutation test. Bonferroni was used to adjust p-values. The correlation analyses were performed by Pearson’s test. Multivariate analyses were also performed using the PCA, PLS-DA and OPLS-DA model. Subsequently, we used binary regression and a linear fitting model to do receiver operating characteristic (ROC) curve analysis to evaluate the performance of the metabolite or metabolite panel for the prediction of PC. P-values < 0.05 or adj.p-values < 0.05 were considered statistically significant.

## 3.3 Results

### 3.3.1 Identification of important metabolites and the metabolic changes

Based on $^{1}$H nuclear magnetic resonance ($^{1}$H-NMR) analysis, 20 metabolites were identified. For direct comparison of the levels of the 20 metabolites, an integrated strategy combining Wilcoxon analysis was used to identify critical metabolites between the PC and the control group. We compared the uinary metabolomic profiles of the two groups, based on the Bonferroni method of p-value adjustment. The analysis revealed a total of 8 significant metabolites (adj.p-value<0.05): guanidinoacetate, L-lactate, L-alanine, phenylacetylglycine, glycine, acetate, formate, and dimethylglycine.

### 3.3.2 Acquisition of the most prominent metabolites, Correlation analysis and ROC analysis

After the overlapping progression, we focused on the five most prominent metabolites: guanidinoacetate, phenylacetylglycine, glycine, L-lactate and L-alanine**.** We found a strong positive correlation between guanidinoacetate and phenylacetylglycine (Pearson’s correlation coefficient; $r$=0.93, p-value<0.001), and moderate positive correlations between L-alanine and L-lactate ($r$=0.65, p-value<0.001), guanidinoacetate and glycine ($r$=0.67, p-value<0.01), and phenylacetylglycine and glycine ($r$=0.64, p-value<0.001).

ROC analysis of significant metabolites in multiple t-test revealed for guanidinoacetate an AUC of 0.77 (sensitivity = 60%, specificity = 88%), phenylacetylglycine an AUC of 0.73 (sensitivity = 74%, specificity = 60%), and glycine an AUC of 0.70 (sensitivity =

72%, specificity = 64%). The AUCs of L-alanine and L-lactate were lower than 0.70, respectively.

Based on a linear fitting model, various combinations were evaluated for their ability to predict PC. The combination of guanidinoacetate, phenylacetylglycine and glycine identified PC with an AUC=0.77, sensitivity = 80%, specificity = 64%. However, while improving the sensitivity from 60% to 80% (p-value=0.03), this combination did not significantly improve the diagnostic probablity of PC. The combination of guanidinoacetate, phenylacetylglycine, glycine, L-alanine and L-lactate showed less performance (AUC=0.65, sensitivity = 52%, specificity = 80%), as did the combinations of L-alanine and L-lactate and others (AUCs < 0.7 with low specificity and sensitivity).

### 3.3.3 Subgroup analysis

To explore the property of the metabolites to separate between different PC stages, we compared the urine levels of the five metabolites L-lactate, L-alanine, glycine, guanidinoacetate and phenylacetylglycine in different subgroups of PC. Three metabolites: glycine, guanidinoacetate and phenylacetylglycine showed significant differences between low GS $\leqslant$ 6 and high GS $\geqslant$ 7 when using the biopsy GS (GS(pre)) or final post-surgery GS (GS(post)) for stratification (ANOVA with Bonferroni-adjusted p-values, $p < 0.05$).

In addition, we found significant differences in the urine levels of glycine, guanidinoacetate and phenylacetylglycine between PSA-groups (low PSA $\leqslant$10 ng/ml and high PSA>20 ng/ml), while L-lactate and L-alanine were not different. Comparison of TNM or risk groups did not reveal significant differences.

### 3.3.4 Analysis of the metabolite interaction networks and corresponding pathways

Based on the degree of interaction cut-off value>2, we found another 16 annotated metabolites potentially interacted with the five metabolites defined above. And we also found 53 different interactions among them. Based on KEGG database analysis, “Glycine, serine, and threoninemetabolism” and ”Aminoacyl-tRNA biosynthesis” were the associated pathways with p-value < 0.05.

## 3.4 Discussion

The present study identified five prominent metabolites: guanidinoacetate, phenylacetylglycine, glycine, L-lactate and L-alanine. NMR-derived urinary metabolomics seem sufficiently robust to detect PC. In comparison with previous studies, the most interesting findings were:

I. The metabolites guanidinoacetate, phenylacetylglycine and glycine were significantly upregulated in urine samples of PC. On the contrary, L-alanine and L-lactate were significantly downregulated. Furthermore, the majority of them

were positively correlated. Especially strong correlations were seen between guanidinoacetate, phenylacetylglycine and glycine.

II. Guanidinoacetate, phenylacetylglycine and glycine urine levels were significantly different between PC patients stratified for low GS ($\leqslant$ 6) and high GS ($\geqslant$ 7).

III. Using the network module, we comprehensively described the potential interaction between the most prominent metabolites. ROC analyses of prominent metabolites revealed a reasonably high diagnostic accuracy of guanidinoacetate, phenylacetylglycine and glycine.

IV. Pathway enrichment analysis indicated “Glycine, Serine and Threonine metabolism” as the most importantly altered pathway. Those results provide evidence for the metabolites, and associated pathway potentially playing an essential role in PC.

V. Here, we reported for the first time that guanidinoacetate, and phenylacetylglycine could be promising novel urine biomarkers for PC.

The limitations of our study are: (1) the cohort size is small, and we lack an external validation cohort; therefore, our results are at risk of overfitting; (2) as the aim of this study was to evaluate the performance of urine $^{1}$H-NMR metabolomics in an Asian cohort, we did not include Caucasian patients for comparison; (3) due to the small cohort we were not able to analyze PC subgroups, e.g. PSA/Gleason Score/Metastases; (4) In addition, we focused on the metabolites in urine. Therefore, we cannot estimate the differences of discrimination ability between the blood sample, urine sample and tissue sample at the same time.

Further research will have to validate the urine metabolite biomarker panel in a larger cohort. Comparison to a matched Caucasian cohort could provide interesting insights into ethnical differences, which would have a severe impact on the clinical implementation of urine metabolomics biomarker in different populations.

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## 5. Attachments

**Abbreviations**

| | |
|---|---|
| AUC | Area under the receiver operating characteristic (ROC) curve |
| CI | Confidence Interval |
| DRE | Digital rectal examination |
| FC | Fold change |
| GAA | Guanidinoacetate |
| GC/MS | Gas chromatography-mass spectrometry |
| GS | Gleason score |
| HMDB | Human Metabolome Database |
| NMR | Nuclear magnetic resonance |
| OPLS-DA | Orthogonal Partial Least Squares discriminant analysis |
| PBS | Phosphate buffer solution |
| PC | Prostate cancer |
| PCA | Principal component analysis |
| PLS-DA | Partial Least Squares discriminant analysis |
| PSA | Prostate specific antigen |
| ROC | Receiver operating characteristic curve |
| STITCH | Search tool for interactions of chemicals |
| TSP | Trimethylsilylpropionic acid-$d_4$ sodium salt |
| TSP | Trimethylsilyl propionate |
| TURP | Transurethral resection of the prostate |
| VIP | Variable importance in projection |

**6. Description of the scientific share of the applicant in the included publications**

Herewith we attest the following author contributions of Bo Yang in the publications:

1) Yang, B.; Liao, G.Q.; Wen, X.F.; Chen, W.H.; Cheng, S.; Stolzenburg, J.U.; Ganzer, R.; Neuhaus, J. Nuclear magnetic resonance spectroscopy as a new approach for improvement of early diagnosis and risk stratification of prostate cancer. *J Zhejiang Univ Sci B* **2017**;*18(11)*:921-33. doi:10.1631/jzus.B1600441.

**Bo Yang:** Conceptualization, Data curation, Formal analysis, Writing-original draft

15.1.2022 Xiaofei WEN
Date / Signature (Xiao-fei Wen)

12.01.2022 Guoqiang Liao
Date / Signature (Guo-qiang Liao)

17.1.2022 Weihua CHEN
Date / Signature (Wei-hua Chen)

12.01.2022 Sheng Cheng
Date / Signature (Sheng Cheng)

Date / Signature (Jens-Uwe Stolzenburg)

04.02.2022
Date / Signature (Roman Ganzer)

11.01.2022
Date / Signature (Jochen Neuhaus)

2) Neuhaus, J.; Yang, B. Liquid Biopsy Potential Biomarkers in Prostate Cancer. *Diagnostics (Basel)* **2018**;*8(4):68*. doi:10.3390/diagnostics8040068.

**Bo Yang:** Data curation, Writing & Editing

11.01.2022 .....
Date / Signature (Jochen Neuhaus)

3) Yang, B.; Zhang, C.; Cheng, S.; Li, G.; Griebel, J.; Neuhaus, J. Novel Metabolic Signatures of Prostate Cancer Revealed by $^{1}$H-NMR Metabolomics of Urine. *Diagnostics* **2021**;*11(2)*:149. doi:10.3390/diagnostics11020149.

Bo Yang: Conceptualization, Data curation, formal analysis, Writing-original draft, Review & Editing

12.01.2022 Chuan Zhang .....
Date / Signature
(Chuan Zhang, shared first authorship)

12.01.2022 Sheng Cheng .....
Date / Signature (Sheng Cheng)

12.01.2022 Gonghui Li .....
Date / Signature (Gonghui Li)

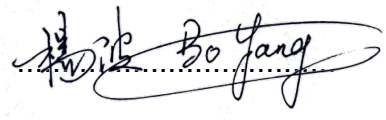
18.01.2022 Jan Griebel .....
Date / Signature (Jan Griebel)

11.01.2022 .....
Date / Signature (Jochen Neuhaus)

## 7. Declaration (Eigenständigkeitserklärung)

**Erklärung über die eigenständige Abfassung der Arbeit**

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar eine Vergütung oder geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, und dass die vorgelegte Arbeit weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt wurde. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt an der Entstehung der vorliegenden Arbeit beteiligt waren. Die aktuellen gesetzlichen Vorgaben in Bezug auf die Zulassung der klinischen Studien, die Bestimmungen des Tierschutzgesetzes, die Bestimmungen des Gentechnikgesetzes und die allgemeinen Datenschutzbestimmungen wurden eingehalten. Ich versichere, dass ich die Regelungen der Satzung der Universität Leipzig zur Sicherung guter wissenschaftlicher Praxis kenne und eingehalten habe.

16, Dec, 2021 ..... 

Datum Unterschrift

## 9. Scientific Publications and Awards

### 9.1. Publications

1. Deping Yang , **Bo Yang** , Yanjun Zhu , Qianlin Xia , Yan Zhang , Xin Zhu , Jianming Guo , Tao Ding , Jianghua Zheng  Circular RNA-DPP4 serves an oncogenic role in prostate cancer progression through regulating miR-195/cyclin D1 axis. Cancer Cell Int. 2021 Jul 16;21(1):379. doi: 10.1186/s12935-021-02062-z.
2. Cheng S, **Yang B**, Xu L, Zheng Q, Ding G, Li G. Vasectomy and prostate cancer risk: a meta-analysis of prospective studies. Carcinogenesis, 2021 Feb 11;42(1):31-37. doi: 10.1093/carcin/bgaa086.
3. **Yang B**, Zhang C, Cheng S, Li G, Griebel J, Neuhaus J. Novel Metabolic Signatures of Prostate Cancer Revealed by $^{1}$H-NMR Metabolomics of Urine. Diagnostics (Basel), 2021 Jan 20;11(2):149. doi: 10.3390/diagnostics11020149.
4. Neuhaus J, **Yang B.** Liquid Biopsy Potential Biomarkers in Prostate Cancer. Diagnostics (Basel). 2018 Sep 21;8(4):68. doi: 10.3390/diagnostics8040068.
5. **Yang B**, Liao GQ, Wen XF, Chen WH, Cheng S, Stolzenburg JU, Ganzer R, Neuhaus J. Nuclear magnetic resonance spectroscopy as a new approach for improvement of early diagnosis and risk stratification of prostate cancer. J Zhejiang Univ Sci B, 2017 Nov.;18(11):921-933. doi:10.1631/jzus.B1600441.

### 9.2. Published abstracts

NO

### 9.3. Awards

Bo Yang, The Experimental study on the SMO specific siRNA targeted treatment of prostate cancer**,** The science and technology progress award of Pudong, Shanghai, China, 2016

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