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# GC-MS metabolomics studies in Prostate Cancer: a novel approach using *in vitro* models

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**Abstract**

Cancer is a serious health problem in the world, both in terms of morbidity and mortality. Prostate cancer (PCa) is the most frequently diagnosed cancer and the second leading cause of cancer death among men in Western countries. Currently, prostate serum antigen (PSA) is the most used biomarker for PCa diagnostic. However, the PSA screening has limited sensitivity and specificity and PSA is not able to differentiate aggressive from indolent PCa.

Metabolomics is a powerful analytical tool with which biomarkers and therapeutic targets can likely be discovered because cancer cells have the capacity to modify many homeostatic systems within the body and consequently change the production, the use and consequently the levels of many metabolites. Metabolomics allows the discovery of biochemical signatures and with these signatures it is possible to investigate several metabolic pathways, and the differences between cancer and healthy metabolic phenotypes.

In this work a GC-MS was used as analytical platform to prove the concept that metabolic alterations were able to discriminate PCa cell lines (22RV1; PC3; DU145; LNCaP) from normal prostate cell line (PNT2). For that, in the first part of the work, we evaluated alteration in the volatiloma (extracellular metabolites) obtained at pH 7 and pH 2. In both approach, volatiloma revealed to be able to differentiate PCa cell lines from normal prostate cell line. The altered VOCs (volatile organic compounds) include ketones, aldehydes and organic acids. In the second part of the work, we evaluated alteration in intracellular metabolites and for that a derivatization protocol was used before GC-MS analysis. Once again the results reveal that metabolic alterations were able to differentiate PCa cell lines from normal prostate cell line. The altered metabolites include amino acids, sugars, steroids, and fatty acids. By evaluating the altered metabolites it was also possible to conclude that the main disrupted metabolic pathways, in consequence of neoplastic progression, were linked to the energetic metabolism, protein metabolism and lipid metabolism.

**Key words:** Prostate cancer; Metabolomics; Cell Lines; Volatile Carbonyl Compounds; Gas Chromatography /Mass Spectrometry.

**Resumo**

O cancro é um grave problema de saúde, em todo o mundo, tanto em termos de morbilidade como de mortalidade. O cancro da próstata (PCa) é o cancro mais frequentemente diagnosticado nos homens e o segundo cancro mais mortal para os homens nos países Ocidentais. Atualmente, o biomarcador mais usado para o diagnóstico desta doença é o antigénio específico da próstata (PSA). Contudo, este biomarcador tem uma sensibilidade e uma especificidade limitadas para o rastreio do cancro da próstata e, além disso, o PSA não é capaz de diferenciar cancros da próstata agressivos de cancros da próstata indolentes.

A metabolómica é uma poderosa ferramenta analítica, que pode ser usada para a descoberta de novos biomarcadores mas também de novos alvos terapêuticos. Isto é possível uma vez que as células cancerígenas têm a capacidade de modificar vários sistemas homeostáticos do organismo e consequentemente alterar a produção, o uso e os níveis de diversos metabolitos. A metabolómica permite descobrir a assinatura metabólica e, assim, utilizando esta assinatura é possível investigar várias vias metabólicas e as diferenças entre o fenótipo cancerígeno e o fenótipo saudável.

Neste trabalho foi usada a técnica de GC-MS como plataforma analítica, como prova de conceito de que as alterações metabólicas são capazes de discriminar linhas celulares de cancro da próstata (22RV1; PC3; DU145; LNCaP) da linha celular de próstata normal (PNT2). Para isso, na primeira parte do nosso trabalho, avaliamos as alterações no volatiloma (metabolitos extracelulares) at pH 7 e a pH 2. Em ambas as abordagens, o volatiloma mostrou ser capaz de diferenciar as linhas celulares de cancro da próstata da linha celular normal. Os compostos orgânicos voláteis (VOCs) alterados incluem cetonas, aldeídos, e ácidos orgânicos. Na segunda parte do trabalho, avaliamos alterações nos metabolitos intracelulares, usando um protocolo de derivatização, prévio á análise por GC-MS. Mais uma vez, os resultados revelaram que as alterações metabólicas foram capazes de diferenciar as linhas celulares cancerígenas da linha celular normal. Os metabolitos alterados incluem aminoácidos, açúcares, esteróides e ácidos gordos. A avaliação dos metabolitos alterados permite concluir que as principais vias metabólicas alteradas, em consequência da progressão neoplásica, são o metabolismo energético, o metabolismo proteico e o metabolismo lipídico.

**Palavras-chave:** Cancro da próstata; Metabolómica; Linhas celulares; Compostos carbonílicos voláteis; Cromatografia gasosa/espetroscopia de massa.

**General Index**

**Abbreviation list**

| | |
|---|---|
| **AAs** | Amino acids |
| **Acetyl-CoA** | Acetyl-coenzyme A |
| **AD** | Androgen-responsivee |
| **Ala** | Alanine |
| **AR** | Androgen receptor |
| **ATP** | Adenosine triphosphate |
| **BPH** | Benign prostate hyperplasia |
| **Cho** | Choline |
| **Cit** | Citrate |
| **$CO_2$** | Carbon dioxide |
| **CoA** | Coenzyme A |
| **Cr** | Creatinine |
| **CR** | Classification rate |
| **CRPC** | Castration-resistant prostate cancer |
| **DCA** | Dichloroacetate |
| **DHEAS** | Dehydroepiandrosterone sulfate |
| **DNA** | Deoxyribonucleic acid |
| **FBS** | Fetal bovine serum |
| **G6P** | Glucose-6-phosphate |
| **G6PD** | Glucose-6-phosphate dehydrogenase |
| **GC** | Gas chromatography |
| **GNMT** | Glycine N-methyltransferase |
| **(GPC + PC)/Cr** | (Glycerol-phosphocholine + phosphoryl-choline)/creatinine |
| **GPE** | Glycerophosphoethanolamine |
| **HCA** | Hierarchical cluster analysis |
| **HG** | High grade |
| **HMDB** | Human metabolome data base |
| **HS-SPME** | Head space – solid phase microextration |
| **KI** | Kovat indices |

| | |
|---|---|
| **Lac/Al** | Lactate/alanine |
| **Lac/Cho** | Lactate/choline |
| **Lac/Cr** | Lactate/Creatine |
| **Lac/(Cho + Cr + Ala)** | Lactate/(choline + creatine + alanine |
| **LC** | Liquid chromatography |
| **LDA** | Linear discriminant analysis |
| **LG** | Low grade |
| **LR** | Logistic regression |
| **LV** | Latent variables |
| **mCRPC** | Metastatic castration-resistant prostate cancer |
| **Min** | Minutes |
| **MRS** | Magnetic resonance spectrometry |
| **MS** | Mass spectrometry |
| **MSTFA** | N-Methyl-N-(trimethylsilyl) trifluoroacetamide |
| **MVA** | Multivariate analysis |
| **NA** | Not available |
| **NaCl** | Sodium chloride |
| **NIST** | National Institute of Standards and Technology |
| **NMR** | Nuclear magnetic resonance |
| **NS** | Not specified |
| **MCCV** | Monte Carlo cross-validation |
| **OPLS-DA** | Orthogonal partial least squares discriminant analysis |
| **3PG** | 3-Phosphoglycerate |
| **6P** | 6-Phosphate |
| **Par** | Pareto |
| **PCA** | Principal component analysis |
| **PCa** | Prostate cancer |
| **PIPOX** | Pipecolic acid oxidase |
| **PLS** | Partial least squares regression |
| **PLA-DA** | Partial least squares regression discriminant analysis |
| **PSA** | Prostate serum antigen |
| **$Q^2$** | Prediction power |
| **QCs** | Quality control samples |
| **Ref.** | References |

| | |
|---|---|
| **RF** | Radom forest |
| **ROC** | Receiver-operator characteristic |
| **ROS** | Reactive oxygen species |
| **RSD** | Relative standard deviation |
| **RT** | Retention time |
| **SARDH** | Sarcosine dehydrogenase |
| **Sens.** | Sensitivity |
| **Spec.** | Specificity |
| **TCA** | Tricarboxylic acid cycle |
| **tCho/Cit** | Total choline/citrate |
| **VIP** | Variable Importance Projection |
| **VOCs** | Volatile organic compounds |

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# 1. Theoretical Introduction

# **Chapter 1:** Biomarker Discovery in Human Prostate Cancer: an Update in Metabolomics Studies (review paper)

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# Biomarker Discovery in Human Prostate Cancer: an Update in Metabolomics Studies

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## Abstract

Prostate cancer (PCa) is the most frequently diagnosed cancer and the second leading cause of cancer death among men in Western countries. Current screening techniques are based on the measurement of serum prostate specific antigen (PSA) levels and digital rectal examination. A decisive diagnosis of PCa is based on prostate biopsies; however, this approach can lead to false-positive and false-negative results. Therefore, it is important to discover new biomarkers for the diagnosis of PCa, preferably noninvasive ones. Metabolomics is an approach that allows the analysis of the entire metabolic profile of a biological system. As neoplastic cells have a unique metabolic phenotype related to cancer development and progression, the identification of dysfunctional metabolic pathways using metabolomics can be used to discover cancer biomarkers and therapeutic targets. In this study, we review several metabolomics studies performed in prostatic fluid, blood plasma/serum, urine, tissues and immortalized cultured cell lines with the objective of discovering alterations in the metabolic phenotype of PCa and thus discovering new biomarkers for the diagnosis of PCa. Encouraging results using metabolomics have been reported for PCa, with sarcosine being one of the most promising biomarkers identified to date. However, the use of sarcosine as a PCa biomarker in the clinic remains a controversial issue within the scientific community. Beyond sarcosine, other metabolites are considered to be biomarkers for PCa, but they still need clinical validation. Despite the lack of metabolomics biomarkers reaching clinical practice, metabolomics proved to be a powerful tool in the discovery of new biomarkers for PCa detection.

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## Introduction

Systems biology applied to cancer research encompasses the "omics" tools, including genomics, transcriptomics, proteomics, and metabolomics, which complement each other and are capable of measuring changes in several entities (genes, transcripts, proteins, or metabolites, respectively) simultaneously, providing an overview of various physiological or pathological conditions [1–3].

Metabolomics can provide an idea of the physiological status of a biological system, and therefore, alterations in the "normal" metabolome may be indicative of disease. These alterations in the "normal" metabolome have the potential to deliver new diagnostic markers for the detection and prognosis of diseases and to monitor the response to therapeutic interventions [4]. Metabolomics also has the potential to give new understanding of the phenotypic changes resultant from genetic alterations, environmental influence, and toxicological influence [5].

The term *metabolomics* includes the assessment of all the endogenous metabolites produced by the organism including small molecule intermediates and end products of biochemical reactions in

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a cell (approximately ≤1500 Da), as well as exogenous metabolites, such as drugs, products from the body flora, and food. Instead of genes and proteins that participate in these biological processes, the metabolites produced are indicators of what is happening in the metabolism of a cell in physiological or pathophysiological conditions. Thus, metabolites can be altered in such diseases as cancer [1,6–9].

As neoplastic cells have a unique metabolic phenotype related to cancer development and progression, the identification of dysfunctional metabolic pathways through metabolomics can be used to identify cancer biomarkers and discover therapeutic targets [5,6,10].

## Prostate Cancer

Prostate cancer (PCa) is the second most diagnosed cancer in men [11], principally affecting men over 50 years old [12], and is the fifth leading cause of cancer-related deaths in men worldwide [11]. Statistically, in 25% of men worldwide with PCa that develop metastatic disease [13], the bones are the principal targets of PCa metastasis [14]. Given that PCa has a long latency period and is potentially curable, it is essential to develop efficient and precocious screening methods for its early detection and characterization [12].

The quantification of prostate serum antigen (PSA) and the digital rectal examination are the most common screening techniques used for PCa diagnosis. However, performing a prostate biopsy is mandatory for a final diagnosis [12]. Serum PSA levels higher than 4.0 ng/ml are a sign of PCa [14], although PSA is not able to differentiate patients with aggressive PCa from those with indolent disease [15]. The value of PSA screening is also controversial because of its limited sensitivity and specificity [1,16]. Recent studies suggested that certain PCa patients may present with PSA levels below 4.0 ng/ml [14]. This fact leads to false negatives, as no reliable cutoff values exist to demonstrate the unequivocal presence of PCa [16,17]. Furthermore, PSA levels may be affected by several other factors, such as age, prostatitis, urinary tract infection, and benign prostate hyperplasia, leading inevitably to false-positive results [14,16,18,19].

The biopsy analysis can also provide false-negative results when the tumor is small; when the cancer cells are distributed heterogeneously; and in early PCa stage when, histologically, the tumor appears benign [20–22]. Thus, samples obtained during the biopsy for histopathologic analysis may not be representative of the cancer [23].

The lack of a consistent biomarker for PCa diagnosis and monitoring highlights the need for novel, specific, sensitive, and cost-effective biomarkers to implement the best treatment approach in a precocious state of the disease [14].

## Altered Metabolism of PCa Cells

In 1920, Otto Warburg discovered that cancer cells, unlike nonmalignant cells, preferentially produce ATP through the glycolytic pathway (anaerobic pathway) instead of the Krebs cycle, even in the presence of oxygen. This capability of cancer cells to sustain high rates of glycolysis for ATP generation is known as the Warburg effect [24–26].

Despite the relevance of the Warburg effect in cancer cells, the Krebs cycle and oxidative phosphorylation also play an important role in many types of cancer, including PCa. Recent evidence suggests that increased citrate oxidation is an important metabolic characteristic in PCa that supports the high cellular energy demand [27]. One of the major functions of prostate cells is the production of citrate, PSA, and polyamines, such as spermine, which are the major components of prostatic fluid. Therefore, prostate cells have a distinct metabolic profile as they produce specific compounds [1,16]. The production of citrate by prostate cells is very high in comparison with other organs [28]. Unlike other cells in the organism, prostate cell metabolism significantly favors citrate synthesis over citrate utilization, which makes the prostate peripheral zone epithelium unique among human cells [29]. Usually, cells degrade citrate in aerobic ATP production, with citrate being oxidized during the Krebs cycle as part of the intermediary metabolism of glucose. However, nonmalignant prostate cells accumulate and secrete citrate. The oxidation of citrate is catalyzed by mitochondrial aconitase (m-aconitase). In normal prostate cells, m-aconitase is inhibited by the high intracellular concentrations of zinc, leading to citrate accumulation (Figure 1) [28,29]. Extensive metabolic alterations occur when prostate cells experience neoplastic transformation. One of the most relevant alterations is citrate oxidation, because cancer cells are unable to accumulate zinc, and without elevated levels of zinc, m-aconitase is no longer inhibited and can catalyze citrate oxidation [2,4,27,28,30]. This transformation of citrate accumulation in healthy prostate cells to oxidized citrate in malignant prostate cells results in more efficient energy production. This is probably an early event in the progression to malignancy and precedes the histopathological identification of malignant cells [27,31,32].

For citrate synthesis, oxaloacetate and acetyl-coenzyme A (acetyl-CoA) are essential, but whereas oxaloacetate is regenerated in the Krebs cycle, acetyl-CoA is consumed. To ensure that cancer cells have the needed energy for rapid proliferation, it is necessary to maintain elevated rates of citrate oxidation, and thus, the availability of acetyl-CoA is required. Some studies suggested that to maintain this accelerated citrate oxidation, alterations in fatty acid metabolism are needed to provide both ATP and acetyl-CoA [27,33,34] (Figure 1).

Beyond the Krebs cycle and glycolysis, glucose also can be degraded by the pentose phosphate pathway. This metabolic pathway provides NADPH and ribose-5-phosphate (important for the synthesis of nucleic acids and nucleotides), thus promoting anabolic reactions and redox homeostasis. In a recent study, Tsouko et al. (2014) demonstrated that androgen receptor (AR) signaling augmented the levels of glucose-6-phosphate dehydrogenase (G6PD) (key enzyme for pentose phosphate pathway), NADPH, and ribose synthesis in hormone-sensitive PCa cells and castrate-resistant PCa (CRPC) cells. After inhibition of mammalian target of rapamycin with rapamycin, the upregulation of G6PD is abolished. Hence, these studies revealed a relationship between the upregulation of G6PD via AR and mammalian target of rapamycin. These results suggested the importance of pentose phosphate pathway for PCa growth [35].

Cell proliferation and intercellular signaling are dependent on increased lipid biosynthesis. Acetyl-CoA also plays an important role in this metabolic alteration because it is a precursor for lipogenesis and cholesterogenesis and can be produced by transformation of citrate in the cytosol [1]. Sterol regulatory element-binding protein–1, an essential transcription factor for lipogenesis, is also implicated in AR transcriptional regulation. Beyond increased lipogenesis, sterol regulatory element-binding protein–1 also increased reactive oxygen species production and the expression of NADPH oxidase, which leads to proliferation, migration, and invasion of PCa cells [36–38]. In PCa cells, the levels of choline and creatine are increased because there is an augmentation of membrane synthesis for cell proliferation [16].

Glutamine also has an important role in the maintenance of lipogenesis, as well as to provide intermediates for the Krebs cycle through glutaminolysis (where glutamine is transformed into glutamate by glutaminase and then glutamate is transformed into

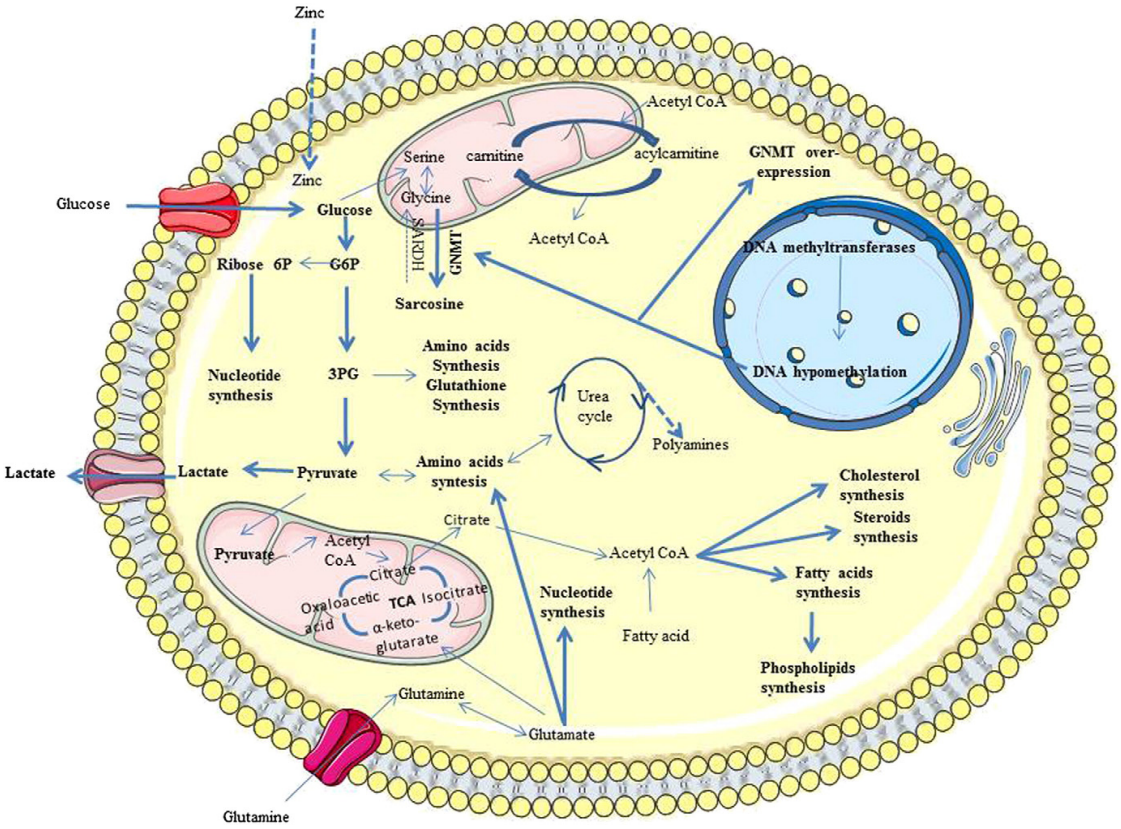


**Figure 1.** Schematic illustration of the most significantly altered metabolic pathways in PCa cells. Dashed lines = downregulated pathway; continuous line = upregulated pathway. Metabolites overexpressed in PCa cells are shown in bold. *TCA*, tricarboxylic acid (cycle); *AAs*, amino acids; *DNA*, deoxyribonucleic acid; GNMT, glycine N-methyltransferase; SARDH, sarcosine dehydrogenase; G6P, glucose-6-phosphate; 6P, 6-phosphate; 3PG, 3-phosphoglycerate; *CoA*, coenzyme A.

α-ketoglutarate). The observation that the glutamine transporter and glutaminase are both overexpressed in PCa cells was proof of the importance of this mechanism in PCa [38–40]. The α-ketoglutarate derivate from glutamine can contribute to the formation of citrate when incorporated into the Krebs cycle (oxidation pathway); however, α-ketoglutarate can also be transformed into citrate by the reversal of the tricarboxylic acid cycle through reductive carboxylation. This alteration of oxidation to reductive carboxylation is promoted by hypoxia and leads to lipid synthesis and tumor growth [41]. The tumor-stromal interactions also have an important role in PCa development. The myofibroblastic microenvironment, formed from the interaction of cancer cells with "cancer-associated fibroblasts", is important for the reverse Warburg effect. Cancer-associated fibroblasts in the myofibroblastic microenvironment undergo the Warburg effect, induced by epithelial cancer cells, and secrete lactate and pyruvate. The lactate and pyruvate are taken up by the PCa cells and used for the Krebs cycle, anabolic metabolism, and cell proliferation [38,42,43].

### *Metabolomics Studies in PCa Model Systems and Biological Fluids*

The most common models and biological fluids used to perform metabolomics studies are tissue and cultured cell lines and human urine, serum/plasma, prostatic fluid/seminal fluid, respectively.

The use of urine as a sample for metabolomics studies has many advantages compared with serum: urine is easier to obtain and handle, needs less sample preparation, and has higher amounts of metabolites and a lower protein content [12,44,45]. Blood plasma/serum has some advantages compared with urine, as the diurnal variation and the intra- and intervariability are lower. However, serum and plasma are more complex matrices than urine, having a higher concentration of proteins, and sample collection is more invasive [5].

Seminal fluids, obtained by ejaculation, come from the seminal vesicles, prostate, and epididymis. Prostatic fluid is collected after prostate massage, and the composition of this biofluid is simpler than seminal fluid [46,47]. The use of seminal/prostatic fluids has some advantages compared with the use of other biofluids, as these samples are richer in prostatic metabolites because the metabolites do not need to cross blood-tissue barriers once they are naturally secreted into the seminal/prostatic fluid. Thus, seminal/prostatic fluids are less affected by confounding factors. However, these biofluids may be difficult to collect in men with erectile dysfunction, and a portion of men may have personal or ethical problems with giving these types of samples [48].

The collection of tissue samples is more invasive than the collection of other matrices; however, the use of matched malignant and normal

**Table 1.** Metabolomic Studies Performed in Urines from PCa Patients

| PCa Subject Group | Control Group | Analytical Platform | Statistical Methods | Total Metabolites Found/Discriminative Metabolites Found | Discriminatory Metabolites/Biomarkers | Metabolic Pathways Dysregulated | Ref. |
|---|---|---|---|---|---|---|---|
| *n*= 13 | *n*= 24 | GS-MS | Binary strings, Similarity coefficients | 91/21 | Butyrolactone, methyl vinyl ketone, methylamine, *N*-ethylformamide, acetonitrile dimethylamino, pyridine, *N*-methyl-formamide, acetaldehyde, acetamide, 1-methyl-piperidine, 1-piperidineacetonitrile, dimethylamine, pyrrole, methacrolein, *N*-*N*-dimethylamine, 3-methyl-pyridine, 2-methyl-1H-pyrrole, 2-octanone, 1-ethyl-1H-pyrrole, 2-n-butylacrolein and methyl propyl disulfide | NS | [65] |
| *n*= 59 | *n*= 51 | LC-MS GS-MS | Wilcoxon *P* test, hierarchical clustering, nonparametric tests | 583/34 | Sarcosine (+) | Alterations in glycine synthesis and degradation | [53] |
| *n*= 106 | *n*= 57 (33 patients with no evidence of malignancy plus 24 HC) | GC-MS | Nonparametric statistical tests and ROC | NS/0 | No relevant differences in sarcosine levels between patients with and without PCa | | [61] |
| *n*= 3 | *n*= 5 | LC-MS | NS | NS/5 | 1.Sarcosine<br>2.Proline<br>3.Kynurenine (+)<br>4.Uracil (+)<br>5.Glycerol 3-phosphate (+) | 1. Alterations in glycine synthesis and degradation<br>Sarcosine is an intermediate compound in the metabolism of choline.<br>2. Alteration in amino acids metabolism<br>3. Alteration in kynurenine pathway<br>4. Alteration in pyrimidine metabolism<br>5. Alteration in energetic metabolism | [55] |
| *n*= 25 PCa patients developing biochemical recurrence | *n*= 29 PCa patients who remained recurrence-free | GC-MS | ROC | 8/2 | Sarcosine (+)<br>Cysteine (+)<br>(in the group developing biochemical recurrence) | Alterations in glycine synthesis and degradation | [56] |
| *n*= 33 | *n*= 23 (13 HC plus 10 patients with BPH) | GC-MS | Nonparametric statistical tests and ROC | NS/1 | Sarcosine (+) | Alterations in glycine synthesis and degradation | [57] |
| *n*= 86 | *n*= 45 | LC-MS | ROC | NS/1 | Diagnostic value of sarcosine was modest; relationship with clinicopathologic parameters was not found | Alterations in glycine synthesis and degradation | [58] |
| *n*= 20 | *n*= 28 (8 patients with BPH plus 20 HC) | GC-MS | PCA<br>ROC | 81/5 | Dihydroxybutanoic acid (+), xylonic acid (+), pyrimidine (–), ribofuranoside(–), and xylopyranose(–) | Alterations in carbohydrate and energy metabolism | [60] |
| *n*= 211 | *n*= 134 | GC-MS | ROC | NS/1 | Sarcosine (+) | Alterations in glycine synthesis and degradation<br>Sarcosine is an intermediate compound in the metabolism of choline. | [59] |
| *n*= 32 | *n*= 32 | LC-MS GC-MS | PCA PLS-DA | 1132/15 | 1. Glycine (–), serine(–), threonine (–), alanine (–)<br>2. Glutamine (–), citrate (–), aconitate (–), succinate (–)<br>3. Sucrose (–), sorbose (–), arabinose (–), arabitol (–), inositol (–), galactaric acid (–)<br>4.Carnitines (–) | 1. Alteration in amino acids metabolism<br>2. Disturbance in energy metabolism<br>3. Dysregulation in carbohydrates degradation<br>4. Alteration in long-chain fatty acids metabolism | [63] |
| *n*= 59 | *n*= 43 | GC-MS | RF<br>LDA | 196/4 | 1. 2,6-dimethyl-7-octen-2-ol (–), 3-octanone (–), 2-octanone (–)<br>2. Pentanal (+) | 1. Increase of utilization of these metabolites for increased energy consumption<br>2. Inflammatory conditions via the excessive production of reactive oxygen species, known to induce lipid peroxidation | [64] |

*BPH*, benign prostatic hypertrophy; *GS-MS*, Gas chromatography–mass spectrometry; *HC*, healthy controls; *LDA*, linear discriminant analysis; *LC-MS*, Liquid chromatography–mass spectrometry; *NS*, not specified; *PCA*, Principal component analysis; *PLS-DA*,Partial least squares discriminant analysis *RF*, random forest; *ROC*, receiver-operator characteristic.
(+): levels increased in PCa; (–): levels decreased in PCa.

adjacent prostate tissue is a good strategy to reduce intraindividual variability in metabolomics studies [49].

*In vitro* models are increasingly used because of interindividual variability, and difficulties enrolling patients in these models are nonexistent. In addition, cell lines have a perfectly defined cell state which allows the analysis of a targeted metabolic status [2,50,51]. However, they do not efficiently simulate the complex cell-cell and cell-matrix interactions occurring within an organism [2,52].

There are two different metabolomics approaches to discover biomarkers for cancer: the top-down approach and the bottom-up approach. Both approaches have advantages and disadvantages. The top-down approach has the advantage of starting the metabolome

**Table 2.** Metabolic Studies in Serum/Plasma from PCa patients

| PCa Subject Group | Control Group | Analytical Platform | Statistical Methods | Total Metabolites Found/ Discriminative Metabolites Found | Discriminatory Metabolites/Biomarkers | Metabolic Pathways Dysregulated | Ref. |
|---|---|---|---|---|---|---|---|
| *n*= 962 | *n*= 1061 HC | GC-MS | Conditional logistic regression | NS/5 | Palmitic acid (+), stearic acid (–), myristic acid (+), linolenic acid (+), eicosapentaenoic acids (+) | Alteration in lipid metabolism | [70] |
| *n*= 85 HG plus *n*= 120 LG | *n*= 114 HC | F I A - M S / M S LC-MS/MS | ROC and logistic regression model | 112/5 | 1. Lysophosphatidyl-choline (C16:0 and C18:0) (–)<br>2. Serotonin (–)<br>3. Aspartic acid (+)<br>4. Ornithine (–) | 1. Alteration in lipid metabolism<br>2. Alteration in growth inhibition and induction of apoptosis<br>3. Alteration in protein biosynthesis<br>4. Ornithine decarboxylase overexpression | [75] |
| *n*= 561 | *n*= 1034 HC | GC-MS<br>LC-MS | Wilcoxon signed rank tests and $\chi^2$ tests | 7/3 | Choline (+), vitamin B2 (+), methylmalonic acid (–) | Alteration in membrane phospholipidic metabolism | [71] |
| *n*= 134 | *n*= 666 HC | LC-MS | ROC | 19/7 | Glutamine (–), alanine (+), valine (–), isoleucine (+), tryptophan (–), ornithine (+), lysine (+) | Alteration in free amino acid metabolism | [73] |
| *n*= 28 Serum from patients developing biochemical recurrence | *n*= 30 Serum from patients with recurrence free 5 years after prostatectomy | LC-MS<br>GC-MS | ROC | 9/3 | Cystathionine (+), homocysteine (+), cysteine (+) | Alteration in methionine metabolism | [56] |
| *n*= 36 Fasting plasma from PCa patients 3 months after the therapy initiation | *n*= 36 Fasting plasma from PCa before starting androgen deprivation therapy | LC-MS<br>GC-MS | *t* tests | 504/56 | 1. DHEAS (–), epiandrosterone sulfate (–), androsterone sulfate (–), cortisol (–), 4-androsten-3β (–), 17β-diol disulfates 1 & 2 (–), 5α-androstan-3β (–) 17β-diol disulfate (–), pregnen-diol disulfate (–), pregn steroid monosulfate (–) and andro steroid monosulfates 1 & 2 (–).<br>2. Cholate (+), glycocholate (+), taurocholate (+), chenodeoxycholate (+), taurochenodeoxycholate (+), ursodeoxycholate (+), hyodeoxycholate (+), deoxycholate (+), taurodeoxycholate glycodeoxycholate (+), glycochenodeoxycholate (+), 7-ketodeoxycholate (+) glycochenodeoxycholate (+), glycolithocholate sulfate (+), taurolithocholate 3-sulfate (+), glycocholenate sulfate (–), and taurocholenate sulfate (–)<br>3. Carnitines (–), ketone bodies (–), dicarboxylic acids<br>4. 2-hydroxybutyrate (–) and branched-chain keto-acid dehydrogenase complex products (–) | 1. Steroids metabolism<br>2. Bile acids and intermediates of bile acid metabolism<br>3. Lipid oxidation<br>4. Markers of insulin resistance | [78] |
| *n*= 290 | *n*= 312 | Fluorometric assay | ROC | 1/1 | Sarcosine (+) | Alterations in glycine synthesis and degradation | [66] |
| *n*= 105 | *n*= 36 | ESI-MS/MS | PCA and HCA | 390/35 | Phosphatidylethano-lamine (+), ether-linked phosphatidylethanola mine (+), ether-linked phosphatidylcholine (+) | Alteration in lipid metabolism | [72] |
| *n*= 1122 (813 serum from patients with nonaggressive PCa plus 309 serum from patients with aggressive PCa (Gleason score 8)) | *n*= 1112 | LC-MS | ROC | NS/1 | Sarcosine (+) | Alterations in glycine synthesis and degradation | [67] |
| *n*= 25 | *n*= 100 HC | Immunoassay | NS | 4/1 | Insulin (+) | Alteration in energetic metabolism | [76] |
| *n*= 64 | *n*= 50 HC | LC-MS | PCA | 480/49 | Azelaic acid, uric acid, tryptophan, lysoPC | Alteration in fatty acids | [69] |

**TABLE 2** (*continued*)

| PCa Subject Group | Control Group | Analytical Platform | Statistical Methods | Total Metabolites Found/ Discriminative Metabolites Found | Discriminatory Metabolites/Biomarkers | Metabolic Pathways Dysregulated | Ref. |
|---|---|---|---|---|---|---|---|
| | | | PLS | | (18:0/0:0), 3-oxo-9,11-tridecadienoic acid, 3-hydroxy-tetradecanedioic acid, 6-hydroxy-pentadecanedioic acid, 5-(2-methylpropyl)-2-oxooxolane-3-carboxylic acid, 5-butyl-2-oxooxolane-3-carboxylic acid, lysoPE (0:0/18:2), LysoPE (18:2/0:0), lysoPC (18:2/0:0), cortolone-3-glucuronide, pregnanetriol glucuronide, androstenedione, decanoic acid, menthol glucuronide, citronellol glucuronide, l-α-amino-1H-pyrrole-1-hexanoic acid, lysoPC (0:0/18:2), phenylalanyl phenylalanine 3β,16α-dihydroxyandrostenone sulfate, 2-tert-butyl-1,4-benzenediol sulfate, indoxyl sulfuric acid,10-dihydroxy-12Z,15Z-octadecadienoic acid,12,13-dihydroxy-9Z, 15Z-octadecadienoic acid, 5,16-dihydroxy-9Z,12Z-octadecadienoic acid, 27-nor-5β-cholestane-3α,7α,12α,24,25-pentol glucuronide, hexadecanedioic acid phenylacetylglutamine, heptadecanoic acid, n-[(3α,5β,7β)-7-hydroxy-24-oxo-3-(sulfooxy)cholan-24-yl]-glycine, n-[(3α,5β,7α)-3-hydroxy-24-oxo-7-(sulfooxy)cholan-24-yl]-glycine, glycochenodeoxycholate-3-sulfate, 5-isopropyl-2-methylphenol, sulfate, 5-carboxy-α-chromanol glucuronide, indole-3-carboxaldehyde, androsterone sulfate, 5α-dihydrotestosterone sulfate and etiocholanolone sulfate | metabolism, amino acids metabolism, lysophospholipids metabolism, and bile acids metabolism and alteration in steroid hormone biosynthesis pathway | |
| *n*= 70 40 LG PCa plus 30 HG PCa | *n*= 32 HC | $^{1}$H-NMR | PCA, OPLS-DA and ROC | NS/4 | 1.Alanine (+), pyruvate(+) 2.Sarcosine (+) glycine (–) | 1. Alteration in energetic metabolism and lipogenesis 2. Alterations in glycine synthesis and degradation | [68] |
| *n*= 29 | *n*= 21 BPH | NMR LC-MS GC-MS | PCA and ROC | 348/53 | 1. Acylcarnitines 2. Choline (glycerol-phospholipids) 3. Arginine | 1. Alteration in fatty acids metabolism 2. Alteration in membrane phospholipidic metabolism 3. Alteration in amino acids metabolism | [74] |

*BPH*, Benign prostatic hypertrophy; *DHEAS*, dehydroepiandrosterone sulfate; *HC*, Healthy Controls; *HCA*, hierarchical clustering analysis; *GS-MS*, Gas chromatography–mass spectrometry; *HG*, high grade; *LC-MS*, Liquid chromatography–mass spectrometry; *LG*, low grade; *NS*, Not specified; *OPLS-DA*, orthogonal partial least squares discriminant analysis; *PCA*, principal component analysis; *PLS*, Partial least squares; *ROC*, Receiver-Operator Characteristic.
(+): levels increased in PCa; (–): levels decreased in PCa.

evaluation using a real sample (urine or plasma) that could be used in a clinical practice. However, urine and plasma are complex biological matrices and have metabolites from different origins, and the metabolites may be more diluted. The advantage of the bottom-up approach (starts with the metabolic analyses of cell lines) is that cultured cell lines are a simpler biological system with less interference factors. In fact, studies with immortalized cultured cells are important to eliminate confounding factors, such as the age of patients, smoking habits, diet, and other diseases that influence the intervariability in plasma and urine. Nevertheless, the findings in cultured cell lines may not be directly extrapolated to the real disease as it is practically impossible to simulate complex cell–cell and cell–matrix interactions in cell cultures of PCa. Both approaches used together can be useful tools to obtain metabolic information that can discriminate the metabolic pathways in PCa cells.

### *Studies with Human Fluids and Model Systems*

*Urine Studies.* Table 1 summarizes the major metabolites and metabolic pathways that have been found to be dysregulated in metabolomics studies performed on urine samples from PCa patients.

One of the most relevant metabolomics studies was performed by Seekumar et al. (2009), where sarcosine (*N*-methylglycine) was discovered as possible biomarker in urine for PCa. Sarcosine, an intermediate product in the synthesis and degradation of glycine, was found to be highly elevated during PCa progression to metastasis and was not detected or was presented at very low concentrations in the urine of healthy individuals [53]. Carcinogenesis alters the biosynthesis of sarcosine, although the importance of sarcosine in carcinogenesis remains unknown. It is known that glycine *N*-methyltransferase (GNMT) has a significant role in the metabolism of PCa tissues. This enzyme catalyzes the conversion of glycine to sarcosine and also participates both in the metabolism of methionine and in gluconeogenesis [54]. Similar results were achieved in other studies performed with different analytical platforms using urine as the matrix (Table 1) [55–59]. However, other studies concluded that urinary sarcosine levels were not significantly different between PCa patients and healthy controls (Table 1) [60,61]. According to Issaq and Veenstra (2011), several causes for such divergences can be associated to different study design and methods, which can have diverse specificities and sensitivities, and also to interindividual differences [62].

Others common metabolic alterations detected in urine from PCa patients are alterations in amino acids, organic acids, sphingolipids, fatty acids, and carbohydrates. Dysregulation of carbohydrate degradation occurs because carbohydrates can be used by cancer

**Table 3.** Metabolomic Studies in Prostatic and Seminal Fluid from PCa Patients

| PCa Subject Group | Control Group | Analytical Platform | Statistical Methods | Total Metabolites Found/Discriminative Metabolites Found | Discriminatory Metabolites/Biomarkers | Metabolic Pathways Dysregulated | Ref. |
|---|---|---|---|---|---|---|---|
| *n*= 13 (prostatic fluid) | *n*= 28 chronic prostatitis, *n*= 28 adenoma *n*= 22 HC | Fluorescence | Student's *t* test and Kolmogorov-Smirnov test | 1/1 | Zinc (–) | Lose capability to accumulated zinc | [79] |
| *n*= 4 (prostatic fluid) | *n*= 10 BPH *n*= 12 | NMR | Multiple regression | NS/3 | 1. Citrate (–) 2. Spermine (–) and myo-inositol (–) | 1. Alteration in energetic metabolism 2. Alteration in polyamines synthesis | [80] |
| *n*= 3 (seminal fluid) | *n*= 3 *n*= 1 BPH | NMR | NS | NS/1 | Citrate (–) | Alteration in energetic metabolism | [46] |
| *n*= 21 (seminal fluid) *n*= 7 (prostatic fluid) | *n*= 16 (seminal fluid) *n*= 17 (prostatic fluid) | NMR | ROC | NS/1 | Citrate (–) | Alteration in energetic metabolism | [81] |
| *n*= 52 (prostatic fluid) | *n*= 26 | NMR | LR and ROC | 9/3 | 1. Citrate(–) 2. Myo-inositol (–) and spermine (–) | 1. Alteration in energetic metabolism 2. Alteration in polyamines synthesis | [82] |

*BPH*, Benign prostatic hypertrophy; *LR*, logistic regression; *NMR*, Nuclear magnetic resonance; *NS*, Not specified; *ROC*, Receiver-operator characteristic.
(+): levels increased in PCa; (–): levels decreased in PCa.

cells for energy production. Alterations in carnitine profiles were also detected; carnitines and their derivatives are important for conservation of regular mitochondrial function as well as the transport of activated long-chain fatty acids from the cytoplasm to the mitochondrial compartment. The results also suggested disturbances in energy metabolism, including the Krebs cycle, which were expected given the Warburg effect and the alteration of the activity of m-aconitase, as previously explained (Table 1) [60,63].

Potentiality of urinary volatile organic compounds to discriminate between PCa samples and controls was also evaluated in several different studies. In these studies, volatile organic compounds were able to differentiate urine from PCa patients and from control individuals (Table 1) [64,65].

*Plasma and Serum Studies.* A summary of metabolomics studies performed on serum and plasma samples can be found in Table 2.

Studies evaluating sarcosine as a biomarker for PCa were performed in serum samples using different analytical platforms [fluorometric assay, liquid chromatography–mass spectrometry (LC-MS), and $^{1}$H-nuclear magnetic resonance (NMR)]. The results showed that PCa samples had elevated levels of sarcosine and could distinguish low-grade from high-grade PCa, suggesting plasmatic level of sarcosine as a good biomarker for PCa (Table 2) [66–68].

Beyond the alteration in sarcosine levels, metabolomics studies of serum/plasma from PCa patients also revealed alterations in fatty acids, amino acids, lysophospholipids, bile acids, and metabolites related to the steroid hormone biosynthesis pathway. The alteration in fatty acids is related to changes in lipid β-oxidation necessary to provide energy for abnormal cell proliferation (Table 2) [69,68,70–75]. Alterations in energetic metabolism are also common [68,76] (Table 2), and increased levels of glucose in serum samples at the time of PCa diagnosis were associated with an increased risk of recurrences after therapy with radical prostatectomy or radiation therapy [77].

Serum and plasma metabolomics studies can also be used to assess alterations in the metabolic profile caused by medical treatment. The metabolic profile of fasting plasma from PCa patients before starting androgen deprivation therapy and 3 months after the therapy initiation was analyzed [78]. As expected, steroid levels decreased during androgen deprivation therapy, whereas the levels of most bile acids and their metabolites increased with therapy. Bile acids have an important role in the control of serum lipids, glycemic regulation, and energy homeostasis. Lower levels of metabolites related to lipid metabolism after 3 months of treatment were also observed. Carnitines, ketones, dicarboxylic acids, and the levels of 2-hydroxybutyrate and branched-chain keto-acid dehydrogenase complex products (biomarkers of insulin resistance) were also present in lower levels after the therapy, indicating a reduction in the catabolic state [78].

*Prostatic Fluid and Seminal Fluid Studies.* Table 3 presents a summary of metabolomics studies performed in prostatic fluid and seminal fluid from PCa patients.

Prostatic and seminal fluids are other biofluids that may be used to perform PCa metabolomics studies to discover alterations in cancer cell metabolism and noninvasive biomarkers for PCa detection.

As explained previously, normal prostate cells have the ability to accumulate zinc and consequently accumulate citrate. However, PCa cells lose this ability. Several metabolomics studies support this theory. The analysis of prostatic and seminal fluid using different analytical platforms (fluorescence technique and NMR) revealed reduced levels of zinc and citrate in PCa groups when compared with the controls (Table 3) [46,79–82]. Kline et al. (2006) also concluded that citrate level tests outperform the PSA test in PCa detection. Additionally, the analysis of citrate in semen has the same efficacy as the analysis of citrate in prostatic secretion for detecting PCa [81].

Another important function of normal prostate cells is the synthesis of polyamines, such as spermine and myo-inositol. The analysis of prostatic and seminal fluid from PCa patients showed significantly decreased levels of spermine and myo-inositol (Table 3) [80,82]. Serkova et al. (2008) also demonstrated that the reduction in citrate, spermine, and myo-inositol levels is independent of the patient's age [82].

*Ex Vivo Tissue Studies.* Table 4 presents a summary of metabolomics studies performed in PCa tissues.

The value of sarcosine as a PCa biomarker was also evaluated in prostate tissue samples using different analytical platforms. The levels of sarcosine were increased in PCa samples (Table 4) [15,53,59,83]. Results also revealed that sarcosine levels were significantly elevated in metastatic PCa and clinically localized PCa tissue samples, whereas in benign samples, sarcosine was not detected. These results indicate that sarcosine may be a good biomarker for monitoring disease progression and aggressiveness [53].

Other metabolites, namely citrate, lactate, and alanine, were frequently altered in PCa tissue samples. These results suggest alterations in citrate synthesis (Krebs cycle) and in energetic metabolism. As previously explained, the PCa cells switch from

**Table 4.** Metabolomic Studies in Prostate Cancer Tissue

| PCa Subject Group | Control Group | Analytical Platform | Statistical Methods | Total Metabolites Found/Discriminative Metabolites Found | Discriminatory Metabolites/Biomarkers | Metabolic Pathways Dysregulated | Ref. |
|---|---|---|---|---|---|---|---|
| *n*= 21 | *n*= 66<br>Benign prostate tissue | MRS | LDA | NS/6 | 1. Citrate (–)<br>2. Taurine (+), glutamate (+) | 1. Reduced citrate synthesis (Krebs cycle)<br>2. Alteration in energy metabolism | [85] |
| *n*= 10<br>Malignant human prostate tissue | *n*= 10<br>BPH | $^{1}$H-NMR | Nonparametric test of Kruskal-Wallis | NS/3 | 1. Citrate (–)<br>2. Choline (+)<br>3. Myo-inositol (+) | 1. Reduced citrate synthesis (Krebs cycle)<br>2. Increased membrane turnover<br>3. Altered membrane metabolism | [86] |
| *n*= 15<br>Adenocarcinoma | *n*= 1<br>HC | $^{1}$H-NMR | Linear regression analysis | NS/2 | 1. Citrate (–)<br>2. Spermine (–) | 1. Reduced citrate synthesis<br>2. Reduced spermine synthesis (amino acid synthesis) | [87] |
| *n*= 27<br>Adenocarcinoma | *n*= 44<br>BPH | MRS | NS | 22/3 | 1. Citrate (–)<br>2. Choline (+)<br>3. Lipid/lysine ratio (+) | 1. Reduced citrate synthesis<br>2. Increased membrane turnover<br>3. Increased cellular proliferation | [88] |
| *n*= 20 | *n*= 33<br>Benign tissue | $^{1}$H-NMR | Nonparametric Spearman correlation coefficients | NS/8 | 1. Choline (+), phosphocholine (+), glycerophospho-choline (+)<br>2. Taurine (+)<br>3. Myo-inositol (+), scyllo-inositol (+)<br>4. Citrate (–), polyamines (–) | 1. Alteration in phospholipid membrane synthesis and hydrolysis<br>2. Alteration in energy metabolism<br>3. Altered membrane metabolism<br>4. Reduced citrate and polyamines synthesis | [89] |
| *n*= 15 | *n*= 32<br>Benign prostatic tissue | $^{1}$H-NMR | Z statistics | NS/5 | Phosphocholine (+), glycerol-phosphocholine (+), phosphor-ethanolamine (+), glycerophospho-ethanolamine (+), ethanolamine (–) | Alterations on phospholipid membrane assembly and catabolism | [92] |
| *n*= 16 | *n*= 82<br>Benign prostate biopsies | $^{1}$H-NMR | NS | NS/2 | 1. Lactate (+)<br>2. Alanine (+) | 1. "Warburg effect"<br>2. Intensification in glycolytic flux and increased protein synthesis in cancer cells | [90] |
| *n*= 18 | *n*= 30 | 1H-NMR | LR | NS/7 | tCho/Cit (+), Cho/Cr (+), GPC + PC)/Cr (+), Lac/Al (+) Cit/Cr (–) | Alterations in citrate synthesis (Krebs cycle), in membrane turnover, and in energetic metabolism | [84] |
| *n*= 12 Localized PCa *n*= 14 metastatic PCa | *n*= 16 Benign tissue adjacent to PCa | LC-MS<br>GC-MS | Wilcoxon *P* test, hierarchical clustering, nonparametric tests | 626/60 | 1. Sarcosine (+)<br>2. Uracil (+)<br>3.Kynurenine (+)<br>4. Glycerol-3-phosphate (+),<br>5. Leucine (+), proline (+) | 1. Alterations in glycine synthesis and degradation Sarcosine is an intermediate compound in the metabolism of choline.<br>2. Alterartion in pyrimidine metabolism<br>3.Alteration in Kynurenine pathway<br>4. Alteration in energetic metabolism<br>5. Alteration in amino acids metabolism | [53] |
| *n*= 27 | *n*= 54 | NMR | NS | NS/NS | Omega-6 PUFA (+) | Alteration in lipid metabolism | [95] |
| *n*= 16<br>patients with chemical failure | *n*= 32<br>Patients without recurrence after prostatectomy | $^{1}$H-NMR | PCA | NS/6 | 1. Spermine<br>2. Myo-inositol, phosphoryl, scyllo-inositol<br>3. Choline<br>4. Glutamate, glutamine | 1. Reduced spermine synthesis (amino acid synthesis)<br>2. Altered membrane metabolism<br>3. Alteration in phospholipid membrane synthesis and hydrolysis<br>4.Alteration in energy metabolism | [93] |
| *n*= 41 | *n*= 108<br>Benign adjacent prostate tissue | $^{1}$H-NMR | Binary logistic regression and multivariate linear regression | 13/6 | 1. Choline compounds (+)<br>2. Myo-inositol (–), scyllo-inosito (+) | 1. Alteration in phospholipid membrane synthesis and hydrolysis<br>2. Altered membrane metabolism | [94] |
| *n*= 92 | *n*= 92 | GC-MS | Nonparametric statistical tests and ROC | NS/1 | Sarcosine (+)<br>(sarcosine levels were not related with tumor stage, grade or biochemical recurrence) | Alterations in glycine synthesis and degradation. Sarcosine is an intermediate compound in the metabolism of choline. | [83] |
| *n*= 331 | *n*= 178 | GC-MS<br>LC-MS | ROC | 469/200 | 1. Sarcosine (+)<br>2. Kynurenine (+)<br>3. Proline (+)<br>4. Uracil (+)<br>5.Glycerol-3-phosphate (+), threonine (+) citrate (–) ADP-ribose (–)<br>15-hydroxyeicosatetraenoic acid(–)<br>6.Polyanines (–) | 1. Alterations in glycine synthesis and degradation Sarcosine is an intermediate compound in the metabolism of choline.<br>2. Alteration in kynurenine pathway<br>3.Alteration in amino acids metabolism<br>4.Alterartion in pyrimidine metabolism<br>5.Alteration in energetic metabolism<br>6.Reduced polyamines synthesis | [15] |
| *n*= 11<br>Localized PCa plus *n*= 10 metastatic PCa | *n*= 11<br>Benign adjacent prostate samples | GC-MS | ROC | NS/1 | Sarcosine (+) (progressive elevation from benign tissue to localized tumors and metastatic disease) | Alterations in glycine synthesis and degradation<br>Sarcosine is an intermediate compound in the metabolism of choline. | [59] |

*(continued on next page)*

**TABLE 4** (*continued*)

| PCa Subject Group | Control Group | Analytical Platform | Statistical Methods | Total Metabolites Found/Discriminative Metabolites Found | Discriminatory Metabolites/Biomarkers | Metabolic Pathways Dysregulated | Ref. |
|---|---|---|---|---|---|---|---|
| *n*= 95 | *n*= 95 Normal adjacent prostate tissue | GC-MS LC-MS | ROC, Univariate Cox regression, Kaplan-Meier analyses, and multivariate Cox regression analyses | 820/9 | 1. Gluconic acid (–), maltotriose (–)<br>2. Aminoadipic acid (+)<br>3. Cerebronic acid (+), glycerophosphoethanol-amine (+), 2-hydroxybehenic acid (+), tricosanoic acid (+)<br>4. Isopentenyl pyrophosphate (+)<br>5. 7-methylguanine (+) | 1. Alteration in carbohydrate metabolism<br>2. Increased fatty acid synthesis<br>3. Alteration in lipid metabolism<br>4. Intermediate in the steroid synthesis pathway, indicates an increase of cholesterol levels<br>5. DNA damage | [49] |
| *n*= 30 LG PCa plus *n*= 81 HG PCa | *n*= 47 Normal adjacent tissue | MRS | PCA, PLS and PLS-DA | 23/2 | 1. Citrate (–)<br>2. Spermine (–) | 1. Reduced citrate synthesis<br>2. Reduced spermine synthesis | [91] |

*BPH*, Benign prostatic hypertrophy; *Cho/Cr*, choline/creatinine; *Cit/Cr*, citrate/creatinine; *GS-MS*, Gas chromatography–mass spectrometry; *(GPC + PC)/Cr*, (glycerol-phosphocholine + phosphoryl-choline)/creatinine; *HC*, Healthy Controls; *HG*, High grade; *Lac/Al*, lactate/alanine; *LC-MS*, Liquid chromatography–mass spectrometry; *LDA*, Linear discriminant analysis; *LG*, Low grade; *LR*, Linear regression *MRS*, magnetic resonance spectroscopy; *NMR*, Nuclear magnetic resonance; *NS*, Not specified; *PCA*, Principal component analysis; *PLS*, partial least squares; *PLS-DA*, partial least squares discriminant analysis; *tCho/Cit*, total choline/citrate.
(+): levels increased in PCa; (–): levels decreased in PCa.

citrate accumulation to citrate oxidation when becoming malignant. PCa cells also undergo the Warburg effect, all of which explain these alterations (Table 4) [15,84–91].

It is also well established that cancer cells have elevated proliferation rates, and this is reflected in alterations in membrane metabolism. Several metabolomics studies performed in PCa tissue revealed an increase of choline levels in PCa samples, which indicates alterations in phospholipid membrane synthesis and hydrolysis (Table 4) [84,86,88,89,92–94]. Because this elevated proliferation rate also increases cell energy requirements, PCa samples also have alterations in lipid metabolism (as lipids may be used by the cells to produce energy) (Table 4) [49,95].

Beyond the study of the metabolic pathways involved in cancer development, it is also important to assess which metabolic pathways are involved in the growth of bone metastases. The results obtained from a study [78] revealed a significant increase in cholesterol levels in bone metastases tissues from PCa patients. The metabolic profile from PCa bone metastases indicates high energy metabolism, which may be related to highly proliferating cells. This conclusion was based on the elevated levels of certain metabolites, such as threonine, glutamate, phenylalanine, citrate, fumarate, glycerol-3-phosphate, and fatty acids. Another relevant metabolic alteration in PCa bone metastases tissue was the elevated levels of myo-inositol-1-phosphate, which may indicate active cell signaling involving inositol-based compounds as second messengers. Inositol-based molecules are related to the activation of protein kinase C, and the activation of this molecule is important for cell proliferation, apoptosis, differentiation, invasion, and angiogenesis. The concentration of sarcosine was increased in bone metastases from PCa and from other cancers, which reveals that sarcosine may not be specific to PCa. Metabolites such as threonine, asparagine, fumarate, and linoleic acid are present in high levels in samples from bone metastases. The levels of these metabolites were also increased in samples of primary prostate tissues from patients with confirmed bone metastases. Linoleic acid, an essential fatty acid, was associated with PCa progression. Furthermore, linoleic acid may also be associated with the inflammatory response because linoleic acid is transformed into arachidonic acid. Arachidonic acid is a precursor for prostaglandins, which have an important role in inflammation [96].

*In Vitro Studies.* Table 5 summarizes general information on *in vitro* metabolomics studies in PCa-derived cell lines.

The biological relevance of sarcosine was evaluated in four immortalized PCa cell lines, in primary benign prostate epithelial cells, and in an immortalized benign prostate epithelial cell line. Sarcosine levels were increased in malignant cell lines when compared with benign cell lines [53,97]. Furthermore, alterations in the expression of the enzymes involved in sarcosine metabolism influence cell proliferation, invasion, and cell death, which suggest the importance of sarcosine in PCa metabolism (Table 5) [53,59]. In an effort to understand the role of sarcosine in PCa progression, Sreekumar et al. (2009) evaluated the role of androgen signaling and the genes ERG and ETV1 (important mediators of PCa progression). The results showed that after treatment with androgens, cell lines that were ERG positive and ETV1 positive had increased GNMT expression and decreased sarcosine dehydrogenase (SARDH) expression [53].

In agreement with the results from metabolomics studies performed in other matrices (urine, plasma/serum, and tissues), presented here previously, alterations in amino acid metabolism were also observed in the studies performed in PCa cell lines. PCa cells lines revealed alterations in the levels of certain amino acids, such as leucine, valine, or isoleucine (Table 5) [97–99].

The increase of lactate and alanine levels in PCa cell lines is frequently observed in metabolomics studies. These alterations suggest changes in cellular energy metabolism (Table 5) [97–100].

As reported for other matrices, metabolomics studies in PCa also revealed that PCa cells experience alterations in membrane metabolism with changes, for example, in choline metabolite levels (Table 5) [98,101]. Androgen signaling has an important role in the development and progression of PCa. In fact, one current therapy for metastatic PCa is the use of antiandrogen agents; however, with the progression of the disease, patients normally develop resistance to this therapy, and it is currently impossible to predict if the cancer will progress into a castration-resistant state. The androgen-responsive cell lines can be characterized by increased levels of spermine, *N*-acetylspermine, serine, threonine, lysine, homocysteine, asparagine, alanine, and glutamic acid, as well as decreased levels of *S*-adenosylmethionine, with a simultaneous increase in levels of its breakdown product, homocysteine. These findings indicate that androgen-responsive cell lines have an elevated methylation activity. Androgen treatment resulted in further perturbations in amino acid metabolism and in a shift toward increased methylation.

**Table 5.** Metabolomic Studies Performed in Human PCa-Derived Cell Lines

| Cancer Cell Lines | Control Group | Analytical Platform | Statistical Methods | Total Metabolites Found/ Discriminative Metabolites Found | Discriminatory Metabolites/Biomarkers | Metabolic Pathways Dysregulated | Ref. |
|---|---|---|---|---|---|---|---|
| AD prostate carcinoma LNCaP cell line | Androgen-independent prostate carcinoma PC-3 cell line | MRS | NS | 3/2 | Uptake of ethanolamine and *N,N'*-dimethylethanolamine (+) (principally in AD cells in presence of androgens) | Alteration in membrane lipid synthesis | [101] |
| VCaP, DU145, 22RV1, and LNCaP | PrEC and RWPE | LC-MS GC-MS | Wilcoxon *P* test, hierarchical clustering, nonparametric tests | 1/1 | Sarcosine (+) | Alterations in glycine synthesis and degradation | [53] |
| Androgen-nonresponsive PC3 and DU145 cell lines Androgen-responsive VCaP (treated with synthetic androgen) and LNCaP cell lines | RWPE | LC-MS | HCA | 1553/674 | Malignant cell lines<br>1. Sarcosine (+)<br>2. Threonine (+), phenylalanine (+), alanine (+), creatine (+),creatinine (+), citrulline (+), tryptophan (–), 1-methyl tryptophan (–) and kyneuric acid (–)<br>Androgen-responsive cell lines<br>3. Serine (+), threonine (+), lysine (+), homocysteine (+), asparagine (+), alanine (+), glutamic acid (+)<br>4. *S*-adenosylmethionine (–), homocysteine (+)<br>Androgen treatment resulted in further perturbations in amino acid metabolism and increased methylation. | 1. Alterations in glycine synthesis and degradation Sarcosine is an intermediate compound in the metabolism of choline.<br>2. Alteration in amino acids metabolism<br>3. Alteration in amino acids metabolism<br>4. Elevated methylation activity | [97] |
| PC3 and LNCaP treated with LY294002 (inhibitor of the PI3K signaling pathway) or 17AAG (inhibitor of the HSP90 protein chaperone) | PC3 and LNCaP untreated | MRS | PCA | NS/24 | After both treatments: lactate (–), alanine (–), fumarate (–)<br>LY294002 treatment: phosphocholine (–), glutathione (–), glutamine (+), valine (+), leucine (+), isoleucine (+)<br>17AAG treatment: phosphocholine (+), citrate (+), glutamine (–), valine (+), leucine (+), isoleucine (+), myo-inositol (+), taurine (+) | PI3K and HSP90 inhibition | [102] |
| Low-invasiveness WPE1-NB14 and high-invasiveness WPE1-NB11 cell lines | RWPE-1 | $^{1}$H-NMR | PLS-DA | NS/10 | 1. Leucine (–), valine (–), isoleucine (–), glutamine (–), glutamate (–), β-hydroxyisovalerate (–)<br>2. Glycine (–)<br>3. Lactate, alanine<br>4. Phosphocholine (+) | 1. Increased protein synthesis and amino acid catabolism<br>2. Alterations in methylation and synthesis and degradation of sarcosine<br>3. Alteration in energetic metabolism;<br>4. Alteration in choline metabolism | [98] |
| Androgen-nonresponsive PC3 and androgen-responsive LNCaP cell lines | PNT1A | $^{1}$H-NMR | Two-way analysis of variance followed by Bonferroni posttest | NS/3 | Glucose consumption (+)<br>PC3 cells:<br>Lactate (+)<br>Alanine (+)<br>Lactate/alanine ratio (+) | Increased levels of oxidative stress in PC3 cells.<br>Androgen-responsive and -nonresponsive PCa cells showed different glycolytic metabolism profiles. | [100] |
| DU145, PC3, and LNCaP (knockdown of GNMT, SARDH, or PIPOX and overexpression of GNMT, SARDH, or PIPOX (convert sarcosine back to glycine) | RWPE | GC-MS | ROC | NS/1 | Overexpression of GNMT: sarcosine (+) (increase in invasion).<br>Knockdown of GNMT: sarcosine (–) (reduction in cell proliferation, invasion, and greater percentage of cell death)<br>Overexpression of SARDH or PIPOX: sarcosine (–) (reduced invasion)<br>Knockdown of SARDH: sarcosine (+) (increase proliferation and invasion)<br>Knockdown of PIPOX: sarcosine (+) (increased invasion) | Alteration in glycine synthesis and degradation | [59] |
| CRPC cell C4-2, 22Rv1 and LNCaP-abl | Androgen receptor positive LNCaP and MDA-PCa-2a and MDA-PCa-2b | LC-MS | HCA | 150/38 | CRPC cells:<br>1. Sugars and intermediates associated with energy metabolism and signaling (+)<br>AD cells<br>2. Carnitines, amino acids, and their methylated derivatives (+) | 1. Alteration in energy metabolism and signaling<br>2. Alteration in amino acid metabolism | [99] |

*(continued on next page)*

**TABLE 5** (*continued*)

| Cancer Cell Lines | Control Group | Analytical Platform | Statistical Methods | Total Metabolites Found/ Discriminative Metabolites Found | Discriminatory Metabolites/Biomarkers | Metabolic Pathways Dysregulated | Ref. |
|---|---|---|---|---|---|---|---|
| Highly metastatic LNCaP-LN3 treated with DCA and poorly metastatic LNCaP treated with DCA | LNCaP-LN3 and LNCaP untreated | NMR | Nonparametric test | NS/3 | LNCaP-LN3 treated with DCA: Lac/Cr (-), Lac/Cho (-), Lac/Al (-), and Lac/(Cho + Cr + Al) (-)<br>LNCaP (poorly metastatic) treated with DCA: no change in lactate/metabolite ratios | Reversion of Warburg effect (LNCaP-LN3 cells respond better to DCA) | [103] |

*AD*, Androgen dependent; *CRPC*, castrate-resistant prostate cancer; *DCA*, Dichloroacetate; *GC-MS*, Gas chromatography–mass spectrometry; *GNMT*, Glycine-N-methyl transferase; *HCA*, Hierarchical clustering analysis; *Lac/Al*, lactate/ alanine; *Lac/Cr*, lactate/creatine; *Lac/Cho*, lactate/choline; *Lac/(Cho + Cr + Ala)*, lactate/(choline + creatine + alanine); *LC-MS*, Liquid chromatography–mass spectrometry; *NMR*, Nuclear magnetic resonance; *MRS*, Magnetic resonance spectroscopy; *NS*, Not specified; *PCA*, Principal component analysis; *PIPOX*, Pipecolic acid oxidase; *PLS-DA*, Partial least squares discriminant analysis; *ROC*, Receiver-operating characteristics analysis; *SARD*, Sarcosine dehydrogenase.
(+), Levels increased in PCa; (-), Levels decreased in PCa.

Androgen-nonresponsive cell lines and androgen-responsive cell lines also have differences in their glycolytic metabolism profiles. Androgen-dependent PCa cells and androgen-independent PCa cells also show differences in membrane lipid synthesis (Table 5) [97,100,101].

Metabolomics studies in cell lines may also be used to evaluate the alterations that occur after a pharmacological therapy. The treatment of PCa cells appears to lead to changes in energetic metabolism and choline metabolism (Table 5) [102]. Dichloroacetate (DCA) is an inhibitor of pyruvate dehydrogenase kinase, and inhibition of this enzyme has the potential to reverse the Warburg effect due to the increased pyruvate uptake into mitochondria. After treatment with this drug, the highly metastatic cells showed significantly lower levels of lactate/metabolite ratios [Lac/Cr, Lac/Cho, Lac/Al, and Lac/(Cho + Cr + Al)], whereas in poorly metastatic cells, no changes in lactate/metabolite ratios were found after the treatment. These findings suggest that highly metastatic cells are more dependent on lactate production (Table 5) [103].

## Conclusions and Future Directions

The introduction of PSA testing has radically altered how PCa is diagnosed and managed. However, this test may lead to a false-positive or false-negative diagnosis. This drawback has given rise to serious efforts toward the discovery of new biomarkers, preferentially noninvasive, which have better specificity and sensitivity.

Because metabolic alterations are the last step in the cellular response to diseases, metabolomics can be successfully used to discover new biomarkers for cancer. In this regard, several studies have been conducted to characterize the metabolic profile of PCa.

One of the major obstacles in data interpretation is that the metabolic profile is influenced by various factors, such as age, diet, drugs, and chronobiological variations, among others. Additional problems include sample preparation, the analytical procedures, and the statistical platforms used. Major differences in these conditions can compromise the comparison of results among different studies and consequently compromise the discovery of new biomarkers. Another intrinsic difficulty with metabolomics studies is the massive amount of data produced that is difficult statistically to analyze. Despite these difficulties, metabolomics is a powerful tool for the discovery of new biomarkers for PCa detection, biomarkers indicative of cancer prognosis, disease progression, and therapeutic response, as well as identifying new therapeutic targets.

The metabolomics studies described in this review revealed different results, but almost all studies in urine, plasma/serum, prostatic fluids, tissues, and cell lines associated PCa with decreased levels of citrate and polyamines and increased levels of choline, lactate, and amino acids.

Further studies are still needed to confirm the results of these studies and identify an inexpensive, noninvasive, sensible, and specific biomarker for PCa.

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## References

[1] Trock BJ (2011). Application of metabolomics to prostate cancer. *Urol Oncol* **29**, 572–581.
[2] Halama A (2014). Metabolomics in cell culture—a strategy to study crucial metabolic pathways in cancer development and the response to treatment. *Arch Biochem Biophys* **564**, 100–109.
[3] He JC, Chuang PY, Ma'ayan A, and Iyengar R (2012). Systems biology of kidney diseases. *Kidney Int* **81**, 22–39.
[4] Ramautar R, Berger R, van der Greef J, and Hankemeier T (2013). Human metabolomics: strategies to understand biology. *Curr Opin Chem Biol* **17**, 841–846.
[5] Monteiro M, Carvalho M, de Lourdes Bastos M, and de Pinho P (2014). Biomarkers in renal cell carcinoma: a metabolomics approach. *Metabolomics* **10**, 1210–1222.
[6] Aboud OA and Weiss RH (2013). New opportunities from the cancer metabolome. *Clin Chem* **59**, 138–146.
[7] Monteiro MS, Carvalho M, Bastos ML, and de Pinho PG (2013). Metabolomics analysis for biomarker discovery: advances and challenges. *Curr Med Chem* **20**, 257–271.
[8] Weiss RH and Kim K (2012). Metabolomics in the study of kidney diseases. *Nat Rev Nephrol* **8**, 22–33.
[9] Monteiro M, Carvalho M, Henrique R, Jeronimo C, Moreira N, de Lourdes Bastos M, and de Pinho PG (2014). Analysis of volatile human urinary metabolome by solid-phase microextraction in combination with gas chromatography–mass spectrometry for biomarker discovery: application in a pilot study to discriminate patients with renal cell carcinoma. *Eur J Cancer* **50**, 1993–2002.
[10] Monteiro MS, Carvalho M, Bastos MdL, and Pinho PG (2015). Potentiality of volatile organic compounds to discriminate patients with cancer by using chemometric tools. Identification and data processing methods in metabolomics: Future Science Ltd; 2015. p. 166–184.
[11] Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, and Bray F (2015). Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* **136**, E359-386.

[12] Rigau M, Olivan M, Garcia M, Sequeiros T, Montes M, Colas E, Llaurado M, Planas J, Torres Id, Morote J, et al (2013). The present and future of prostate cancer urine biomarkers. *Int J Mol Sci* **14**, 12620–12649.

[13] Aoun Fouad, Peltier Alexandre, and van Velthoven Roland (2014). A Comprehensive Review of Contemporary Role of Local Treatment of the Primary Tumor and/or the Metastases in Metastatic Prostate Cancer. *BioMed Research International, vol. 2014, Article ID 501213, 12 pages, 2014* **2014**, 501213. http://dx.doi.org/10.1155/2014/501213.

[14] Dimakakos Andreas, Armakolas Athanasios, and Koutsilieris Michael (2014). Novel Tools for Prostate Cancer Prognosis, Diagnosis, and Follow-Up. *BioMed Research International, vol. 2014, Article ID 890697, 9 pages, 2014* **2014**, 890697. http://dx.doi.org/10.1155/2014/890697.

[15] McDunn JE, Li Z, Adam KP, Neri BP, Wolfert RL, Milburn MV, Lotan Y, and Wheeler TM, et al (2013). Metabolomic signatures of aggressive prostate cancer. *Prostate* **73**, 1547–1560.

[16] Roberts MJ, Schirra HJ, Lavin MF, and Gardiner RA (2011). Metabolomics: a novel approach to early and noninvasive prostate cancer detection. *Korean J Urol* **52**, 79–89.

[17] Barry MJ (2009). Screening for prostate cancer—the controversy that refuses to die. *N Engl J Med* **360**, 1351–1354.

[18] Abrate A, Lughezzani G, Gadda GM, Lista G, Kinzikeeva E, Fossati N, Larcher A, Dell'Oglio P, Mistretta F, and Buffi N, et al (2014). Clinical use of [−2]proPSA (p2PSA) and its derivatives (%p2PSA and Prostate Health Index) for the detection of prostate cancer: a review of the literature. *Korean J Urol* **55**, 436–445.

[19] Link RE, Shariat SF, Nguyen CV, Farr A, Weinberg AD, Morton RA, Richardson B, Bernard D, and Slawin KM (2004). Variation in prostate specific antigen results from 2 different assay platforms: clinical impact on 2304 patients undergoing prostate cancer screening. *J Urol* **171**, 2234–2238.

[20] Wu CL, Jordan KW, Ratai EM, Sheng J, Adkins CB, Defeo EM, Jenkins BG, Ying L, McDougal WS, and Cheng LL (2010). Metabolomic imaging for human prostate cancer detection. *Sci Transl Med* **2**, 16ra18.

[21] Andriole GL, Crawford ED, Grubb III RL, Buys SS, Chia D, Church TR, Fouad MN, Isaacs C, Kvale PA, Reding DJ, et al (2012). Prostate cancer screening in the randomized Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial: mortality results after 13 years of follow-up. *J Natl Cancer Inst* **104**, 125–132.

[22] Spur EM, Decelle EA, and Cheng LL (2013). Metabolomic imaging of prostate cancer with magnetic resonance spectroscopy and mass spectrometry. *Eur J Nucl Med Mol Imaging* **40**(Suppl. 1), S60–S71.

[23] Ukimura O, Coleman JA, de la Taille A, Emberton M, Epstein JI, Freedland SJ, Giannarini G, Kibel AS, Montironi R, Ploussard G, et al (2013). Contemporary role of systematic prostate biopsies: indications, techniques, and implications for patient care. *Eur Urol* **63**, 214–230.

[24] Warburg O (1956). On the origin of cancer cells. *Science* **123**, 309–314.

[25] Warburg OPK and Negelein F (1924). Über den Stoffwechsel der Carcinom-Zelle. *Biochem Z* **152**, 319–344.

[26] Soga T (2013). Cancer metabolism: key players in metabolic reprogramming. *Cancer Sci* **104**, 275–281.

[27] Pertega-Gomes N and Baltazar F (2014). Lactate transporters in the context of prostate cancer metabolism: what do we know? *Int J Mol Sci* **15**, 18333–18348.

[28] Costello LC and Franklin RB (1991). Concepts of citrate production and secretion by prostate. 1. Metabolic relationships. *Prostate* **18**, 25–46.

[29] Costello LC and Franklin RB (2009). Prostatic fluid electrolyte composition for the screening of prostate cancer: a potential solution to a major problem. *Prostate Cancer Prostatic Dis* **12**, 17–24.

[30] Dakubo GD, Parr RL, Costello LC, Franklin RB, and Thayer RE (2006). Altered metabolism and mitochondrial genome in prostate cancer. *J Clin Pathol* **59**, 10–16.

[31] Costello LC, Liu Y, Franklin RB, and Kennedy MC (1997). Zinc inhibition of mitochondrial aconitase and its importance in citrate metabolism of prostate epithelial cells. *J Biol Chem* **272**, 28875–28881.

[32] Costello LC and Franklin RB (2000). The intermediary metabolism of the prostate: a key to understanding the pathogenesis and progression of prostate malignancy. *Oncology (Williston Park)* **59**, 269–282.

[33] Zha S, Ferdinandusse S, Hicks JL, Denis S, Dunn TA, Wanders RJ, Luo J, De Marzo AM, and Isaacs WB (2005). Peroxisomal branched chain fatty acid beta-oxidation pathway is upregulated in prostate cancer. *Prostate* **63**, 316–323.

[34] Liu Y, Zuckier LS, and Ghesani NV (2010). Dominant uptake of fatty acid over glucose by prostate cells: a potential new diagnostic and therapeutic approach. *Anticancer Res* **30**, 369–374.

[35] Tsouko E, Khan AS, White MA, Han JJ, Shi Y, Merchant FA, Sharpe MA, Xin L, and Frigo DE (2014). Regulation of the pentose phosphate pathway by an androgen receptor-mTOR–mediated mechanism and its role in prostate cancer cell growth. *Oncogenesis* **3**, e103.

[36] Huang WC, Zhau HE, and Chung LW (2010). Androgen receptor survival signaling is blocked by anti-beta2-microglobulin monoclonal antibody via a MAPK/lipogenic pathway in human prostate cancer cells. *J Biol Chem* **285**, 7947–7956.

[37] Huang WC, Li X, Liu J, Lin J, and Chung LW (2012). Activation of androgen receptor, lipogenesis, and oxidative stress converged by SREBP-1 is responsible for regulating growth and progression of prostate cancer cells. *Mol Cancer Res* **10**, 133–142.

[38] Lucarelli G, Rutigliano M, Galleggiante V, Giglio A, Palazzo S, Ferro M, Simone C, Bettocchi C, Battaglia M, and Ditonno P (2015). Metabolomic profiling for the identification of novel diagnostic markers in prostate cancer. *Expert Rev Mol Diagn* **15**, 1211–1224.

[39] Wang Q, Hardie RA, Hoy AJ, van Geldermalsen M, Gao D, Fazli L, Sadowski MC, Balaban S, Schreuder M, Nagarajah R, et al (2015). Targeting ASCT2-mediated glutamine uptake blocks prostate cancer growth and tumour development. *J Pathol* **236**, 278–289.

[40] Pan T, Gao L, Wu G, Shen G, Xie S, Wen H, Yang J, Zhou Y, Tu Z, and Qian W (2015). Elevated expression of glutaminase confers glucose utilization via glutaminolysis in prostate cancer. *Biochem Biophys Res Commun* **456**, 452–458.

[41] Li Z and Zhang H (2015). Reprogramming of glucose, fatty acid and amino acid metabolism for cancer progression. *Cell Mol Life Sci* **73**(2), 377–392.

[42] Pavlides S, Whitaker-Menezes D, Castello-Cros R, Flomenberg N, Witkiewicz AK, Frank PG, Casimiro MC, Wang C, Fortina P, Addya S, et al (2009). The reverse Warburg effect: aerobic glycolysis in cancer associated fibroblasts and the tumor stroma. *Cell Cycle* **8**, 3984–4001.

[43] Fiaschi T, Marini A, Giannoni E, Taddei ML, Gandellini P, De Donatis A, Lanciotti M, Serni S, Cirri P, and Chiarugi P (2012). Reciprocal metabolic reprogramming through lactate shuttle coordinately influences tumor-stroma interplay. *Cancer Res* **72**, 5130–5140.

[44] Wilkosz J, Brys M, and Rozanski W (2011). Urine markers and prostate cancer. *Cent Eur J Urol* **64**, 9–14.

[45] Zhang T, Watson DG, Wang L, Abbas M, Murdoch L, Bashford L, Ahmad I, Lam NY, Ng AC, and Leung HY (2013). Application of holistic liquid chromatography–high resolution mass spectrometry based urinary metabolomics for prostate cancer detection and biomarker discovery. *PLoS One* **8**, e65880.

[46] Averna TA, Kline EE, Smith AY, and Sillerud LO (2005). A decrease in 1H nuclear magnetic resonance spectroscopically determined citrate in human seminal fluid accompanies the development of prostate adenocarcinoma. *J Urol* **173**, 433–438.

[47] Kumar V, Dwivedi DK, and Jagannathan NR (2014). High-resolution NMR spectroscopy of human body fluids and tissues in relation to prostate cancer. *NMR Biomed* **27**, 80–89.

[48] Roberts MJ, Richards RS, Gardiner RA, and Selth LA (2015). Seminal fluid: a useful source of prostate cancer biomarkers? *Biomark Med* **9**, 77–80.

[49] Jung K, Reszka R, Kamlage B, Bethan B, Stephan C, Lein M, and Kristiansen G (2013). Tissue metabolite profiling identifies differentiating and prognostic biomarkers for prostate carcinoma. *Int J Cancer* **133**, 2914–2924.

[50] Zhang A, Sun H, Xu H, Qiu S, and Wang X (2013). Cell metabolomics. *OMICS* **17**, 495–501.

[51] Cuperlovic-Culf M, Barnett DA, Culf AS, and Chute I (2010). Cell culture metabolomics: applications and future directions. *Drug Discov Today* **15**, 610–621.

[52] Keshari KR, Sriram R, Van Criekinge M, Wilson DM, Wang ZJ, Vigneron DB, Peehl DM, and Kurhanewicz J (2013). Metabolic reprogramming and validation of hyperpolarized 13C lactate as a prostate cancer biomarker using a human prostate tissue slice culture bioreactor. *Prostate* **73**, 1171–1181.

[53] Sreekumar A, Poisson LM, Rajendiran TM, Khan AP, Cao Q, Yu J, Laxman B, Mehra R, Lonigro RJ, Li Y, et al (2009). Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression. *Nature* **457**, 910–914.

[54] Cernei N, Heger Z, Gumulec J, Zitka O, Masarik M, Babula P, Eckschlager T, Stiborova M, Kizek R, and Adam V (2013). Sarcosine as a potential prostate cancer biomarker—a review. *Int J Mol Sci* **14**, 13893–13908.

[55] Jiang Y, Cheng X, Wang C, and Ma Y (2010). Quantitative determination of sarcosine and related compounds in urinary samples by liquid chromatography with tandem mass spectrometry. *Anal Chem* **82**, 9022–9027.

[56] Stabler S, Koyama T, Zhao Z, Martinez-Ferrer M, Allen RH, Luka Z, Loukachevitch LV, Clark PE, Wagner C, and Bhowmick NA (2011). Serum methionine metabolites are risk factors for metastatic prostate cancer progression. *PLoS One* **6**, e22486.

[57] Bianchi F, Dugheri S, Musci M, Bonacchi A, Salvadori E, Arcangeli G, Cupelli V, Lanciotti M, Masieri L, and Serni S, et al (2011). Fully automated solid-phase microextraction-fast gas chromatography–mass spectrometry method using a new ionic liquid column for high-throughput analysis of sarcosine and *N*-ethylglycine in human urine and urinary sediments. *Anal Chim Acta* **707**, 197–203.

[58] Cao DL, Ye DW, Zhang HL, Zhu Y, Wang YX, and Yao XD (2011). A multiplex model of combining gene-based, protein-based, and metabolite-based with positive and negative markers in urine for the early diagnosis of prostate cancer. *Prostate* **71**, 700–710.

[59] Khan AP, Rajendiran TM, Ateeq B, Asangani IA, Athanikar JN, Yocum AK, Mehra R, Siddiqui J, Palapattu G, Wei JT, et al (2013). The role of sarcosine metabolism in prostate cancer progression. *Neoplasia* **15**, 491–501.

[60] Wu H, Liu T, Ma C, Xue R, Deng C, Zeng H, and Shen X (2011). GC/MS-based metabolomic approach to validate the role of urinary sarcosine and target biomarkers for human prostate cancer by microwave-assisted derivatization. *Anal Bioanal Chem* **401**, 635–646.

[61] Jentzmik F, Stephan C, Miller K, Schrader M, Erbersdobler A, Kristiansen G, Lein M, and Jung K (2010). Sarcosine in urine after digital rectal examination fails as a marker in prostate cancer detection and identification of aggressive tumours. *Eur Urol* **58**, 12–18 [discussion 20–11].

[62] Issaq HJ and Veenstra TD (2011). Is sarcosine a biomarker for prostate cancer? *J Sep Sci* **34**, 3619–3621.

[63] Struck-Lewicka W, Kordalewska M, Bujak R, Yumba Mpanga A, Markuszewski M, Jacyna J, Matuszewski M, Kaliszan R, and Markuszewski MJ (2015). Urine metabolic fingerprinting using LC-MS and GC-MS reveals metabolite changes in prostate cancer: A pilot study. *J Pharm Biomed Anal* **111**, 351–361.

[64] Khalid T, Aggio R, White P, De Lacy Costello B, Persad R, Al-Kateb H, Jones P, Probert CS, and Ratcliffe N (2015). Urinary volatile organic compounds for the detection of prostate cancer. *PLoS One* **10**, e0143283.

[65] Steve Smith PW, Redding Jemma, Ratcliffe Norman M, and Probert Chris S J (2010). Application of similarity coefficients to predict disease using volatile organic compounds. *IEEE Sens J* **10**, 92–96.

[66] Lucarelli G, Fanelli M, Larocca AM, Germinario CA, Rutigliano M, Vavallo A, Selvaggi FP, Bettocchi C, Battaglia M, and Ditonno P (2012). Serum sarcosine increases the accuracy of prostate cancer detection in patients with total serum PSA less than 4.0 ng/ml. *Prostate* **72**, 1611–1621.

[67] Koutros S, Meyer TE, Fox SD, Issaq HJ, Veenstra TD, Huang WY, Yu K, Albanes D, Chu LW, Andriole G, et al (2013). Prospective evaluation of serum sarcosine and risk of prostate cancer in the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial. *Carcinogenesis* **34**, 2281–2285.

[68] Kumar D, Gupta A, Mandhani A, and Sankhwar SN (2015). Metabolomics-derived prostate cancer biomarkers: fact or fiction? *J Proteome Res* **14**, 1455–1464.

[69] Zang X, Jones CM, Long TQ, Monge ME, Zhou M, Walker LD, Mezencev R, Gray A, McDonald JF, and Fernandez FM (2014). Feasibility of detecting prostate cancer by ultraperformance liquid chromatography–mass spectrometry serum metabolomics. *J Proteome Res* **13**, 3444–3454.

[70] Crowe FL, Allen NE, and Appleby PN, et al (2008). Fatty acid composition of plasma phospholipids and risk of prostate cancer in a case–control analysis nested within the European Prospective Investigation into Cancer and Nutrition. *Am J Clin Nutr* **88**, 1353–1363.

[71] Johansson M, Van Guelpen B, Vollset SE, Hultdin J, Bergh A, Key T, Midttun O, Hallmans G, Ueland PM, and Stattin P (2009). One-carbon metabolism and prostate cancer risk: prospective investigation of seven circulating B vitamins and metabolites. *Cancer Epidemiol Biomarkers Prev* **18**, 1538–1543.

[72] Zhou X, Mao J, Ai J, Deng Y, Roth MR, Pound C, Henegar J, Welti R, and Bigler SA (2012). Identification of plasma lipid biomarkers for prostate cancer by lipidomics and bioinformatics. *PLoS One* **7**, e48889.

[73] Miyagi Y, Higashiyama M, Gochi A, Akaike M, Ishikawa T, Miura T, Saruki N, Bando E, Kimura H, Imamur F, et al (2011). Plasma free amino acid profiling of five types of cancer patients and its application for early detection. *PLoS One* **6**, e24143.

[74] Giskeodegard GF, Hansen AF, Bertilsson H, Gonzalez SV, Kristiansen KA, Bruheim P, Mjos SA, Angelsen A, Bathen TF, and Tessem MB (2015). Metabolic markers in blood can separate prostate cancer from benign prostatic hyperplasia. *Br J Cancer* **113**, 1712–1719.

[75] Osl M, Dreiseitl S, Pfeifer B, Weinberger K, Klocker H, Bartsch G, Schafer G, Tilg B, Graber A, and Baumgartner C (2008). A new rule-based algorithm for identifying metabolic markers in prostate cancer using tandem mass spectrometry. *Bioinformatics* **24**, 2908–2914.

[76] Pandeya DR, Mittal A, Sathian B, and Bhatta B (2014). Role of hyperinsulinemia in increased risk of prostate cancer: a case control study from Kathmandu Valley. *Asian Pac J Cancer Prev* **15**, 1031–1033.

[77] Wright JL, Plymate SR, Porter MP, Gore JL, Lin DW, Hu E, and Zeliadt SB (2013). Hyperglycemia and prostate cancer recurrence in men treated for localized prostate cancer. *Prostate Cancer Prostatic Dis* **16**, 204–208.

[78] Saylor PJ, Karoly ED, and Smith MR (2012). Prospective study of changes in the metabolomic profiles of men during their first three months of androgen deprivation therapy for prostate cancer. *Clin Cancer Res* **18**, 3677–3685.

[79] Zaichick VY, Sviridova TV, and Zaichick SV (1996). Zinc concentration in human prostatic fluid: normal, chronic prostatitis, adenoma and cancer. *Int Urol Nephrol* **28**, 687–694.

[80] Lynch MJ and Nicholson JK (1997). Proton MRS of human prostatic fluid: correlations between citrate, spermine, and myo-inositol levels and changes with disease. *Prostate* **30**, 248–255.

[81] Kline EE, Treat EG, Averna TA, Davis MS, Smith AY, and Sillerud LO (2006). Citrate concentrations in human seminal fluid and expressed prostatic fluid determined via 1H nuclear magnetic resonance spectroscopy outperform prostate specific antigen in prostate cancer detection. *J Urol* **176**, 2274–2279.

[82] Serkova NJ, Gamito EJ, Jones RH, O'Donnell C, Brown JL, Green S, Sullivan H, Hedlund T, and Crawford ED (2008). The metabolites citrate, myo-inositol, and spermine are potential age-independent markers of prostate cancer in human expressed prostatic secretions. *Prostate* **68**, 620–628.

[83] Jentzmik F, Stephan C, Lein M, Miller K, Kamlage B, Bethan B, Kristiansen G, and Jung K (2011). Sarcosine in prostate cancer tissue is not a differential metabolite for prostate cancer aggressiveness and biochemical progression. *J Urol* **185**, 706–711.

[84] van Asten JJ, Cuijpers V, Hulsbergen-van de Kaa C, Soede-Huijbregts C, Witjes JA, Verhofstad A, and Heerschap A (2008). High resolution magic angle spinning NMR spectroscopy for metabolic assessment of cancer presence and Gleason score in human prostate needle biopsies. *MAGMA* **21**, 435–442.

[85] Hahn P, Smith IC, Leboldus L, Littman C, Somorjai RL, and Bezabeh T (1997). The classification of benign and malignant human prostate tissue by multivariate analysis of 1H magnetic resonance spectra. *Cancer Res* **57**, 3398–3401.

[86] Garcia-Segura JM, Sanchez-Chapado M, Ibarburen C, Viano J, Angulo JC, Gonzalez J, and Rodriguez-Vallejo JM (1999). In vivo proton magnetic resonance spectroscopy of diseased prostate: spectroscopic features of malignant versus benign pathology. *Magn Reson Imaging* **17**, 755–765.

[87] Cheng LL, Wu C, Smith MR, and Gonzalez RG (2001). Non-destructive quantitation of spermine in human prostate tissue samples using HRMAS 1H NMR spectroscopy at 9.4 T. *FEBS Lett* **494**, 112–116.

[88] Swindle P, McCredie S, Russell P, Himmelreich U, Khadra M, Lean C, and Mountford C (2003). Pathologic characterization of human prostate tissue with proton MR spectroscopy. *Radiology* **228**, 144–151.

[89] Swanson MG, Vigneron DB, Tabatabai ZL, Males RG, Schmitt L, Carroll PR, James JK, Hurd RE, and Kurhanewicz J (2003). Proton HR-MAS spectroscopy and quantitative pathologic analysis of MRI/3D-MRSI-targeted postsurgical prostate tissues. *Magn Reson Med* **50**, 944–954.

[90] Tessem MB, Swanson MG, Keshari KR, Albers MJ, Joun D, Tabatabai ZL, Simko JP, Shinohara K, Nelson SJ, Vigneron DB, et al (2008). Evaluation of lactate and alanine as metabolic biomarkers of prostate cancer using 1H HR-MAS spectroscopy of biopsy tissues. *Magn Reson Med* **60**, 510–516.

[91] Giskeodegard GF, Bertilsson H, Selnaes KM, Wright AJ, Bathen TF, Viset T, Halgunset J, Angelsen A, Gribbestad IS, and Tessem MB (2013). Spermine and citrate as metabolic biomarkers for assessing prostate cancer aggressiveness. *PLoS One* **8**, e62375.

[92] Swanson MG, Keshari KR, Tabatabai ZL, Simko JP, Shinohara K, Carroll PR, Zektzer AS, and Kurhanewicz J (2008). Quantification of choline- and ethanolamine-containing metabolites in human prostate tissues using 1H HR-MAS total correlation spectroscopy. *Magn Reson Med* **60**, 33–40.

[93] Maxeiner A, Adkins CB, Zhang Y, Taupitz M, Halpern EF, McDougal WS, Wu CL, and Cheng LL (2010). Retrospective analysis of prostate cancer recurrence potential with tissue metabolomic profiles. *Prostate* **70**, 710–717.

[94] Stenman K, Stattin P, Stenlund H, Riklund K, Grobner G, and Bergh A (2011). H HRMAS NMR derived bio-markers related to tumor grade, tumor cell fraction, and cell proliferation in prostate tissue samples. *Biomark Insights* **6**, 39–47.

[95] Stenman K, Hauksson JB, Grobner G, Stattin P, Bergh A, and Riklund K (2009). Detection of polyunsaturated omega-6 fatty acid in human malignant prostate tissue by 1D and 2D high-resolution magic angle spinning NMR spectroscopy. *MAGMA* **22**, 327–331.

[96] Thysell E, Surowiec I, Hornberg E, Crnalic S, Widmark A, Johansson AI, Stattin P, Bergh A, Moritz T, Antti H, et al (2010). Metabolomic characterization of human prostate cancer bone metastases reveals increased levels of cholesterol. *PLoS One* **5**, e14175.

[97] Putluri N, Shojaie A, Vasu VT, Nalluri S, Vareed SK, Putluri V, Vivekanandan-Giri A, Byun J, Pennathur S, Sana TR, et al (2011). Metabolomic profiling reveals a role for androgen in activating amino acid metabolism and methylation in prostate cancer cells. *PLoS One* **6**, e21417.

[98] Teahan O, Bevan CL, Waxman J, and Keun HC (2011). Metabolic signatures of malignant progression in prostate epithelial cells. *Int J Biochem Cell Biol* **43**, 1002–1009.

[99] Kaushik AK, Vareed SK, Basu S, Putluri V, Putluri N, Panzitt K, Brennan CA, Chinnaiyan AM, Vergara IA, Erho N, et al (2014). Metabolomic profiling identifies biochemical pathways associated with castration-resistant prostate cancer. *J Proteome Res* **13**, 1088–1100.

[100] Vaz CV, Alves MG, Marques R, Moreira PI, Oliveira PF, Maia CJ, and Socorro S (2012). Androgen-responsive and nonresponsive prostate cancer cells present a distinct glycolytic metabolism profile. *Int J Biochem Cell Biol* **44**, 2077–2084.

[101] Mintz A, Wang L, and Ponde DE (2008). Comparison of radiolabeled choline and ethanolamine as probe for cancer detection. *Cancer Biol Ther* **7**, 742–747.

[102] Lodi A and Ronen SM (2011). Magnetic resonance spectroscopy detectable metabolomic fingerprint of response to antineoplastic treatment. *PLoS One* **6**, e26155.

[103] Kailavasan M, Rehman I, Reynolds S, Bucur A, Tozer G, and Paley M (2014). NMR-based evaluation of the metabolic profile and response to dichloroacetate of human prostate cancer cells. *NMR Biomed* **27**, 610–616.

# **Chapter 2:** Aims

## Aims

Prostate cancer is the second most diagnosed cancer in men, with 1.1 million new cases diagnosed in all world, in 2012 (1). The quantification of prostate serum antigen (PSA) and the digital rectal examination are the most common screening techniques used for PCa diagnosis. Nevertheless, to reach a final diagnosis it is mandatory to perform a prostate biopsy (PB) (2). However, PSA screening is controversy due to its limited sensitivity and specificity. PSA is specific for prostate diseases, in general, but not specific for PCa (3-5). Therefore, it is urgent and necessary discover new biomarkers in order to establish a confident, rapid, specific, noninvasive and economic methodology for early PCa diagnosis and prognosis. In this sense, metabolomics has been increasingly playing an important role in the discovery of new biomarkers. Many metabolomic studies have been conducted in multiple matrices (cells, tissues and biofluids) in order to improve diagnostic approaches, revealing promising results (6-15). However, the studies related with VOCs, for this specific pathology, are limited.

In order to contribute to the discovery of new PCa biomarkers and to better understand the prostatic cancer mechanism, the aims of this study are based on the realization of a complex metabolomic study, using four different prostatic cancer cell lines and one normal prostate cell line. In order to achieve this goal, the present work was subdivided in the following tasks:

1) Application of a metabolomic approach based on HS-SPME/GC-MS analytical technique to study the exometabolome (VOCs) of 4 prostate cancer cell lines (PC3, 22RV1, LNCaP, DU145 cell lines) with different grades and features and a non-tumoral cell line (PNT2 cell line), as well as to search for significant differences between them;
2) Study of the exometabolome at two different pHs (pH 2 and 7) in order to infer what is the ideal pH for the extraction of volatile organic compounds and discrimination of potential biomarkers;
3) Screening, identification and statistical analysis of VOCs that contribute for separation between cell lines and consequent discovery of potential biomarkers;
4) Application of a metabolomic approach based on GC-MS technique to reveal the endometabolome (intracellular metabolites such as AAs, organic acids; fatty acids, sugars and steroids) of the all prostate cell lines (tumorals and non-tumoral), as well as to search for significant differences between them;

5) Screening, identification and statistical analysis of intracellular compounds (AAs, organic acids; fatty acids, sugars and steroids) that contribute for separation between cell lines and consequent discovery of potential biomarkers;
6) Integration of the results obtained through the two GC-MS techniques, in order to better understand the metabolic pathways changed in prostate cancer;

As far as we know, this is the first time that a complex metabolomics study is performed *in vitro*, not having been executed any study of VOCs profiling in *in vitro* prostate cancer cell models.

# 2. Experimental Work

Prostate cancer (PCa) is the second most diagnosed cancer in men, and is the fifth leading cause of cancer-related deaths in men worldwide (1). In 2014, in the United States, 233 000 men were diagnosed with PCa and 29 480 men died due to this cancer. (16). The five-year survival for men with metastatic disease is very low (28%) and this occurs because of the development of resistance to castration, nerveless PCa has a long latency period and is potentially curable (2).

Age, diet, genetics, the pre-existence of sexually transmitted infections, the exposure to toxic metals, polychlorinated biphenyls and polycyclic aromatic hydrocarbons are some of the factors involved in the development of PCa (17-20).

Currently, prostate serum antigen (PSA) is the most used biomarker for PCa diagnostic. However, the PSA screening has limited sensitivity and specificity (17, 21, 22) and PSA is not able to differentiate aggressive from indolent PCa (3, 4). Therefore, discovery of new biomarkers for PCa diagnostic is urgent and has the potential for substantial public health benefit.

An ideal cancer biomarker must be monitored in biological samples obtained by noninvasive procedure and inexpensive to allow frequent measurements, sensitive and specific for a particular cancer, and should appear altered in early stages of the disease. Additionally, it must be capable to depict the evolution of the tumor and the metastatic onset or spread (17).

Metabolomics is a powerful analytical tool with which biomarkers and therapeutic targets can likely be discovered as cancer cells have the capacity to modify many homeostatic systems within the body and, consequently, change the production, the use and the levels of many metabolites (23, 24). Metabolomics allows the discovery of biochemical signatures and with these signatures it is possible to investigate several metabolic pathways, and the differences between cancer and healthy metabolic phenotypes (23). An early intervention is possible using metabolomics since it is believed that metabolic alterations precede neoplastic proliferation. Thus, in theory, it is possible to detect early and avoid cancer development and to reduce neoplastic proliferation and invasion of local or distant tissues by precocious treatment (3).

In the design of experimental and analytical models of cancer, it is very important to be aware that metabolic profile can be altered by other factors not related to the cancer, like, age, diet, drugs, chronobiological variations, among others, being very important to control these effects in order to obtain reliable results, the cell lines are the ideal model to overcome these problems (23). The advantages of using cell lines in metabolomics

studies are the nonexistence of interindividual variability or difficulties enrolling patient controls in the studies. In fact, in cell lines studies there are less confounding factors as, for example, age, concurrent pathologies, diet, tabagic habits and influence from different tissues. In addition, cell lines have a perfectly defined cell state which allows the analysis of a targeted metabolic status. Metabolic alterations that do not appear in studies using animal models or human subjects due to sample biological complexity are reveled. Cell lines are less complicated to control, less expensive and the results are easier to interpret and more reproducible when compared with other samples like biofluids or tissue from humans or animal models. Also, ethical problems inherent to the use of animal models or human subjects are obviated (25-27). However, none of these models are perfect because it is practically impossible to simulate complex cell–cell and cell–matrix interactions in cell cultures of prostate cancer and these interactions are very important for metabolic alterations that happen with tumor progression (26, 28).

Volatile organic compounds (VOCs) correspond to a carbon-based chemical group, with low molecular weight and high volatility. These compounds are emitted from the human body and can reflect the metabolic condition of an individual. VOCs, odorous and non-odorous, vary in relative abundance with age, gender, health status, lifestyle behaviors, as well as, with genetic background (29, 30). VOCs, prove to have a great potential to provide new biomarkers of cancer detection. Some works have already focused on the application of VOCs analyses to different cancers, namely breast, lung, head and neck, esophago-gastric, skin, colorectal, liver and renal (31-38). In these works, several different matrices were used to the identification of VOCs, one of the most used matrix was breath, however other matrices were used with success, namely, cell lines, urine, plasma, gastric content and fecal samples (39-43).VOCs analyses were also applicated in PCa with success. In these studies, VOCs were able to differentiate urine from PCa patients and control individuals (7, 44). In these works the analytical platform used was GC-MS. For GC separation, it is necessary to use elevated temperatures and the metabolites must be volatilized in the injector port. For these reasons, GC can only be used in thermally stable compounds. GC-MS has the highest resolving power, but can only be used to analyze volatile compounds and certain substances, such as fatty acids and organic acids, with low molecular weights, being an ideal technique to VOCs analysis (45-47). GC-MS, compared to LC-MS, has higher sensitivity, robustness and reproducibility, and the identification of unknown metabolites is possible using the extensive spectrum databases available, which is a very important tool to identify unknown compounds (45).

# **Chapter 3:** Volatile Metabolic Profile of Prostate Cancer Cells vs Normal Prostate Cells

## Materials and Methods

### Chemicals

All chemicals and reagents were analytical grade. RPMI-1640 medium, and 4-fluorobenzaldeyde (used as internal standard) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The antibiotic mixture penicillin/streptomycin (10000 U/mL/10000 µg/mL), heat inactivated fetal bovine serum (FBS) and trypsin 0.25%-EDTA were purchased from GIBCO Invitrogen (Barcelona, Spain). Hydrochloric acid (HCl) and sodium hydrogencarbonate were obtained from Merck (Darmstadt, Germany). Sodium chloride (NaCl) was from VWR (Leuven, Belgium).

### Cell Culture

PCa immortalized cell lines (PC3, 22RV1, DU145 and LNCaP) and normal prostate epithelium immortalized cell line (PNT2) were provided by Portuguese Oncology Institute-Porto (Table 1). All cell lines were grown in RPMI-1640 supplemented with 10% of FBS and 1% of penicillin/streptomycin. All cell lines were maintained at 37°C and 5% $CO_2$. Cells were grown to 80% confluency, before passage.

**Table 1:** Characteristics of prostate cell lines used in this study.

| | PNT2 | 22RV1 | PC3 | DU145 | LNCaP |
|---|---|---|---|---|---|
| **Organism** | *Homo sapiens* | *Homo sapiens* | *Homo sapiens* | *Homo sapiens* | *Homo sapiens* |
| **Age** | 33 years | NA | 62 years | 69 years | 50 years |
| **Ethnicity** | NA | NA | Caucasian | Caucasian | Caucasian |
| **Tissue** | Prostate | Prostate | Prostate; derived from metastatic site: bone | Prostate; derived from metastatic site: brain | Prostate; derived from metastatic site: left supraclavicular lymph node |
| **Morphology** | Epithelial | Epithelial | Epithelial | Epithelial | Epithelial |
| **Culture Properties** | Adherent | Adherent | Adherent | Adherent | Adherent |

| | PNT2 | 22RV1 | PC3 | DU145 | LNCaP |
|---|---|---|---|---|---|
| **Disease** | Healthy | Carcinoma | Grade IV, adenocarcinoma | Carcinoma | Carcinoma |
| **Tumorigenic** | No | Yes | Yes | Yes | Yes |
| **AR expression** | Yes | Yes | No | No | Yes |
| **Metastatic potential** | — | NA | High | Moderate | Low |

NA: Not available; AR: Androgen receptor

*VOCs collection from extracellular medium*

All cell lines were plated in 75 $cm^2$ culture flasks and grown to 100% confluency in RPMI-1640 medium. After the cells had reached 100% confluency the medium was discarded and replaced with 15 mL of fresh RMPI-1640 medium, and left to grow for 48h, together with three controls (cellular medium without cells). After the 48h, the extracellular medium from flasks with cells and without cells (controls) was collected, centrifuged (2000 x g for 10 minutes at 4 ºC), the supernatant separated in two aliquots (one to use for volatile profiling at pH 7 and other to use for volatile profiling at pH 2) and immediately frozen at -80 ºC until analysis. The same procedure, for all cell lines (four PCa cell lines and one normal cell line), was used for the four consecutive passages (passage number 3, 4, 5 and 6) each passage was performed in triplicate, resulting in a total of 60 experiments.

*VOCs extraction from cell lines*

The method used was based on those previously developed by Monteiro et al (2014), with some modifications. Briefly, stored samples were thawed slowly at low temperatures, in order to minimize the loss of VOCs. All samples were analyzed at the pH of the medium culture around pH 7 (pH between 7.395 and 7.956) and at pH 2 (pH between 1.846 and 2.466). For acidification a fixed volume of 5M HCl was used (2.5 µL for pH 7 controls; 50 µL for pH 2 samples; and 52 µL for pH 2 controls). For GC–MS analysis 2 mL of sample were put into a 10 mL glass vial added with the internal standard (4-fluorobenzaldehyde, 10 µL with a concentration of 10 µg/mL) and salt (NaCl, 0.59 g). For the extraction a DVB/PDMS fiber coating was used, with 9 min of incubation and 24 min of extraction at 68°C with agitation (38).

## GC–MS system and data acquisition

*GC–MS analysis*

The GC-MS conditions for VOCs analysis were previously optimized by Monteiro et al. (2014). A Scion-436 gas chromatograph coupled to a Bruker SQ (single quadrupole) equipped with a SCION SQ ion trap mass detector and a Bruker Daltonics MS workstation software version 6.8, with a VF-5 MS (30 m x 0.25 mm x 0.25 µm) column (Varian) was used. A CombiPAL automatic autosampler (Varian, Palo Alto, CA) was used and experimental conditions were previously described (38). The carrier gas used was helium C-60 (Gasin, Portugal) (flow of 1 ml/min) and the injector port was heated to 230 °C. The analysis was performed in Full Scan mode. The oven temperature was fixed at 40 °C for 1 min, then increasing to 250 °C (rate 5 °C/min), held for 5 min, then increasing to 300 °C (rate 5 °C/min) and held for 1 min. The transfer line temperature was 280 °C, manifold temperature was 50 °C and the trap temperature was 180 °C. The mass range was 40 - 350 m/z, with a scan rate of 6 scan/s. All samples were injected randomly.

To ensure reproducibility, quality control samples (QCs) were injected three times *per* day. These QCs were a pool of all samples (the samples from the five cell lines and the respective controls) (48), this pool was aliquoted (2 mL vials) and immediately frozen at -80 ºC until analysis.

*Statistical analysis*

Prior to statistical analysis of results, all chromatograms were pre-processed: baseline correction (used to the raw data before peak detection to remove random noise and baseline shift), peak detection (the aim is to detect all peaks avoiding as possible false positives), chromatogram deconvolution (to decompose overlapped chromatographic signals), and alignment (to adjust for any minor variation in retention time), for these corrections the program MZmine was used (49), the parameters used to accomplish these were: RT range 2.8-34.0 min; m/z range 50-250; MS data noise level $1.0\times10^4$; m/z tolerance 0.5; chromatogram baseline level $1.0\times10^3$; peak duration range 0.02-0.30 min. Also all ions with a RSD (relative standard deviation) greater than 30% as well as ions (m/z) coming from the column, the fiber and responsible for temporal interference were removed from the matrix. The obtained data was also normalized for the total area of the chromatograms (division of peak area for the sum of the areas of all peaks). The statistical treatment includes an unsupervised (PCA) and a supervised analyzes (PLS-DA), after a

pareto (Par) scaling, to determine which VOCs were significantly different between PCa lines and normal cell line. Model robustness was estimated by $R^2X$ (variance explained by the X matrix, i.e. GC-MS data), $R^2Y$ (variance explained by the Y matrix, i.e., sample class) and $Q^2$ (goodness of prediction). All VOCs with VIP (Variable Importance Projection) values greater than one were considered potential relevant VOCs for the separation among cell lines. For these relevant compounds an univariate analysis was performed, using Shapiro-Wilk test (to determine normality distribution of data), and unpaired Student's t-test with Welch correction (for normal distribution) test or unpaired Mann-Whitney test (for non-normal distribution) to calculated the *p* value. In addition the percentage of variation, uncertainty of the variation quotient, as well as, the effect size and its uncertainty, were calculated (50). Bonferroni correction was used to adjust p-values for multiple comparisons by setting the significance cut off to *p* value (0.05) divided by the number of compounds simultaneously tested in univariate statistical analyses.

Finally, to confirm the robustness of the PLS-DA models, a randomized Monte Carlo cross-validation (MCCV) (7 blocks, 500 runs) was performed (using a software developed in the University of Aveiro), (51) obtaining the prediction power ($Q^2$) and confusion matrices for original and permuted data for all PLS-DA models. When a minimal overlapping of the distribution of original and permuted classes was obtained, PLS-DA models were considered robust. Classification rates, specificity (spec.) and sensitivity (sens.) were also acquired for all PLS-DA models through a receiver operating characteristic (ROC) map.

## Results

*VOCs analysis obtained at pH 7*

After data acquisition by GC-MS, the data were used to perform the multivariate analyses, namely PCA and PLS-DA. A total of 239 features were detected in the chromatograms obtained at pH 7.

The reproducibility of the analytical method is confirmed by the QCs projection on axis 1 and 2 (Figure 1) (all QCs samples are grouped (orange color). Furthermore, the multivariate analyses prove that VOCs are able to discriminate PCa cell lines from normal prostate cell line and between the different PCa cell lines. This discriminant capability is observed not only in PLS-DA analysis but also in PCA (Figure 2).

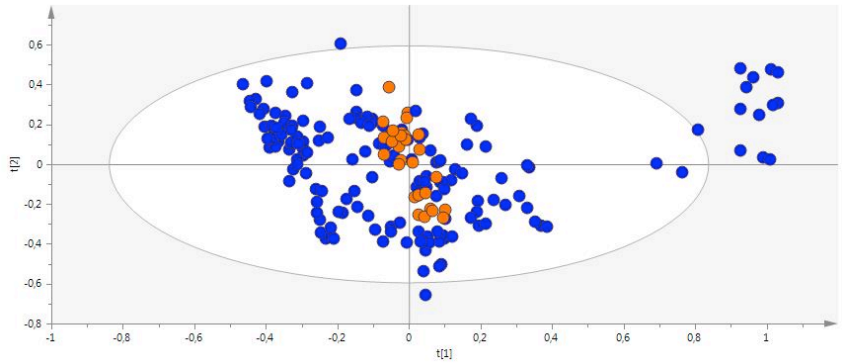


**Figure 1:** PCA with QCs samples (in orange) and VOCs from all cell lines and controls (blue) ($R^2X$ = 0.357)

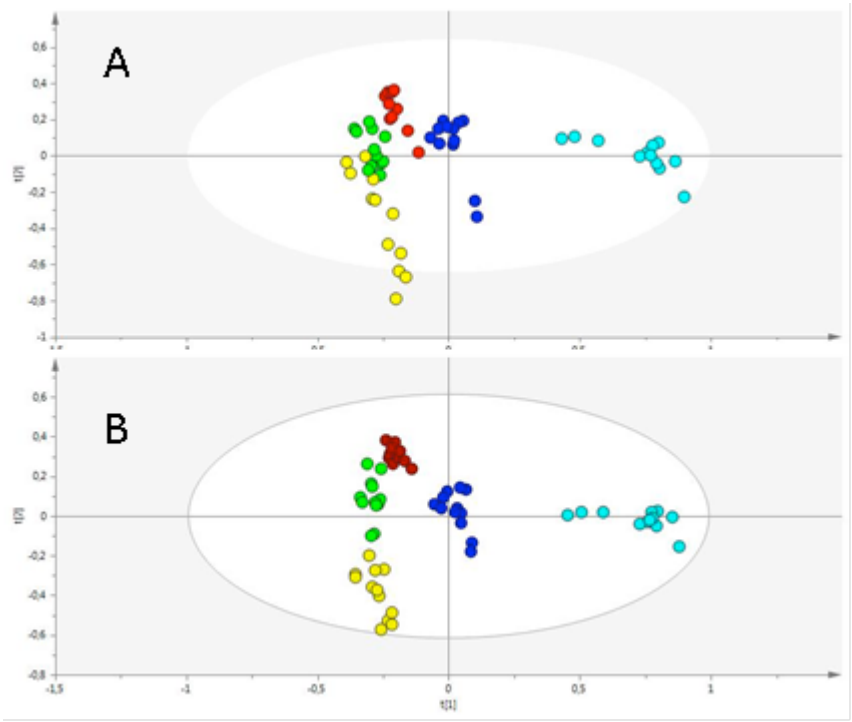


**Figure 2**: PCA (A) ($R^2X$ = 0.445) and PLS-DA (B) with VOCs from all cells line ($R^2X$ = 0.438; $R^2Y$ = 0.459; $Q^2$ = 0.44) (PNT2 (normal cells): green; PC3 (PCa cells): light blue; DU145 (PCa cells): red; LNCaP (PCa cells): yellow; 22RV1 (PCa cells): dark blue)

To evaluate which VOCs were responsible for this separation, each cancer cell line was compared separately with the normal cell line, namely 22RV1 vs PNT2, PC3 vs PNT2,

DU145 vs PNT2 and LNCaP vs PNT2. An optimal separation between PCa cell lines and normal cell line was observed (Figure 3).

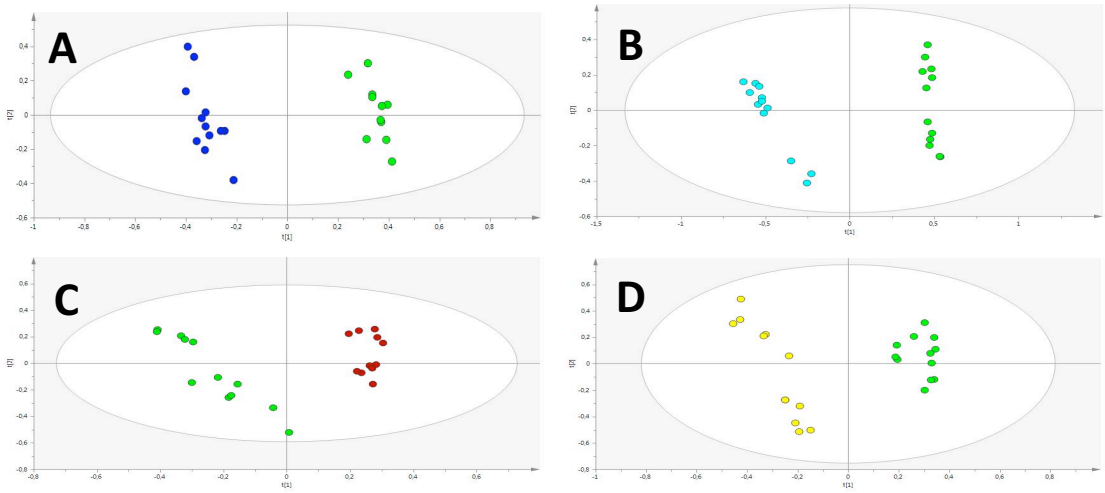


**Figure 3:** PLS-DA from PCa cell line vs normal cell line. **A:** 22RV1 (PCa cells) (dark blue) vs PNT2 (normal cells) (green) ($R^2X$=0.436; $R^2Y$=0.989; $Q^2$=0.967). **B:** PC3 (PCa cells) (light blue) vs PNT2 (normal cells) (green) ($R^2X$=0.667; $R^2Y$=0.985; $Q^2$=0.973). **C:** DU145 (PCa cells) (red) vs PNT2 (normal cells) (green) ($R^2X$=0.401; $R^2Y$=0.959; $Q^2$=0.884). **D:** LNCaP (PCa cells) (yellow) vs PNT2 (normal cells) (green) ($R^2X$=0.486; $R^2Y$=0.97; $Q^2$=0.931).

All VOCs with VIP (Variable Importance Projection) values greater than one were considered potentially relevant for the separation among cell lines. Hence, a total of 23 VOCs were considered relevant to differentiate 22RV1 from PNT2; 16 VOCs were considered relevant to differentiate PC3 from PNT2; 27 VOCs were considered relevant to differentiate DU145 from PNT2 and 21 were considered relevant to differentiate LNCaP from PNT2. The identification of VOCs selected by the statistical approaches was done by using the National Institute of Standards and Technology (NIST 14) data base spectra library and by comparing experimental Kovats indexes and kovats indexes from literature. To confirm the importance of these metabolites univariate analysis was performed to calculate the *p* value, the variation quotient and the effect size.

After univariate analysis the VOCs that were taken into account, were those with $p<0.05$; relevant value of variation quotient; and relevant effect size. Therefore, a total of eight VOCs proved to be relevant to differentiate 22RV1 from PNT2, eight VOCs proved to be relevant to differentiate PC3 from PNT2, seven VOCs proved to be relevant to differentiate DU145 from PNT2 and seven proved to be relevant to differentiate LNCaP from PNT2. All these results are shown in detail in Table 2 (22RV1 vs PNT2), Table 3 (PC3 vs PNT2), Table 4 (DU145 vs PNT2) and Table 5 (LNCaP vs PNT2).These VOCs

include ketones, alcohols, aldehydes, among others. From these VOCs two stand out, namely, 1,3-benzothiazole and pentadecan-2-one because they reveal to be important for the separation among all PCa cell lines and normal cell line. However, several other VOCs were identified and were also able to discriminate between cancer and normal cell lines, namely, 3,7-Dimethyloct-7-en-1-ol (22RV1 vs PNT2, PC3 vs PNT2, DU145 vs PNT2), 2-methylundecanal, (PC3 vs PNT2, DU145 vs PNT2 and LNCaP vs PNT2), 2,7-dimethyloctan-1-ol (PC3 vs PNT2), 2-(1-4-Methylcyclohex-3-en-1-yl)propan-2-ol (PC3 vs PNT2), decan-1-ol (DU145 vs PNT2), 1-methoxypropan-2-yl acetate (LNCaP vs PNT2), and 1,4-xylene (LNCaP vs PNT2). Some unidentified VOCs were also important for the discrimination between cancer and normal cell lines (Table 2; Table 3; Table 4; Table 5) (Figure 4; Figure 5; Figure 6; Figure 7).

**Table 2:** List of metabolites selected in PLS-DA of 22RV1 (PCa cells) vs PNT2 (normal cells) (VIP>1) as potentially important for discrimination between PCa and normal cell lines obtained at pH 7.

| Chemical name (IUPAC) or common name | RT | Characteristic ions | KI from literature | Experimental KI | MS-R match | Cas number | *p* value | Variation % (uncertainty) | Effect size (uncertainty) |
|---|---|---|---|---|---|---|---|---|---|
| 3-Methylbut-3-en-1-ol | 3.57 | 67/68 | 730 | 749 | 881 | 763-32-6 | >0.05 | ↑↓ 7.3 ± 5.4 | ↑↓ |
| 1-Methoxypropan-2-yl acetate | 6.47 | 58/72 | 850 | 863 | 911 | 108-65-6 | >0.05 | ↓ 30.4± 16.7 | ↓ 0.85± 0.81 |
| Unknown | 8.3 | 58/69 | NA | 930 | NA | NA | >0.05 | ↓ | ↓ |
| Unknown | 10.87 | 56 | NA | 1016 | NA | NA | 0.0204 | ↓ 62.5± 47.4 | ↓ |
| 2,6-Dimethyloct-7-en-2-ol | 12.5 | 67 | 1064 | 1070 | 851 | 18479-58-8 | >0.05 | ↑ | ↑ |
| Nonan-2-one | 13.06 | 58 | 1092 | 1088 | 900 | 821-55-6 | >0.05 | ↓ | ↓ |
| Unknown | 14.21 | 55 | NA | 1127 | NA | NA | >0.05 | ↓80.1 ± 47.2 | ↓ 1.12± 0.83 |
| 2,7-Dimethyloctan-1-ol (dihydro citronellol) | 14.26 | 56 69 | 1130 | 1128 | 727 | 15250-22-3 | >0.05 | ↓ | ↓ |
| **Naphthalene** | **15.83** | **51/102/127** | **1182** | **1182** | **914** | **91-20-3** | **0.0011[P]** | **↑ 49.2 ± 8.8** | **↑ 1.76± 0.92** |
| 6-Ethyl-2-methyldecane | 16.32 | 57/71/85 | 1185 | 1198 | 878 | 62108-21-8 | >0.05 | ↑ | ↑ |
| **3,7-Dimethyloct-7-en-1-ol (α-citronellol)** | **16.71** | **67/81** | **1214** | **1212** | **791** | | **0.0096** | **↓48.8± 20.3** | **↓ 1.26± 0.85** |
| **1,3-Benzothiazole** | **16.98** | **135/108** | **1229** | **1222** | **896** | **95-16-9** | **<0.0001[P]** | **↓ 75.0 ±6.8** | **↓ 6.91 ± 2.10** |
| **Unknown** | **17.03** | **69/ 67** | **NA** | **1224** | **NA** | **NA** | **0.0011[P]** | **↓ 58.3± 22.2** | **↓ 1.46± 0.88** |
| **Unknown** | **19.51** | **140/125/57** | **NA** | **1313** | **NA** | **NA** | **0.0024** | **↑14.6± 3.8** | **↑1.43± 0.87** |
| **Unknown** | 20.18 | 72 | NA | 1338 | NA | NA | >0.05 | ↓ | ↓ |
| 2-Methylpropyl 3-hydroxy-2,2,4-trimethylpentanoate | 20.33 | 56 71 | 1331 | 1344 | 783 | NA | >0.05 | ↓ | ↓ |
| 5-Pentyloxolan-2-one or γ-Nonanoic lactone | 20.63 | 85 | 1363 | 1355 | 818 | 104-61-0 | >0.05 | ↓ | ↓ |
| Unknown | 23.12 | 158 | NA | 1452 | NA | NA | >0.05 | ↓ | ↓ |
| Unknown | 23.34 | 85 | NA | 1461 | NA | NA | >0.05 | ↓ | ↓ |

| Chemical name (IUPAC) or common name | RT | Characteristic ions | KI from literature | Experimental KI | MS-R match | Cas number | *p* value | Variation % (uncertainty) | Effect size (uncertainty) |
|---|---|---|---|---|---|---|---|---|---|
| **Unknown** | **24.1** | **58 119** | **NA** | **1491** | **NA** | **NA** | **<0.0001[P]** | **↑63.5± 4.7** | **↑4.03 ± 1.38** |
| Hexadecane | 26.6 | 57 85 | 1600 | 1596 | 907 | 544-76-3 | >0.05 | ↑ | ↑ |
| **Pentadecan-2-one** | **28.78** | **58 59 71** | **1698** | **1689** | **835** | **2345-28-0** | **<0.0001[P]** | **↑203.5 ± 5.4** | **↑7.34 ± 2.22** |
| **Unknown** | **28.87** | **57 85** | **NA** | **1693** | **NA** | **NA** | **0.0003[P]** | **↑58.6 ±10.1** | **↑1.76 ± 0.92** |

KI: Kovat indices; not available; [P] Alterations remaining significant after Bonferroni correction, with cutoff *p* value of $2.17 \times 10^{-3}$ (0.05 divided by 23 analyzed VOCs); RT: Retention Time.

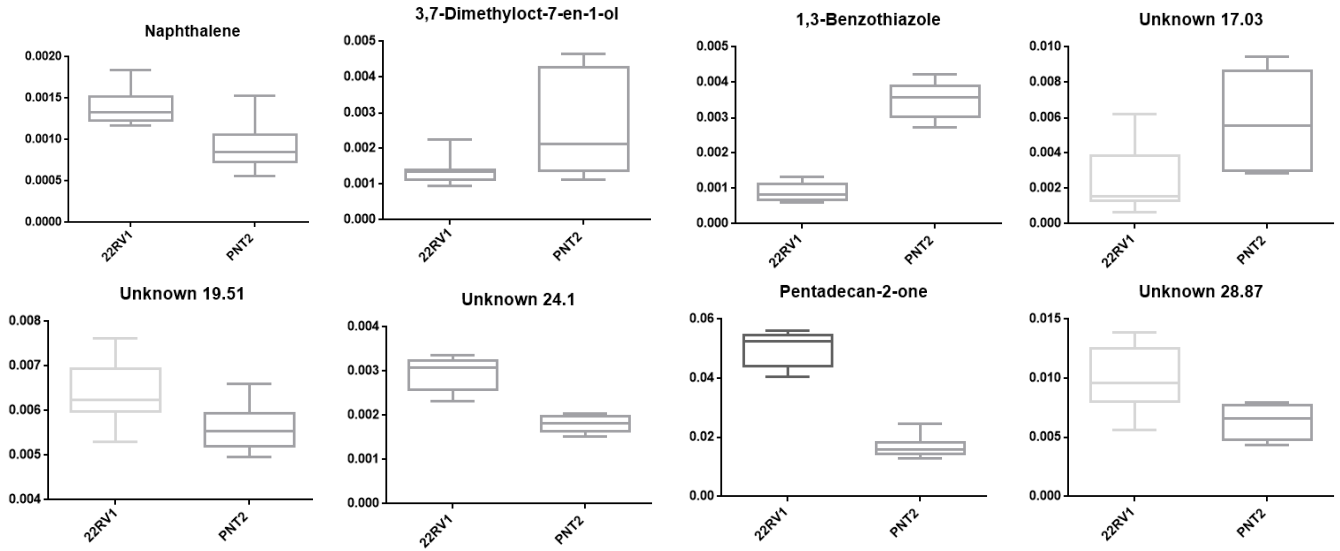


**Figure 4:** Boxplots from the metabolites important for the separation between 22RV1 (PCa cells) and PNT2 (normal cells), after univariate analysis, obtained at pH 7.

**Table 3:** List of metabolites selected in PLS-DA of PC3 (PCa cells) vs PNT2 (normal cells) (VIP>1) as potentially important for discrimination between PCa and normal cell lines obtained at pH 7.

| Chemical name (IUPAC) or common name | RT | Characteristic ions | KI from literature | Experimental KI | MS-R match | Cas number | *p* value | Variation % (uncertainty) | Effect size (uncertainty) |
|---|---|---|---|---|---|---|---|---|---|
| 1,4-Xylene | 6.57 | 91 | 865 | 867 | 907 | 106-42-3 | >0.05 | ↓ 10.74± 6.87 | ↓ |
| Unknown | 10.87 | 56 | NA | 1016 | NA | NA | >0.05 | ↓ | ↓ |
| 2,6-Dimethyloct-7-en-2-ol | 12.5 | 67 | 1064 | 1070 | 851 | 18479-58-8 | >0.05 | ↓ | ↓ |
| Nonan-2-one | 13.06 | 58 | 1092 | 1088 | 900 | 821-55-6 | >0.05 | ↓ | ↓ |
| Unknown | 14.21 | 55 | NA | 1127 | NA | NA | >0.05 | ↓ | ↓ |
| **2,7-Dimethyloctan-1-ol** | **14.26** | **56 69** | **1130** | **1128** | **727** | **15250-22-3** | **<0.0001[P]** | **↓ 74.48± 7.9** | **↓ 5.91± 1.84** |
| **2-(1-4-Methylcyclohex-3-en-1-yl)propan-2-ol (α-Terpineol)** | **16.14** | **93/136** | **1190** | **1192** | **869** | **10482-56-1** | **<0.0001[P]** | **↓58.47± 4.51** | **↓7.22± 2.18** |
| **3,7-Dimethyloct-7-en-1-ol (α-citronellol)** | **16.71** | **67/81** | **1212** | **1212** | **791** | **6812-78-8** | **<0.0001[P]** | **↓58± 21.18** | **↓ 1.52± 0.88** |
| **1,3-Benzothiazole** | **16.98** | **135/108** | **1229** | **1222** | **896** | 95-16-9 | **<0.0001[P]** | **↓ 70.22 ±7.35** | **↓7.03 ± 2.13** |
| Unknown | 17.03 | 69/67 | NA | 1224 | NA | NA | >0.05 | ↓ | ↓ |
| **2-Methylundecanal** | **18.86** | **58/71** | **1306** | **1289** | **810** | **110-41-8** | **0.0029[P]** | **↑58.67±11** | **↑1.54± 0.89** |
| Unknown | 20.18 | 72 | NA | 1338 | NA | NA | >0.05 | ↓ | ↓ |
| 2-Methylpropyl 3-hydroxy-2,2,4-trimethylpentanoate | 20.33 | 56/71 | 1331 | 1344 | 783 | NA | >0.05 | ↓ | ↓ |
| **Unknown** | **24.1** | **58/119** | **NA** | **1491** | **NA** | **NA** | **<0.0001[P]** | **↑177.78± 6.14** | **↑6.03 ± 1.87** |
| **Pentadecan-2-one** | **28.78** | **58/59/71** | **1698** | **1689** | **835** | **2345-28-0** | **<0.0001[P]** | **↑848.29 ± 6.06** | **↑10.53 ±3.07** |

| Chemical name (IUPAC) or common name | RT | Characteristic ions | KI from literature | Experimental KI | MS-R match | Cas number | *p* value | Variation % (uncertainty) | Effect size (uncertainty) |
|---|---|---|---|---|---|---|---|---|---|
| **Unknown** | **28.87** | **57/85** | **NA** | **1693** | **NA** | **NA** | **<0.0001[P]** | **↑ 181.87 ±4.63** | **↑ 8.1 ± 2.42** |

KI: Kovat indices; NA: not available; [P] Alterations remaining significant after Bonferroni correction, with cutoff *p* value of $3.12x10^{-3}$ (0.05 divided by 16 analyzed VOCs); RT: retention time.

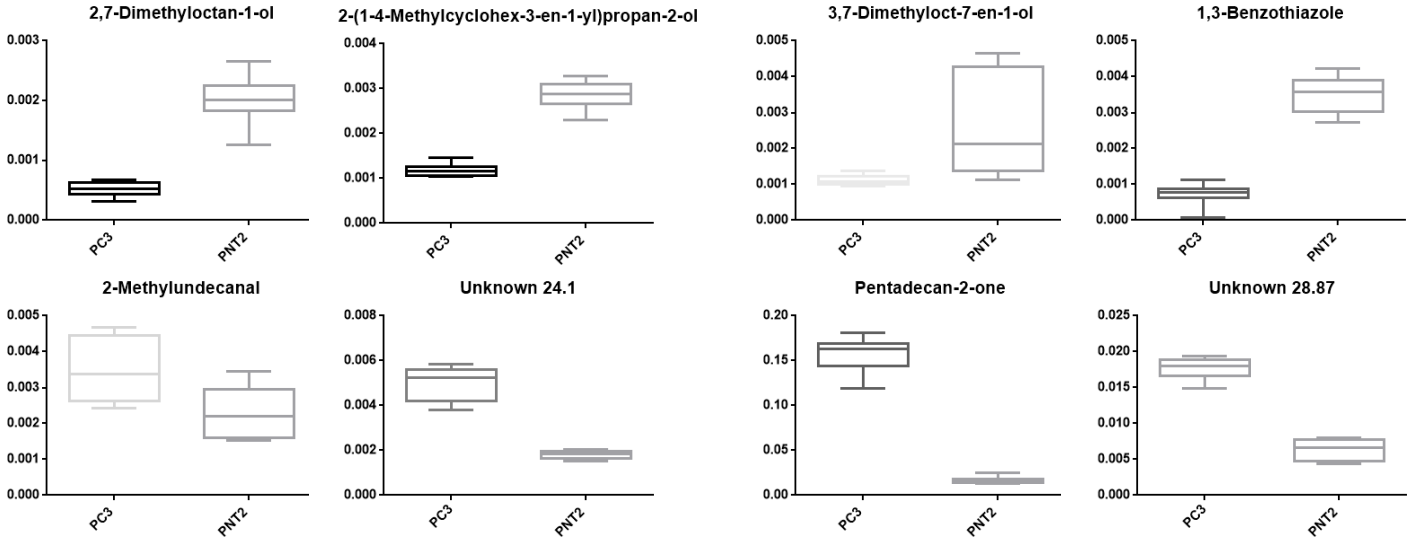


**Figure 5:** Boxplots from the metabolites important for the separation between PC3 (PCa cells) and PNT2 (normal cells), after univariate analysis, obtained at pH 7.

**Table 4:** List of metabolites selected in PLS-DA of DU145 (PCa cells) vs PNT2 (normal cells) (VIP>1) as potentially important for discrimination between PCa and normal cell lines obtained at pH 7.

| Chemical name (IUPAC) or common name | RT | Characteristic ions | KI from literature | Experimental KI | MS-R match | Cas number | *p* value | Variation % (uncertainty) | Effect size (uncertainty) |
|---|---|---|---|---|---|---|---|---|---|
| 4-Methylpentan-2-one | 3.71 | 57 | 735 | 755 | 862 | 108-10-1 | >0.05 | ↑ 45.57 ±28.50 | ↑ |
| 4-Methylpent-3-en-2-one | 4.81 | 55 83 | 798 | 798 | 869 | 141-79-7 | >0.05 | ↓ 22.18± 13.60 | ↓ |
| 1-Methoxypropan-2-yl acetate | 6.47 | 58 72 | 850 | 863 | 911 | 108-65-6 | >0.05 | ↑ 72.03±8.56 | ↑ 1.11±0.85 |
| 1,4-Xylene | 6.57 | 91 | 865 | 867 | 907 | 106-42-3 | >0.05 | ↑ 89.15±32.29 | ↑ |
| **Unknown** | **9.22** | **59** | **NA** | **961** | **NA** | **NA** | **0.0414** | **↓ 32.26± 15.95** | **↓ 0.92± 0.83** |
| Unknown | 9.95 | 58/85 | NA | 985 | NA | NA | >0.05 | ↓ 20.36± 12.46 | ↓ |
| Unknown | 10.87 | 56 | NA | 1016 | NA | NA | >0.05 | ↓ 62.5± 47.4 | ↓ 0.76± 0.80 |
| 4,6-Dimethylheptan-2-one | 11.91 | 78/84 | 1045 | 1050 | 836 | 19549-80-5 | >0.05 | ↓ | ↓ |
| 2,6-Dimethyloct-7-en-2-ol | 12.5 | 67 | 1064 | 1070 | 851 | 18479-58-8 | >0.05 | ↑ 46.05±30.73 | ↑ |
| Nonan-2-one | 13.06 | 58 | 1092 | 1088 | 900 | 821-55-6 | >0.05 | ↓ | ↓ |
| Unknown | 14.21 | 55 | NA | 1127 | NA | NA | >0.05 | ↑ | ↑ |
| 2,7-Dimethyloctan-1-ol | 14.26 | 56/69 | 1130 | 1128 | 727 | 15250-22-3 | >0.05 | ↑ | ↑ |
| Unknown | 14.62 | 55 | NA | 1141 | NA | NA | >0.05 | ↑ | ↑ |
| **3,7-Dimethyloct-7-en-1-ol** | **16.71** | **67/81** | **1214** | **1212** | **791** | **6812-78-8** | **<0.001[P]** | **↓ 73.13± 23.15** | **↓ 1.86± 0.95** |
| **1,3-Benzothiazole** | **16.98** | **135/108** | **1229** | **1222** | **896** | **95-16-9** | **0.0348** | **↑ 23.35±8.56** | **↑ 1.02±0.84** |
| **Unknown** | **17.03** | **69/67** | **NA** | **1224** | **NA** | **NA** | **0.0010[P]** | **↓ 23.26±6.27** | **↓ 1.63± 0.91** |

| Chemical name (IUPAC) or common name | RT | Characteristic ions | KI from literature | Experimental KI | MS-R match | Cas number | *p* value | Variation % (uncertainty) | Effect size (uncertainty) |
|---|---|---|---|---|---|---|---|---|---|
| **Decan-1-ol** | **18.3** | **55/69** | **1257** | **1269** | **888** | **112-30-1** | **0.0251** | **↑ 18.24±6.65** | **↑ 1.01±0.84** |
| **2-Methylundecanal** | **18.86** | **58/71** | **1306** | **1289** | **810** | **110-41-8** | **0.0037** | **↓ 31.88± 10.31** | **↓ 1.41± 0.88** |
| Unknown | 19.51 | 140/125/57 | NA | 1313 | NA | NA | >0.05 | ↓ 9.18±5.37 | ↓ |
| 2-Methylpropyl 3-hydroxy-2,2,4-trimethylpentanoate | 20.33 | 56/71 | 1331 | 1344 | 783 | NA | 0.0267 | ↑ 32.07± 16.86 | ↑ |
| 5-Pentyloxolan-2-one (γ-Nonanoic lactone) | 20.63 | 85 | 1363 | 1355 | 818 | 104-61-0 | >0.05 | ↑ 65.35± 36.32 | ↑ |
| Unknown | 23.12 | 158 | NA | 1452 | NA | NA | >0.05 | ↑ 64.14± 37.74 | ↑ |
| **Unknown** | **23.97** | **57/69** | **NA** | **1486** | **NA** | **NA** | **0.0338** | **↑ 20.39± 7.80** | **↑ 0.96± 0.84** |
| Unknown | 24.1 | 58/119 | NA | 1491 | NA | NA | >0.05 | ↑ | ↑ |
| Hexadecane | 26.6 | 57/85 | 1600 | 1596 | 907 | 544-76-3 | >0.05 | ↑ 31.52± 18.52 | ↑ |
| **Pentadecan-2-one** | **28.78** | **58/59/71** | **1698** | **1689** | **835** | **2345-28-0** | **0.0007[P]** | **↑ 58.66 ±10.04** | **↑ 1.89 ± 0.96** |
| Unknown | 28.87 | 57/85 | NA | 1693 | NA | NA | >0.05 | ↓ | ↓ |

KI: Kovat indices; NA: not available; [P] Alterations remaining significant after Bonferroni correction, with cutoff *p* value of $1.85X10^{-3}$ (0.05 divided by 27 analyzed VOCs); RT: retention time.

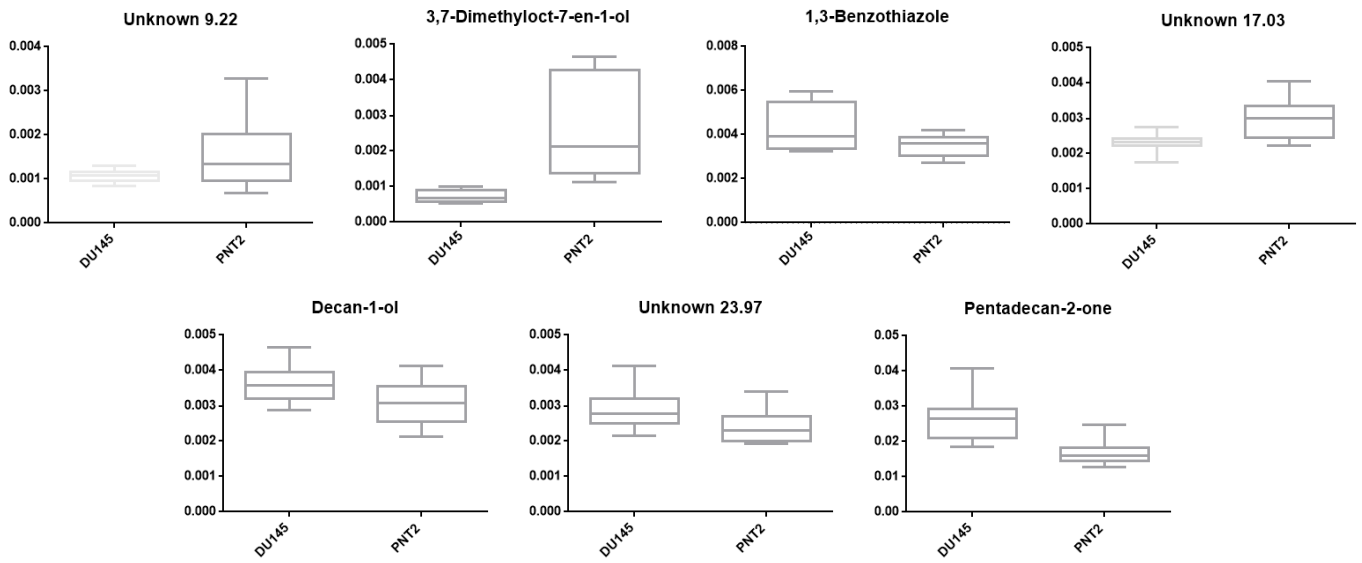


**Figure 6:** Boxplots from the metabolites important for the separation between DU145 (PCa cells) and PNT2 (normal cells), after univariate analysis, obtained at pH 7.

**Table 5:** List of metabolites selected in PLS-DA of LNCaP (PCa cells) vs PNT2 (normal cells) (VIP>1) as potentially important for discrimination between PCa and normal cell lines obtained at pH 7.

| Chemical name (IUPAC) or common name | RT | Characteristic ions | KI from literature | Experimental KI | MS-R match | Cas number | *p* value | Variation % (uncertainty) | Effect size (uncertainty) |
|---|---|---|---|---|---|---|---|---|---|
| **3-Methylbut-3-en-2-ol** | **2.95** | **58** | **737** | **725** | **724** | **10473-14-0** | **0.0018[P]** | **↑ 34.78±7.88** | **↑ 1.48±0.88** |
| **1-Methoxypropan-2-yl acetate** | **6.47** | **58/72** | **850** | **863** | **911** | **108-65-6** | **0.0447** | **↑ 70.57±18.49** | **↑ 1.11±0.83** |
| **1,4-Xylene** | **6.57** | **91** | **865** | **867** | **907** | **106-42-3** | **0.0011[P]** | **↑ 182.61± 24.11** | **↑ 1.56±0.89** |
| Unknown | 8.3 | 58/69 | NA | 930 | NA | NA | >0.05 | ↑ 11.59±10.16 | ↑ |
| **Unknown** | **9.95** | **58/85** | **NA** | **985** | **NA** | **NA** | **0.0204** | **↑ 30.56±10.07** | **↑ 1.04±0.83** |
| Unknown | 10.87 | 56 | NA | 1016 | NA | NA | >0.05 | ↓ 55.61± 31.41 | ↓ |
| 4,6-Dimethylheptan-2-one | 11.91 | 78/84 | 1045 | 1050 | 836 | 19549-80-5 | >0.05 | ↑ 11.34±8.23 | ↑ |
| Nonan-2-one | 13.06 | 58 | 1092 | 1088 | 900 | 821-55-6 | >0.05 | ↑ | ↑ |
| Unknown | 14.21 | 55 | NA | 1127 | NA | NA | >0.05 | ↑ 38.6±26.35 | ↑ |
| 2,7-Dimethyloctan-1-ol | 14.26 | 56/69 | 1130 | 1128 | 727 | 15250-22-3 | >0.05 | ↓ 32.61± 28.71 | ↓ |
| Benzyl acetate | 15.15 | 150 | 1164 | 1159 | 801 | 140-11-4 | >0.05 | ↑ 159.11± 37.11 | ↑ 0.94± 0.82 |
| 6-Ethyl-2-methyldecane | 16.32 | 57/71/85 | 1185 | 1198 | 878 | 62108-21-8 | >0.05 | ↑ 20.83± 10.32 | ↑ |
| Decanal | 16.44 | 56/57/70 | 1206 | 1203 | 880 | 112-31-2 | 0.0068 | ↑ 83.27± 29.23 | ↑ |
| 3,7-Dimethyloct-7-en-1-ol | 16.71 | 67/81 | 1214 | 1212 | 791 | 6812-78-8 | >0.05 | ↑ 137.92± 28.06 | ↑ 1.14± 0.84 |
| **1,3-Benzothiazole** | **16.98** | **135/108** | **1229** | **1222** | **896** | **95-16-9** | **<0.0001[P]** | **↓ 69.91± 7.11** | **↓ 5.96±1.86** |
| **Unknown** | **17.03** | **69/67** | **NA** | **1224** | **NA** | **NA** | **0.0023[P]** | **↓ 34.1±15.37** | **↓ 1.05± 0.83** |
| **2-Methylundecanal** | **18.86** | **58/71** | **1306** | **1289** | **810** | **110-41-8** | **0.0045** | **↑ 71.77±15.15** | **↑ 1.37±0.86** |
| Unknown | 19.51 | 140/125/57 | NA | 1313 | NA | NA | >0.05 | ↑ | ↑ |

| Chemical name (IUPAC) or common name | RT | Characteristic ions | KI from literature | Experimental KI | MS-R match | Cas number | *p* value | Variation % (uncertainty) | Effect size (uncertainty) |
|---|---|---|---|---|---|---|---|---|---|
| 2-Methylpropyl 3-hydroxy-2,2,4-trimethylpentanoate | 20.33 | 56/71 | 1331 | 1344 | 783 | NA | >0.05 | ↓ | ↓ |
| **Unknown** | **24.1** | **58/119** | **NA** | **1491** | **NA** | **NA** | **0.0016[P]** | **↑ 36.55±7.49** | **↑ 1.63±0.9** |
| **Pentadecan-2-one** | **28.78** | **58/59/71** | **1698** | **1689** | **835** | **2345-28-0** | **<0.0001[P]** | **↑ 73.12 ±9.26** | **↑ 2.27 ±1.01** |

KI: Kovat indices; NA: not available; [P] Alterations remaining significant after Bonferroni correction, with cutoff *p* value of $2.38X10^{-3}$ (0.05 divided by 21 analyzed VOCs); RT: retention time.

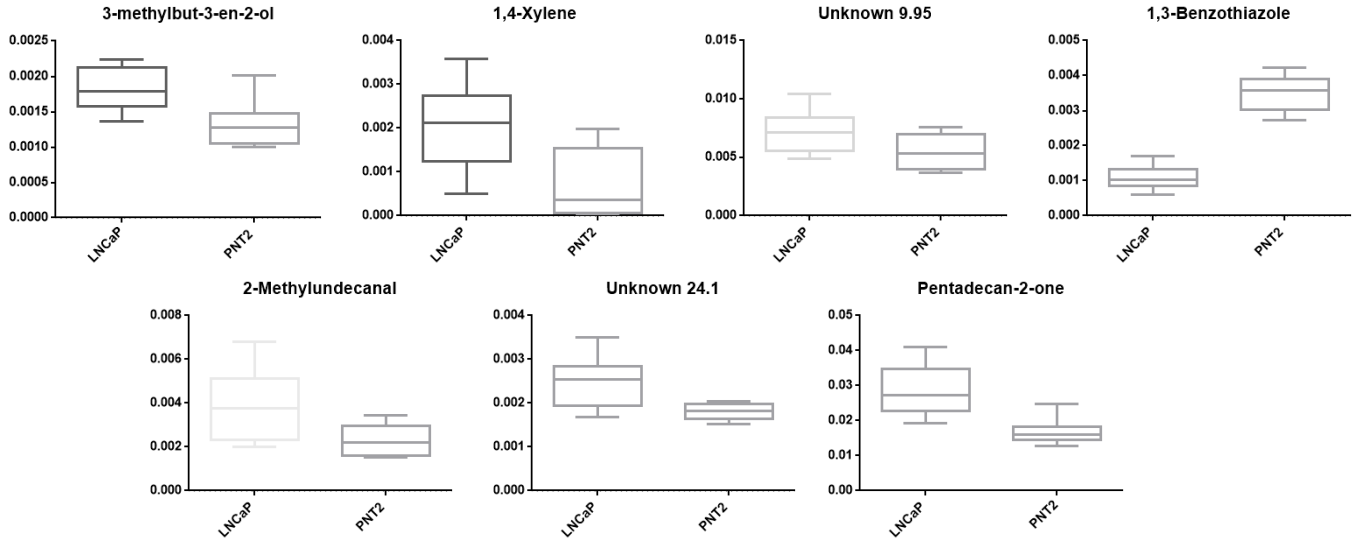


**Figure 7:** Boxplots from the metabolites important for the separation between LNCaP (PCa cells) and PNT2 (normal cells), after univariate analysis, obtained at pH 7.

A new PLS-DA models using only discriminant metabolites (Figure 8) was performed. To prove the robustness of the models, a MCCV validation was performed for all comparisons, using GC-MS full data but also using just the discriminant metabolites described before. The results of this validation prove that all created models are robust for the discrimination between PCa cell lines and normal cell line (Table 6 and Figure 9).

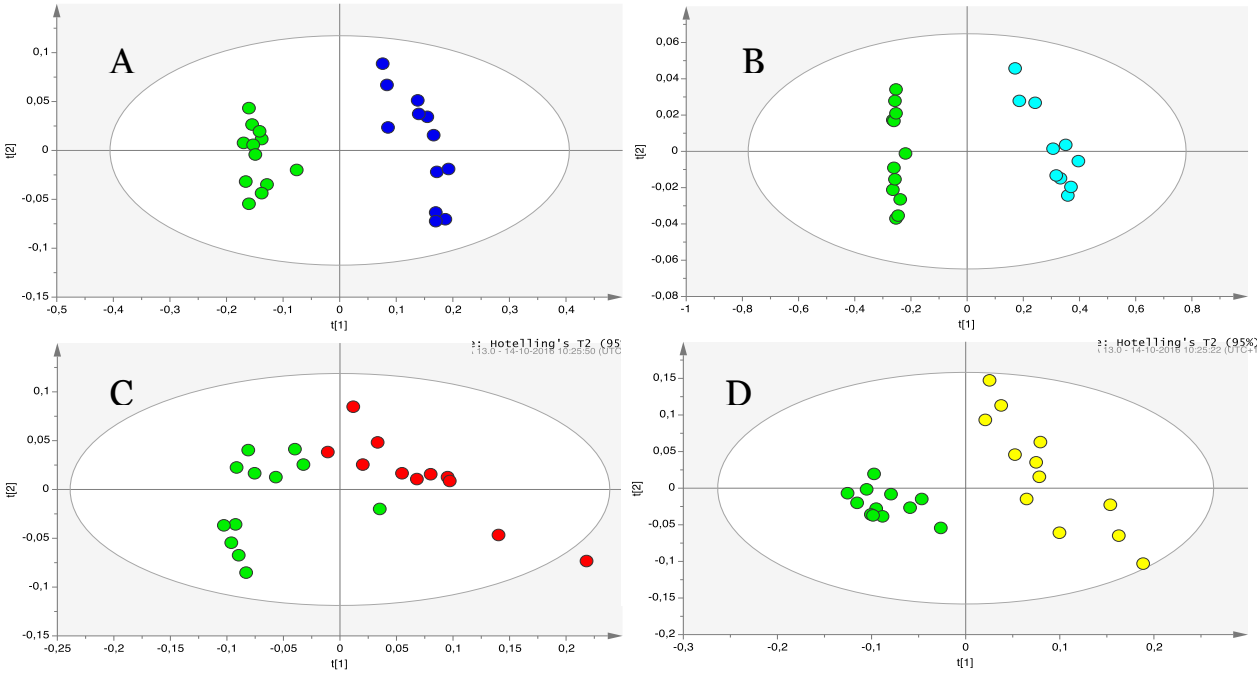


**Figure 8:** PLS-DA from PCa cell line vs normal cell line with the set of discriminant VOCs. **A.** 22RV1 (PCa cells) (dark blue) vs PNT2 (normal cells) (green) ($R^2X$=0.889; $R^2Y$=0.971; $Q^2$=0.957). **B.** PC3 (PCa cells) (light blue) vs PNT2 (normal cells) (green) ($R^2X$=0.987; $R^2Y$=0.979; $Q^2$=0.974). **C.** DU145 (PCa cells) (red) vs PNT2 (normal cells) (green) ($R^2X$=0.831; $R^2Y$=0.738; $Q^2$=0.66). **D.** LNCaP (PCa cells) (yellow) vs PNT2 (normal cells) (green) ($R^2X$=0.891; $R^2Y$=0.94; $Q^2$=0.925).

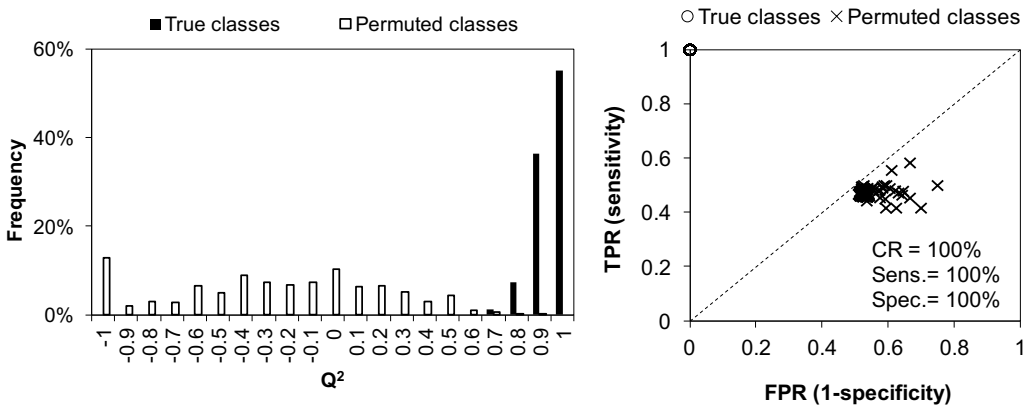


**Figure 9:** $Q^2$ distribution and ROC plot of true and permuted classes obtained by Monte Carlo cross validation for 22RV1 (PCa cells) vs PNT2 (normal cells) obtained at pH 7.

**Table 6:** MCCV parameters of true and permuted classes obtained for pH 7 when considering GC-MS full data and the set of discriminant VOCs.

| Models | True classes | | | | | Permuted classes | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | LV | $Q^2$ | CR (%) | Sens. (%) | Spec. (%) | LV | $Q^2$ | CR (%) | Sens. (%) | Spec. (%) |
| ***GC-MS full data*** | | | | | | | | | | |
| 22RV1 vs PNT2 | 5 | 0.91 | 100 | 100 | 100 | 1 | -0.28 | 47 | 47 | 47 |
| PC3 vs PNT2 | 2 | 0.99 | 100 | 100 | 100 | 1 | -0.29 | 48 | 49 | 48 |
| DU145 vs PNT2 | 3 | 0.94 | 100 | 99 | 100 | 1 | -0.42 | 47 | 44 | 50 |
| LNCaP vs PNT2 | 4 | 0.90 | 100 | 100 | 100 | 1 | -0.29 | 48 | 48 | 48 |
| ***Set of discriminant VOCs*** | | | | | | | | | | |
| 22RV1 vs PNT2 | 1 | 0.94 | 100 | 100 | 100 | 1 | -0.26 | 48 | 48 | 47 |
| PC3 vs PNT2 | 1 | 0.98 | 100 | 100 | 100 | 1 | -0.14 | 49 | 44 | 54 |
| DU145 vs PNT2 | 1 | 0.72 | 87 | 100 | 75 | 1 | -0.34 | 47 | 42 | 51 |
| LNCaP vs PNT2 | 2 | 0.96 | 100 | 100 | 100 | 1 | -0.32 | 45 | 45 | 45 |

LV: no. of latent variables; $Q^2$: median predictive power; CR: classification rate; sens.: sensitivity, spec.: specificity.

*VOCs analysis obtained at pH 2*

The same samples analyzed at pH 7 were also analyzed at pH 2. It is important to use two different pH levels because variations of pH can lead to the alteration of the VOCs ionization state, consequently VOCs that are undetected at pH 7, can be detected at pH 2. Despite the alteration of pH, the analytical procedure and the statistical treatment used for VOCs obtained at pH 2 were the same used for pH 7. A total of 221 features were detected in the chromatograms from pH 2 samples.

The multivariate analysis of chromatograms obtained at pH 2, shows that the analytical method is reproducible, which can be seen in Figure 10 (all QCs samples are projected together in the space formed by the first three PCA axes). Furthermore, the multivariate analyses prove that VOCs obtained at pH 2 are also able to discriminate PCa cell lines from normal prostate cell line and between the different PCa cell lines, this discriminant capability is observed not only in PLS-DA analysis but also in PCA (Figure 11).

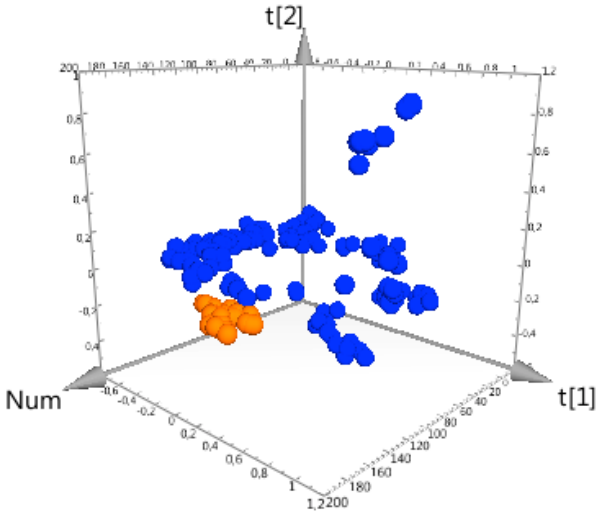


**Figure 10:** PCA with QCs samples (in orange) and VOCs from all cell lines and controls (blue) obtained at pH 2 ($R^2X$ = 0.562).

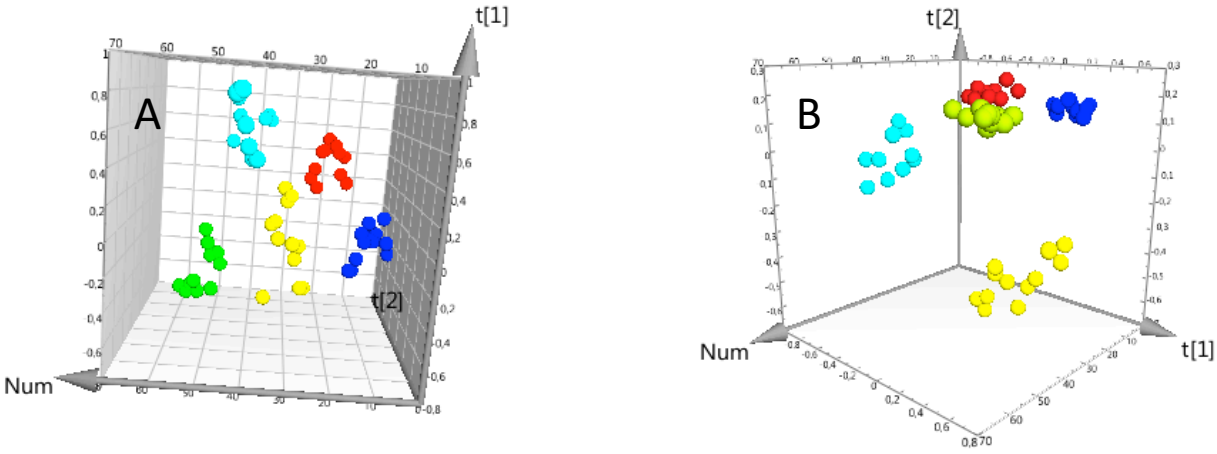


**Figure 11:** PCA (A) ($R^2X$ = 0.582) and PLS-DA (B) ($R^2X$ = 0.526; $R^2Y$ = 0.457; $Q^2$ = 0.437) with VOCs from all cells line obtained at pH 2 (PNT2 (normal cells): green; 22RV1 (PCa cells): dark blue; PC3 (PCa cells): light blue; DU145 (PCa cells): red; LNCaP (PCa cells): yellow).

To evaluate which were the VOCs responsible for this separation all cancer cell lines were compared with the normal cell line, namely 22RV1 vs PNT2, PC3 vs PNT2, DU145 vs PNT2 and LNCaP vs PNT2. An optimal separation between PCa cell lines and normal cell line was observed (Figure 12).

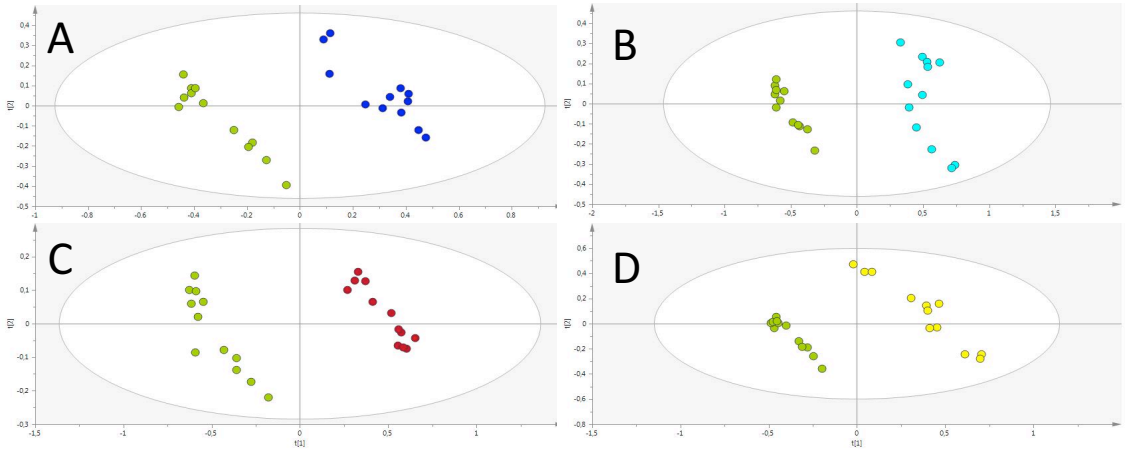


**Figure 12:** PLS-DA from PCa vs PNT2 obtained at pH 2. **A.** 22RV1 (PCa cells) (dark blue) vs PNT2 (normal cells) (green) ($R^2X$=0.586; $R^2Y$=0.976; $Q^2$=0.956). **B.** PC3 (PCa cells) (light blue) vs PNT2 (normal cells) (green) ($R^2X$=0.721; $R^2Y$=0.976; $Q^2$=0.957). **C.** DU145 (PCa cells) (red) vs PNT2 (normal cells) (green) ($R^2X$=0.788; $R^2Y$=0.987; $Q^2$=0.969). **D.** LNCaP (PCa cells) (yellow) vs PNT2 (normal cells) (green) ($R^2X$=0.729; $R^2Y$=0.989; $Q^2$=0.983).

Despite some VOCs detected at pH 2 are the same detected at pH 7, as expected, the pH alteration promoted the detection of different VOCs that were not detected at pH 7, principally organic acids. As previously explained all VOCs with VIP values greater than one were considered potentially relevant for the separation among cell lines (a total of 32 VOCs for 22RV1 vs PNT2, 25 VOCs for PC3 vs PNT2, 21 VOCs for DU145 vs PNT2 and 24 for LNCaP vs PNT2). To confirm the importance of these metabolites univariate analysis was performed to calculate the *p* value, the variation quotient and the effect size. In Table 7 (22RV1 vs PNT2), Table 8 (PC3 vs PNT2), Table 9 (DU145 vs PNT2) and Table 10 (LNCaP vs PNT2) are shown, in detail, all these results.

After univariate analysis the VOCs that were taken into account, were those with $p<0.05$; relevant value of variation quotient; and relevant effect size. Therefore, a total of 11 VOCs proved to be relevant to differentiate 22RV1 from PNT2, 19 VOCs proved to be relevant to differentiate PC3 from PNT2, 13 VOCs proved to be relevant to differentiate DU145 from PNT2 and 13 proved to be relevant to differentiate LNCaP from PNT2. Comparing to pH 7, the statistical analyses of results of pH 2, revealed more discriminant VOCs. From these VOCs eight stand out, namely, cyclohexanone, 4-methylheptan-2-one, 2-methylpentane-1,3-diol, 4-methylbenzaldehyde, 1-(3,5-dimethylfuran-2-yl) ethanone, methyl benzoate, nonanoic acid and decanoic acid because they reveal to be important for the separation between all PCa cell lines and normal cell line. Other VOCs revealed to be more specific of each cell line, only allowing discrimination between some cell lines, namely, 4-methylpent-3-en-2-one (22RV1 vs PNT2, PC3 vs PNT2, DU145 vs PNT2), 5-methylheptan-2-one (PC3 vs PNT2, DU145 vs PNT2 and LNCaP vs PNT2), phenylethanol (22RV1 vs PNT2, PC3 vs PNT2, DU145 vs PNT2), 4-methylnonanoic acid (22RV1 vs PNT2 and LNCaP vs PNT2), benzyl acetate (22RV1 vs PNT2 and LNCaP vs PNT2), 5-methyl-2-propan-2-ylcyclohexan-1-ol (PC3 vs PNT2 and LNCaP vs PNT2), 1-ethoxypentane (DU145 vs PNT2 and LNCaP vs PNT2), methyl nonanoate (22RV1 vs PNT2), 2-ethoxy-2-methylbutane (PC3 vs PNT2), hexanoic acid (PC3 vs PNT2), phenylmethanol (PC3 vs PNT2), 2,4-dimethylheptan-1-ol (PC3 vs PNT2), benzoic acid (PC3 vs PNT2), 6-pentyloxan-2-one (PC3 vs PNT2). Some unidentified VOCs were also important for the discrimination between cancer and normal cell lines (Table 7; Table 8; Table 9; Table 10) (Figure 13; Figure 14; Figure 15; Figure 16)

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**Table 7:** List of metabolites selected in PLS-DA of 22RV1 (PCa cells) vs PNT2 (normal cells) (VIP>1) as potentially important for discrimination between PCa and normal cell lines obtained at pH 2.

| Chemical name (IUPAC) or common name | RT | Characteristic ions | KI from literature | Experimental KI | MS-R match | Cas number | *p* value | Variation % (uncertainty) | Effect size (uncertainty) |
|---|---|---|---|---|---|---|---|---|---|
| 2-Ethoxy-2-methylbutane | 2.49 | 59 | 728 | 707 | 683 | 919-94-8 | >0.05 | ↓ 26.6± 19.9 | ↓ |
| Unknown | 4.18 | 70 | NA | 773 | NA | NA | >0.05 | ↑ | ↑ |
| **4-Methylpent-3-en-2-one** | **4.81** | **55/83/98** | **798** | **798** | **855** | **141-79-7** | **<0.0001[P]** | **↓ 47.2± 7.1** | **↓ 3.44± 1.24** |
| **Cyclohexanone** | **7.2** | **98/70** | **894** | **891** | **904** | **108-94-1** | **<0.0001[P]** | **↓ 81.1± 7.0** | **↓ 7.72±2.32** |
| 2-Methylheptan-2-ol | 7.25 | 59 | 885 | 893 | 748 | 625-25-2 | >0.05 | ↑ | ↑ |
| **4-Methylheptan-2-one** | **8.44** | **58/59** | **943** | **934** | **928** | **6137-06-0** | **<0.0001[P]** | **↓ 28.6±6.3** | **↓ 2.10±0.97** |
| Hexanoic acid | 10 | 87 | 990 | 987 | 959 | 142-62-1 | >0.05 | ↓ | ↓ |
| **2-Methylpentane-1,3-diol** | **10.65** | **57/89** | **1005** | **1009** | **682** | **149-31-5** | **0.0105** | **↓ 10.7± 3.8** | **↓ 1.17± 0.84** |
| Phenylmethanol | 11.33 | 77 | 1036 | 1031 | 799 | 100-51-6 | >0.05 | ↑ 35.5±19.6 | ↑ |
| 2,4-Dimethylheptan-1-ol | 11.91 | 57 | 1030 | 1050 | 731 | 18450-73-2 | >0.05 | ↑ 32.7±20.1 | ↑ |
| **Phenylethanol** | **12.14** | **77/122** | **1055** | **1058** | **847** | **1517-69-7** | **<0.0001[P]** | **↓ 38.1 ±7.9** | **↓ 2.36 ±1.02** |
| **4-Methyl-benzaldehyde** | **12.82** | **91/119/120** | **1095** | **1080** | **953** | **104-87-0** | **0.0022** | **↓ 10.6±3.0** | **↓ 1.47± 0.88** |
| **1-(3,5-Dimethylfuran-2-yl) ethanone** | **12.99** | **123/138** | **1057** | **1086** | **760** | **22940-86-9** | **<0.0001[P]** | **↓ 47.8± 5.3** | **↓ 4.79± 1.56** |
| **Methyl benzoate** | **13.15** | **77/105/136** | **1094** | **1091** | **921** | **93-58-3** | **<0.0001[P]** | **↓ 42.2± 3.9** | **↓ 5.51± 1.74** |
| **Benzyl acetate** | **15.15** | **90/135** | **1164** | **1159** | **770** | **140-11-4** | **<0.0001[P]** | **↓ 35.17±6.1** | **↓ 2.75±1.10** |
| Benzoic acid | 15.33 | 122 | 1170 | 1165 | 837 | 65-85-0 | >0.05 | ↓ | ↓ |

| Chemical name (IUPAC) or common name | RT | Characteristic ions | KI from literature | Experimental KI | MS-R match | Cas number | *p* value | Variation % (uncertainty) | Effect size (uncertainty) |
|---|---|---|---|---|---|---|---|---|---|
| 5-Methyl-2-propan-2-ylcyclohexan-1-ol (DL-menthol) | 15.66 | 71/75/81 | 1175 | 1176 | 914 | 2216-51-5 | >0.05 | ↓ | ↓ |
| **Methyl nonanoate** | **17.09** | **87/129** | **1225** | **1226** | **745** | **1731-84-6** | **<0.0001[P]** | **↑366.0± 7.9** | **↑6.46±1.98** |
| 4-Methyloctanoic acid | 17.22 | 99 | 1208 | 1231 | 882 | 54947-74-9 | 0.0447 | ↓ 30.4± 20.7 | ↓ |
| Ethyl 2-phenylacetate | 17.44 | 164 | 1246 | 1238 | 918 | 101-97-3 | >0.05 | ↓ 31.9± 19.8 | ↓ |
| Unknown | 17.52 | 96 | NA | 1241 | NA | NA | >0.05 | ↓ | ↓ |
| Unknown | 17.71 | 81 | NA | 1248 | NA | NA | >0.05 | ↓ | ↓ |
| 5-Butyloxolan-2-one (γ-octalactone) | 17.79 | 85 | 1261 | 1251 | 866 | 104-50-7 | >0.05 | ↓ 18.1± 15.2 | ↓ |
| **Nonanoic acid** | **18.33** | **73/115** | **1273** | **1279** | **909** | **112-05-0** | **<0.0001[P]** | **↓ 55.6± 8.8** | **↓ 3.47± 1.25** |
| **Unknown** | **18.55** | **66/117** | **NA** | **1278** | **NA** | **NA** | **<0.0001[P]** | **↓ 94.9± 11.8** | **↓ 6.05± 1.88** |
| **4-Methylnonanoic acid** | **19.5** | **60/113** | **1308** | **1313** | **798** | **45019-28** | **<0.0001[P]** | **↑1217.1±11.7** | **↑5.79± 1.81** |
| 5-Pentyloxolan-2-one (γ-Nonanoic lactone) | 20.63 | 85 | 1363 | 1355 | 890 | 104-61-0 | >0.05 | ↓ | ↓ |
| **Decanoic acid** | **20.94** | **60/73/129** | **1373** | **1367** | **931** | **334-48-5** | **0.0027** | **↑71.1± 14.0** | **↑1.47± 0.88** |
| Unknown | 22.2 | 73 | NA | 1416 | NA | NA | >0.05 | ↓ 20.9± 17.1 | ↓ |
| 5-hexyloxolan-2-one (γ-decalactone) | 23.32 | 85 | 1470 | 1460 | 909 | 706-14-9 | >0.05 | ↑ | ↑ |
| Undec-2-enoic acid | 23.83 | 99 | 1479 | 1481 | 635 | 15790-94-0 | >0.05 | ↓ | ↓ |
| 6-Pentyloxan-2-one (δ-decalactone) | 23.97 | 99 | 1496 | 1486 | 863 | 705-86-2 | >0.05 | ↓ | ↓ |

KI: Kovat indices; NA: not available; [P] Alterations remaining significant after Bonferroni correction, with cutoff *p* value of $1.56X10^{-3}$ (0.05 divided by 32 analyzed VOCs).

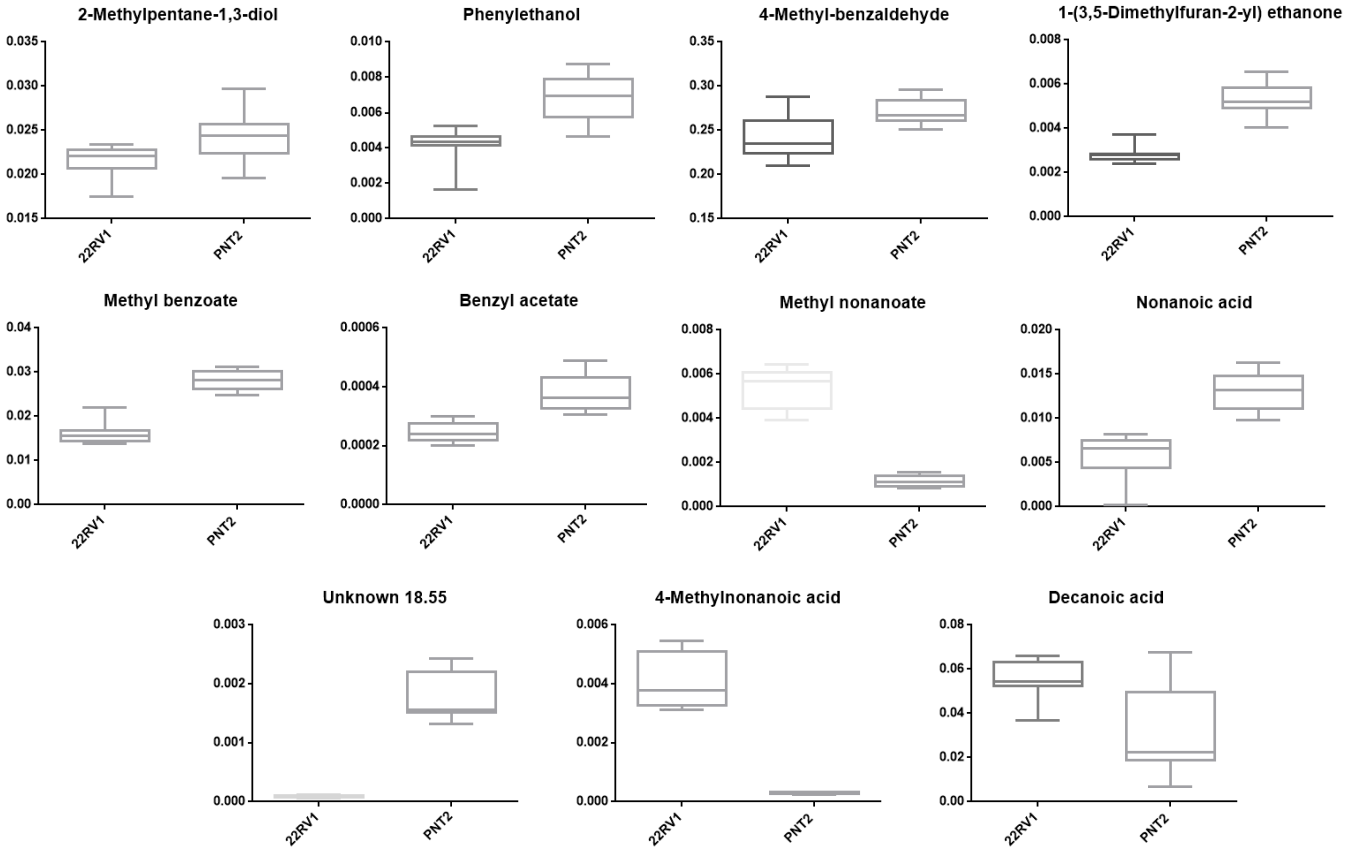


**Figure 13:** Boxplots from the metabolites important for the separation between 22RV1 (PCa cells) and PNT2 (normal cells), after univariate analysis, at pH 2.

**Table 8:** List of metabolites selected in PLS-DA of PC3 (PCa cells) vs PNT2 (normal cells) (VIP>1) as potentially important for discrimination between PCa and normal cell lines obtained at pH 2.

| Chemical name (IUPAC) or common name | RT | Characteristic ions | KI from literature | Experimental KI | MS-R match | Cas number | *p* value | Variation % (uncertainty) | Effect size (uncertainty) |
|---|---|---|---|---|---|---|---|---|---|
| **2-Ethoxy-2-methylbutane** | **2.49** | **59** | **728** | **707** | **683** | **919-94-8** | **0.0342** | **⇓38.26± 19.77** | **⇓ 0.94±0.81** |
| 1-Ethoxypentane | 3.63 | 59/70 | 760 | 752 | 786 | 17952-11-3 | 0.0056 | ⇑ | ⇑ |
| Unknown | 4.18 | 70 | NA | 773 | NA | NA | >0.05 | ⇓34.73± 20.54 | ⇓ |
| **4-Methylpent-3-en-2-one** | **4.81** | **55/83/98** | **798** | **798** | **855** | **141-79-7** | **<0.0001[P]** | **⇓ 62.38± 7.88** | **⇓ 4.54± 1.5** |
| **Cyclohexanone** | **7.2** | **98/70** | **894** | **891** | **904** | **108-94-1** | **<0.0001[P]** | **⇓ 50.48± 5.88** | **⇓ 4.53±1.5** |
| **4-Methylheptan-2-one** | **8.44** | **58/59** | **943** | **934** | **928** | **6137-06-0** | **0.0003[P]** | **⇓ 38.37±8.84** | **⇓ 2.11±0.98** |
| **5-Methylheptan-2-one** | **9.35** | **58/71** | **971** | **965** | **714** | **18217-12-4** | **<0.0001[P]** | **⇓ 51.58± 6.11** | **⇓ 4.48± 1.48** |
| **Hexanoic acid** | **10** | **87** | **990** | **987** | **959** | **142-62-1** | **0.0092** | **⇓40 ±16.66** | **⇓ 1.18±0.84** |
| **2-Methylpentane-1,3-diol** | **10.65** | **57/89** | **1005** | **1009** | **682** | **149-31-5** | **<0.0001[P]** | **⇓ 45.1± 5.81** | **⇓ 3.95± 1.36** |
| **Phenylmethanol** | **11.33** | **77** | **1036** | **1031** | **799** | **100-51-6** | **0.0189** | **⇓ 33.69± 15.3** | **⇓ 1.04± 0.83** |
| **2,4-Dimethylheptan-1-ol** | **11.91** | **57** | **1030** | **1050** | **731** | **18450-73-2** | **0.0210** | **⇓ 35.32 ±16.5** | **⇓ 1.02 ±0.83** |
| **Phenylethanol** | **12.14** | **77/122** | **1055** | **1058** | **847** | **1517-69-7** | **<0.0001[P]** | **⇓ 69.81 ±8.12** | **⇓ 5.2 ±1.66** |
| **4-Methylbenzaldehyde** | **12.82** | **91/119/120** | **1095** | **1080** | **953** | **104-87-0** | **<0.0001[P]** | **⇓ 38.0±2.98** | **⇓ 6.20± 1.92** |
| **1-(3,5-Dimethylfuran-2-yl) ethanone** | **12.99** | **123/138** | **1057** | **1086** | **760** | **22940-86-9** | **<0.0001[P]** | **⇓ 41.51± 5.09** | **⇓ 4.06± 1.38** |
| **Methyl benzoate** | **13.15** | **77/105/136** | **1094** | **1091** | **921** | **93-58-3** | **<0.0001[P]** | **⇓ 53.12± 4.1** | **⇓ 6.95± 2.11** |
| **Benzoic acid** | **15.33** | **122** | **1170** | **1165** | **837** | **65-85-0** | **0.0331** | **⇓ 37.17±17.66** | **⇓ 1.01±0.82** |

| Chemical name (IUPAC) or common name | RT | Characteristic ions | KI from literature | Experimental KI | MS-R match | Cas number | *p* value | Variation % (uncertainty) | Effect size (uncertainty) |
|---|---|---|---|---|---|---|---|---|---|
| **5-Methyl-2-propan-2-ylcyclohexan-1-ol (DL-menthol)** | **15.66** | **71/75/81** | **1175** | **1176** | **914** | **2216-51-5** | **0.0068** | **⇓ 45.82±18.48** | **⇓ 1.27±0.85** |
| **Unknown** | **17.52** | **96** | **NA** | **1241** | **NA** | **NA** | **0.0321** | **⇓ 30.2±14.74** | **⇓ 0.95±0.82** |
| 5-Butyloxolan-2-one (γ-Octalactone) | 17.79 | 85 | 1261 | 1251 | 866 | 104-50-7 | >0.05 | ⇓ 28.49± 17.02 | ⇓ |
| **Nonanoic acid** | **18.33** | **73/115** | **1273** | **1279** | **909** | **112-05-0** | **<0.0001[P]** | **⇓ 59.85± 7.41** | **⇓ 4.54± 1.5** |
| **Unknown** | **18.55** | **66/117** | **NA** | **1278** | **NA** | **NA** | **<0.0001[P]** | **⇓ 91.07± 11.48** | **⇓ 5.74± 1.8** |
| **Decanoic acid** | **20.94** | **60/73/129** | **1373** | **1367** | **931** | **334-48-5** | **<0.0001[P]** | **⇑ 250.44± 18.1** | **⇑ 2.42± 1.03** |
| **Unknown** | **22.2** | **73** | **NA** | **1416** | **NA** | **NA** | **0.0004[P]** | **⇓ 38.38± 10.98** | **⇓ 1.71±0.91** |
| 5-hexyloxolan-2-one | 23.32 | 85 | 1470 | 1460 | 909 | 706-14-9 | >0.05 | ⇑ | ⇑ |
| **6-Pentyloxan-2-one (δ-decalactone)** | **23.97** | **99** | **1496** | **1486** | **863** | **705-86-2** | **0.0006[P]** | **⇓ 39.93±11.98** | **⇓ 1.64±0.9** |

KI: Kovat indices; NA: not available; [P] Alterations remaining significant after Bonferroni correction, with cutoff *p* value of $2.00X10^{-3}$ (0.05 divided by 25 analyzed VOCs); RT: retention time.

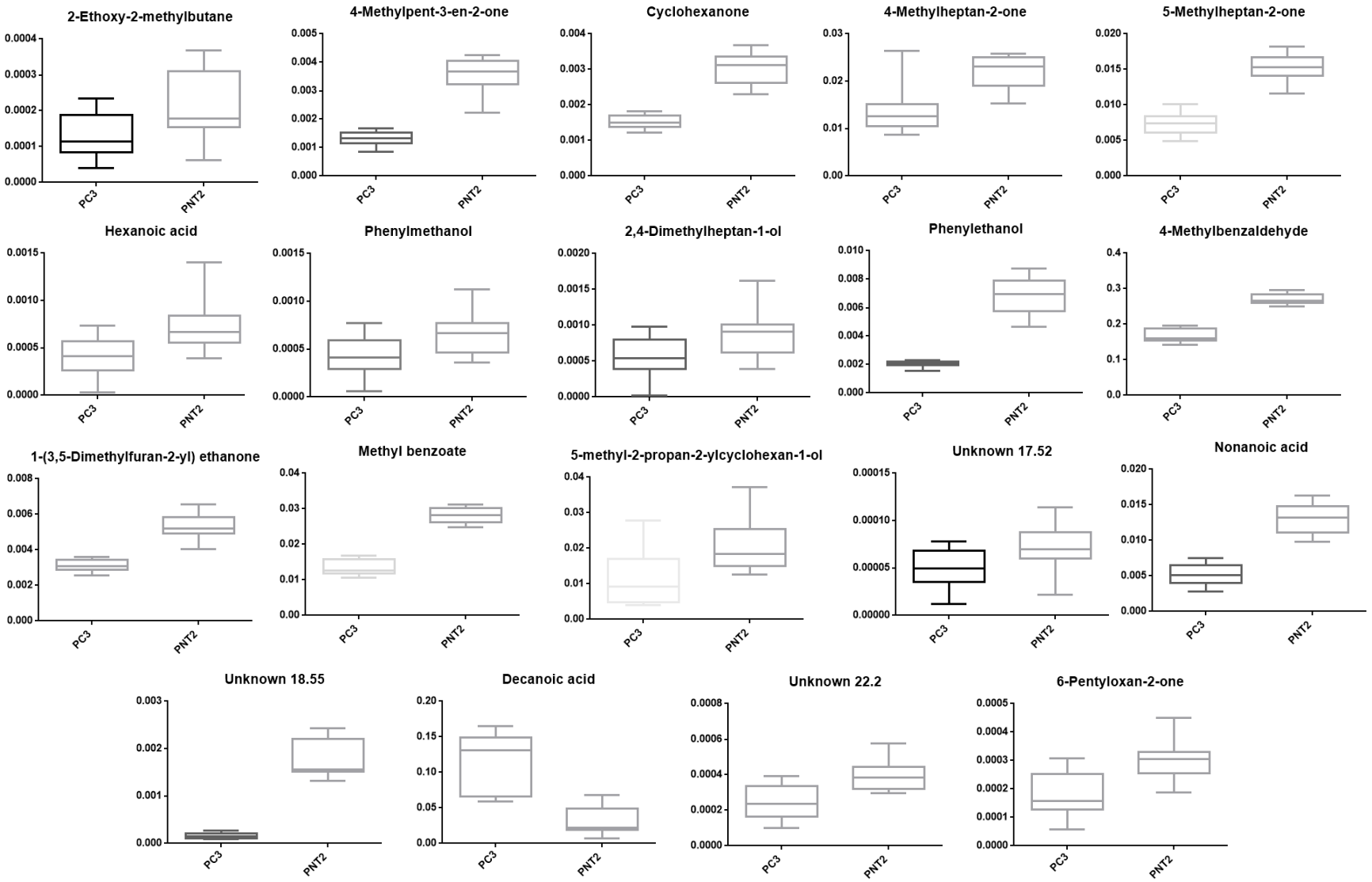


**Figure 14:** Boxplots from the metabolites important for the separation between PC3 (PCa cells) and PNT2 (normal cells), after univariate analysis, at pH 2.

**Table 9:** List of metabolites selected in PLS-DA of DU145 (PCa cells) vs PNT2 (normal cells) (VIP>1) as potentially important for discrimination between PCa and normal cell lines obtained at pH 2.

| Chemical name (IUPAC) or common name | RT | Characteristic ions | KI from literature | Experimental KI | MS-R match | Cas number | *p* value | Variation % (uncertainty) | Effect size (uncertainty) |
|---|---|---|---|---|---|---|---|---|---|
| **1-Ethoxypentane** | **3.63** | **59/70** | **760** | **752** | **786** | **17952-11-3** | **0.0010[p]** | **⇓ 31.16± 9.21** | **⇓1.58±0.89** |
| **4-Methylpent-3-en-2-one** | **4.81** | **55/83/98** | **798** | **798** | **855** | **141-79-7** | **<0.0001[p]** | **⇓ 48.54± 9.03** | **⇓2.8±1.11** |
| **Cyclohexanone** | **7.2** | **98/70** | **894** | **891** | **904** | **108-94-1** | **<0.0001[p]** | **⇓ 82.41± 7.28** | **⇓7.59±2.28** |
| **4-Methylheptan-2-one** | **8.44** | **58/59** | **943** | **934** | **928** | **6137-06-0** | **<0.0001[p]** | **⇓ 36.06±6.93** | **⇓2.51±1.05** |
| **5-Methylheptan-2-one** | **9.35** | **58/71** | **971** | **965** | **714** | **18217-12-4** | **<0.0001[p]** | **⇓ 27.47±5.97** | **⇓2.1±0.97** |
| Hexanoic acid | 10 | 87 | 990 | 987 | 959 | 142-62-1 | >0.05 | ⇓ | ⇓ |
| **2-Methylpentane-1,3-diol** | **10.65** | **57/89** | **1005** | **1009** | **682** | **149-31-5** | **<0.0001[p]** | **⇓ 25.79± 3.95** | **⇓2.95± 1.14** |
| Phenylmethanol | 11.33 | 77 | 1036 | 1031 | 799 | 100-51-6 | >0.05 | ⇑ | ⇑ |
| 2,4-Dimethylheptan-1-ol | 11.91 | 57 | 1030 | 1050 | 731 | 18450-73-2 | >0.05 | ⇓ | ⇓ 82.41± 7.28 |
| **Phenylethanol** | **12.14** | **77/122** | **1055** | **1058** | **847** | **1517-69-7** | **<0.0001[p]** | **⇓ 38.79 ±8.76** | **⇓ 2.16 ±0.99** |
| **4-Methylbenzaldehyde** | **12.82** | **91/119/120** | **1095** | **1080** | **953** | **104-87-0** | **<0.0001[p]** | **⇓ 32.04±2.39** | **⇓ 6.29± 1.94** |
| **1-(3,5-Dimethylfuran-2-yl)ethanone** | **12.99** | **123/138** | **1057** | **1086** | **760** | **22940-86-9** | **<0.0001[p]** | **⇓ 46.45± 6.01** | **⇓ 3.96± 1.36** |
| **Methyl benzoate** | **13.15** | **77/105/136** | **1094** | **1091** | **921** | **93-58-3** | **<0.0001[p]** | **⇓ 51.06± 5.58** | **⇓ 4.84± 1.57** |
| Benzoic acid | 15.33 | 122 | 1170 | 1165 | 837 | 65-85-0 | >0.05 | ⇓ | ⇓ |
| **5-methyl-2-propan-2-ylcyclohexan-1-ol (DL-menthol)** | **15.66** | **71/75/81** | **1175** | **1176** | **914** | **2216-51-5** | **0.0447** | **⇓ 27.4±14.89** | **⇓ 0.84±0.81** |

| Chemical name (IUPAC) or common name | RT | Characteristic ions | KI from literature | Experimental KI | MS-R match | Cas number | *p* value | Variation % (uncertainty) | Effect size (uncertainty) |
|---|---|---|---|---|---|---|---|---|---|
| **Nonanoic acid** | **18.33** | **73/115** | **1273** | **1279** | **909** | **112-05-0** | **<0.0001[P]** | **↓ 31.32± 6.29** | **↓ 2.33± 1.02** |
| 5-Pentyloxolan-2-one | 20.63 | 85 | 1363 | 1355 | 890 | 104-61-0 | >0.05 | ↓13.92±11.08 | ↓ |
| **Decanoic acid** | **20.94** | **60/73/129** | **1373** | **1367** | **931** | **334-48-5** | **<0.0001[P]** | **↑268.37± 8.66** | **↑5.21±1.67** |
| Unknown | 22.2 | 73 | NA | 1416 | NA | NA | >0.05 | ↓ | ↓ |
| 5-hexyloxolan-2-one (γ - Decalactone) | 23.32 | 85 | 1470 | 1460 | 909 | 706-14-9 | >0.05 | ↑ | ↑ |
| 6-Pentyloxan-2-one (δ-decalactone) | 23.97 | 99 | 1496 | 1486 | 863 | 705-86-2 | >0.05 | ↓ | ↓ |

KI: Kovat indices; NA: not available; [P] Alterations remaining significant after Bonferroni correction, with cut off *p* value of $2.38 \times 10^{-3}$ (0.05 divided by 21 analyzed VOCs); RT: retention time.

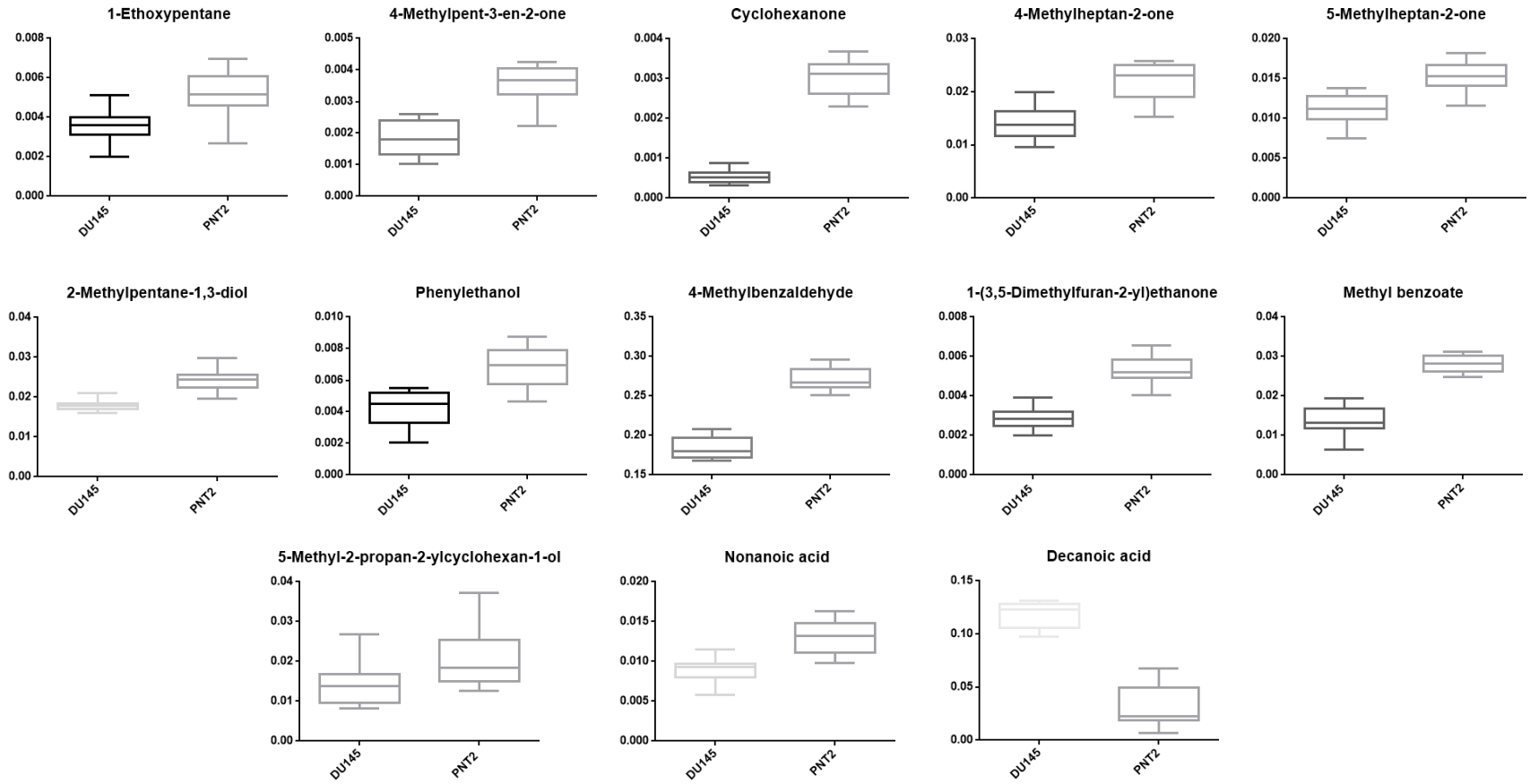


**Figure 15:** Boxplots from the metabolites important for the separation between DU145 (PCa cells) and PNT2 (normal cells), after univariate analysis, obtained at pH 2.

**Table 10:** List of metabolites selected in PLS-DA of LNCaP (PCa cells) vs PNT2 (normal cells) (VIP>1) as potentially important for discrimination between PCa and normal cell lines obtained at pH 2.

| Chemical name (IUPAC) or common name | RT | Characteristic ions | KI from literature | Experimental KI | MS-R match | Cas number | *p* value | Variation % (uncertainty) | Effect size (uncertainty) |
|---|---|---|---|---|---|---|---|---|---|
| 2-Ethoxy-2-methylbutane | 2.49 | 59 | 728 | 707 | 683 | 919-94-8 | >0.05 | ↓ | ↓ |
| **1-Ethoxypentane** | **3.63** | **59/70** | **760** | **752** | **786** | **17952-11-3** | **0.0001[p]** | **↓ 35.34± 8.69** | **↓ 1.95± 0.95** |
| 4-Methylpentan-2-one | 3.71 | 58 | 735 | 755 | 907 | 108-10-1 | >0.05 | ↑ | ↑ |
| **Cyclohexanone** | **7.2** | **98/70** | **894** | **891** | **904** | **108-94-1** | **<0.0001[p]** | **↓ 80.15± 7.33** | **↓ 7.19±2.18** |
| **4-Methylheptan-2-one** | **8.44** | **58/59** | **943** | **934** | **928** | **6137-06-0** | **0.0014[p]** | **↓ 26.3±7.86** | **↓ 1.52±0.88** |
| **5-Methylheptan-2-one** | **9.35** | **58/71** | **971** | **965** | **714** | **18217-12-4** | **0.0014[p]** | **↓ 20.36±5.95** | **↓ 1.5±0.88** |
| Hexanoic acid | 10 | 87 | 990 | 987 | 959 | 142-62-1 | >0.05 | ↑ | ↑ |
| **2-Methylpentane-1,3-diol** | **10.65** | **57/89** | **1005** | **1009** | **682** | **149-31-5** | **<0.0001[p]** | **↓ 24.26±5.53** | **↓ 1.97± 0.95** |
| Phenylmethanol | 11.33 | 77 | 1036 | 1031 | 799 | 100-51-6 | >0.05 | ↑ | ↑ |
| 2,4-Dimethylheptan-1-ol | 11.91 | 57 | 1030 | 1050 | 731 | 18450-73-2 | >0.05 | ↑ | ↑ |
| **4-Methylbenzaldehyde** | **12.82** | **91/119/120** | **1095** | **1080** | **953** | **104-87-0** | **0.0005[p]** | **↓ 18.39±4.31** | **↓ 1.85± 0.93** |
| **1-(3,5-Dimethylfuran-2-yl) ethanone** | **12.99** | **123/138** | **1057** | **1086** | **760** | **22940-86-9** | **0.0003[p]** | **↓ 21.78± 5.5** | **↓ 1.75± 0.91** |
| **Methyl benzoate** | **13.15** | **77/105/136** | **1094** | **1091** | **921** | **93-58-3** | **<0.0001[p]** | **↓ 22.45± 4.27** | **↓ 2.33± 1.01** |
| **Benzyl acetate** | **15.15** | **90/135** | **1164** | **1159** | **770** | **140-11-4** | **0.0090** | **↑ 23.73±7.0** | **↑ 1.19±0.84** |
| Benzoic acid | 15.33 | 122 | 1170 | 1165 | 837 | 65-85-0 | >0.05 | ↑ | ↑ |
| **5-methyl-2-propan-2-ylcyclohexan-1-ol (DL-menthol)** | **15.66** | **71/75/81** | **1175** | **1176** | **914** | **2216-51-5** | **0.0019[p]** | **↓ 42.1±13.39** | **↓ 1.57±0.89** |

| Chemical name (IUPAC) or common name | RT | Characteristic ions | KI from literature | Experimental KI | MS-R match | Cas number | *p* value | Variation % (uncertainty) | Effect size (uncertainty) |
|---|---|---|---|---|---|---|---|---|---|
| Ethyl 2-phenylacetate | 17.44 | 164 | 1246 | 1238 | 918 | 101-97-3 | >0.05 | ↑ | ↑ |
| **Nonanoic acid** | **18.33** | **73/115** | **1273** | **1279** | **909** | **112-05-0** | **<0.0001[P]** | **↓ 66.28± 7.67** | **↓ 5.09± 1.64** |
| **4-Methylnonanoic acid** | **19.5** | **60/113** | **1308** | **1313** | **798** | **45019-28** | **<0.0001[P]** | **↑ 3242.3±10.9** | **↑ 65.79± 2.07** |
| 5-Pentyloxolan-2-one | 20.63 | 85 | 1363 | 1355 | 890 | 104-61-0 | >0.05 | ↓ | ↓ |
| **Decanoic acid** | **20.94** | **60/73/129** | **1373** | **1367** | **931** | **334-48-5** | **0.0122** | **↑ 74.5± 19.0** | **↑ 1.13± 0.83** |
| Unknown | 22.2 | 73 | NA | 1416 | NA | NA | >0.05 | ↑ | ↑ |
| 5-hexyloxolan-2-one | 23.32 | 85 | 1470 | 1460 | 909 | 706-14-9 | >0.05 | ↑ | ↑ |
| 6-Pentyloxan-2-one | 23.97 | 99 | 1496 | 1486 | 863 | 705-86-2 | >0.05 | ↑ | ↑ |

KI: Kovat indices; NA: not available; [P] Alterations remaining significant after Bonferroni correction, with cut-off *p* value of $2.08 \times 10^{-3}$ (0.05 divided by 24 analyzed VOCs) RT: retention time.

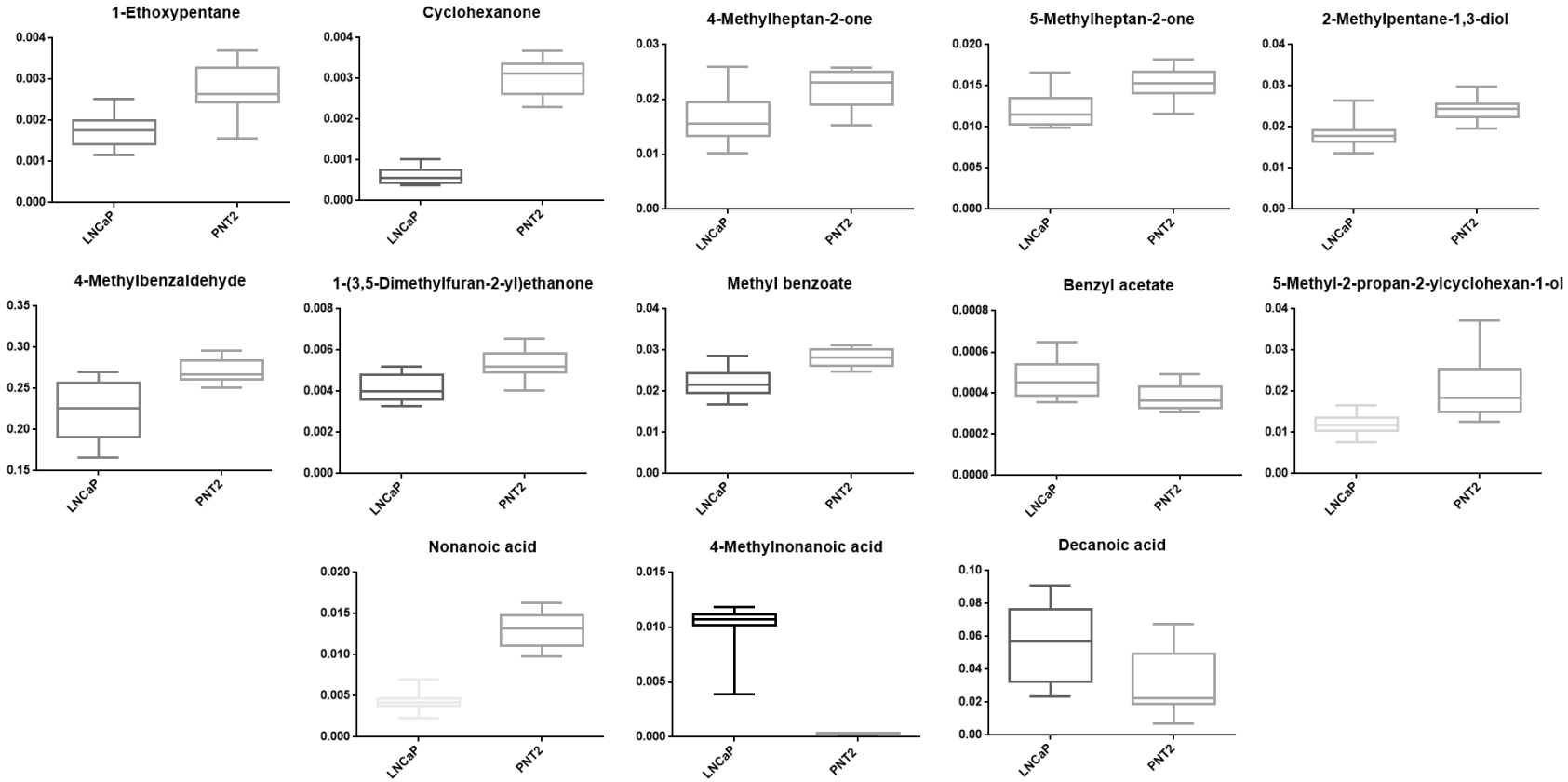


**Figure 16:** Boxplots from the metabolites important for the separation between LNCaP ( PCa cells) and PNT2 (normal cells), after univariate analysis, obtained at pH 2.

New PLS-DA models using just these set of discriminant metabolites (Figure 17) were performed. To prove the robustness of the models a MCCV validation was performed for all comparisons, using GC-MS full data and also using discriminant VOCs obtained at pH 2 described before. Results of these validations prove that all created models are robust for the discrimination between PCa cell lines and normal cell line (Table 11).

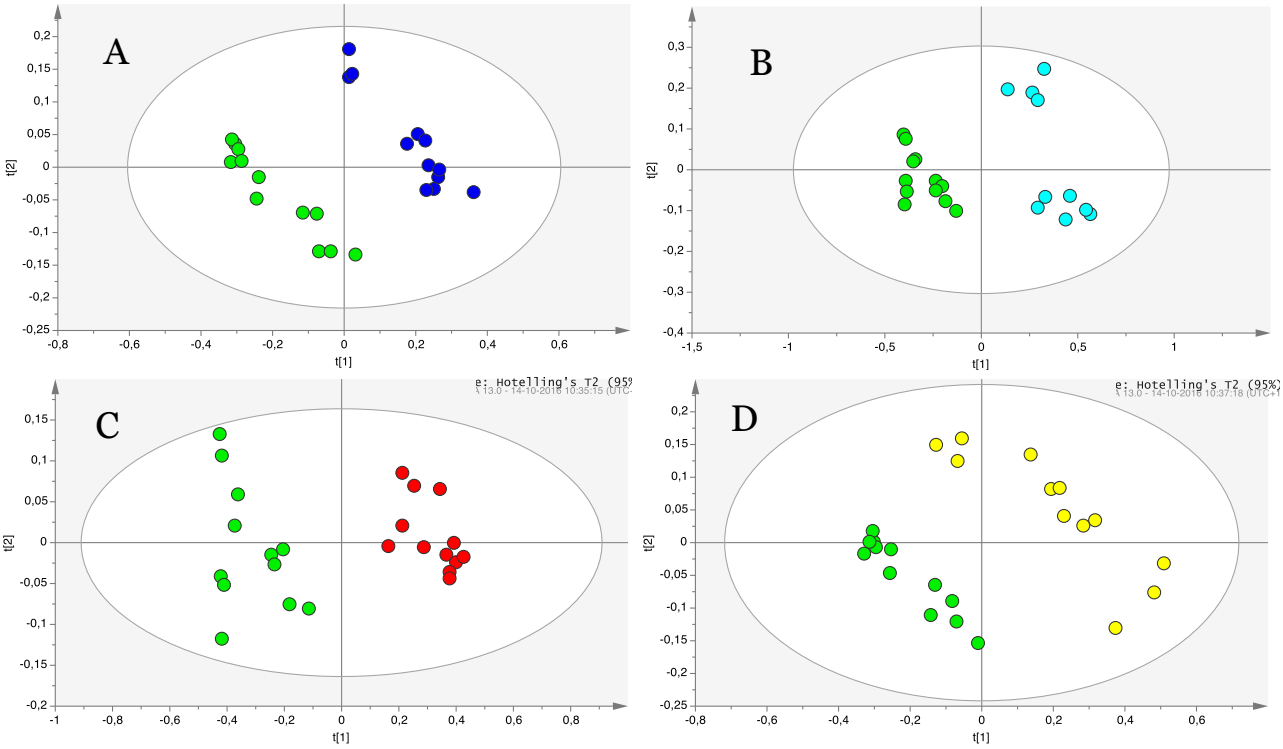


**Figure 17:** PLS-DA from PCa cell line vs normal cell line with the set of discriminant VOCs obtained at pH 2. **A.** 22RV1 (PCa cells) (dark blue) vs PNT2 (normal cells)(green) ($R^2X$=0.824; $R^2Y$=0.977; $Q^2$=0.945). **B.** PC3 (PCa cells) (light blue) vs PNT2 (normal cells) (green) ($R^2X$=0.924; $R^2Y$=0.945; $Q^2$=0.919). **C.** DU145 (PCa cells) (red) vs PNT2 (normal cells) (green) ($R^2X$=0.927; $R^2Y$=0.933; $Q^2$=0.9). **D.** LNCaP (PCa cells) (yellow) vs PNT2 (normal cells) (green) ($R^2X$=0.868; $R^2Y$=0.945; $Q^2$=0.926).

**Table 11**: MCCV parameters of true and permuted classes obtained for pH 2 when considering GC-MS full data and the set of discriminant VOCs.

| Models | True classes | | | | | Permuted classes | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | LV | $Q^2$ | CR (%) | Sens. (%) | Spec. (%) | LV | $Q^2$ | CR (%) | Sens. (%) | Spec. (%) |
| ***GC-MS full data*** | | | | | | | | | | |
| 22RV1 vs PNT2 | 1 | 0.99 | 100 | 100 | 100 | 1 | -0.34 | 47 | 47 | 47 |
| PC3 vs PNT2 | 1 | 0.98 | 100 | 100 | 100 | 1 | -0.31 | 49 | 48 | 49 |
| DU145 vs PNT2 | 1 | 0.98 | 100 | 100 | 100 | 1 | -0.34 | 49 | 49 | 49 |
| LNCaP vs PNT2 | 1 | 0.98 | 100 | 100 | 100 | 1 | -0.39 | 47 | 48 | 46 |
| ***Set of discriminant VOCs*** | | | | | | | | | | |
| 22RV1 vs PNT2 | 1 | 0.97 | 100 | 100 | 100 | 1 | -0.21 | 48 | 49 | 47 |
| PC3 vs PNT2 | 1 | 0.96 | 100 | 100 | 100 | 1 | -0.27 | 46 | 47 | 45 |
| DU145 vs PNT2 | 1 | 0.93 | 100 | 100 | 100 | 1 | -0.24 | 47 | 47 | 46 |
| LNCaP vs PNT2 | 2 | 0.94 | 100 | 100 | 100 | 1 | -0.20 | 46 | 46 | 47 |

LV – no. of latent variables, $Q^2$ – median predictive power, CR – classification rate, sens. – sensitivity, spec. – specificity.

## Discussion

*Comparative analysis of VOCs in PCa and normal cell lines obtained at pH 7*

In this work we showed the potentiality of VOCs at physiological pH to discriminate different PCa cell lines from normal prostate cell line, indicating that the volatilome may be a valuable source of biomarkers for PCa detection. The results revealed significantly altered VOCs in all PCa cell lines when compared with normal cell line, namely 1,3-benzothiazole (decreased in the extracellular medium of all PCa cell lines except DU145) and pentadecan-2-one (increased in all PCa cell lines). However, considering the different VOCs it is possible to discriminate PCa with different grades of aggressiveness, for example, 2,7-dimethyloctan-1-ol and 2-(1-4-methylcyclohex-3-en-1-yl)propan-2-ol, are significantly altered specifically in high metastatic potential cell line (PC3); the significant increase of decan-1-ol levels is a characteristic alteration of cell line with moderated metastatic potential (DU145); and the significant alteration of 3-methylbut-3-en-2-ol**,** 1-methoxypropan-2-yl acetate and 1,4-xylene levels is characteristic of cell line with low

metastatic potential (LNCaP). It is also possible to discriminate between androgen-responsive cell lines from androgen-nonresponsive cell lines using 3,7-Dimethyloct-7-en-1-ol, since this metabolite is just significantly decreased in the androgen-nonresponsive cell lines (PC3, DU145 and 22RV1). Theoretically 22RV1 is an androgen receptor positive cell line, however, since the culture medium was not supplement with androgen we theorize that this cell line develop androgen independency for a mechanism similar to what happens in clinical practice after hormone deprivation therapy. This phenome was already addressed by other work groups (52).

The integration of VOCs in metabolic pathways is, at the moment hard to performed, because volatilomic is a recent study approach, however using the human metabolome data base (HMDB), a free use date base that contains 41,993 metabolite entries, it is possible to see if some of those metabolites found in our samples were already found by other researchers in biological matrices. Table 12 does an interconnection between our results (significantly altered identified metabolites) and this date base (53-55).

**Table 12:** Significantly altered metabolites important for the separation between PCa and normal cell lines, after univariate analysis, obtained at pH 7.

| Chemical name | HMDB number | Matrices previously found | Cellular locations |
|---|---|---|---|
| **3-Methylbut-3-en-2-ol** | HMDB39779 | NA | Cytoplasm Extracellular |
| **1-Methoxypropan-2-yl acetate** | NA | NA | NA |
| **1,4-Xylene** | HMDB59924 | Feces; Saliva | Membrane |
| **2,7-Dimethyloctan-1-ol** | NA | NA | NA |
| **2-(1-4-methylcyclohex-3-en-1-yl)propan-2-ol** | HMDB37171 | NA | Extracellular Membrane |
| **Naphthalene** | HMDB29751 | Feces; Salive | Membrane |
| **3,7-Dimethyloct-7-en-1-ol** | HMDB37171 | NA | Extracellular Membrane |
| **Decan-1-ol** | HMDB11624 | Feces | Extracellular Membrane |
| **1,3-Benzothiazole** | HMDB32930 | NA | Cytoplasm Extracellular |
| **2-Methylundecanal** | HMDB31734 | NA | Membrane |
| **Pentadecan-2-one** | HMDB31081 | Saliva | Membrane |

NA: not available; HMDB: Human Metabolome Database.

From all significantly altered metabolites pentadecan-2-one is the VOCs that changes more and is specific for the cells (does not exist in the cellular medium). Pentadecan-2-one is a ketone, and the increase of ketones levels was previously reported for PCa and other cancers (7, 37, 44, 56, 57). The production of pentadecan-2-one may be explained by the increased fatty acid oxidation and increased protein metabolism, characteristic of cancer which leads to ketones production (58).

The alteration in aldehydes (e.g. 2-methylundecanal) is also a common alteration related with cancer, including PCa (7, 37, 44, 59). The alteration in aldehyde profiling can be explained by an alteration in lipid peroxidation, which is induced by the increased of ROS levels characteristic of cancer cell metabolism and inflammation. Beyond lipid peroxidation, aldehydes can also result from amino acid and carbohydrate catabolism. Other possible explanation for the aldehydes levels variation can be related to the aldehyde dehydrogenase (responsible for the aldehydes oxidation to carboxylic acids) activity (increased activity of these enzyme leads to reduction in aldehydes levels and down-regulation of these enzyme leads to increase in aldehydes levels) (7, 58-60).

The hydrocarbon metabolism is well described in cancer metabolism. In our study, several alcohols were altered in PCa cell lines, namely, 3-methylbut-3-en-2-ol 3,7-Dimethyloct-7-en-1-ol, 2,7-dimethyloctan-1-ol, 2-(1-4-methylcyclohex-3-en-1-yl)propan-2-ol, and decan-1-ol, this alteration may be due to the alteration in hydrocarbon metabolism once alcohols are end-products of this metabolism (61, 62). Other well described characteristic of cancer cells is their rapid growth; this rapid growth implies an increase of cellular membrane synthesis. The alcohols may be metabolized, by the cell to carboxylic acids and these used to the synthesis of cellular membrane precursors (63).

We also observed an alteration in aromatic hydrocarbon (1,4-xylene) the presence of these molecules was previously related with the presence of ROS (58, 64). Naphthalene, a cycloalkane, was significantly altered, the presence of these molecules is also related with the presence of ROS and oxidative stress (61). Significant alteration in 1,4-xylene levels was previously detected in exhaled breath of PCa patients (65).

Our results were concordant with the results of previously volatiloma analysis of PCa urine samples since some compounds significantly altered in this study belong to the same classes (ketones, alcohols, aldehydes) of compounds significantly altered in previously studies, however since, analytical conditions were different (e. g. different equipment, fiber, column and temperature gradient programme) the direct comparison of the results is not possible (7, 66).

*Comparative analysis of VOCs in PCa and normal cell lines obtained at pH 2*

Like VOCs analyzed at physiological pH, the volatilome obtained at pH 2 is also able to differentiate PCa cell lines from normal prostate cell line, taking into account the quality of the two models ($R^2X$ = 0.526; $R^2Y$ = 0.457; $Q^2$ = 0.437 for pH 2 vs $R^2X$ = 0.445; $R^2Y$ = 0.463; $Q^2$ = 0.446 for pH 7) the discriminant capability of these two models is similar. However some of the discriminate metabolites were not the same in the two models, so the acidification of the samples markedly influences the detected volatilome (e. g. detection of organic acids).

VOCs namely cyclohexanone, 4-methylheptan-2-one, 2-methylpentane-1,3-diol, 4-methylbenzaldehyde, 1-(3,5-dimethylfuran-2-yl)ethanone, methyl benzoate, nonanoic acid and decanoic acid are significantly altered in all PCa cell lines when compared with normal cell line. These VOCs are significantly decreased in extracellular medium of all PCa cell lines except decanoic acid that is increased in all PCa cell lines. Also, some VOCs namely, 2-ethoxy-2-methylbutane, hexanoic acid, phenylmethanol, 2,4-Dimethylheptan-1-ol, benzoic acid, and 6-pentyloxan-2-one, can discriminate PCa with different aggressiveness, for example, 2-ethoxy-2-methylbutane, hexanoic acid, phenylmethanol, 2,4-Dimethylheptan-1-ol, benzoic acid, and 6-pentyloxan-2-one, are significantly decreased specifically in high metastatic potential cell line (PC3) and, 1-ethoxypentane, is significantly decreased specifically in moderated and low metastatic potential cell line (DU145 and LNCaP, respectively). 4-Methylpent-3-en-2-one and Phenylethanol can discriminate androgen-responsive cell lines from androgen-nonresponsive cell lines, these metabolites are just significantly decreased in the androgen-nonresponsive cell lines (PC3, DU145 and 22RV1).

Some of the VOCs significantly altered in the comparison PCa vs normal prostate cell lines are produced by cells (do not exist in the cellular medium) namely**,** 4-methylnonanoic acid, hexanoic acid and 6-pentyloxan-2-one.

The majority of the VOCs significantly altered were decreased in cancer cell lines in comparison with normal cell line this observation was previously made in other metabolomics studies with urine from PCa patients (7, 67). The cancer cells may use these metabolites for their metabolic processes more extensively then normal prostate cells, converting these VOCs in other metabolites than cannot be analyzed by our methodology (7, 67).

As performed for pH 7, Table 13, make an interconnection between our results at pH 2 (significantly altered identified metabolites) and HMDB. As for pH 7 it is possible to see if some of those metabolites found in our samples at pH 2 were already found by other researchers in biological matrices (53-55).

**Table 13:** Significantly altered metabolites important for the separation between PCa and normal cell lines, after univariate analysis, obtained at pH 2.

| Chemical name | HMDB number | Matrices previously found | Cellular locations |
|---|---|---|---|
| **2-Ethoxy-2-methylbutane** | NA | NA | NA |
| **1-Ethoxypentane** | NA | NA | NA |
| **4-Methylpent-3-en-2-one** | HMDB31563 | Feces<br>Saliva | Cytoplasm<br>Extracellular |
| **Cyclohexanone** | HMDB03315 | Feces | NA |
| **4-Methylheptan-2-one** | NA | NA | NA |
| **5-Methylheptan-2-one** | NA | NA | NA |
| **Hexanoic acid** | HMDB00535 | Blood<br>Cerebrospinal Fluid<br>Feces<br>Saliva<br>Urine | Cytoplasm<br>Extracellular<br>Membrane |
| **2-Methylpentane-1,3-diol** | NA | NA | NA |
| **2,4-Dimethylheptan-1-ol** | NA | NA | NA |
| **Phenylethanol** | HMDB32619 | Feces<br>Saliva | Cytoplasm<br>Extracellular |
| **4-Methylbenzaldehyde** | HMDB29638 | Feces<br>Saliva | Cytoplasm<br>Extracellular |
| **1-(3,5-Dimethylfuran-2-yl)ethanone** | HMDB32159 | NA | Cytoplasm<br>Extracellular |
| **Methyl benzoate** | HMDB33968 | NA | Cytoplasm<br>Extracellular |
| **Benzyl acetate** | HMDB31310 | Saliva | Cytoplasm<br>Extracellular |

| Chemical name | HMDB number | Matrices previously found | Cellular locations |
|---|---|---|---|
| **Benzoic acid** | HMDB01870 | Blood<br>Feces<br>Saliva<br>Urine | Cytoplasm<br>Extracellular<br>Endoplasmic reticulum |
| **5-methyl-2-propan-2-ylcyclohexan-1-ol** | HMDB03352 | Blood<br>Feces<br>Saliva<br>Urine | Extracellular<br>Membrane |
| **Methyl nonanoate** | HMDB31264 | NA | Extracellular<br>Membrane |
| **Nonanoic acid** | HMDB00847 | Feces<br>Saliva | Extracellular<br>Membrane |
| **4-Methylnonanoic acid** | HMDB34849 | NA | Extracellular<br>Membrane |
| **Decanoic acid** | HMDB00511 | Blood<br>Breast Milk<br>Saliva<br>Urine | Extracellular<br>Membrane |
| **6-Pentyloxan-2-one** | HMDB37116 | NA | Membrane |

NA: not available; HMDB: Human Metabolome Database

Some VOCs previously associated with cancer in other metabolomics studies namely, phenylmethanol, nonanoic acid, decanoic acid were also significantly altered in this work (68, 69).

Several VOCs significantly altered in pH 2 belong to the same class of the VOCs significantly altered in pH 7, namely ketones (cyclohexanone; 4-methylheptan-2-one; 5-methylheptan-2-one; 1-(3,5-dimethylfuran-2-yl)ethanone; 6-pentyloxan-2-one; and 4-methylpent-3-en-2-one), aldehydes (4-methylbenzaldehyde) and alcohols (2-methylpentane-1,3-diol; phenylethanol; 5-methyl-2-propan-2-ylcyclohexan-1-ol; phenylmethanol; 2,4-dimethylheptan-1-ol).

Significantly alteration in some ethers (1-ethoxypentane and 2-ethoxy-2-methylbutane) and organic acids (methyl benzoate; benzyl acetate; nonanoic acid; 4-methylnonanoic acid; decanoic acid; hexanoic acid) were also observed. Ethers can be found naturally in

fats and may be in the origin of acids and alcohols during lipid hydrolysis, which may explain the reduction of these VOCs levels, observed in PCa cell lines (62).

Alteration in organic acids was previously described in urine and serum of PCa patients (67, 69). Organic acids can be involved in several biological processes, including cell signaling, energy storage, energy source, and cellular membrane integrity, so the alteration in these VOCs indicated that cancer cells have alteration in energy and lipid metabolism (67).

# **Chapter 4:** Analysis of Amino Acids, Fatty Acids, Steroids and Sugars in PCa and Normal Prostate Cell Lines

## Materials and Methods

### Chemicals

All chemicals and reagents were analytical grade.

RPMI-1640 medium, phosphate buffered saline 1% (PBS), norvaline, methyl linolelaidate, desmosterol and N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) were purchased from Sigma-Aldrich Co (St. Louis, MO, USA). The antibiotic mixture penicillin/streptomycin (10000 U/mL/10000 µg/mL), heat inactivated fetal bovine serum (FBS) and trypsin 0.25%-EDTA were purchased from GIBCO Invitrogen (Barcelona, Spain). Sodium hydrogencarbonate was obtained from Merck (Darmstadt, Germany) and methanol was from VWR (Leuven, Belgium).

### Cell Culture

PCa immortalized cell lines (PC3, 22RV1, DU145 and LNCaP) and normal prostate epithelium immortalized cell line (PNT2) were provided by Portuguese Oncology Institute-Porto (see Table 1, Chapter 3) for detail information about cell lines). All cell lines were grown in RPMI-1640 medium supplemented with 10% of FBS and 1% of penicillin-streptomycin. All cell lines were maintained at 37°C under 5% $CO_2$. Cells grow to 80% confluency, before passage.

*Samples collection*

All cell lines were plated and grown to 100% confluency in RPMI-1640 medium. Forty eight hours after cells reach 100% confluency the medium was collected for VOCs analysis (see Chapter 3) and cells were washed with PBS solution. The PBS is rejected and the cells were scrapped with ice cold methanol and transferred to falcons in ice and centrifuged at 3000 x g for 10 minutes at 4 ºC. After this, the methanol was separated from the pellet and immediately frozen at -80 ºC until analysis. The same procedure, for all cell lines, was used for the four consecutive passages, (passage 3 to 6) each passage was performed in triplicate, resulting in a total of 60 experiments.

*Metabolites extraction from cell lines*

AA, fatty acids, sugars and steroids may not be analyzed directly by gas chromatography because of their low volatility and polarity and need to be previously derivatized. The method used was previously developed by Pereira et al. (2012), with some adaptations.

Briefly, the samples were centrifuged (3000 x g for 10 minutes at 4 ºC) (to ensure the total exclusion of any remains pellet) transferred to a glass vial and the internal standards (10µL/mL) norvaline, methyl linolelaidate and desmosterol were added. One mL of each sample was evaporated under a nitrogen stream and 50 µL of dichloromethane plus 50 µL of the derivatization reagent, MSTFA, was added to the residue. The vial was vortexed and heated for 30 min at 80 °C. 50 µL of remain residue was transfer to the glass vial used for GC–MS analysis (70). All extractions and analysis were performed in triplicate.

**GC–MS system and data acquisition**

*GC–MS analysis*

The GC–MS conditions were based on those previously optimized by Pereira et al. (2012) with some adaptations. An EVOQ-436 gas chromatograph equipped with a Bruker Triple Quadrupole mass detector and a Bruker MS workstation software version 8.2 were used. The chromatographic separation was accomplished using a column Rxi-5Sil MS (30 m x 0.25 mm x 0.25 µm) column (Restek). A CombiPAL automatic autosampler (Bruker) was used for all experiments. One µL of sample was injected using split mode (ratio 1/10), the carrier gas used was helium C-60 (Gasin, Portugal) (flow of 1 ml/min) and the injector port was heated to 250 °C. The analysis was performed in Full Scan mode. The oven temperature was fixed at 70 °C for 2 min, increasing to 250 °C (rate 15 °C/min), held for 2 min, finally increasing to 300 °C (rate 10 °C/min) and held for 8 min. The transfer line temperature was 250 °C and manifold temperature was 40 °C. The mass ranged from 50 to 1000 m/z. The emission current was 50 µA and the electron multiplier was set in relative mode to an auto tune procedure. All mass spectra were acquired in the electron impact (EI) mode (70). To ensure reproducibility of the methodology, quality control samples (QCs) were injected (48), four times *per* day. To produce these QCs a pool of all samples (from the five cell lines) were made, then this pool was immediately frozen at -80 ºC until analysis.

*Statistical analysis*

The statistical approach used was the same used for VOCs profiling (see Chapter 3).

Prior to statistical analysis all chromatograms were pre-processed: baseline correction, peak detection, chromatogram deconvolution, and alignment, for these corrections the program MZmine was used (49), the parameters used to accomplish these were: RT

range 3.60-24.0 min; m/z range 50-500; MS data noise level $1.0\times10^4$; m/z tolerance 0.3; chromatogram baseline level $2.0\times10^4$; peak duration range 0.02-0.30 min. All ions (m/z) with a RSD greater than 50% (48), as well as ions (m/z) coming from the column, were removed. The obtained data was next normalized for the total area of the chromatograms. The statistical treatment included an unsupervised (PCA) and a supervised analysis (PLS-DA) to determine which compounds were significantly different between PCa cell lines and normal cell line. To confirm the results, univariate analysis was performed for all metabolites relevant for the separation among cell lines, by calculating the *p* value, percentage of variation and uncertainty of the variation quotient, as well as, the effect size and its uncertainty (50).

Bonferroni correction was used to adjust p-values for multiple comparisons by setting the significance cutoff to *p* value (0.05) divided by the number of compounds simultaneously tested in univariate statistical analyses.

To confirm the robustness of the models, a MCCV was used (51). The identification of compounds selected by statistical approaches was done by using the National Institute of Standards and Technology (NIST 14) data base spectra library kovat indices and also by using standards.

## Results

After GC-MS analysis, the obtained data were used to perform multivariate analyses, namely PCA and PLS-DA. A total of 150 features were detected in the chromatograms.

The reproducibility of the analytical method is confirmed by the QCs projection on axis 1 and 2 (Figure 18) (all QCs samples are grouped (orange color). Furthermore, the multivariate analyses prove that VOCs are able to discriminate PCa cell lines from normal prostate cell line and between the different PCa cell lines. This discriminant capability is observed not only in PLS-DA analysis but also in PCA (Figure 19).

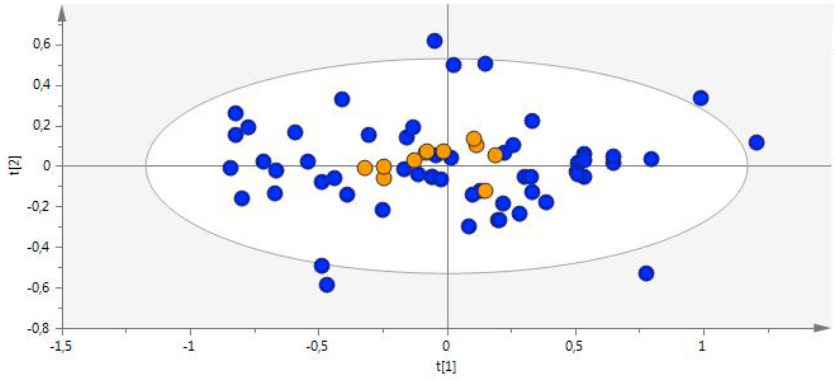


**Figure 18:** PCA with QCs samples (orange) and all cell lines samples and controls (blue) ($R^2X$ = 0.652).

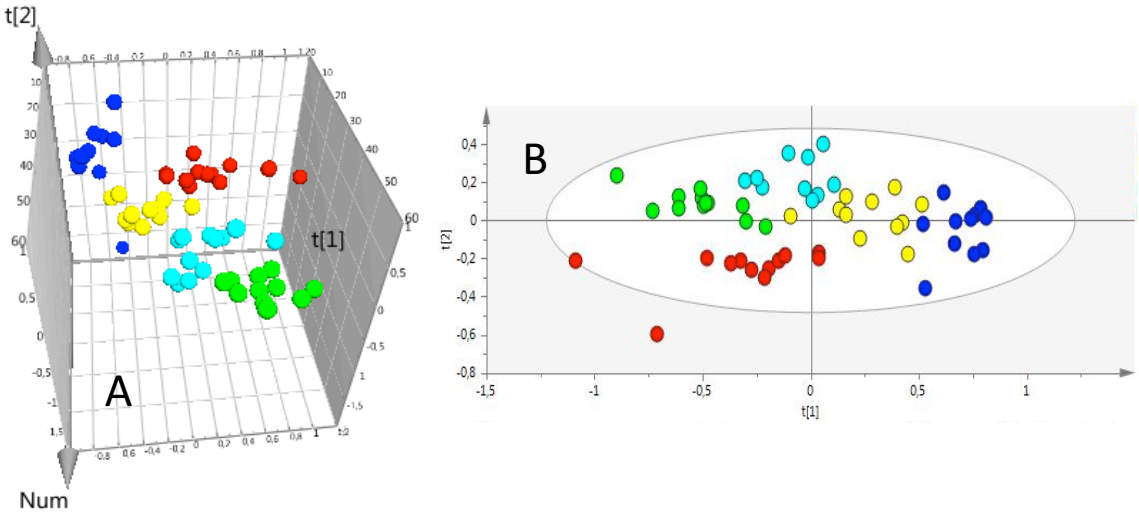


**Figure 19:** PCA (A) ($R^2X$ = 0.314) and PLS-DA (B) ($R^2X$ = 0.618; $R^2Y$ = 0.386; $Q^2$ = 0.35) with metabolites from all cells line (PNT2 (normal cells): dark blue: green; 22RV1 (PCa cells): dark blue; PC3 (PCa cells): light blue; DU145 (PCa cells): red; LNCaP(PCa cells): yellow;).

To evaluate which metabolites were responsible for this separation, all cancer cell lines were compared with the normal cell line, namely 22RV1 vs PNT2, PC3 vs PNT2, DU145 vs PNT2 and LNCaP vs PNT2. An optimal separation between each PCa cell lines and normal cell line was observed (Figure 20).

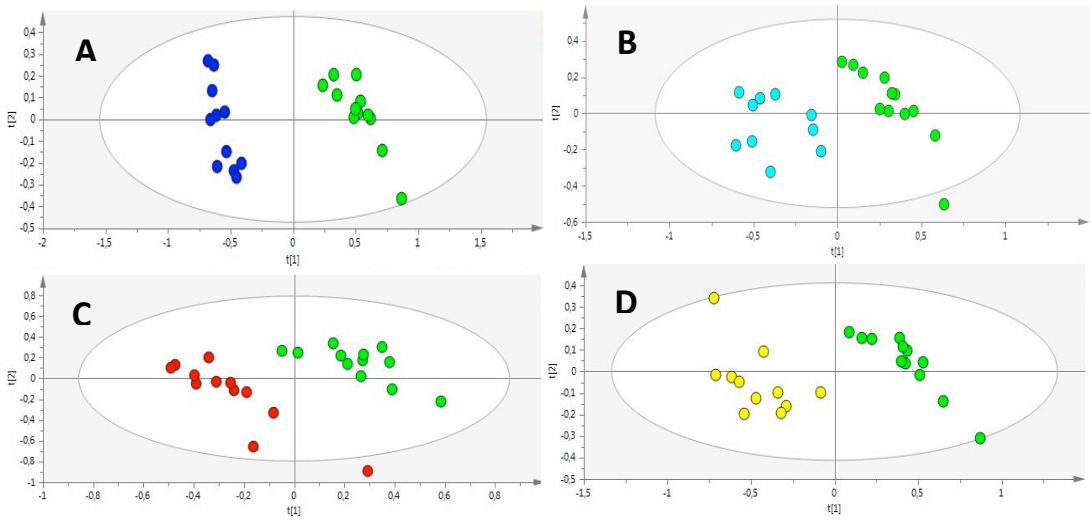


**Figura 20:** PLS-DA from PCa vs PNT2. **A.** 22RV1 (PCa cells) (dark blue) vs PNT2 (normal cells) (green) ($R^2X$=0.724; $R^2Y$=0.978; $Q^2$=0.964). **B.** PC3 (PCa cells) (light blue) vs PNT2 (normal cells) (green) ($R^2X$=0.709; $R^2Y$=0.887; $Q^2$=0.817). **C.** DU145 (PCa cells) (red) vs PNT2 (normal cells) (green) ($R^2X$=0.722; $R^2Y$=0.916; $Q^2$=0.879). **D.** LNCaP (PCa cells) (yellow) vs PNT2 (normal cells) (green) ($R^2X$=0.734; $R^2Y$=0.932; $Q^2$=0.894).

All metabolites with VIP values greater than one were considered potential relevant for the separation among cell lines. Hence, a total of 30 metabolites were considered relevant to differentiate 22RV1 from PNT2, 37 metabolites were considered relevant to differentiate PC3 from PNT2, 46 metabolites were considered relevant to differentiate DU145 from PNT2 and 38 were considered relevant to differentiate LNCaP from PNT2. To confirm the importance of these metabolites, univariate analysis was performed to calculate the *p* value, the variation quotient and the effect size. After univariate analysis the VOCs that were taken into account, were those with $p<0.05$; relevant value of variation quotient; and relevant effect size. Therefore, a total of 22 VOCs proved to be relevant to differentiate 22RV1 from PNT2, 20 VOCs proved to be relevant to differentiate PC3 from PNT2, 24 VOCs proved to be relevant to differentiate DU145 from PNT2 and 26 proved to be relevant to differentiate LNCaP from PNT2. All these results are shown detail in Table 14 (22RV1 vs PNT2), Table 15 (PC3 vs PNT2), Table 16 (DU145 vs PNT2) and Table 17 (LNCaP vs PNT2).

After univariate analysis, metabolites with $p<0.05$ and relevant variations quotient and effect size values were considered important for the separation among cell lines and potential biomarkers for PCa. These metabolites include amino acids, fatty acids, steroids and sugars. From these metabolites eight stand out, namely, ethanolamine, lactic acid, L-

valine, L-leucine, L-threonine, lyxofuranose, and L-tyrosine, because they revealed to be important for the separation among all PCa cell lines and normal cell line. However, several other metabolites were identified and were also able to discriminate between different cancer cell lines and normal cell lines, namely, L-alanine, (PC3 vs PNT2; DU145 vs PNT2; LNCaP vs PNT2), 3-hydroxyisovaleric acid (PC3 vs PNT2; DU145 vs PNT2; LNCaP vs PNT2), urea, (22RV1 vs PNT2; PC3 vs PNT2; DU145 vs PNT2), phenylalanine, (22RV1 vs PNT2; PC3 vs PNT2; LNCaP vs PNT2), ribofuranose, (22RV1 vs PNT2; PC3 vs PNT2; LNCaP vs PNT2) talofuranose, (22RV1 vs PNT2; DU145 vs PNT2; LNCaP vs PNT2), sorbose, (22RV1 vs PNT2; DU145 vs PNT2; LNCaP vs PNT2), cholesterol, (22RV1 vs PNT2; PC3 vs PNT2; LNCaP vs PNT2), glycerol, (DU145 vs PNT2; LNCaP vs PNT2), 2-hydroxyiminohexanoic acid, (22RV1 vs PNT2; LNCaP vs PNT2), palmitoleic acid (22RV1 vs PNT2; DU145 vs PNT2), methyl 2-acetamido-2-deoxy-3-O-methyl-a-D-galactopyranoside, (DU145 vs PNT2; LNCaP vs PNT2), 2-butenoic acid, (DU145 vs PNT2), erythrotetrofuranose, (DU145 vs PNT2), palmitic acid (LNCaP vs PNT2), 13-octadecenoic acid (LNCaP vs PNT2). Some unidentified metabolites were also important for the discrimination between cancer and normal cell lines (tables 14, 15, 16 and 17) (figures 21, 22, 23 and 24).

**Table 14:** List of metabolites selected in PLS-DA of 22RV1 (PCa cells) vs PNT2 (normal cells) (VIP>1) as potentially important for discrimination between PCa and normal cell lines

| Name | RT | Characteristic ions | KI from literature | Experimental KI or standards | MS-R match | Cas number | *p* value | Variation % (uncertainty) | Effect size (uncertainty) |
|---|---|---|---|---|---|---|---|---|---|
| **Ethanolamine**, 2TMS derivative | **5.08** | **102/147** | **1021** | **1027** | **890** | **17165-52-5** | **<0.0001[P]** | **↓97.18± 18.37** | **↓ 3.8±1.35** |
| **Lactic Acid**, 2TMS derivative[*] | **5.43** | **73/147** | **1066** | **1057** | **927** | **17596-96-2** | **<0.0001[P]** | **↓ 84.79± 7.44** | **↓7.43±2.29** |
| **Glycine**, 2TMS derivative | **6.14** | **102/147/204** | **—** | **S** | **950** | **7364-42-3** | **0.0004[P]** | **↑ 834776.33 ± 190.83** | **↑0.46 ± 0.80** |
| **ß-Alanine**, 2TMS derivative | **6.9** | **102/176** | **1190** | **1186** | **899** | **17891-86-0** | **<0.0001[P]** | **↓ 83.07 ± 21.15** | **↓ 2.52± 1.07** |
| **2-Butenoic acid**, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester | 6.96 | 73/147 | 1186 | 1192 | 793 | 55590-70-0 | >0.05 | ↑ 29.61 ± 20.94 | ↑ |
| **L-Valine**, 2TMS derivative | **7.17** | **144/218** | **—** | **S** | **920** | **7364-44-5** | **<0.0001[P]** | **↓ 92.03± 12.16** | **↓5.20± 1.7** |
| **Urea,** 2TMS derivative | **7.43** | **73/147** | **1249** | **1219** | **921** | **18297-63-7** | **0.0002[P]** | **↓36.52± 9.04** | **↓ 1.93± 0.97** |
| **L-Leucine**, 2TMS derivative | **7.94** | **102/158** | **—** | **S** | **931** | **7364-46-7** | **<0.0001[P]** | **↓ 72.24± 10.06** | **↓ 4.29 ± 1.47** |
| **L-Proline**, 2TMS derivative | 8.01 | 73/142/216 | **—** | S | 909 | 7364-47-8 | >0.05 | ↑ 21.11 ± 17.59 | ↑ |
| **Unknown** | **8.54** | **147/204** | **NA** | **1277** | **NA** | **NA** | **0.0309** | **↑55.75±17.47** | **↑1.04± 0.84** |
| **L-Threonine**, 2TMS derivative | **8.77** | **73/117/130** | **—** | **S** | **737** | **7536-82-5** | **0.0187** | **↓37.40± 17.94** | **↓1.02±0.84** |
| **Glycerol**, 3TMS derivative | 8.92 | 103/147/205 | 1289 | 1297 | 789 | 6787-10-6 | >0.05 | ↑ 32.43 ± 19.85 | ↑ |
| **L-Aspartic acid**, 3TMS derivative | 9.93 | 100 | **—** | S | 890 | 55268-53-6 | >0.05 | ↓ | ↓ |
| **L- Glutamine**, 3TMS derivative | **10.73** | **73/75/246** | **—** | **S** | **570** | **70591-28-5** | **<0.0001[P]** | **↑122.43± 13.48** | **↑2.36± 1.04** |

| Name | RT | Characteristic ions | KI from literature | Experimental KI or standards | MS-R match | Cas number | *p* value | Variation % (uncertainty) | Effect size (uncertainty) |
|---|---|---|---|---|---|---|---|---|---|
| **Phenylalanine**, 2TMS derivative | **10.84** | **73/147/218** | **—** | **S** | **876** | **2899-52-7** | **0.0075** | **↑29.96 ± 8.45** | **↑1.23 ± 0.87** |
| Unknown | 11.77 | 73 | NA | 1646 | NA | NA | >0.05 | ↓54.68± 39.46 | ↓ |
| **Ribofuranose**, tetrakis(trimethylsilyl) ether | **12.09** | **73/147/217** | **1671** | **1690** | **864** | **NA** | **0.0011[P]** | **↓37.95± 11.34** | **↓1.60±0.91** |
| **Lyxofuranose**, tetrakis(trimethylsilyl) ether | **12.15** | **73/147/217** | **1671** | **1698** | **859** | **NA** | **<0.0001[P]** | **↓57.89± 13.12** | **↓2.43±1.06** |
| **Talofuranose,** pentakis(trimethylsilyl) ether | **12.87** | **73/103/147/191** | **1823** | **1803** | **720** | **NA** | **0.0003[P]** | **↓47.78± 11.98** | **↓2.00±0.98** |
| **Sorbose**, 5TMS derivative | **13.02** | **103/217** | **1867** | **1826** | **809** | **NA** | **<0.0001[P]** | **↓73.09± 10.30** | **↓4.22±1.45** |
| **L-Tyrosine**, 3TMS derivative* | **13.06** | **73/100/218/280** | **—** | **S** | **923** | **51220-73-6** | **0.0002[P]** | **↑43.50 ± 7.52** | **↑1.92 ± 0.96** |
| **Allopyranose**, 5TMS derivative | 13.24 | 217 | 1829 | 1859 | 862 | NA | >0.05 | ↑ | ↑ |
| **Palmitic Acid**, TMS derivative | 13.77 | 73/132/129/313 | **—** | S | 935 | 55520-89-3 | >0.05 | ↓13.54± 6.80 | ↓0.86± 0.83 |
| **Galacturonic acid**, 5TMS derivative | 14.04 | 75 | 1943 | 1924 | 729 | NA | >0.05 | ↑115.65± 78.05 | ↑ |
| **Unknown** | **14.25** | **73/145/311** | **NA** | **1934** | **NA** | **NA** | **<0.0001[P]** | **↑222.97± 14.87** | **↑3.03± 1.17** |
| Unknown | 14.42 | 117 | NA | 1942 | NA | NA | >0.05 | ↓ | ↓ |
| **Unknown** | **14.94** | **117/129/339** | **NA** | **1966** | **NA** | **NA** | **0.0106** | **↑25.74± 8.04** | **↑1.16± 0.85** |
| Unknown | 15.14 | 117/129/145/341 | NA | 1975 | NA | NA | 0.0252 | ↑24.74± 8.62 | ↑1.04± 0.84 |
| **9-Hexadecenoic acid**, TMS derivative (palmitoleic acid) | **15.8** | **55/75/145** | **2027** | **2006** | **701** | **NA** | **<0.0001[P]** | **↑284.19± 18.01** | **↑2.81± 1.13** |
| **Cholesterol**, TMS derivative | **23.06** | **129/329/368** | **—** | **S** | **912** | **1856-05-9** | **0.0004[P]** | **↑110.38± 15.40** | **↑1.96± 0.97** |

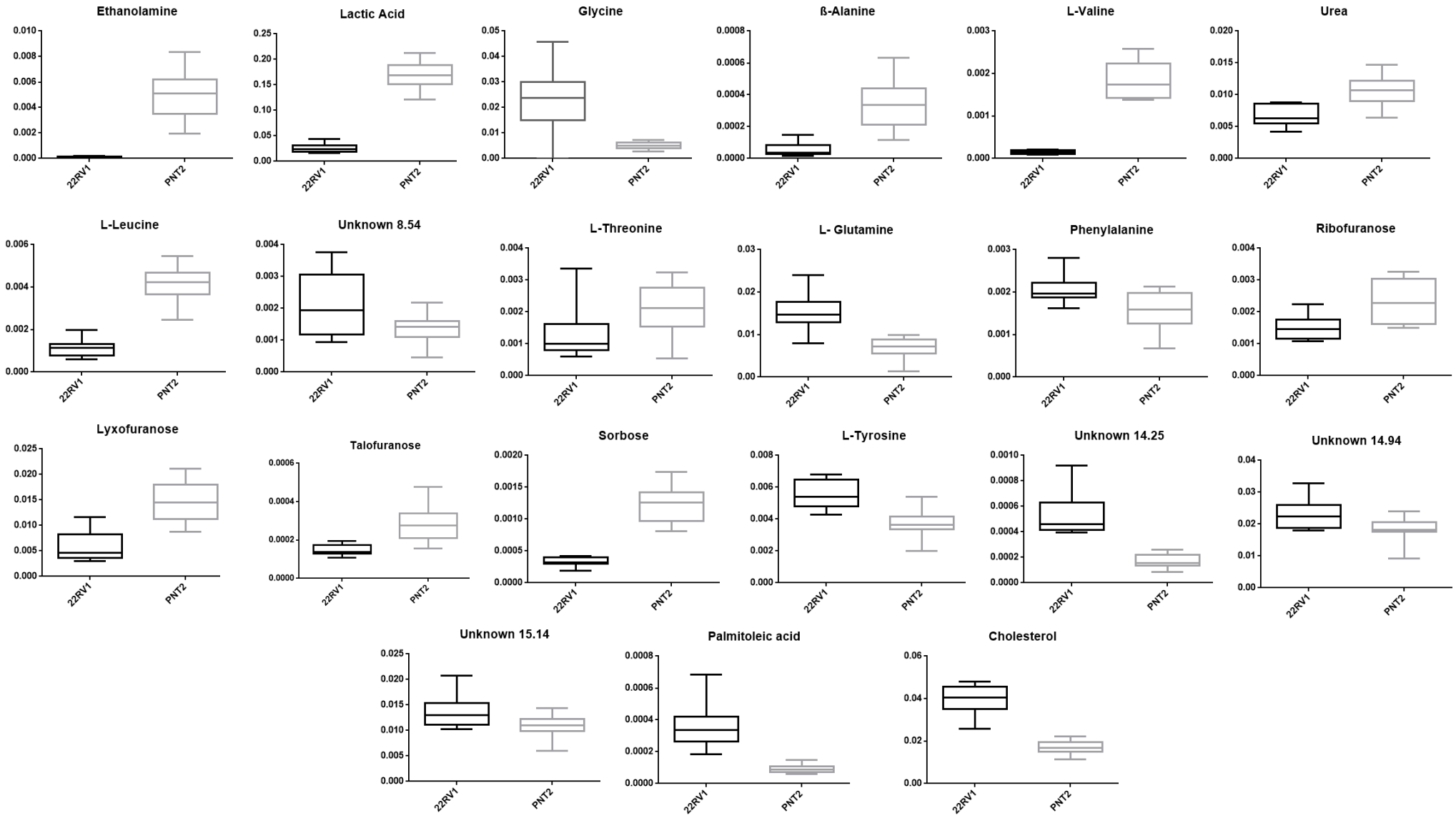


**Figure 21:** Boxplots from the metabolites important for the separation between 22RV1 (PCa cells) and PNT2 (normal cells), after univariate analysis.

**Table 15:** List of metabolites selected in PLS-DA of PC3 (PCa cells) vs PNT2 (normal cells) (VIP>1) as potentially important for discrimination between PCa and normal cell lines

| Name | RT | Characteristic ions | KI from literature | Experimental KI or standards | MS-R match | Cas number | *p* value | Variation % (uncertainty) | Effect size (uncertainty) |
|---|---|---|---|---|---|---|---|---|---|
| Unknown | 4.56 | 69/140 | NA | 983 | NA | NA | >0.05 | ↑ 69.1 ± 27.4 | ↑ 0.90± 0.87 |
| **Ethanolamine,** 2TMS derivative | **5.08** | **102/147** | **1021** | **1027** | **890** | **17165-52-5** | **0.0052** | **↓ 54.1± 21.8** | **↓ 1.41±0.93** |
| **Lactic Acid,** 2TMS derivative | **5.43** | **73/147** | **—** | **S** | **927** | **17596-96-2** | **<0.0001[p]** | **↓ 31.8± 6.2** | **↓ 2.38±1.10** |
| **L-Alanine,** 2TMS derivative | **5.94** | **116/147/190** | **—** | **S** | **935** | **27844-07-1** | **0.0034** | **↓ 24.9±8.5** | **↓ 1.18± 0.90** |
| **Glycine,** 2TMS derivative | **6.14** | **102/147/204** | **—** | **S** | **950** | **7364-42-3** | **0.0015** | **↑ 111.1 ± 27.0** | **↑ 1.35 ± 0.93** |
| **Sarcosine,** 2TMS derivative | 6.32 | 73 | 1161 | 1134 | 845 | 7364-43-4 | >0.05 | ↓ | ↓ |
| **β-Alanine,** 2TMS derivative | **6.9** | **102/176** | **1190** | **1186** | **899** | **17891-86-0** | **0.0066** | **↑ 78.5± 18.5** | **↑ 1.43 ± 0.94** |
| **2-Butenoic acid**, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester | 6.96 | 73/147 | 1186 | 1192 | 793 | 55590-70-0 | >0.05 | ↑ 28.8 ± 12.2 | ↑ |
| **3-Hydroxyisovaleric acid,** 2TMS derivative | **7.1** | **73/131** | **1216** | **1202** | **937** | **55124-90-8** | **<0.0001[p]** | **↑ 802.1 ± 48.5** | **↑ 1.84± 1.00** |
| **L-Valine**, 2TMS derivative | **7.17** | **144/218** | **—** | **S** | **920** | **7364-44-5** | **<0.0001[p]** | **↓ 62.10± 15.2** | **↓ 2.40± 1.10** |
| **Urea**, 2TMS derivative | **7.43** | **73/147** | **—** | **S** | **921** | **18297-63-7** | **<0.0001[p]** | **↓ 45.1± 9.4** | **↓ 2.36± 1.09** |
| **L-Leucine**, 2TMS derivative | **7.94** | **102/158** | **—** | **S** | **931** | **7364-46-7** | **0.0420** | **↓ 25.2±12.6** | **↓ 1.00 ± 0.88** |

| Name | RT | Characteristic ions | KI from literature | Experimental KI or standards | MS-R match | Cas number | *p* value | Variation % (uncertainty) | Effect size (uncertainty) |
|---|---|---|---|---|---|---|---|---|---|
| **L-Proline**, 2TMS derivative | 8.01 | 73/142/216 | — | **S** | 909 | 7364-47-8 | >0.05 | ↑ | ↑ |
| **Unknown** | **8.54** | **147/204** | **NA** | **1277** | **NA** | **NA** | **0.0006**[p] | **↓ 44.7±12.7** | **↓ 1.67±0.97** |
| **L-Threonine**, 2TMS derivative | **8.77** | **73/117/130** | — | **S** | **737** | **7536-82-5** | **0.0104** | **↓ 39.3± 16.3** | **↓1.18±0.90** |
| L-Aspartic acid, 3TMS derivative | 9.93 | 100 | — | S | NA | NA | >0.05 | ↑ | ↑ |
| **L- Glutamine**, 3TMS derivative | 10.73 | 73/75/246 | — | S | 570 | 70591-28-5 | >0.05 | ↑35.7± 19.3 | ↑0.73± 0.86 |
| **Phenylalanine,** 2TMS derivative | **10.84** | **73/147/218** | — | **S** | **876** | **2899-52-7** | **0.0056** | **↓35.6±13.2** | **↓1.34±0.92** |
| **1,2-Benzisothiazol-3-amine**, TMS derivative | 11.7 | 207 | 1605 | 1637 | 626 | NA | >0.05 | ↑ | ↑ |
| Unknown | 11.77 | 73 | NA | 1646 | NA | NA | >0.05 | ↓37.5±36.7 | ↓ |
| **Ribofuranose,** tetrakis(trimethylsilyl) ether | **12.09** | **73/147/217** | **1671** | **1690** | **864** | **NA** | **0.0028** | **↓36.9±12.5** | **↓1.41±0.93** |
| **Lyxofuranose,** tetrakis(trimethylsilyl) ether | **12.15** | **73/147/217** | **1671** | **1698** | **859** | **NA** | **0.0017** | **↓37.9±12.2** | **↓1.53±0.95** |
| **Tridecanoic acid,** TMS derivative | 12.43 | 117 | 1705 | 1739 | 688 | 169597-14-2 | >0.05 | ↑ | ↑ |
| **Sorbose**, 5TMS derivative | 13.02 | 103/217 | 1867 | 1826 | 809 | NA | >0.05 | ↓21.2±13.2 | ↓ |
| **L-Tyrosine**, 3TMS derivative | **13.06** | **73/100/218/280** | — | **S** | **923** | **51220-73-6** | **0.0322** | **↑27.8± 10.5** | **↑1.04± 0.89** |

| Name | RT | Characteristic ions | KI from literature | Experimental KI or standards | MS-R match | Cas number | *p* value | Variation % (uncertainty) | Effect size (uncertainty) |
|---|---|---|---|---|---|---|---|---|---|
| **Allopyranose**, 5TMS derivative | 13.24 | 217 | 1829 | 1859 | 862 | NA | >0.05 | ↑ | ↑ |
| **Allofuranose**, pentakis(trimethylsilyl) ether | 13.36 | 147 | 1853 | 1877 | 785 | NA | >0.05 | ↑93.0± 69.3 | ↑ |
| **Unknown** | **13.61** | **73/117/129** | **NA** | **1904** | **NA** | **NA** | **<0.0001[P]** | **↑182.7± 16.8** | **↑2.97± 1.22** |
| **Palmitic Acid**, TMS derivative | 13.77 | 73/132/129/313 | — | S | 935 | 55520-89-3 | >0.05 | ↑ | ↑ |
| **Galacturonic acid**, 5TMS derivative | 14.04 | 75 | 1943 | 1924 | 729 | NA | >0.05 | ↑ | ↑ |
| **Unknown** | **14.25** | **73/145/311** | **NA** | **1934** | **NA** | **NA** | **0.0008[P]** | **↑132.5± 18.4** | **↑2.18± 1.06** |
| Unknown | 14.94 | 117/129/339 | NA | 1966 | NA | NA | >0.05 | ↓16.4±9.5 | ↓ |
| **Unknown** | **15.14** | **117/129/145/341** | **NA** | **NA** | **NA** | **18748-91-9** | **0.0032** | **↓29.5±9.8** | **↓1.48±0.94** |
| **Unknown** | **16.78** | **75/129** | **NA** | **2051** | **NA** | **NA** | **<0.0001[P]** | **↓56.5±9.1** | **↓3.52±1.35** |
| **Methyl 2-acetamido-2-deoxy-3-O-methyl-a-D-galactopyranoside**, 2TMS | 17.94 | 73/117 | 2134 | 2105 | 660 | 56196-89-5 | >0.05 | ↑196.9± 50.0 | ↑1.05± 0.89 |
| **Cholesterol**, TMS derivative | **23.06** | **129/329/368** | — | **S** | **912** | **1856-05-9** | **<0.0001[P]** | **↑65.9± 7.6** | **↑3.01± 1.23** |

KI: Kovat indices; S: Identified using standards; NA: not available; [P] Alterations remaining significant after Bonferroni correction, with cutoff *p* value of $1.39 \times 10^{-3}$ (0.05 divided by 36 analyzed metabolites).

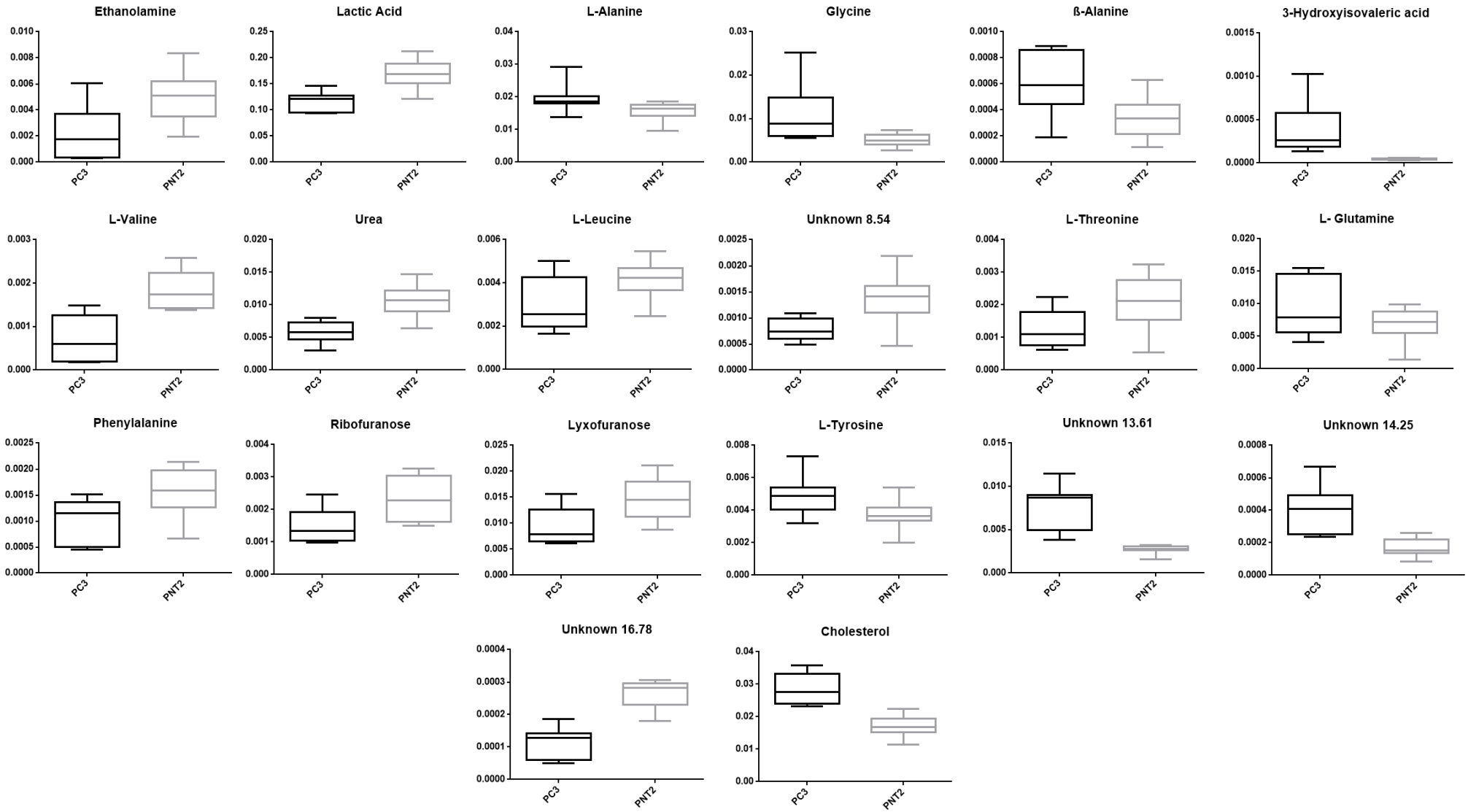


**Figure 22:** Boxplots from the metabolites important for the separation between PC3 (PCa cells) and PNT2 (normal cells), after univariate analysis

**Table 16:** List of metabolites selected in PLS-DA of DU145 (PCa cells) vs PNT2 (normal cells) (VIP>1) as potentially important for discrimination between PCa and normal cell lines

| Name | RT | Characteristic ions | KI from literature | Experimental KI or standards | MS-R match | Cas number | *p* value | Variation % (uncertainty) | Effect size (uncertainty) |
|---|---|---|---|---|---|---|---|---|---|
| **Ethanolamine**, 2TMS derivative | **5.08** | **102/147** | **1021** | **1027** | **890** | **17165-52-5** | **<0.0001[p]** | **⇓80.14± 18.04** | **⇓2.92±1.31** |
| **Lactic Acid**, 2TMS derivative | **5.43** | **73/147** | **—** | **S** | **927** | **17596-96-2** | **0.0213** | **⇓ 18.06±7.62** | **⇓1.03±0.85** |
| **L-Alanine**, 2TMS derivative | **5.94** | **116/147/190** | **—** | **S** | **935** | **27844-07-1** | **<0.0001[p]** | **⇓ 53.75±8.88** | **⇓3.26± 1.20** |
| **Glycine**, 2TMS derivative | 6.14 | 102/147/204 | — | S | 950 | 7364-42-3 | >0.05 | ⇑ | ⇑ |
| **Hydracrylic acid**, 2TMS derivative | 6.41 | 127 | 1151 | 1140 | 842 | 55162-32-8 | >0.05 | ⇑ | ⇑ |
| **ß-Alanine**, 2TMS derivative | **6.9** | **102/176** | **1190** | **1186** | **899** | **17891-86-0** | **<0.0001[p]** | **⇑ 729.64± 20.99** | **⇑ 2.95± 1.14** |
| **2-Butenoic acid**, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester | **6.96** | **73/147** | **1186** | **1192** | **793** | **55590-70-0** | **<0.0001[p]** | **⇑ 130.36 ± 12.10** | **⇑ 2.57±1.06** |
| **3-Hydroxyisovaleric acid, 2TMS derivative** | **7.1** | **73/131** | **1216** | **1202** | **937** | **55124-90-8** | **<0.0001[p]** | **⇑ 789.83 ± 18.65** | **⇑ 3.37± 1.23** |
| **L-Valine**, 2TMS derivative | **7.17** | **144/218** | **—** | **S** | **920** | **7364-44-5** | **<0.0001[p]** | **⇓ 58.58± 11.82** | **⇓2.76± 1.10** |
| **Urea**, 2TMS derivative | **7.43** | **73/147** | **1249** | **1219** | **921** | **18297-63-7** | **<0.0001[p]** | **⇓ 47.59± 9.66** | **⇓ 2.31± 1.01** |
| **L-Leucine**, 2TMS derivative | **7.94** | **102/158** | **—** | **S** | **931** | **7364-46-7** | **<0.0001[p]** | **⇓ 45.52± 10.07** | **⇓ 2.97 ± 1.16** |

| Name | RT | Characteristic ions | KI from literature | Experimental KI or standards | MS-R match | Cas number | *p* value | Variation % (uncertainty) | Effect size (uncertainty) |
|---|---|---|---|---|---|---|---|---|---|
| **L-Proline**, 2TMS derivative | 8.01 | 73/142/216 | — | S | 909 | 7364-47-8 | >0.05 | ⇓ | ⇓ |
| **Unknown** | **8.54** | **147/204** | **NA** | **1277** | **NA** | **NA** | **<0.0001[p]** | **⇓ 56.86± 15.31** | **⇓ 2.05± 0.97** |
| **L-Threonine**, 2TMS derivative | **8.77** | **73/117/130** | — | **S** | **737** | **7536-82-5** | **0.0499** | **⇓ 26.44± 13.94** | **⇓0.86±0.81** |
| **Glycerol**, 3TMS derivative | **8.92** | **103/147/205** | **1289** | **1297** | **789** | **6787-10-6** | **0.0005[p]** | **⇓ 60.06± 18.39** | **⇓ 1.84±0.93** |
| **Erythrotetrofuranose,** tris-O-(trimethylsilyl) | **9.74** | **73/129217** | **1326** | **1387** | **673** | **NA** | **0.0001[p]** | **⇓ 39.31± 9.51** | **⇓ 2.03± 0.96** |
| **L-Aspartic acid**, 3TMS derivative | 9.93 | 100 | — | S | NA | NA | >0.05 | ⇑ | ⇑ |
| **Creatinine**, N,N,O-tris(trimethylsilyl) | 10.26 | 75 | 1445 | 1447 | 758 | NA | >0.05 | ⇓ 51.11± 46.5 | ⇓ |
| **L- Glutamine**, 3TMS derivative | 10.73 | 73/75/246 | — | S | 570 | 70591-28-5 | >0.05 | ⇓ | ⇓ |
| **Phenylalanine**, 2TMS derivative | 10.84 | 73/147/218 | — | S | 876 | 2899-52-7 | 0.0447 | ⇓20.14±10.71 | ⇓ |
| **1,2-Benzisothiazol-3-amine**, TMS derivative | 11.7 | 207 | 1605 | 1637 | 626 | NA | >0.05 | ⇑ | ⇑ |
| Unknown | 11.77 | 73 | NA | 1646 | NA | NA | >0.05 | ⇓ | ⇓ |
| **Lyxofuranose**, tetrakis(trimethylsilyl) ether | **12.15** | **73/147/217** | **1671** | **1898** | **859** | **NA** | **0.0001[p]** | **⇓42.84± 10.66** | **⇓2.01±0.96** |

| Name | RT | Characteristic ions | KI from literature | Experimental KI or standards | MS-R match | Cas number | *p* value | Variation % (uncertainty) | Effect size (uncertainty) |
|---|---|---|---|---|---|---|---|---|---|
| **Tridecanoic acid**, TMS derivative | 12.43 | 117 | 1705 | 1739 | 688 | 169597-14-2 | >0.05 | ↑63.28±58.62 | ↑ |
| **Talofuranose**, pentakis(trimethylsilyl) ether | **12.87** | **73/103/147/191** | **1823** | **1803** | **720** | **NA** | **0.0224** | **↓25.94±11.29** | **↓1.04±0.83** |
| **Sorbose**, 5TMS derivative | **13.02** | **103/217** | **1867** | **1826** | **809** | **NA** | **0.0009[p]** | **↓37.57±11.5** | **↓1.59±0.89** |
| **L-Tyrosine**, 3TMS derivative | **13.06** | **73/100/218/280** | **—** | **S** | **923** | **51220-73-6** | **0.0406** | **↓20.27±8.1** | **↓0.9±0.81** |
| **Allopyranose**, 5TMS derivative | 13.24 | 217 | 1829 | 1859 | 862 | NA | >0.05 | ↑ | ↑ |
| **Allofuranose**, pentakis(trimethylsilyl) ether | 13.36 | 147 | 1853 | 1877 | 785 | NA | >0.05 | ↑ | ↑ |
| **Unknown** | **13.61** | **73/117/129** | **NA** | **1904** | **NA** | **NA** | **0.0018** | **↑82.93± 11.28** | **↑2.05± 0.97** |
| **Palmitic Acid**, TMS derivative | 13.77 | 73/132/129/313 | **—** | S | 935 | 55520-89-3 | >0.05 | ↑ | ↑ |
| **Galacturonic acid**, 5TMS derivative | 14.04 | 75 | 1943 | 1924 | 729 | NA | >0.05 | ↑ | ↑ |
| Unknown | 14.21 | 131 | NA | 1932 | NA | NA | >0.05 | ↑47.15± 41.17 | ↑ |
| **Unknown** | **14.25** | **73/145/311** | **NA** | **1934** | **NA** | **NA** | **0.0078** | **↑70.10±16.23** | **↑1.26±0.85** |
| Unknown | 14.42 | 117 | NA | 1942 | NA | NA | >0.05 | ↑63.22±58.62 | ↑ |
| **Unknown** | **14.47** | **117/327** | **NA** | **1944** | **NA** | **NA** | **0.0002[p]** | **↑71.71±10.89** | **↑1.91±0.94** |

| Name | RT | Characteristic ions | KI from literature | Experimental KI or standards | MS-R match | Cas number | *p* value | Variation % (uncertainty) | Effect size (uncertainty) |
|---|---|---|---|---|---|---|---|---|---|
| **Unknown** | **14.94** | **117/129/339** | **NA** | **1966** | **NA** | **NA** | **0.0014** | **↑27.91±7.39** | **↑1.31±0.87** |
| Unknown | 15.14 | 117/129/145/341 | NA | 1975 | NA | NA | >0.05 | ↑11.09± 7.51 | ↑ |
| **9-Hexadecenoic acid**, TMS derivative (palmitoleic acid) | **15.8** | **55/75/145** | **2027** | **2006** | **701** | **NA** | **0.0048** | **↑85.75±23.12** | **↑1.02± 0.83** |
| Unknown | 17.3 | 55 | NA | 2075 | NA | NA | >0.05 | ↑ | ↑ |
| **Methyl 2-acetamido-2-deoxy-3-O-methyl-a-D-galactopyranoside**, 2TMS | **17.94** | **73/117** | **2134** | **2105** | **660** | **56196-89-5** | **0.0037** | **↑88.07± 16.92** | **↑1.42± 0.87** |
| **6,9-Octadecadiynoic acid, methyl ester** | 18.87 | 77 | 2112 | 2148 | 637 | 56847-03-1 | >0.05 | ↑ | ↑ |
| Unknown | 19.00 | 73/117 | NA | 2154 | NA | NA | <0.0001[P] | ↑326.66± 19.57 | ↑2.5± 1.04 |
| **Cholesterol,** TMS derivative | 23.06 | 129/329/368 | — | S | 912 | 1856-05-9 | >0.05 | ↑20.85± 12.10 | ↑ |

KI: Kovat indices; S: Identified using standards; NA: not available; [P] Alterations remaining significant after Bonferroni correction, with cutoff *p* value of $1.14x10^{-3}$ (0.05 divided by 44 analyzed metabolites).

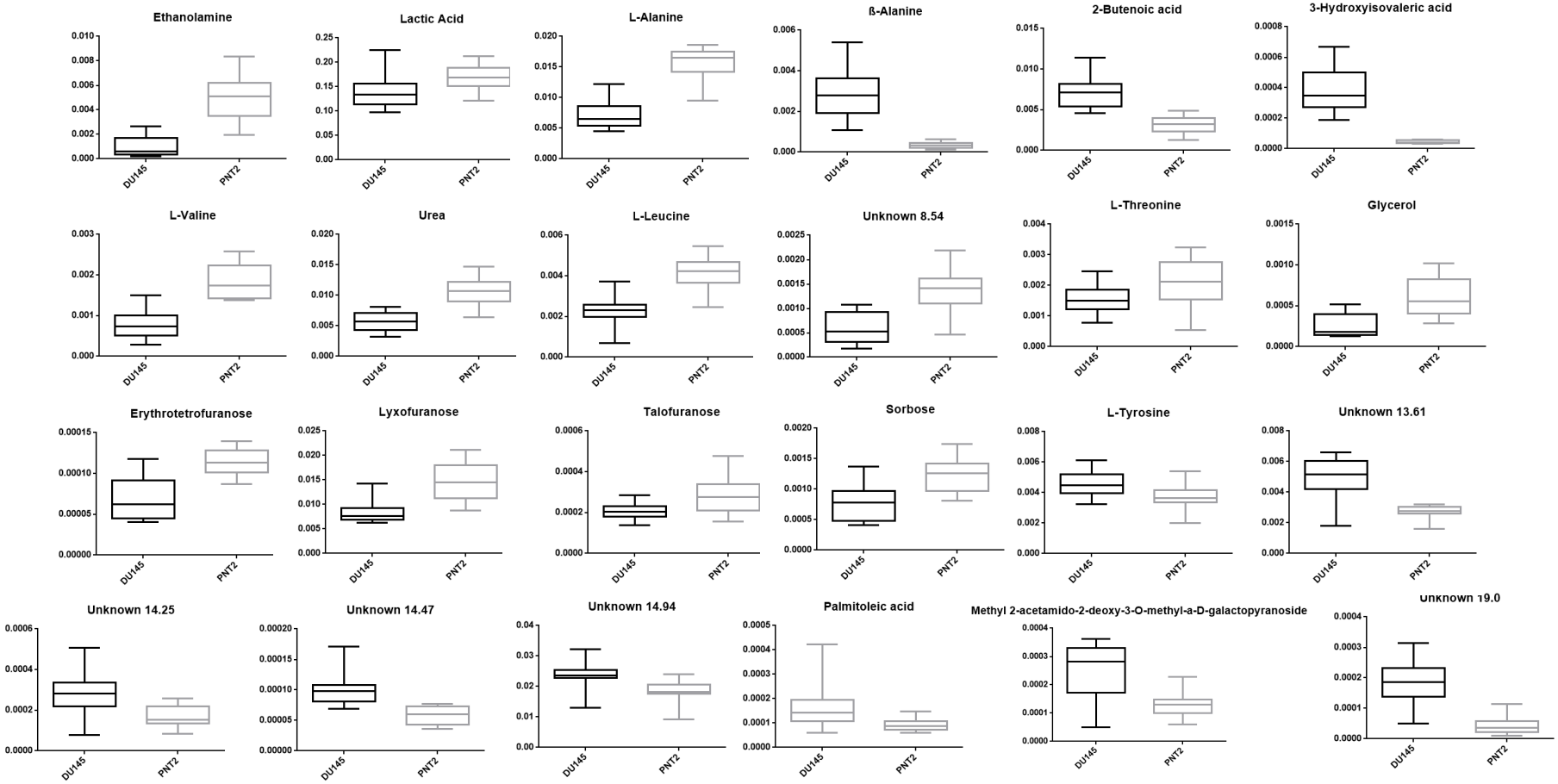


**Figure 23:** Boxplots from the metabolites important for the separation between DU145 (PCa cells) and PNT2 (normal cells), after univariate analysis.

**Table 17:** List of metabolites selected in PLS-DA of LNCaP (PCa cells) vs PNT2 (normal cells) (VIP>1) as potentially important for discrimination between PCa and normal cell lines

| Name | RT | Characteristic ions | KI from literature | Experimental KI or standards | MS-R match | Cas number | *p* value | Variation % (uncertainty) | Effect size (uncertainty) |
|---|---|---|---|---|---|---|---|---|---|
| Unknown | **4.56** | **69/140** | **NA** | **983** | **NA** | **NA** | **0.0001[p]** | **⇓ 70.35± 22.44** | **⇓ 1.82± 0.94** |
| **Ethanolamine**, 2TMS derivative | **5.08** | **102/147** | **1021** | **1027** | **890** | **17165-52-5** | **<0.0001[p]** | **⇓89.19± 17.26** | **⇓ 3.47±1.28** |
| **Lactic Acid**, 2TMS derivative | **5.43** | **73/147** | **—** | **S** | **927** | **17596-96-2** | **<0.0001[p]** | **⇓ 55.39± 7.88** | **⇓3.83±1.36** |
| **L-Alanine**, 2TMS derivative | **5.94** | **116/147/190** | **—** | **S** | **935** | **27844-07-1** | **0.0171** | **⇓ 24.11± 9.88** | **⇓ 1.13± 0.85** |
| **Glycine,** 2TMS derivative | **6.14** | **102/147/204** | **—** | **S** | **950** | **7364-42-3** | **0.0042** | **⇑ 118.14 ± 20.13** | **⇑ 1.57 ± 0.91** |
| **ß-Alanine**, 2TMS derivative | **6.9** | **102/176** | **1190** | **1186** | **899** | **17891-86-0** | **<0.0001[p]** | **⇓ 87.83 ± 21.39** | **⇓ 2.73± 1.12** |
| **2-Butenoic acid**, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester | 6.96 | 73/147 | 1186 | 1192 | 793 | 55590-70-0 | >0.05 | ⇑ 20.13 ± 14.41 | ⇑ |
| **3-Hydroxyisovaleric acid, 2TMS derivative** | **7.1** | **73/131** | **1216** | **1202** | **937** | **55124-90-8** | **<0.0001[p]** | **⇑ 3161.67 ± 34.43** | **⇑ 2.40± 1.04** |
| **L-Valine**, 2TMS derivative | **7.17** | **144/218** | **—** | **S** | **920** | **7364-44-5** | **<0.0001[p]** | **⇓ 71.40± 10.70** | **⇓3.92± 1.38** |
| **Urea**, 2TMS derivative | 7.43 | 73/147 | 1249 | 1219 | 921 | 18297-63-7 | >0.05 | ⇑ 8.39 ± 7.20 | ⇑ |
| **L-Leucine**, 2TMS derivative | **7.94** | **102/158** | **—** | **S** | **931** | **7364-46-7** | **<0.0001[p]** | **⇓ 51.55±9.06** | **⇓ 2.97 ± 1.16** |

| Name | RT | Characteristic ions | KI from literature | Experimental KI or standards | MS-R match | Cas number | *p* value | Variation % (uncertainty) | Effect size (uncertainty) |
|---|---|---|---|---|---|---|---|---|---|
| **L-Proline**, 2TMS derivative | 8.01 | 73/142/216 | — | S | 909 | 7364-47-8 | >0.05 | ⇓ | ⇓ |
| **Unknown** | **8.54** | **147/204** | **NA** | **1277** | **NA** | **NA** | **0.0321** | **⇑93.47± 25.21** | **⇑1.07± 0.84** |
| **p-Toluic acid**, TMS derivative | 8.64 | 65/119/193 | 1282 | 1282 | 818 | NA | >0.05 | ⇓16.04± 10.59 | ⇓ |
| **L-Threonine,** 2TMS derivative | **8.77** | **73/117/130** | **—** | **S** | **737** | **7536-82-5** | **0.0445** | **⇓ 27.58± 14.13** | **⇓0.88±0.82** |
| **Glycerol,** 3TMS derivative | **8.92** | **103/147/205** | **1289** | **129** | **789** | **6787-10-6** | **0.0004[p]** | **⇓ 58.18± 15.77** | **⇓1.97±0.97** |
| **L-Aspartic acid**, 3TMS derivative | 9.93 | 100 | — | S | NA | NA | >0.05 | ⇓25.32±22.2 | ⇓ |
| **L- Glutamine**, 3TMS derivative | **10.73** | **73/75/246** | **—** | S | **570** | **70591-28-5** | **<0.0001[p]** | **⇑108.67± 12.83** | **⇑2.29± 1.03** |
| **Phenylalanine,** 2TMS derivative | **10.84** | **73/147/218** | **—** | S | **876** | **2899-52-7** | **0.0036** | **⇓42.45±13.45** | **⇓1.59±0.91** |
| **1,2-Benzisothiazol-3-amine**, TMS derivative | 11.7 | 207 | 1605 | 1637 | 626 | NA | >0.05 | ⇓ | ⇓ |
| Unknown | 11.77 | 73 | NA | 1646 | NA | NA | >0.05 | ⇓40.90±32.77 | ⇓ |
| **Ribofuranose**, tetrakis(trimethylsilyl) ether | **12.09** | **73/147/217** | **1671** | **1690** | **864** | **NA** | **0.0004[p]** | **⇓42.26±10.73** | **⇓1.96±0.96** |
| **Lyxofuranose**, tetrakis(trimethylsilyl) ether | **12.15** | **73/147/217** | **1671** | **1898** | **859** | **NA** | **<0.0001[p]** | **⇓48.11±11.13** | **⇓2.23±1.02** |
| **Talofuranose,** pentakis(trimethylsilyl) ether | **12.87** | **73/103/147/191** | **1823** | **1803** | **720** | **NA** | **0.0056** | **⇓29.01±16.04** | **⇓0.85±0.83** |

| Name | RT | Characteristic ions | KI from literature | Experimental KI or standards | MS-R match | Cas number | *p* value | Variation % (uncertainty) | Effect size (uncertainty) |
|---|---|---|---|---|---|---|---|---|---|
| **Sorbose**, 5TMS derivative | **13.02** | **103/217** | **1867** | **1826** | **809** | **NA** | **<0.0001[P]** | **⩛64.31±10.68** | **⩛3.41±1.26** |
| **L-Tyrosine,** 3TMS derivative | **13.06** | **73/100/218/280** | **—** | **S** | **923** | **51220-73-6** | **<0.0001[P]** | **⩛44.38±9.74** | **⩛2.28±1.03** |
| **Allopyranose**, 5TMS derivative | 13.24 | 217 | 1829 | 1859 | 862 | NA | >0.05 | ⩛ | ⩛ |
| **Allofuranose**, pentakis(trimethylsilyl) ether | 13.36 | 147 | 1853 | 1877 | 785 | NA | >0.05 | ⩛ | ⩛ |
| Unknown | **13.61** | **73/117/129** | **NA** | **1904** | **NA** | **NA** | **<0.0001[P]** | **⩚64.27± 7.16** | **⩚2.80± 1.13** |
| **Palmitic Acid**, TMS derivative | **13.77** | **73/132/129/313** | **—** | **S** | **935** | **55520-89-3** | **0.0424** | **⩚21.27± 8.34** | **⩚0.95± 0.84** |
| **Galacturonic acid**, 5TMS derivative | 14.04 | 75 | 1943 | 1924 | 729 | NA | >0.05 | ⩚ | ⩚ |
| Unknown | 14.94 | 117/129/339 | NA | 1966 | NA | NA | >0.05 | ⩚15.5± 7.07 | ⩚ |
| Unknown | **15.14** | **117/129/145/341** | **NA** | **1975** | **NA** | **NA** | **0.0256** | **⩚20.39± 7.37** | **⩚1.02± 0.84** |
| Unknown | **15.62** | **57/69/84/** | **NA** | **1997** | **NA** | **NA** | **<0.0001[P]** | **⩚147.83± 10** | **⩚3.53± 1.29** |
| Unknown | 16.28 | 117 | NA | 2028 | NA | NA | >0.05 | ⩚25.39± 16.87 | ⩚ |
| **Methyl 2-acetamido-2-deoxy-3-O-methyl-a-D-galactopyranoside**, 2TMS | **17.94** | **73/117** | **2134** | **2105** | **660** | **56196-89-5** | **0.0050** | **⩚374.03± 35.88** | **⩚1.57± 0.91** |
| **13-Octadecenoic acid**, TMS derivative | **19.72** | **69/75/117** | **2228** | **2187** | **634** | **NA** | **<0.0001[P]** | **⩚560.66± 18.06** | **⩚3.54± 1.29** |
| **Cholesterol**, TMS derivative | **23.06** | **129/329/368** | **—** | **S** | **912** | **1856-05-9** | **0.0004[p]** | **⩚73.42± 14.39** | **⩚1.58± 0.91** |

KI: Kovat Indices; S: Identified using standards; NA: not available; [P] Alterations remaining significant after Bonferroni correction, with cutoff *p* value of $1.37 \times 10^{-3}$ (0.05 divided by 38 analyzed metabolites).

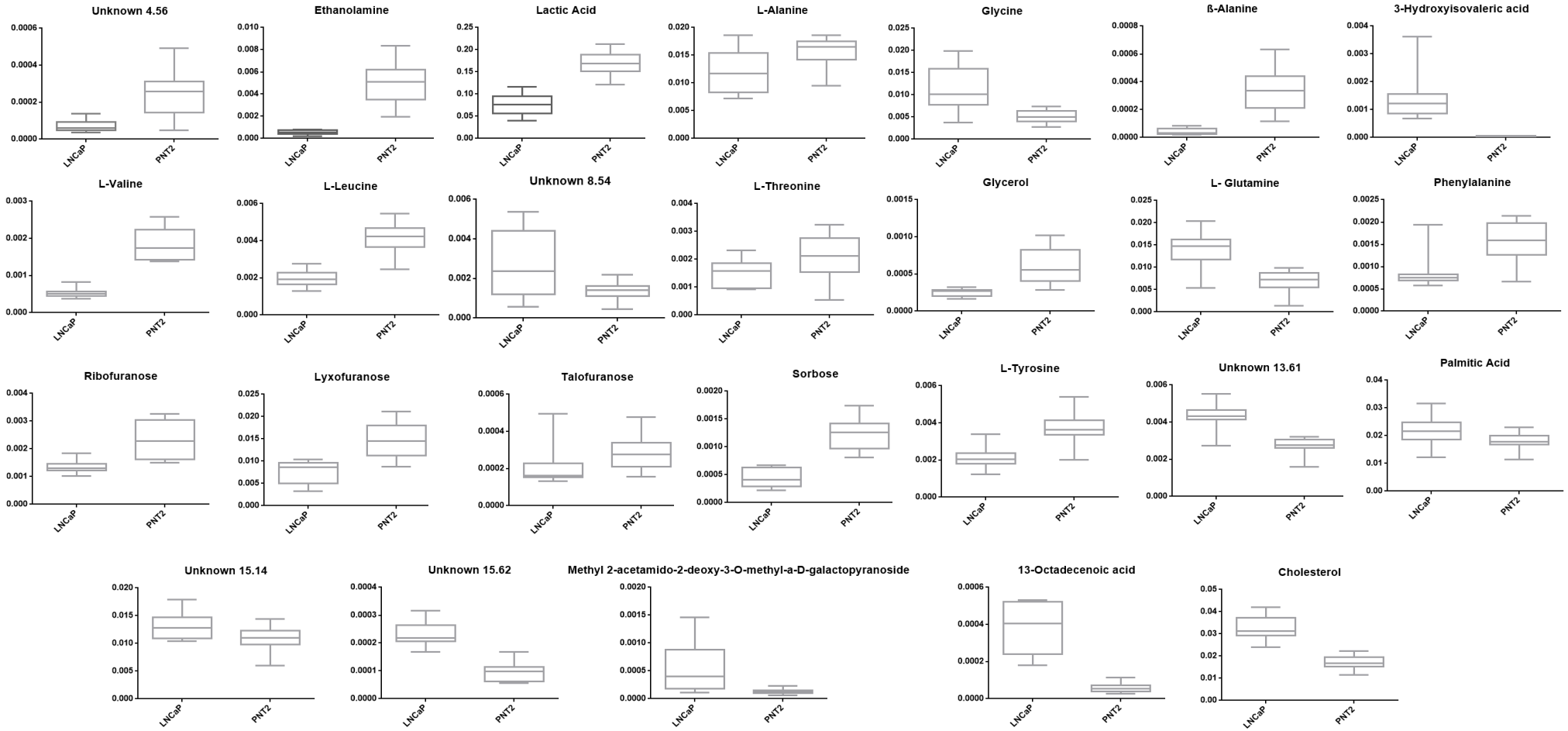


**Figure 24:** Boxplots from the metabolites important for the separation between LNCaP (PCa cells) and PNT2 (normal cells), after univariate analysis.

New PLS-DA models using only discriminant metabolites (Figure 25) was performed. To prove the robustness of the models, a MCCV validation was performed for all comparisons, using GC-MS full data but also using just the discriminant metabolites described before, was also performed new PLS-DA models using just these set of discriminant metabolites (Figure 25). The results of these validations proved that all created models are robust for the discrimination between PCa cell lines and normal cell line (Table 18).

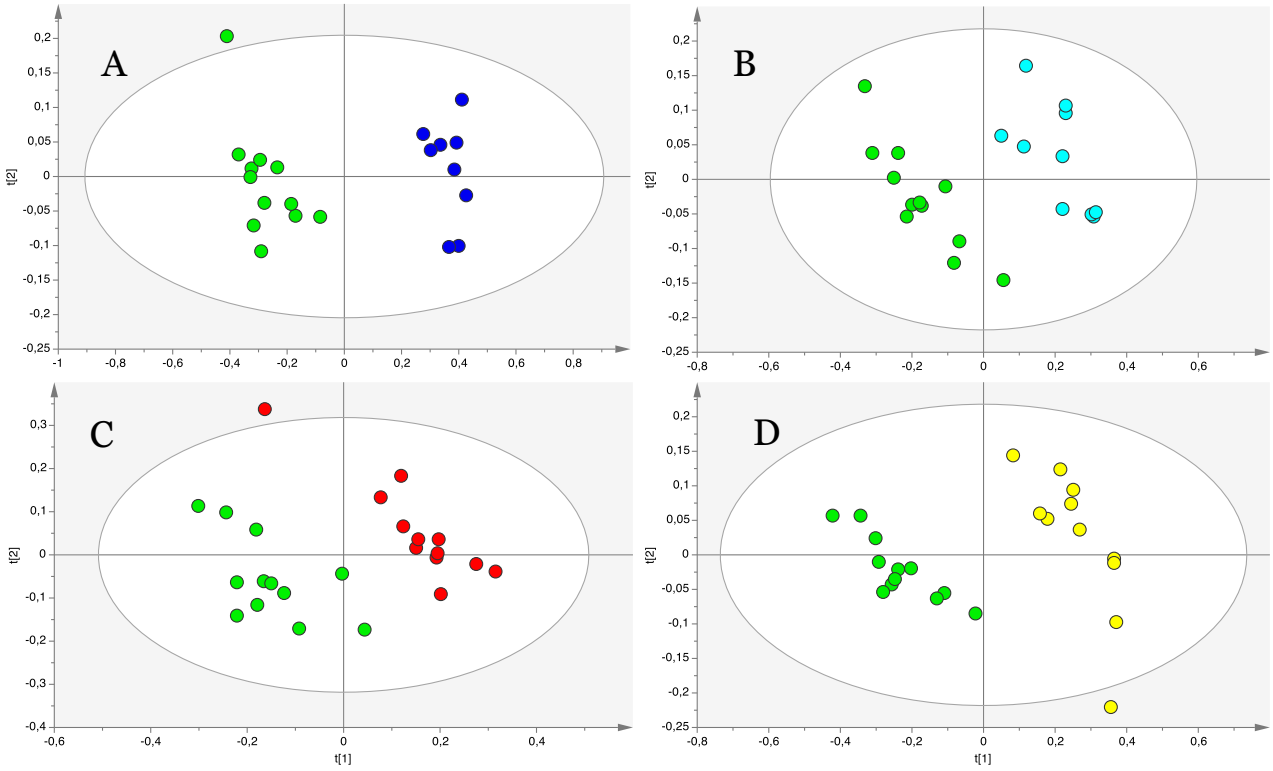


**Figure 25:** PLS-DA from PCa cell line vs normal cell line with the set of discriminant metabolites. **A.** 22RV1 (PCa cells) (dark blue) vs PNT2 (normal cells) (green) ($R^2X$=0.845; $R^2Y$=0.962; $Q^2$=0.947). **B.** PC3 (PCa cells) (light blue) vs PNT2 (normal cells) (green) ($R^2X$=0.77; $R^2Y$=0.932; $Q^2$=0.895). **C.** DU145 (PCa cells) (red) vs PNT2 (normal cells) (green) ($R^2X$=0.855; $R^2Y$=0.903; $Q^2$=0.864). **D.** LNCaP (PCa cells) (yellow) vs PNT2 (normal cells) (green) ($R^2X$=0.814; $R^2Y$=0.938; $Q^2$=0.919).

**Table 18:** MCCV parameters of true and permuted classes obtained for derivatized metabolites when considering GC-MS full data and the set of discriminant compounds.

| Models | True classes | | | | | Permuted classes | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | LV | $Q^2$ | CR (%) | Sens. (%) | Spec. (%) | LV | $Q^2$ | CR (%) | Sens. (%) | Spec. (%) |
| ***GC-MS full data*** | | | | | | | | | | |
| 22RV1 vs PNT2 | 4 | 0.93 | 100 | 100 | 100 | 1 | -0.22 | 45 | 45 | 45 |
| PC3 vs PNT2 | 1 | 0.87 | 98 | 95 | 100 | 1 | -0.38 | 47 | 47 | 46 |
| DU145 vs PNT2 | 2 | 0.93 | 100 | 100 | 100 | 1 | -0.26 | 47 | 47 | 47 |
| LNCaP vs PNT2 | 4 | 0.92 | 100 | 100 | 100 | 1 | -0.25 | 47 | 45 | 49 |
| ***Set of discriminant compounds*** | | | | | | | | | | |
| 22RV1 vs PNT2 | 1 | 0.97 | 100 | 99 | 100 | 1 | -0.30 | 48 | 48 | 47 |
| PC3 vs PNT2 | 2 | 0.93 | 100 | 100 | 100 | 1 | -0.32 | 48 | 47 | 49 |
| DU145 vs PNT2 | 1 | 0.94 | 100 | 100 | 100 | 1 | -0.30 | 47 | 47 | 47 |
| LNCaP vs PNT2 | 2 | 0.96 | 100 | 100 | 100 | 1 | -0.27 | 49 | 47 | 50 |

LV: no. of latent variables; $Q^2$: median predictive power; CR: classification rate; sens.: sensitivity; spec.: specificity.

## Discussion

In this work, we revealed the potentiality of metabolome for discrimination different PCa cell lines from normal prostate cell line, which indicates that the metabolome may be a valuable source of biomarkers for PCa detection in the future. The results revealed metabolites significantly decreased in all PCa cell lines when compared with normal cell line, namely ethanolamine, lactic acid, L-valine, L-leucine, L-threonine, lyxofuranose, and L-tyrosine significantly increased in all PCa cell lines. However, considering different metabolites is possible to discriminate PCa with different aggressiveness, for example, glycerol is significantly decrease in low (LNCaP) and moderated (DU145) metastatic potential cell line (but not in in high metastatic cell line (PC3)) and methyl 2-acetamido-2-deoxy-3-O-methyl-a-D-galactopyranoside is significantly increased in DU145 and in LNCaP but not in PC3; the significant increase of 2-butenoic acid levels and the significant decrease of erythrotetrofuranose levels are characteristic alterations of cell line with moderated metastatic potential (DU145); and the significant increase of palmitic acid, and 13-octadecenoic acid are characteristic of LNCaP, a cell line with low metastatic potential.

It is also possible discriminate between androgen-responsive from androgen-nonresponsive PCa cell lines, using urea, since this metabolite is significantly decreased in androgen-nonresponsive PCa cell lines (22RV1, PC3 and DU145); significant increase of palmitic acid, and (E)-13-octadecenoic acid are others characteristic of androgen-responsive PCa cell line (LNCaP).

All living cells have in common most of the biochemical pathways in the body, for example, metabolic pathways involving energy and amino acid catabolism occurs similarly in all cells. However, cancer cells have unique features, consequence of their extremely proliferative capacity and resistance to apoptosis and, therefore, most certainly have some metabolic pathways altered (enhanced or diminished) (23). This theory was evaluated in this work, and the results show, as expected, alteration in several metabolic pathways, mostly energetic metabolism, lipid metabolism or protein metabolism.

As previously explained (see Chapter 1), the prostate cell has a unique metabolic profile, once one of the major functions of these cells is production and accumulation of citrate (components of the prostatic fluid) (71). Unlike other human cells, the prostate cell does not use, preferentially, citrate in Krebs cycle, for ATP production (aerobic ATP production), even in the presence of oxygen, prostate cell produces ATP mainly by the glucose oxidation which leads to pyruvate production and, consequently, to lactate production. However, prostate cells that undergo neoplastic transformation lose this ability to citrate accumulation and use citrate for Krebs cycle (26, 72-74). This well described alteration may explain the reduction of the levels of lactate observed in this study.

The significant increase in glutamine levels is another indicator of possible alterations in Krebs cycle related with PCa once, through glutaminolysis, delivers intermediates (α-ketoglutarate) for the Krebs cycle. Glutamine is also important for lipogenesis (75, 76).

The alteration in energetic metabolism, related with PCa, is also observed, in the significant levels alteration of several carbohydrates, namely ribofuranose, lyxofuranose, talofuranose, and sorbose, since carbohydrates can be used by cancer cells for energy production. The described alterations in carbohydrates were also observed in other metabolomics PCa studies (13, 66).

Mucin are glycoconjugates, and the increase in their levels is associated with cancer, once these compounds are involved in signaling cell growth and survival, inducing tumor progression (77). These important roles of mucin in cancer may explain the significant increase in the mucin fragment methyl 2-acetamido-2-deoxy-3-O-methyl-a-D-galactopyranoside (78) observed in our study.

The alteration in sarcosine levels in PCa is one of the most studied metabolic alterations related with PCa and one of the most promising potential biomarkers for PCa (6). In this work, we were unable to confirm the significant increase of sarcosine levels related with PCa, however, a significant alteration in glycine levels was observed. Glycine is a precursor of sarcosine so the alteration of this metabolite may suggest alteration in the metabolic pathway involved in sarcosine synthesis. Glycine is also an important protein precursor and provide C2N subunit for purines synthesis (79).

Beyond glycine, several other amino acids were altered in PCa samples, namely, alanine, valine, leucine, threonine, phenylalanine and tyrosine, which may indicate the increased protein synthesis. The increase in protein metabolism is essential to cancer cell maintain the elevated proliferative state. This metabolic alteration was previously focused in other metabolomics PCa studies (66, 80, 81).

The urea cycle uses ammonia to produce urea, as urea is less toxic than ammonia. The main source of ammonia is the degradation of proteins and, as previously explained, cancer cells have increased requirement of proteins which may explain the decreased levels of urea in PCa cells observed in our study.

Other important indicator of alteration in proteins metabolism observed in our study is the significant increase of 3-hydroxyisovaleric acid levels. This leucine metabolite has an important role in protein metabolism once 3-hydroxyisovaleric acid stimulates protein synthesis and prevents protein catabolism (82).

The elevated cholesterol levels observed in PCa cells was previously described in other metabolic studies using prostatic tissue from PCa patients (13, 83). The PCa cells have the ability to synthetise cholesterol, furthermore, a study performed by Awad et al (2001) revealed that *in vitro* supplementation with cholesterol increase cell proliferation, migration, and invasion in PCa cell lines (84). The relevant role of cholesterol in PCa metabolism may be due to the increased need of membrane biosynthesis in cancer cells, the need of growth factor signaling and cholesterol may have an important role in castration resistance of PCa, once cholesterol can be converted *in vivo* to androgens (83).

The alteration in lipid metabolism, particularly, on phospholipid membrane constituents assembly and catabolism, is confirmed by the significant decreased ethanolamine levels. Ethanolamine is one of the principal components of cellular membrane, once ethanolamine is the precursor and the degradation product of phosphatidylethanolamine (abundant phosphoglyceride of human cellular membrane). The alteration in ethanolamine levels in PCa cells was previously observed by other authors (85, 86). Mintz et al (2008),

using radiolabel ethanolamine, demonstrated that PCa cells shown an increase in ethanolamine uptake when compared with normal prostate cells (principally in androgen-responsive cells in presence of androgens) (85-87). Glycerol is another important metabolite involved in lipid metabolism, once glycerol is present in the composition of triglycerides and phospholipids. The catabolism of these lipids, to produce energy, leads to the formation of glycerol, and glycerol can also be converted to glucose (the principal cellular source of energy). Therefore, the dysregulation in glycerol levels is indicative of alteration in lipid metabolism and of the high energy demand characteristic of cancer cells, to sustain its high proliferation state (53-55).

Significant alterations in organic acids, namely 13-octadecenoic acid and 2-butenoic acid, was also observed in our study, as well as in other PCa metabolomics studies (67, 69, 88). Fatty acids can be produced by the β-oxidation of lipids to produce energy but can be involved in other cellular mechanisms, namely cell signaling and cellular membrane integrity, so the alteration in these metabolites indicates that cancer cells have alteration in energetic and lipid metabolism (67, 69, 88).

In this study we also observed a significant increase in palmitic acid levels in PCa samples. The increase of palmitic acid levels, in plasma, was previously associated, in a prospective case-control study, with the risk of PCa (9). Palmitic acid is produced *in vivo* from other fatty acids and from *de novo* lipogenesis, so the significant increase of palmitic acid levels is indicative of alteration in lipid metabolism principally the increase in fatty acid synthesis (9). Besides palmitic acid, palmitoleic acid is another fatty acid significantly increases in PCa cells.

# 3. Main Conclusions

## Conclusion and future perspectives

Currently, PCa diagnostic is performed by using PSA as biomarker, however, this biomarker has low sensibility and specificity. These drawbacks have given rise to serious efforts to the discovery of new biomarkers, preferentially noninvasive, that have better specificity and sensitivity. A novel biomarker is required to be specific for PCa and not altered or expressed in other human tissues or in other diseases, which will bring major benefits for patient health. Since metabolic alterations are the last step in cellular response to diseases, metabolomics can be successfully used to discover new biomarkers for cancer.

In this study, we evaluated the alterationsin prostate exometabolome, more specifically the volatilome (Chapter 3) and intrametaboloma (Chapter 4) caused by the cancer development, using different PCa cell lines with different aggressiveness and hormone dependent and hormone independent. The results prove that, in both approaches, metabolomics alterations are capable of discriminating the different cell lines, therefore is a powerful tool to discover new biomarkers for PCa.

In the third Chapter of this work, we identified several altered VOCs related to PCa. Most of them were low molecular weight compounds such as ketones, aldehydes and organic acids, indicating alteration in lipid and energy metabolism of PCa cells.

In this Chapter was performed a comparative analysis of VOCs in PCa and normal cell lines obtained at pH 7 and pH 2 and the results in both show some potential biomarkers for PCa were discover. In pH 7 the VOCs that stand out as potential biomarkers for PCa was pentadecan-2-one which was increased in all PCa cell lines when compared with normal cell line. It was also possible to discriminate PCa with different grades of aggressiveness using, 2,7-dimethyloctan-1-ol and 2-(1-4-methylcyclohex-3-en-1-yl)propan-2-ol, which are significantly altered specifically in high metastatic potential cell line (PC3), significant increase in decan-1-ol levels is a characteristic alteration of cell line with moderated metastatic potential (DU145) and the significant alteration of 3-methylbut-3-en-2-ol**,** 1-methoxypropan-2-yl acetate and 1,4-xylene levels is characteristic of cell line with low metastatic potential (LNCaP). Moreover, was also possible the discrimination between androgen-responsive cell lines from androgen-nonresponsive cell lines using 3,7-dimethyloct-7-en-1-ol, since this metabolite is just significantly decreased in the androgen-nonresponsive cell lines (PC3, DU145 and 22RV1). In the results of the comparative analysis of VOCs in PCa and normal cell lines obtained at pH 2 the VOCs

that stand out were, cyclohexanone, 4-methylheptan-2-one, 2-methylpentane-1,3-diol, 4-methylbenzaldehyde, 1-(3,5-dimethylfuran-2-yl)ethanone, methyl benzoate, nonanoic acid and decanoic acid which were significantly altered in all PCa cell lines when compared with normal cell line. It was also possible to discriminate PCa with different grades of aggressiveness using 2-ethoxy-2-methylbutane, hexanoic acid, 1-phenylmethanol, 2,4-dimethylheptan-1-ol, benzoic acid, and 6-pentyloxan-2-one, are significantly decreased specifically in high metastatic potential cell line (PC3) and, 1-ethoxypentane, is significantly decreased specifically in moderated and low metastatic potential cell line (DU145 and LNCaP, respectively). Moreover, was also possible the discrimination between androgen-responsive cell lines from androgen-nonresponsive cell lines using 4-methylpent-3-en-2-one and 1-phenylethanol can discriminate androgen-responsive cell lines from androgen-nonresponsive cell lines, these metabolites are just significantly decreased in the androgen-nonresponsive cell lines (PC3, DU145 and 22RV1).

The integration of VOCs in specific metabolic pathways is still very difficult, needing more studies to accomplish this goal. Moreover, it was also evaluated the influence of pH in the volatilome. Acidification of the samples markedly influences it (e. g. detection of organic acids) but the quality of the PCA and PLS-DA models produced were not markedly different.

The results of the evaluation of alterations in intrametaboloma were concordant with the results of the evaluation of volatiloma, in both approach, the results reveal alterations in lipid and energy metabolism of PCa cells. Alteration in protein metabolism was also observed in intrametaboloma. The alteration in these metabolic pathways can be explained by the well-known features of cancer cells, namely, their extremely proliferative, growth and migration capacity, and resistance to apoptosis. To sustain these elevated proliferation rates, the energy demand increases, as well as the protein synthesis. Alterations in lipid metabolism have also an important paper in the sustain of these elevated proliferation rates, once intercellular signaling are dependent on increased lipid biosynthesis, as well as, the increased cellular membrane synthesis is dependent on increased lipid biosynthesis and lipids also can be used for the cell to produce energy. The discriminative metabolites include ethanolamine, lactic acid, L-valine, L-leucine, L-threonine, lyxofuranose, and L-tyrosine, metabolites significantly altered in all PCa cell lines when compared with normal cell line; significant decreased in glycerol levels and the significantly increased in methyl 2-acetamido-2-deoxy-3-methyl-galactopyranoside levels is characteristic of low (LNCaP) and moderated (DU145) metastatic potential cell line (but

not in in high metastatic potential cell line (PC3); the significant increase of 2-butenoic acid levels and the significant decrease of erythrotetrofuranose levels are characteristic alterations of cell line with moderated metastatic potential (DU145); and the significant increase of palmitic acid, and 13-octadecenoic acid are characteristic of LNCaP, a cell line with low metastatic potential. It is also possible discriminate between androgen-responsive from androgen-nonresponsive PCa cell lines, using urea, since this metabolite is significantly decreased in androgen-nonresponsive PCa cell lines (22RV1, PC3 and DU145); significant increase of palmitic acid, and 13-octadecenoic acid are others characteristic of androgen-responsive PCa cell line (LNCaP).

Up to date and according to the scientific literature, the most promising biomarker for PCa is sarcosine. In our work, once we not have available sarcosine standard, the formal identification of these metabolite was not accomplish, so we were unable to confirmed the value of sarcosine as PCa biomarker. However, we observed significant alteration in glycine levels and once sarcosine occurs as an intermediate product in the synthesis and degradation of this amino acid, we can conclude that this metabolic pathway is altered in consequence of PCa.

The evaluation of these latter compounds (aminoacids, sugars, fatty acids and steroids) has the advantage to be easier to relate alterations in these metabolites with known metabolic pathways. Allowing the discover of potential biomarkers for PCa but also, allowing a better understand of which are the most important metabolic pathways altered in consequence of cancer development and progression and, consequently, may permit the discover of new therapeutic targets and the development of new therapeutic approaches. However, the study of the alterations in exometaboloma has the advantage of being potentially easier to translate these results for real samples, like urine, once these metabolites are secreted by cells.

One of the advantages of metabolomics is that it allows improve the sensibility and specificity of biomarkers by using not just one metabolite but a panel of several metabolites, so it is possible with the interaction from results of different studies to achieve better discriminant capability.

One limitation of the present study is that some compounds were not identified using standards, and despite, our best effort to correctly identify these metabolites, the formal identification of compounds is only accomplished by using standards.

It is important to emphasize that for a possible use in the clinical practice is mandatory to prove the translability of results for real samples. Once it is practically impossible to

simulate complex cell–cell and cell–matrix interactions in cell cultures of prostate cancer and these interactions are very important for metabolic alterations that happen with tumor progression, so the translability of results for real samples is not guaranteed.

It is also necessary to develop more work with the objective of better understand the cellular source and the metabolic pathways responsible for the production and consumption of the VOCs.

Despite the limitation of the study, the results reveal the potential of metabolomics, in both, extra and intracellular, approaches to discriminated between groups (PCa vs normal prostate cell lines), which may lead to the identification of new biomarkers for PCa. However, further studies are still needed to confirmed our results, and find out an inexpensive, noninvasive, sensible and specific biomarker for prostate cancer.

# 4. References

## References


1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. International journal of cancer Journal international du cancer. 2015;136(5):E359-86.
2. Rigau M, Olivan M, Garcia M, Sequeiros T, Montes M, Colas E, et al. The present and future of prostate cancer urine biomarkers. International journal of molecular sciences. 2013;14(6):12620-49.
3. Roberts MJ, Schirra HJ, Lavin MF, Gardiner RA. Metabolomics: a novel approach to early and noninvasive prostate cancer detection. Korean journal of urology. 2011;52(2):79-89.
4. Trock BJ. Application of metabolomics to prostate cancer. Urologic oncology. 2011;29(5):572-81.
5. Schalken J, Dijkstra S, Baskin-Bey E, van Oort I. Potential utility of cancer-specific biomarkers for assessing response to hormonal treatments in metastatic prostate cancer. Therapeutic advances in urology. 2014;6(6):245-52.
6. Sreekumar A, Poisson LM, Rajendiran TM, Khan AP, Cao Q, Yu J, et al. Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression. Nature. 2009;457(7231):910-4.
7. Khalid T, Aggio R, White P, De Lacy Costello B, Persad R, Al-Kateb H, et al. Urinary Volatile Organic Compounds for the Detection of Prostate Cancer. PloS one. 2015;10(11):e0143283.
8. Giskeodegard GF, Hansen AF, Bertilsson H, Gonzalez SV, Kristiansen KA, Bruheim P, et al. Metabolic markers in blood can separate prostate cancer from benign prostatic hyperplasia. British journal of cancer. 2015;113(12):1712-9.
9. Crowe FL, Allen NE, Appleby PN, Overvad K, Aardestrup IV, Johnsen NF, et al. Fatty acid composition of plasma phospholipids and risk of prostate cancer in a case-control analysis nested within the European Prospective Investigation into Cancer and Nutrition. The American journal of clinical nutrition. 2008;88(5):1353-63.
10. Kline EE, Treat EG, Averna TA, Davis MS, Smith AY, Sillerud LO. Citrate concentrations in human seminal fluid and expressed prostatic fluid determined via 1H nuclear magnetic resonance spectroscopy outperform prostate specific antigen in prostate cancer detection. The Journal of urology. 2006;176(5):2274-9.
11. Serkova NJ, Gamito EJ, Jones RH, O'Donnell C, Brown JL, Green S, et al. The metabolites citrate, myo-inositol, and spermine are potential age-independent markers of prostate cancer in human expressed prostatic secretions. The Prostate. 2008;68(6):620-8.
12. McDunn JE, Li Z, Adam KP, Neri BP, Wolfert RL, Milburn MV, et al. Metabolomic signatures of aggressive prostate cancer. The Prostate. 2013;73(14):1547-60.
13. Jung K, Reszka R, Kamlage B, Bethan B, Stephan C, Lein M, et al. Tissue metabolite profiling identifies differentiating and prognostic biomarkers for prostate carcinoma. International journal of cancer Journal international du cancer. 2013;133(12):2914-24.
14. Vaz CV, Alves MG, Marques R, Moreira PI, Oliveira PF, Maia CJ, et al. Androgen-responsive and nonresponsive prostate cancer cells present a distinct glycolytic metabolism profile. The international journal of biochemistry & cell biology. 2012;44(11):2077-84.
15. Kaushik AK, Vareed SK, Basu S, Putluri V, Putluri N, Panzitt K, et al. Metabolomic profiling identifies biochemical pathways associated with castration-resistant prostate cancer. Journal of proteome research. 2014;13(2):1088-100.

16. Aoun F, Peltier A, van Velthoven R. A comprehensive review of contemporary role of local treatment of the primary tumor and/or the metastases in metastatic prostate cancer. BioMed research international. 2014;2014:501213.
17. Dimakakos A, Armakolas A, Koutsilieris M. Novel tools for prostate cancer prognosis, diagnosis, and follow-up. BioMed research international. 2014;2014:890697.
18. Caini S, Gandini S, Dudas M, Bremer V, Severi E, Gherasim A. Sexually transmitted infections and prostate cancer risk: a systematic review and meta-analysis. Cancer epidemiology. 2014;38(4):329-38.
19. Doolan G, Benke G, Giles G. An update on occupation and prostate cancer. Asian Pacific journal of cancer prevention : APJCP. 2014;15(2):501-16.
20. Mandair D, Rossi RE, Pericleous M, Whyand T, Caplin ME. Prostate cancer and the influence of dietary factors and supplements: a systematic review. Nutrition & metabolism. 2014;11:30.
21. Abrate A, Lughezzani G, Gadda GM, Lista G, Kinzikeeva E, Fossati N, et al. Clinical use of [-2]proPSA (p2PSA) and its derivatives (%p2PSA and Prostate Health Index) for the detection of prostate cancer: a review of the literature. Korean journal of urology. 2014;55(7):436-45.
22. Link RE, Shariat SF, Nguyen CV, Farr A, Weinberg AD, Morton RA, et al. Variation in prostate specific antigen results from 2 different assay platforms: clinical impact on 2304 patients undergoing prostate cancer screening. The Journal of urology. 2004;171(6 Pt 1):2234-8.
23. Aboud OA, Weiss RH. New opportunities from the cancer metabolome. Clinical chemistry. 2013;59(1):138-46.
24. Monteiro MS, Carvalho M, Bastos MdL, Pinho PG. Potentiality of volatile organic compounds to discriminate patients with cancer by using chemometric tools. Identification and Data Processing Methods in Metabolomics. Future Science Book Series: Future Science Ltd; 2015. p. 166-84.
25. Cuperlovic-Culf M, Barnett DA, Culf AS, Chute I. Cell culture metabolomics: applications and future directions. Drug discovery today. 2010;15(15-16):610-21.
26. Halama A. Metabolomics in cell culture--a strategy to study crucial metabolic pathways in cancer development and the response to treatment. Archives of biochemistry and biophysics. 2014;564:100-9.
27. Zhang A, Sun H, Xu H, Qiu S, Wang X. Cell metabolomics. Omics : a journal of integrative biology. 2013;17(10):495-501.
28. Keshari KR, Sriram R, Van Criekinge M, Wilson DM, Wang ZJ, Vigneron DB, et al. Metabolic reprogramming and validation of hyperpolarized 13C lactate as a prostate cancer biomarker using a human prostate tissue slice culture bioreactor. The Prostate. 2013;73(11):1171-81.
29. Schmidt K, Podmore I. Current Challenges in Volatile Organic Compounds Analysis as Potential Biomarkers of Cancer. Journal of biomarkers. 2015;2015:981458.
30. Shirasu M, Touhara K. The scent of disease: volatile organic compounds of the human body related to disease and disorder. Journal of biochemistry. 2011;150(3):257-66.
31. Li J, Peng Y, Liu Y, Li W, Jin Y, Tang Z, et al. Investigation of potential breath biomarkers for the early diagnosis of breast cancer using gas chromatography-mass spectrometry. Clinica chimica acta; international journal of clinical chemistry. 2014;436:59-67.
32. Poli D, Goldoni M, Corradi M, Acampa O, Carbognani P, Internullo E, et al. Determination of aldehydes in exhaled breath of patients with lung cancer by means of on-fiber-derivatisation SPME-GC/MS. Journal of chromatography B, Analytical technologies in the biomedical and life sciences. 2010;878(27):2643-51.

33. Leunis N, Boumans ML, Kremer B, Din S, Stobberingh E, Kessels AG, et al. Application of an electronic nose in the diagnosis of head and neck cancer. The Laryngoscope. 2014;124(6):1377-81.
34. Kumar S, Huang J, Abbassi-Ghadi N, Spanel P, Smith D, Hanna GB. Selected ion flow tube mass spectrometry analysis of exhaled breath for volatile organic compound profiling of esophago-gastric cancer. Analytical chemistry. 2013;85(12):6121-8.
35. Abaffy T, Moller MG, Riemer DD, Milikowski C, DeFazio RA. Comparative analysis of volatile metabolomics signals from melanoma and benign skin: a pilot study. Metabolomics. 2013;9(5):998-1008.
36. Altomare DF, Di Lena M, Porcelli F, Trizio L, Travaglio E, Tutino M, et al. Exhaled volatile organic compounds identify patients with colorectal cancer. The British journal of surgery. 2013;100(1):144-50.
37. Mochalski P, Sponring A, King J, Unterkofler K, Troppmair J, Amann A. Release and uptake of volatile organic compounds by human hepatocellular carcinoma cells (HepG2) in vitro. Cancer cell international. 2013;13(1):72.
38. Monteiro M, Carvalho M, Henrique R, Jeronimo C, Moreira N, de Lourdes Bastos M, et al. Analysis of volatile human urinary metabolome by solid-phase microextraction in combination with gas chromatography-mass spectrometry for biomarker discovery: application in a pilot study to discriminate patients with renal cell carcinoma. European journal of cancer. 2014;50(11):1993-2002.
39. Fu XA, Li M, Knipp RJ, Nantz MH, Bousamra M. Noninvasive detection of lung cancer using exhaled breath. Cancer medicine. 2014;3(1):174-81.
40. Amal H, Ding L, Liu BB, Tisch U, Xu ZQ, Shi DY, et al. The scent fingerprint of hepatocarcinoma: in-vitro metastasis prediction with volatile organic compounds (VOCs). International journal of nanomedicine. 2012;7:4135-46.
41. Arasaradnam RP, McFarlane MJ, Ryan-Fisher C, Westenbrink E, Hodges P, Thomas MG, et al. Detection of colorectal cancer (CRC) by urinary volatile organic compound analysis. PloS one. 2014;9(9):e108750.
42. Kumar S, Huang J, Cushnir JR, Spanel P, Smith D, Hanna GB. Selected ion flow tube-MS analysis of headspace vapor from gastric content for the diagnosis of gastro-esophageal cancer. Analytical chemistry. 2012;84(21):9550-7.
43. de Meij TG, Larbi IB, van der Schee MP, Lentferink YE, Paff T, Terhaar Sive Droste JS, et al. Electronic nose can discriminate colorectal carcinoma and advanced adenomas by fecal volatile biomarker analysis: proof of principle study. International journal of cancer Journal international du cancer. 2014;134(5):1132-8.
44. Steve Smith PW, Jemma Redding, Norman M. Ratcliffe, and Chris S. J. Probert. Application of Similarity Coefficients to Predict Disease Using Volatile Organic Compounds. IEEE SENSORS JOURNAL. 2010;10:92 - 6.
45. Wang JH, Byun J, Pennathur S. Analytical approaches to metabolomics and applications to systems biology. Seminars in nephrology. 2010;30(5):500-11.
46. Lenz EM, Wilson ID. Analytical strategies in metabonomics. Journal of proteome research. 2007;6(2):443-58.
47. Márcia Sá M, Márcia C, Maria de Lourdes B, Paula Guedes de P. Potentiality of volatile organic compounds to discriminate patients with cancer by using chemometric tools. Identification and Data Processing Methods in Metabolomics. Future Science Book Series: Future Science Ltd; 2015. p. 166-84.

48. Mastrangelo A, Ferrarini A, Rey-Stolle F, Garcia A, Barbas C. From sample treatment to biomarker discovery: A tutorial for untargeted metabolomics based on GC-(EI)-Q-MS. Analytica chimica acta. 2015;900:21-35.
49. Pluskal T, Castillo S, Villar-Briones A, Oresic M. MZmine 2: modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. BMC bioinformatics. 2010;11:395.
50. Berben L, Sereika SM, Engberg S. Effect size estimation: methods and examples. International journal of nursing studies. 2012;49(8):1039-47.
51. Graca G, Duarte IF, Barros AS, Goodfellow BJ, Diaz SO, Pinto J, et al. Impact of prenatal disorders on the metabolic profile of second trimester amniotic fluid: a nuclear magnetic resonance metabonomic study. Journal of proteome research. 2010;9(11):6016-24.
52. Marcias G, Erdmann E, Lapouge G, Siebert C, Barthelemy P, Duclos B, et al. Identification of novel truncated androgen receptor (AR) mutants including unreported pre-mRNA splicing variants in the 22Rv1 hormone-refractory prostate cancer (PCa) cell line. Human mutation. 2010;31(1):74-80.
53. Wishart DS, Tzur D, Knox C, Eisner R, Guo AC, Young N, et al. HMDB: the Human Metabolome Database. Nucleic acids research. 2007;35(Database issue):D521-6.
54. Wishart DS, Jewison T, Guo AC, Wilson M, Knox C, Liu Y, et al. HMDB 3.0--The Human Metabolome Database in 2013. Nucleic acids research. 2013;41(Database issue):D801-7.
55. Wishart DS, Knox C, Guo AC, Eisner R, Young N, Gautam B, et al. HMDB: a knowledgebase for the human metabolome. Nucleic acids research. 2009;37(Database issue):D603-10.
56. Dunja Zimmermann MH, Mary Pat Moyer, Jurgen Nolte, and Jorg Ingo Baumbach. Determination of volatile products of human colon cell line metabolism by GC/MS analysis. Metabolomics. 2007;3:13-7.
57. Sponring A, Filipiak W, Ager C, Schubert J, Miekisch W, Amann A, et al. Analysis of volatile organic compounds (VOCs) in the headspace of NCI-H1666 lung cancer cells. Cancer biomarkers : section A of Disease markers. 2010;7(3):153-61.
58. Wojciech F, Pawel M, Anna F, Clemens A, Raquel C, Davis CE, et al. A compendium of volatile organic compounds (VOCs) released by human cell lines. Curr Med Chem. 2016.
59. Filipiak W, Sponring A, Mikoviny T, Ager C, Schubert J, Miekisch W, et al. Release of volatile organic compounds (VOCs) from the lung cancer cell line CALU-1 in vitro. Cancer cell international. 2008;8:17.
60. Davies MP, Barash O, Jeries R, Peled N, Ilouze M, Hyde R, et al. Unique volatolomic signatures of TP53 and KRAS in lung cells. British journal of cancer. 2014;111(6):1213-21.
61. Haick H, Broza YY, Mochalski P, Ruzsanyi V, Amann A. Assessment, origin, and implementation of breath volatile cancer markers. Chemical Society reviews. 2014;43(5):1423-49.
62. Hakim M, Broza YY, Barash O, Peled N, Phillips M, Amann A, et al. Volatile organic compounds of lung cancer and possible biochemical pathways. Chemical reviews. 2012;112(11):5949-66.
63. Zhang Y, Gao G, Liu H, Fu H, Fan J, Wang K, et al. Identification of volatile biomarkers of gastric cancer cells and ultrasensitive electrochemical detection based on sensing interface of Au-Ag alloy coated MWCNTs. Theranostics. 2014;4(2):154-62.
64. Wallqvist A, Monks A, Rabow AA, Thanki N, Shoemaker RH, Covell DG. Mining the NCI screening database: explorations of agents involved in cell cycle regulation. Progress in cell cycle research. 2003;5:173-9.
65. Peng G, Hakim M, Broza YY, Billan S, Abdah-Bortnyak R, Kuten A, et al. Detection of lung, breast, colorectal, and prostate cancers from exhaled breath using a single array of nanosensors. British journal of cancer. 2010;103(4):542-51.

66. Struck-Lewicka W, Kordalewska M, Bujak R, Yumba Mpanga A, Markuszewski M, Jacyna J, et al. Urine metabolic fingerprinting using LC-MS and GC-MS reveals metabolite changes in prostate cancer: A pilot study. Journal of pharmaceutical and biomedical analysis. 2015.
67. Wu H, Liu T, Ma C, Xue R, Deng C, Zeng H, et al. GC/MS-based metabolomic approach to validate the role of urinary sarcosine and target biomarkers for human prostate cancer by microwave-assisted derivatization. Analytical and bioanalytical chemistry. 2011;401(2):635-46.
68. Kwak J, Gallagher M, Ozdener MH, Wysocki CJ, Goldsmith BR, Isamah A, et al. Volatile biomarkers from human melanoma cells. Journal of chromatography B, Analytical technologies in the biomedical and life sciences. 2013;931:90-6.
69. Zang X, Jones CM, Long TQ, Monge ME, Zhou M, Walker LD, et al. Feasibility of detecting prostate cancer by ultraperformance liquid chromatography-mass spectrometry serum metabolomics. Journal of proteome research. 2014;13(7):3444-54.
70. Pereira DM, Vinholes J, de Pinho PG, Valentao P, Mouga T, Teixeira N, et al. A gas chromatography-mass spectrometry multi-target method for the simultaneous analysis of three classes of metabolites in marine organisms. Talanta. 2012;100:391-400.
71. Costello LC, Franklin RB. Concepts of citrate production and secretion by prostate. 1. Metabolic relationships. The Prostate. 1991;18(1):25-46.
72. Costello LC, Franklin RB. Prostatic fluid electrolyte composition for the screening of prostate cancer: a potential solution to a major problem. Prostate cancer and prostatic diseases. 2009;12(1):17-24.
73. Pertega-Gomes N, Baltazar F. Lactate transporters in the context of prostate cancer metabolism: what do we know? International journal of molecular sciences. 2014;15(10):18333-48.
74. Ramautar R, Berger R, van der Greef J, Hankemeier T. Human metabolomics: strategies to understand biology. Current opinion in chemical biology. 2013;17(5):841-6.
75. Lucarelli G, Rutigliano M, Galleggiante V, Giglio A, Palazzo S, Ferro M, et al. Metabolomic profiling for the identification of novel diagnostic markers in prostate cancer. Expert review of molecular diagnostics. 2015;15(9):1211-24.
76. Wang Q, Hardie RA, Hoy AJ, van Geldermalsen M, Gao D, Fazli L, et al. Targeting ASCT2-mediated glutamine uptake blocks prostate cancer growth and tumour development. The Journal of pathology. 2015;236(3):278-89.
77. Kufe DW. Mucins in cancer: function, prognosis and therapy. Nature reviews Cancer. 2009;9(12):874-85.
78. Kohata K, Abbas SA, Matta KL. Synthetic mucin fragments: methyl 3-O-(2-acetamido-2-deoxy-beta-D-galactopyranosyl-beta-D- 3-O-(2-acetamido-2-deoxy-3-O-beta-D-galactopyranosyl-beta-D-glucopyranosyl)-beta-D-galactoyranoside. pyranosyl)-beta-D-galactopyranoside. Carbohydrate research. 1984;132(1):127-35.
79. Cernei N, Heger Z, Gumulec J, Zitka O, Masarik M, Babula P, et al. Sarcosine as a potential prostate cancer biomarker--a review. International journal of molecular sciences. 2013;14(7):13893-908.
80. Miyagi Y, Higashiyama M, Gochi A, Akaike M, Ishikawa T, Miura T, et al. Plasma free amino acid profiling of five types of cancer patients and its application for early detection. PloS one. 2011;6(9):e24143.
81. Teahan O, Bevan CL, Waxman J, Keun HC. Metabolic signatures of malignant progression in prostate epithelial cells. The international journal of biochemistry & cell biology. 2011;43(7):1002-9.
82. Eley HL, Russell ST, Baxter JH, Mukerji P, Tisdale MJ. Signaling pathways initiated by beta-hydroxy-beta-methylbutyrate to attenuate the depression of protein synthesis in skeletal muscle

in response to cachectic stimuli. American journal of physiology Endocrinology and metabolism. 2007;293(4):E923-31.
83. Thysell E, Surowiec I, Hornberg E, Crnalic S, Widmark A, Johansson AI, et al. Metabolomic characterization of human prostate cancer bone metastases reveals increased levels of cholesterol. PloS one. 2010;5(12):e14175.
84. Awad AB, Fink CS, Williams H, Kim U. In vitro and in vivo (SCID mice) effects of phytosterols on the growth and dissemination of human prostate cancer PC-3 cells. European journal of cancer prevention : the official journal of the European Cancer Prevention Organisation. 2001;10(6):507-13.
85. Zhou X, Mao J, Ai J, Deng Y, Roth MR, Pound C, et al. Identification of plasma lipid biomarkers for prostate cancer by lipidomics and bioinformatics. PloS one. 2012;7(11):e48889.
86. Swanson MG, Keshari KR, Tabatabai ZL, Simko JP, Shinohara K, Carroll PR, et al. Quantification of choline- and ethanolamine-containing metabolites in human prostate tissues using 1H HR-MAS total correlation spectroscopy. Magnetic resonance in medicine : official journal of the Society of Magnetic Resonance in Medicine / Society of Magnetic Resonance in Medicine. 2008;60(1):33-40.
87. Mintz A, Wang L, Ponde DE. Comparison of radiolabeled choline and ethanolamine as probe for cancer detection. Cancer biology & therapy. 2008;7(5):742-7.
88. Saylor PJ, Karoly ED, Smith MR. Prospective study of changes in the metabolomic profiles of men during their first three months of androgen deprivation therapy for prostate cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2012;18(13):3677-85.