EXPERT REVIEWS

Metabolomic profiling for the identification of novel diagnostic markers in prostate cancer

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Metabolomic profiling offers a powerful methodology for understanding the perturbations of biochemical systems occurring during a disease process. During neoplastic transformation, prostate cells undergo metabolic reprogramming to satisfy the demands of growth and proliferation. An early event in prostate cell transformation is the loss of capacity to accumulate zinc. This change is associated with a higher energy efficiency and increased lipid biosynthesis for cellular proliferation, membrane formation and cell signaling. Moreover, recent studies have shown that sarcosine, an *N*-methyl derivative of glycine, was significantly increased during disease progression from normal to localized to metastatic prostate cancer. Mapping the metabolomic profiles to their respective biochemical pathways showed an upregulation of androgen-induced protein synthesis, an increased amino acid metabolism and a perturbation of nitrogen breakdown pathways, along with high total choline-containing compounds and phosphocholine levels. In this review, the role of emerging biomarkers is summarized, based on the current understanding of the prostate cancer metabolome.

Keywords: androgen receptor • biomarker • metabolomics • prostate cancer • sarcosine

Prostate cancer (PCa) is the most common male malignancy, and the second leading cause of cancer death in American men, behind only lung cancer. Recent estimates have calculated that in 2015, 220,800 new cases will be diagnosed and 27,540 patients will die of PCa in the USA [1]. PCa is usually suspected on the basis of digital rectal examination (DRE) and/or prostate-specific antigen (PSA) levels. However, this biomarker is organ- but not cancer-specific, and may be elevated in many non-malignant clinical conditions. Indeed, because of the low specificity of PSA, up to 75% of men with PSA levels of 2–10 ng/ml and/or a suspicious DRE have a negative first biopsy [2,3]. Because early diagnosis improves treatment efficacy and the quality of life, as well as reducing the cost for disease management, new biomarkers have been evaluated to increase the sensitivity and specificity for PCa diagnosis and prognosis, and reduce the number of unnecessary biopsies. These biomarkers include Prostate Cancer Antigen 3 (PCA3), the TMPRSS2-ERG gene fusion, Spondin 2, and circulating tumor cells [4–10].

Many studies suggest that an altered metabolism is involved in the development of cancer [11,12]. Moreover, many genes implicated in cancer pathogenesis play an important role in controlling cell metabolism [13].

High-throughput analysis of low-molecular-weight metabolites allows global assessment of a cellular state in normal and pathological conditions. Metabolomics is the comprehensive analysis of the complete set of metabolic products in a cell, tissue, organ or organism [14,15]. This approach can be used to define the 'metabolic fingerprint' of a tumor and identify novel biomarkers that may be potentially useful for both early diagnosis and monitoring the therapeutic response. Metabolite-based biomarkers are currently used in clinical practice for PCa diagnostic imaging. For example, ^{18}F-fluorodeoxyglucose and ^{18}F-fluorocholine are two positron-emission tomography radiotracers commonly utilized for PCa detection, based

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Table 1. Advantages and disadvantages for nuclear magnetic resonance and mass spectrometry.

	Nuclear magnetic resonance	Mass spectrometry
Detection limits	Micromolar at typical observation frequencies. Nanomolar using cryoprobes	Picomolar with standard techniques. Much lower with special techniques
Sample handling	Whole sample analyzed in one measurement	Different conditions for different classes of metabolites
Analytic reproducibility	Very high	Low
Sample preparation	Minimal	Extensive
Sensitivity	Lower	Higher
Cost	Low	High
Availability of databases	Not yet comprehensive	Comprehensive

on an elevated glucose and choline metabolism in malignant tissue compared with normal tissue [16–18]. In addition, other metabolic compounds that have been correlated with PCa progression are under-screening for diagnostic imaging in preclinical models [19–21].

In this article, we review the role of emerging biomarkers, based on the current understanding of the PCa metabolome.

Methods used for metabolomic profiling

Multiple analytic platforms have been used to profile metabolites in biological samples. These include HPLC, nuclear magnetic resonance (NMR), mass spectrometry (MS, which requires an initial separation of metabolites by gas or liquid chromatography – GC/MS and LC/MS) and ELISA [22–25]. HPLC identifies compounds based solely on their chromatographic retention time; the main limitation of this technique is the need for external standards.

MS and NMR have evolved as the most common techniques in metabolomics studies, but each has advantages and limitations (Table 1). Advantages of NMR include minimal sample preparation, very high analytical reproducibility, low cost, the possibility to quantify metabolites, and the identification of unknown intermediates. The high reproducibility of NMR-based techniques and the high sensitivity and selectivity of MS-based techniques make these methods superior to other analytical techniques for metabolomic profiling. Compared with NMR, MS is superior for the identification of secondary metabolites. Moreover, the use of different operational principles in MS technologies, such as different ionization techniques and mass analyzer technology, increases the number of metabolites that can be detected. Even if MS has a much higher sensitivity in the detection of metabolites compared with NMR, major limitations include more expensive sample preparation and low analytical reproducibility. In addition, the derivatization processes that are used to improve analytical capabilities can result in metabolites degradation.

The high reproducibility and noninvasive characteristics of NMR offer advantages in metabolomics research. In particular, any metabolite pathway investigated *in vitro* by MRI can be studied *in vivo* by means of magnetic resonance spectroscopy. Moreover, an NMR-based metabolomics approach involving isotope labeled tracers, such as ^{13}C and ^{15}N can be used to monitor the flow of compounds through metabolic pathways.

ELISA is limited by the need for specific antibodies, and it can be challenging to generate these, especially for small molecules. Moreover, some problems have been reported for the type of samples used for analysis. For example, for sarcosine evaluation, commercial fluorometric assays are available that use sarcosine oxidase (SO), which is quite specific for sarcosine detection (there is some cross reactivity with sarcosine analogs, such as ethyl or methyl-glycine, but they show a much poorer activity). The problem with fluorometric assays arises when these methods are used for measurements of sarcosine in urine because of the large amount of reducing equivalents, which cause a diminished response. This is because sarcosine oxidase (SO) generates H_2O_2 that is subsequently utilized by horseradish peroxidase. In urine, much of the H_2O_2 is diverted to oxidation of the reducing substances, with the result that the stoichiometry of Red Probe oxidation for sarcosine is much <1. This problem has not been reported for serum.

Finally, a novel technique called dynamic nuclear polarization (hyperpolarization) of 13C-labeled cell substrates has recently been introduced as a noninvasive and well-tolerated method for visualizing alterations in tumor metabolism. In particular, Nelson *et al.* [26] conducted a first-in-man study to evaluate the safety and feasibility of hyperpolarized [1-13C] pyruvate as an imaging agent in PCa. This technique revealed tumors in all patients included in the study; moreover, three-dimensional imaging revealed tumor cells in regions of the prostate that had been deemed tumor-free using conventional imaging methods. Imaging living systems with hyperpolarized agents can yield a >10,000-fold enhanced signal compared with conventional magnetic resonance imaging, and could provide a

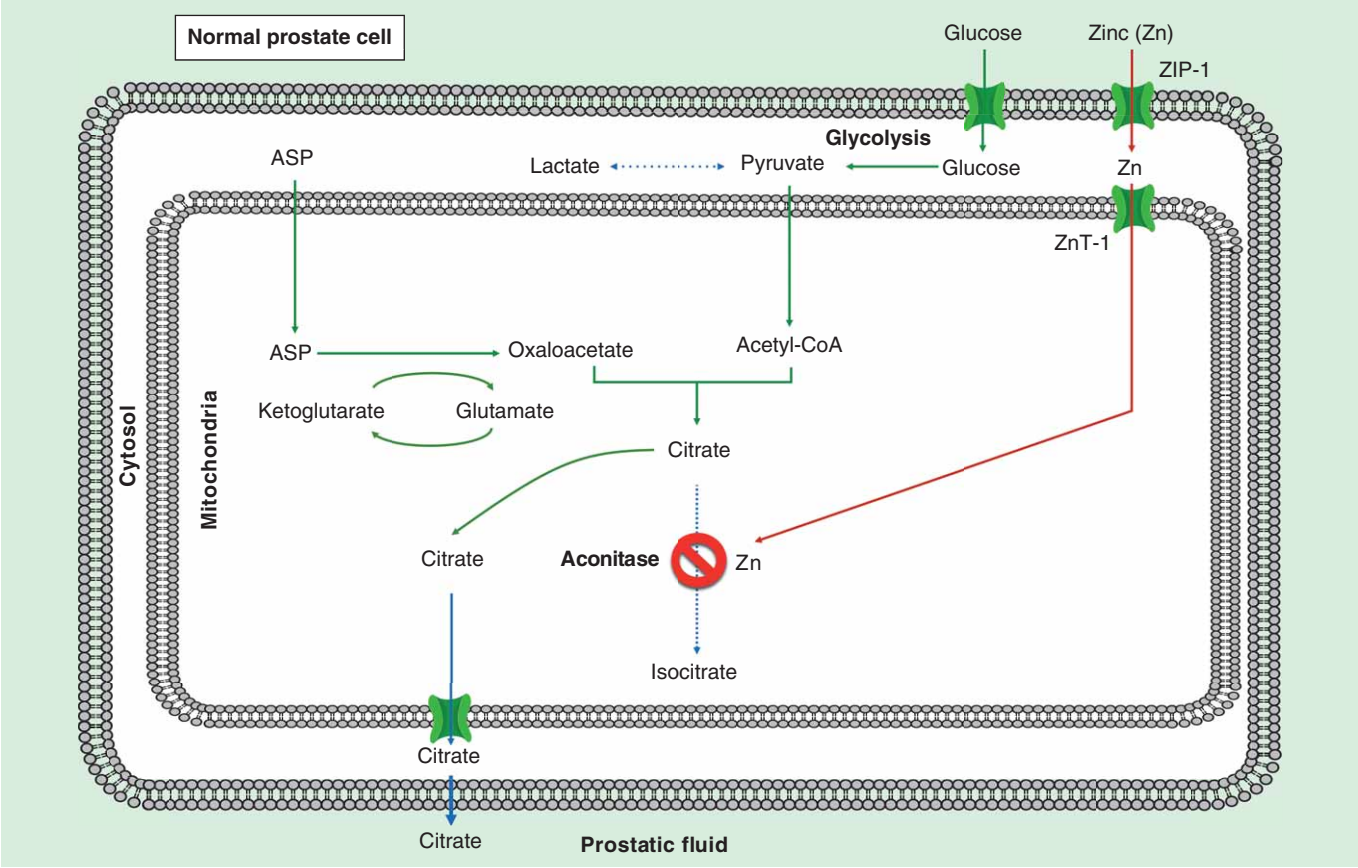

Figure 1. Citrate production in a normal prostate cell.

new tool for early PCa detection and treatment response monitoring.

General metabolic characteristics of normal & tumor prostate cells

Many studies have shown that normal prostate cells have a particular metabolic profile characterized by an increased production of citrate and polyamines that are components of the prostatic fluid. According to Costello *et al.* [27], the capability of normal prostate cells to produce and secrete high levels of citrate requires a particular regulation of metabolic pathways that are not generally observed in other normal human cells. Moreover, the accumulation of zinc in these citrate-producing cells is an important factor associated with prostate metabolic regulation. In normal eukaryotic cells, glucose is converted via glycolysis to pyruvate, which enters the mitochondria and is oxidized to acetyl-CoA. Next, a two-carbon acetyl group from acetyl-CoA is transferred to the four-carbon acceptor compound (oxaloacetate) to form a six-carbon compound (citrate). The citrate then goes through a series of chemical transformations (Krebs cycle), losing two carboxyl groups as CO_2, while four carbons are conserved as oxaloacetate that is therefore regenerated. In normal prostate cells, citrate is an end product of metabolism, rather than a metabolic intermediate [28]. This characteristic is due to the inhibition by zinc of the mitochondrial enzyme aconitase, which truncates the Krebs cycle at the first step of citrate oxidation [28]. Therefore, for citrate to accumulate in prostate cells, its upstream intermediates (namely acetyl-CoA and oxaloacetate) must be available. Acetyl-CoA derives from glucose metabolism via glycolysis, whereas oxaloacetate is produced through the aspartate-glutamate-citrate pathway [29]. This metabolic adaptation has an important role in the cell energy balance. In fact, compared with other cell types, citrate-producing prostate cells produce about 60% less ATP from complete glucose oxidation (Figure 1).

During neoplastic transformation, prostate cells undergo metabolic reprogramming to satisfy the demands of growth and proliferation. An early event in prostate cells transformation is the loss of capacity to accumulate zinc, due in part to a genetic alteration in the expression of zinc transporters [30]. This change leads to the restoration of mitochondrial aconitase activity and consequent citrate oxidation via the Krebs cycle, and an increased generation of ATP. Therefore, in PCa cells, the loss of zinc accumulation is associated with a higher energy efficiency, as a consequence of the citrate metabolism by means of the Krebs cycle that produces the additional 24 ATP from complete glucose oxidation.

Zinc is a cofactor of >300 enzymes that have structural, catalytic and regulatory roles. Two families of zinc transporters have been identified: the ZIP (Zrt/IRT-like proteins) family,

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Figure 2. Major biological roles of zinc.
PCa: Prostate cancer.

which imports zinc from extracellular compartments; and the ZnT (Zn Transporter) family, which exports zinc and/or redistributes it intracellularly in mitochondria and lysosomes. Among the 14 mammalian ZIP proteins, only four members (ZIP1-ZIP4) have been identified on the human cell plasma membrane. All these proteins are significantly downregulated in PCa as compared with normal tissue [31–35]. Moreover, it has been shown that ZIP1 and ZIP4 overexpression in DU145 and PC3 PCa cell lines reduces their tumorigenic potential and inhibits cell proliferation and invasiveness [36,37]. Conversely, high levels of the ZnT-1 transporter have been reported in LNCaP and PC-3 cell lines, in accordance with its role as a zinc exporting protein [38].

In addition to its inhibitory effects on mitochondrial aconitase, this metal ion has a role in regulating other biological processes, such as cell division, intracellular signaling, apoptosis, cell migration, and invasion (Figure 2). Liang *et al.* [39] showed that an accumulation of zinc in PCa cells resulted in a marked inhibition of cell growth. Flow cytometric analyses revealed a dramatic increase of the cancer cell population in the G2/M phase, and a decreased proportion of cells in the S phase, indicating a G2/M phase arrest. These findings were associated with an increase in apoptosis, and in mRNA levels of p21 (Waf1/Cip1/Sdi1), in both LNCaP and PC-3 cells [39]. Moreover, these authors suggested that the inhibitory effect of zinc could be mediated by its interaction with the $p13^{suc1}$ subunit of Cdc2 kinase [39]. Uzzo *et al.* [40] demonstrated that physiological levels of zinc inhibit NF-kB activity in DU-145 and PC-3 human PCa cells. NF-kB is a nuclear transcription factor that regulates the expression of multiple genes involved in tumor growth, metastasis, and angiogenesis. Its inhibition after treatment of cancer cells with zinc was associated with a reduced expression of VEGF, IL-6, IL-8, and MMP-9. Conversely, Golovine *et al.* [41] showed that a zinc depletion in PCa cells caused an increased expression of these tumorigenic cytokines via the NF-kB-dependent pathway. Zinc deficiency was also associated with high levels of PKB/Akt and Mdm2 phosphorylation, and with a reduced nuclear accumulation of p53 and p21. The authors concluded that the Akt-p21 signaling axis was responsible for cell survival in zinc deficiency PCa cells [42]. It has been shown that elevated zinc concentrations have pro-apoptotic effects, because zinc is able to induce the mitochondrial release of cytochrome c, the activation of caspase-9 and caspase-3, and the cleavage of poly(ADP-ribose) polymerase [43,44]. Moreover, treatment of PCa cells with this ion reduces the expression of intercellular adhesion molecules, such as Intercellular adhesion molecule 1 (ICAM-1) [40], impairs cancer cell ability to invade Matrigel [45], and inhibits the activity of Aminopeptidase N, a protease that degrades collagen type IV and plays a role in invasion and metastasis [46].

Another important metabolic adaptation observed in PCa cells is the increased lipid biosynthesis for cellular proliferation, membrane formation and cell signaling. In particular, *de novo* lipogenesis and cholesterogenesis are sustained by the conversion in the cytosol of citrate to acetyl-CoA by ATP citrate lyase (Figure 3). These findings are consistent with the results of recent studies that have demonstrated androgen-regulation and overexpression of a number of fatty acids and cholesterol synthesis enzymes in PCa [47,48]. In this context, it has been reported that sterol regulatory element-binding protein-1 (SREBP-1) – a critical transcription factor for lipogenesis – is involved in the transcriptional regulation of androgen receptor (AR) and formation of fatty acids through an altered expression of fatty acid synthase [49]. In addition, Huang *et al.* [50] showed that sterol regulatory element-binding protein-1 (SREBP-1) induced PCa cell proliferation, migration, and invasion by activating lipogenesis and through an increased production of reactive oxygen species and NADPH oxidase 5 expression.

Other metabolic changes involve choline- and ethanolamine-containing metabolites, which are the main precursors and degradation products of membrane phospholipids. High levels of phosphocholine, phosphoethanolamine, and glycerophosphocholine have been observed in PCa and these findings are consistent with active membrane remodeling and cellular proliferation processes [51].

Using an unbiased functional genetic approach to investigate metabolic adaptations that differentiate Pca cells from normal cells, Ros *et al.* [52] identified two genes (PRKAB1 and PFKFB4) that were selectively required for PCa cells survival.

PRKAB1 encodes for a regulatory subunit of AMP-activated protein kinase (AMPK), a highly conserved sensor of the cellular energy status that protects cells from stresses that cause ATP depletion by switching off ATP-consuming biosynthetic

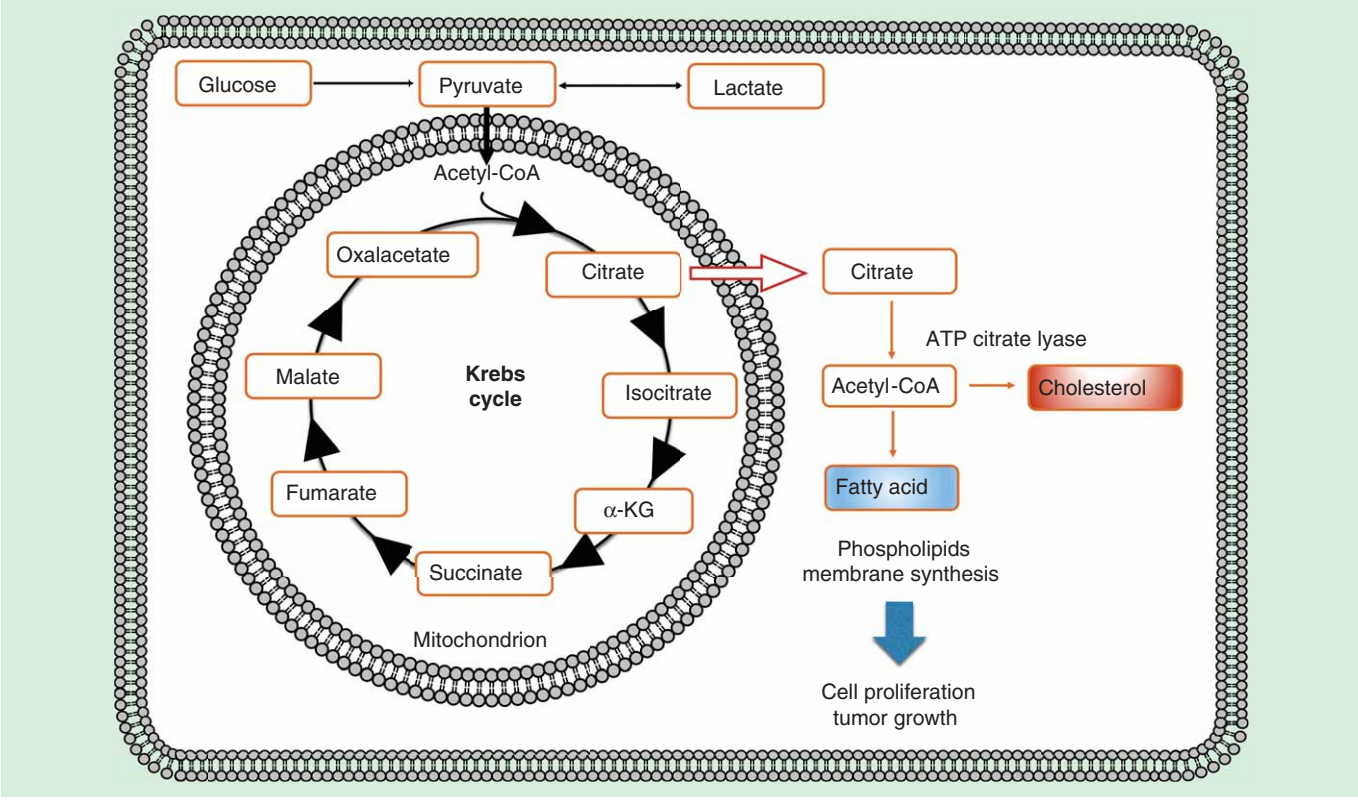

Figure 3. *De novo* lipogenesis and cholesterogenesis in a prostate cancer cell.
DMGDH: Dimethylglycine dehydrogenase; GNMT: Glycine-N-methyltransferase; SARDH: Sarcosine dehydrogenase; AR and ERG denote the binding sites for the androgen receptor and ERG transcription factor, respectively.

pathways. It has been shown that mice with a loss of LKB1 – an AMPK upstream kinase – develop atypical hyperplasia and prostate intraepithelial neoplasia (PIN) [53]. The pivotal role of the LKB1-AMPK axis in controlling oncogenic pathways is mainly due its crosstalk with the PI3K, mTOR, and MAPK pathways [54]. In addition, it was shown that AR signaling promoted PCa mitochondrial biogenesis and growth through an AMPK signaling cascade [55].

We recently demonstrated that LKB1 expression was significantly decreased in human PCa and that LKB1 protein levels decreased throughout advancing prostate carcinogenesis, with a significant reduction already evident in high-grade PIN lesions and a complete loss in adenocarcinomas [56]. Moreover, we showed the existence of an inverse correlation between the activity of the LKB1-AMPK pathway and p38 MAPK signaling, and suggested that LKB1 might be used as a predictive marker of therapeutic response to p38 inhibitors in PCa patients [56].

The second gene identified in the study by Ros *et al.* [52] was PFKFB4, which encodes for 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4, an isoform of the glycolytic enzyme phosphofructokinase 2. PFKFB4 mRNA was found highly increased in metastatic PCa compared with localized tumors and this enzyme was shown to have a fundamental role in PCa cells survival by controlling glycolysis and antioxidant production.

Another important anaplerotic source by which PCa cells support the pools of Krebs cycle intermediates and lipogenesis is glutamine that is converted to glutamate and then to α-ketoglutarate (glutaminolysis). To sustain the glutamine metabolism, PCa cells increase the expression of the glutamine transporter ASCT2 (SLC1A5), and of glutaminase, an enzyme that converts glutamine to glutamate [57,58].

Finally, it has been shown that lactate and alanine concentrations are significantly elevated in PCa compared with benign prostate tissue [59], and these findings suggest that a metabolic shift occurs, characterized by an increased glycolysis and lactate production regardless of oxygen availability (the Warburg effect). In addition to glycolysis, alternative glucose metabolic pathways have a fundamental role in promoting cancer cell growth. Among these, the pentose phosphate pathway (PPP) is a pathway that, starting from glucose-6-phosphate, generates precursors for nucleotide biosynthesis and NADPH for anabolic reactions and redox homeostasis maintenance [60,61]. Glucose-6-phosphate dehydrogenase (G6PDH) is the rate-limiting enzyme of the PPP, and recent studies have showed that G6PDH levels and metabolism through this pathway are increased in PCa [52,62]. Recently, Tsouko *et al.* [62] demonstrated that G6PDH, NADPH, and ribose synthesis were all increased by AR signaling in PCa. Moreover, the increased flux through the PPP was sustained

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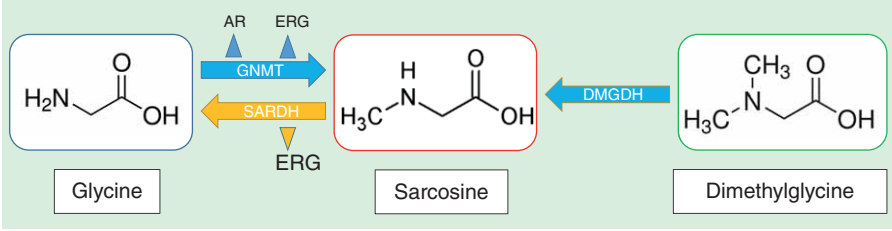

Figure 4. Diagram illustrating enzymes involved in the synthesis of sarcosine.
DMGDH: Dimethylglycine dehydrogenase; GNMT: Glycine-N-methyltransferase; SARDH: Sarcosine dehydrogenase; AR and ERG denote the binding sites for the androgen receptor and ERG transcription factor, respectively.

by the mammalian target of rapamycin (mTOR)-mediated upregulation of G6PDH.

The tumor micro-environment plays a fundamental role in tumor initiation, progression and metastasis. In this regard, 'cancer-associated fibroblasts' (CAF) actively interact with neoplastic cells and form a myofibroblastic microenvironment that promotes cancer growth and survival. Pavlides *et al.* [63] hypothesized that epithelial cancer cells could induce the Warburg effect in neighboring CAFs and that these stromal cells, after myofibroblastic differentiation, could secrete lactate and pyruvate. Epithelial cancer cells could then uptake these metabolites and incorporate them into Krebs cycle for efficient energy production. These authors termed this new model of tumor-stroma metabolic cross-talk a 'Reverse Warburg Effect' [63].

This model was validated in PCa in a study by Fiaschi *et al.* [64] that demonstrated the existence of a reciprocal metabolic reprogramming between CAFs and PCa cells. In particular, these authors showed that after activation, CAFs exhibit a Warburg metabolic shift that is redox and HIF-1 dependent, with the extrusion of lactate by the MCT4 transporter. This catabolite shuttles back to PCa cells, where it is uploaded through the MCT1 transporter, and used for fueling Krebs cycle, as well as anabolic processes and cell proliferation.

Exploring the prostate cancer metabolome

In a proof-of-concept study published in 2009 in Nature, sarcosine, an *N*-methyl derivative of glycine, was identified as a metabolite that shows a progressive increase in benign, through localized, to metastatic PCa [65]. Using a combination of liquid and gas chromatography-based MS, Sreekumar *et al.* [65] profiled 1126 metabolites across 262 different prostate-related samples (tissue, urine, and serum samples). Class-specific coordinated metabolite patterns were examined using the Oncomine Concept Map, a bioinformatics tool that permits systematic linkages of metabolomic signatures to molecular concepts, generating novel hypotheses about the biological progression of PCa. Mapping the metabolomic profiles to their respective biochemical pathways showed an up-regulation of androgen-induced protein synthesis, an increased amino acid metabolism and a perturbation of nitrogen breakdown pathways, along with high total choline-containing compounds and phosphocholine levels [66,67]. Moreover, the metabolomic profiles for compounds that were over-expressed in metastatic samples demonstrated a strongly elevated methyltransferase activity. This enhanced methylation potential was supported by a significant elevation in the levels of S-adenosyl methionine (SAM), the involvement of the SET domain containing proteins and an increased histone-lysine *N*-methyltransferase activity in the metastatic samples.

These findings were in accordance with previous studies that showed an increase in the enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2)-mediated histone methyl transferase activity in PCa [68–72]. Among the perturbed metabolites, sarcosine was significantly increased during disease progression from normal through localized to metastatic PCa, and was detectable in urine. One of the major pathways for sarcosine generation involves the transfer of the methyl group from SAM to glycine, a reaction catalyzed by glycine-*N*-methyltransferase (GNMT; Figure 4). Using siRNA directed against GNMT, Sreekumar *et al.* showed that sarcosine generation was important for the cell invasion process. Similar findings were reported after knockdown of dimethylglycine dehydrogenase, a gene that encodes for another sarcosine-generating enzyme. Unlike GNMT, knockdown of sarcosine dehydrogenase in benign prostate epithelial cells (RWPE cell line) resulted in the induction of an invasive phenotype. These results were confirmed in a subsequent study that also showed that elevated levels of this metabolite boost tumor progression, mediating PCa cell invasion and intravasation *in vivo* [73].

A recent study reported higher expression levels of GNMT in PCa compared with normal tissues and showed that patients with a high cytoplasmic enzyme expression had significantly lower disease-free survival rates than patients with low levels [74]. Moreover, a persistent activation of the AR signaling and chromosomal rearrangements that result in a high level expression of ETS gene family members (ERG, ETV1) have been shown to be common events in PCa progression [6,75–78]. Chromatin immunoprecipitation sequencing revealed a direct binding of the AR and ERG to the GNMT promoter, while knockdown of TMPRSS2–ERG gene fusion in the VCaP cell line resulted in a decrease of sarcosine levels. Therefore, these findings directly link activation of the sarcosine pathway to AR and ETS gene fusion regulation.

Sreekumar *et al.* [65] also validated the increased levels of four additional metabolites in prostate-derived samples. In particular, levels of cysteine, glutamic acid, glycine and thymine, all increased during progression from benign to localized PCa to metastatic disease, while citrate levels were reduced upon disease progression.

To evaluate the potential of sarcosine as a novel diagnostic biomarker for PCa, levels were measured in urine from patients at risk for PCa who underwent prostate biopsy. Receiver operating characteristics (ROC) curve analysis demonstrated a modest predictive performance of urinary sarcosine in

differentiating biopsy-positive from biopsy-negative patients. However, when the analysis was restricted to patients with PSA values in the gray zone of 2–10 ng/ml, urinary sarcosine outperformed PSA in differentiating biopsy-positive PCa patients from biopsy-negative controls, with an area under the curve (AUC) of 0.69 (95% CI: 0.55–0.84) compared with an AUC of 0.53 (95% CI: 0.37–0.69) for total PSA.

In the wake of these findings, many studies were published to evaluate the potential role of sarcosine in PCa. Sreekumar *et al.* [79] validated their results in an independent cohort of patients and confirmed their previous results of higher levels of sarcosine in PCa patients. Cao *et al.* [80] evaluated sarcosine levels in urine supernatants and sediments and used PCa antigen 3 (PCA3) and %fPSA as comparators. Regardless of the specimen type, sarcosine was significantly higher in PCa patients than in controls, but there was no correlation with the Gleason score or clinical stage. Moreover, ROC curve analysis showed that none of the sarcosine algorithms (AUC = 0.69) had a significantly higher diagnostic power than that of serum PSA (AUC = 0.53) nor significantly lower than PCA3 (AUC = 0.70) and %fPSA (AUC = 0.71). However, when sarcosine was combined with PCA3 or %fPSA, the combined model had a higher predictive value (AUC = 0.77 and AUC = 0.76, respectively). These results suggest that even if the predictive power of urinary sarcosine alone is modest, in association with other tumor markers it could increase the sensitivity and specificity for PCa diagnosis. Later studies provided evidence that serum sarcosine had a higher predictive value (AUC = 0.66) than total PSA (AUC = 0.53) and %fPSA (AUC = 0.58) in detecting PCa in patients with total serum PSA <4 ng/ml [81]. Moreover, it was shown that serum sarcosine had the largest AUC in predicting low-grade, low-PSA PCa, suggesting that this marker may be a further tool, not only for diagnosing PCa in patients with normal PSA values but also for selecting candidates for active surveillance [9,81]. In addition, elevated circulating sarcosine levels have been demonstrated in patients with metastatic castration-resistant PCa compared with patients with non-metastatic disease [82]. In particular, Kaplan–Meier curves demonstrated clear differences in overall survival (OS) and progression-free survival (PFS) between patients with high versus low serum sarcosine levels. At multivariate analysis, this metabolite remained an independent prognostic indicator of outcome for OS and PFS.

In the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial, a positive association was identified between elevated serum sarcosine and PCa [83]. In particular, the findings of this large prospective study suggest that serum sarcosine can be an early biomarker of PCa, specifically of non-aggressive disease, and can have a stronger effect among men with diabetes and among smokers [83]. Recently, Mondul *et al.* [84] reported the results of a prospective metabolomics profiling study of PCa within the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study. Serum metabolomics profiling was performed in 200 cases of incident PCa (including 100 aggressive cases) matched with 200 controls. A strong inverse association between energy and lipid metabolites and aggressive PCa was observed. Inositol-1-phosphate glycerophospholipids and fatty acids (such as leoyl-linoleoyl-glycerophosphoinositol, 1-stearoylglycerophosphoglycerol, stearate, and docosadienoate) showed the strongest inverse association. Moreover, high levels of thyroxine and trimethylamine oxide, and low levels of citrate and α-ketoglutarate, were associated with aggressive disease.

Androgens and AR play an important role not only in the development and function of normal prostate but also in the development and progression of PCa [75,76]. Hormonal therapies for advanced PCa target AR-mediated functions by suppressing the production of androgens and/or androgen binding to the AR. Although these therapies often result in a period of clinical regression, they are not curative because castration-resistant disease (CRPC) progression occurs. Moreover, much evidence has shown that CRPC continues to require AR signaling, despite the reduction in circulating androgen levels [85–97].

In the past years, it has become clear that different mechanisms occur through which AR can be inappropriately reactivated, also in the presence of AR antagonists. In general, the theories addressing the mechanisms by which CRPC develops incorporate the concept of a continued AR signaling via alternative pathways, or suggest AR-independent mechanisms [97,98]. The resistance to AR antagonists is a poorly understood CRPC characteristic. One explanation may be that AR antagonists can no longer compete for AR binding due to increased intracellular testosterone levels or molecular changes that have increased the AR affinity for its ligands. Recent data suggest that testosterone and other androgens can be biosynthesized de novo within PCa from cholesterol or other ubiquitous precursors through the expression of genes encoding for steroidogenic enzymes [85–89]. Amplification of the AR gene has been proposed as another mechanism for CRPC and highlights the strong selective pressure for continued AR signaling as PCa evolves in a depleted androgen environment [90–92]. It leads to an overexpression of the receptor, hypersensitivity to androgenic ligands and could explain the development of resistance to androgen deprivation therapy (ADT). Moreover, patients with AR-amplified tumors have a longer response duration to androgen deprivation therapy (ADT) and an increased likelihood of response to second-line hormone treatment, as compared with those without AR amplification [99]. Functional analysis of mutant AR has shown that the majority of AR mutations are of gain-of-function type [100]. This type of mutations is considered to hypersensitize the receptor to lower concentrations of testosterone and/or make it responsive to other steroid hormones. Mutations in the AR can induce structural changes that might lead to an alteration in the ligand-binding domain and trans-activation specificities of the receptor. The first AR mutation of this type was discovered in the LNCaP cell line. The substitution of alanine for threonine at position 877 (T877A) generates a receptor that is activated by various non-androgenic steroid hormones, such as estradiol and progesterone [101]. In a similar way, the L701H mutant receptor (leucine-to-histidine substitution at amino acid 701) has a reduced affinity for

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dihydrotestosterone but binds other adrenal corticosteroids, particularly the glucocorticoids cortisol and cortisone [102]. An alternative mechanism of resistance to AR antagonists, such as bicalutamide is that this molecule can no longer function as an antagonist but becomes an agonist. For example, the AR mutation in codon 741 allows bicalutamide to function as an agonist and this mutation has been found in patients treated with this molecule, and in LNCaP cells after long-term culture with bicalutamide [103,104]. Moreover, alterations in the balance between AR coactivators and corepressors may result in a growth advantage for PCa cells, and recent studies have shown that two corepressors (NcoR and SMRT) can contribute to the antagonist activity of bicalutamide. The removal of these proteins from the nucleus of cancer cells may convert bicalutamide into an agonist, even if other data indicate that bicalutamide functions as an antagonist because it fails to mediate coactivators recruitment [105,106]. The relative frequency of these events and their relationship to clinical drug resistance remain to be defined. In any case, a better understanding of the molecular basis of AR antagonist resistance is important for the discovery of more effective antagonists.

Other mechanisms that might have a role in the development of CRPC are based on the concept that AR can be activated in a ligand-independent fashion or that AR signaling can be bypassed by alternative pathways. Growth factors, such as IGF-1, keratinocyte growth factor, and EGF can activate the AR, allowing the transactivation of AR target genes in depleted androgen conditions. It has been shown that in androgen-independent PCa cells, luteinizing hormone-releasing hormone (LHRH) analogues interfere with some of the intracellular events activated by the growth factors, such as receptor expression or receptor phosphorylation. Moreover, LHRH analogs counteract the pro-migratory and pro-invasion activity of IGF-1, interfering with the induction of lamellopodia, the cytoskeleton organization, integrins expression, and regulation of antiapoptotic molecules [107–110]. So even after the failure of bicalutamide monotherapy, the introduction of an LHRH analog can exert a direct antitumor effect by reducing both the proliferation and the metastatic behavior of PCa cells, in part by interfering with the activity of the growth factors. Taken together, these findings suggest that AR antagonists can generate a strong selective pressure for mutations that enhance AR activity, inducing the upregulation of alternative pathways that are responsible for AR reactivation or that bypass the AR completely, but that are still able to respond to second-line hormonal treatment. On the basis of these results, targeting the AR pathway continues to have an important role in the treatment of PCa, although drug resistance mechanisms have been described also for novel agents that have recently been introduced in clinical practice (such as abiraterone and enzalutamide).

Considering the central role of the androgens/AR axis in every phase of the natural history of PCa, Putluri *et al.* [111] explored the biochemical alterations mediated by androgens in PCa cell lines. To profile the androgen-regulated metabolome in PCa, the authors employed LC-MS to evaluate the levels of metabolites in benign, androgen-responsive (VCaP and LNCaP), and androgen-non responsive (DU145 and PC3) PCa cell lines. The comparison of metabolomics profiles between benign and PCa cell lines showed important differences in the levels of many amino acids. In particular, cancer cell lines showed elevated levels of sarcosine, threonine, phenylalanine, and alanine, as well as high levels of nitrogen metabolism components (namely creatine, creatinine, citrulline, and *N*-acetyl-spermine), indicating an increased amino acid utilization. Metabolomic profiles that differentiated androgen-responsive from androgen-independent PCa cells revealed differences in histidine, serine, threonine, alanine, asparagine, aspartic acid, glutamine, glutamic acid, and compounds of tryptophan metabolism (like kynurenine and kynurenic acid) [112]. Interestingly, in androgen-sensitive cell lines, levels of SAM were reduced in association with an increased production of its breakdown product, homocysteine. Moreover, the metabolic signature that distinguished hormone-sensitive from hormone-refractory cell lines showed a similar pattern in the enriched metabolic activity that differentiates localized from metastatic PCa. To better define the metabolic pathways influenced by androgens exposure, the authors carried out an unbiased analysis of VCap cell lines after treatment with R1881 (methyltrienolone, a synthetic androgen) for 24 and 48 h. Again the metabolic profile of androgen-treated VCaP showed an increase in amino acids and components of the nitrogen metabolism pathway. In addition, androgen treatment caused a reduction in SAM levels with a concomitantly increased production of methylated metabolites, such as *N*-methylglycine (sarcosine), 2-methyl glutaric acid, dimethyl glycine, and methyl valine. Taken together, these findings indicate that androgens have an important role in regulating the PCa amino acid metabolism, and in altering the methylation potential in accordance with the increased expression of methyltransferases like EZH2 during PCa progression [68–72]. In a subsequent study, using well-characterized cell lines models for metastatic PCa, Kaushik *et al.* [113] defined the biochemical pathways associated with CRPC. In particular, the network generated using the Oncomine Concept Map showed a significant enrichment of UDP-glucuronosyltransferase (UGT) activity, sucrose metabolism, and pentose/glucuronate interconversions. UGT-related pathways play an important role in androgens metabolism [114,115], and MS analysis demonstrated reduced levels of glucuronic acid in CRPC cells, indicating an increased use for the glucuronidation reactions involved in androstane-3α,17β-diol-G glucuronides synthesis. Supporting these findings, previous studies had shown a higher expression of UGT2B15 and UGT2B17 in CRPC cells [116,117]. Therefore, to evaluate the prognostic value of the UGT-associated pathways, Kaushik *et al.* [113] examined three independent gene expression datasets and investigated whether UGT2B15, UGT2A1, and UGT2B28 (three genes associated with UGT activity) had any correlation with PCa biochemical recurrence.

Interestingly, Kaplan–Meier curves showed that patients with PCa characterized by higher combined expression levels for these three genes had a reduced biochemical PFS compared with patients with lower levels.

PCa is a heterogeneous disease, and an important goal in clinical practice is to distinguish patients with aggressive disease from those with an indolent tumor [118–120]. In this scenario, a recent study identified metabolomic signatures of PCa associated with aggressive biological characteristics and progression potential [121]. In particular, this study identified a set of biochemical compounds associated to aggressiveness, through a combination of surgical and pathological findings, such as the Gleason score, extracapsular extension, seminal vesicle involvement, and lymph nodes metastases. This analysis, performed on 331 PCa tissue samples and 178 tumor-free prostates, confirmed the findings of early studies, showing that PCa has a distinct metabolic signature characterized by increased levels of amino acids and peptides, carnitine, lipids, membrane-remodeling intermediates, and stress pathway metabolites. Moreover, PCa tissue showed a reduction in metabolites associated with normal prostate function, namely simple sugars, polyamines, and citrate. Forty metabolites were identified as associated with an aggressive disease and these compounds were able to stratify this population with a high cancer risk into two distinct subsets of aggressive tumors. The first group was characterized by elevated levels of four-carbon Krebs cycle intermediates, while the second group showed an increased production of NAD^+ and choline phosphate. The real clinical significance of this differentiation needs to be addressed, but these findings suggest that different metabolic pathways are deregulated in PCa, and that the molecular stratification of patients according to their genomic and/or metabolomic profiles can have important implications for prognosis and response to therapy.

Expert commentary

Metabolomic profiling offers a powerful methodology for understanding the perturbations of biochemical systems occurring during a disease process. However, metabolomics analysis is a static tool that cannot provide information regarding the direction or enzymatic activity of a pathway. Therefore, a challenging aspect of this technology is the need to integrate it with other omics, with the aim of identifying cellular networks with a critical role in cancer development and progression [122,123]. Overall, most metabolomics studies on PCa have outlined the metabolic signature characterizing this tumor, and the global profiling associated with transition from hormone-sensitive to CRPC. Other studies are needed to identify metabolic intermediates that can distinguish indolent from aggressive tumors. In this regard, preliminary studies have shown that sarcosine had a higher predictive value than total PSA and free PSA in detecting PCa in patients with total serum PSA <4 ng/ml [81]. Moreover, it was demonstrated that sarcosine generated the largest AUC in predicting low grade, low-PSA PCa, suggesting that this marker could be a further tool, not only for diagnosing PCa in normal PSA value patients but also for selecting candidates for non-aggressive therapies and active surveillance. In addition, the role of this metabolite as a biomarker for predicting progression and survival in patients with advanced disease was explored. In particular, recent findings suggest that this N-methyl derivative of glycine could be used as a prognostic indicator of outcome in terms of PFS and OS, in patients treated with docetaxel-based chemotherapy [82]. Taken together, these results suggest that sarcosine may distinguish slow-growing PCa from forms prone to a rapid, lethal spread and that this new biomarker may be potentially useful not only for early diagnosis but also for monitoring therapeutic efficacy. Other metabolites, such as choline, glycerophospholipids, lactate, and intermediates of different metabolic pathways are currently under investigation as novel potential biomarkers. The metabolomic approach in PCa research is still in its infancy but it shows potential for widespread clinical applications ranging from diagnosis to prognosis, permitting analysis of different biofluids (including serum and urine), and use in diagnostic imaging. In particular, the development of novel metabolomics-based analytical methods to quantify metabolites in urine will provide simple, fast and sensitive tools for early and non-invasive cancer detection, therapy monitoring and clinical outcome prediction. Current advances in metabolomics have been applied for the definition of PCa metabolic profiles, with the aim of identifying novel clinically useful biomarkers with a better diagnostic performance than PSA.

In the next future, the combination and integration of data derived from different platforms and other omics approaches should define the complex regulatory networks characterizing PCa, and bring us one more step toward personalized medicine and individualized treatment.

Five-year view

The field of cancer metabolomics is evolving very rapidly. Recent discoveries have cast new light on regulation processes of the cell metabolism, and novel biochemical pathways are under investigation. However, beyond the amazing advances described in this research field, many unanswered questions need to be addressed in the next years. For example:

- Does a specific metabolomic profile of cancer cells exist?
- Do cancer cells use different metabolic programs?
- Which oncogenes are involved in the regulation of specific metabolic reactions?
- For which nutrients do cancer cells exhibit a preferential uptake?
- Is the tumor cell metabolism influenced by external factors?
- Can we develop drugs targeting the cancer metabolism with clinical efficacy?

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Many of these questions will find an answer in the next years. We now know that the PCa metabolome is characterized by an increased amino acid metabolism and a perturbation of nitrogen breakdown pathways, along with an increased methyltransferase activity. A better understanding of the programs underlying this metabolic phenotype will be critical for the identification of novel therapeutic targets. In this context, it has recently been shown that the inhibition of G6PDH caused a significant decrease in prostate and renal cancer cell growth, and an increased sensitivity to cisplatin-induced cytotoxicity [61,62].

A number of metabolic pathways will be explored in the next years. Many of these pathways are potential therapeutic targets and may serve as diagnostic and prognostic biomarkers.

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The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Key issues

- Prostate cancer (PCa) is the most common male malignancy. Recent estimates have calculated that in 2015, 220,800 new cases will be diagnosed and 27,540 patients will die of PCa in the USA.
- Normal prostate cells have a particular metabolic profile characterized by an increased production of citrate and polyamines that are components of the prostatic fluid.
- In PCa, the normal citrate-producing cells are metabolically transformed into citrate-oxidizing cells that lose the ability to accumulate zinc.
- The PCa metabolome is characterized by an increased amino acid metabolism and a perturbation of nitrogen breakdown pathways, along with high total choline-containing compounds and phosphocholine levels.
- Sarcosine, an *N*-methyl derivative of glycine, was identified as a metabolite that shows a progressive increase in benign, through localized, to metastatic PCa.
- Androgens have an important role in regulating the PCa amino acid metabolism, and in altering the methylation potential, in accordance with the increased expression of methyltransferases like EZH2.
- A significantly enriched UDP-glucuronosyltransferase activity, as well as sucrose metabolism and pentose/glucuronate interconversions, has been described in castration-resistant disease.

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