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Application of Metabolomics to Prostate Cancer

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Abstract

The prostate has long been known to exhibit unique metabolite profiles. In the last decade, advances in nuclear magnetic resonance spectroscopy and mass spectrometry have been applied toward identifying metabolic alterations in prostate cancer that may provide clinically useful biomarkers. As with genomics and proteomics, advances in technology and bioinformatics have led to the application of metabolomic profiling to prostate cancer – the high throughput evaluation of a large complement of metabolites in the prostate and how they are altered by disease perturbations. Recently, high profile publications have drawn attention to the potential of metabolomic analysis to identify biomarkers for early detection or disease progression from readily accessible body fluids as well as tissue specimens from biopsy and surgery. This review will examine applications of metabolomics to prostate cancer and highlight clinical associations and potential challenges.

Introduction

More than 240,000 men in the United States will develop prostate cancer in 2011, and more than 33,000 will die of their disease [1]. Worldwide the number of prostate cancer cases is approaching one million, and it is the sixth leading cause of cancer deaths in men. Both incidence and mortality are increasing in many traditionally low risk countries in Asia, Central and Eastern Europe [2]. Testing with serum prostate specific antigen (PSA) has contributed to decreases in prostate cancer mortality in many developed countries, but the test and the diagnostic paradigm suffer from a number of problems including low specificity of PSA, inability to specify a cut-point below which cancer is unlikely, non-trivial false-negative rate for prostate biopsy, and over-diagnosis and over-treatment of relatively indolent tumors with low potential for morbidity or death if left untreated. Furthermore, the results of two randomized trials that demonstrated only modest mortality benefit associated with PSA screening have added to the controversy concerning the early detection paradigm for prostate cancer [3].

For men diagnosed with prostate cancer a number of algorithms or nomograms primarily based on tumor pathology and PSA are available to predict the likely clinical outcome. Although these prediction tools generally work well, there is still significant variability in outcomes for men at both the low and high end of the risk spectrum. For example, at least 30–50% of men assessed as very low risk in a stringent program of active surveillance will go on to require treatment [4], while among high risk men with Gleason 8–10 tumors, 15

year prostate cancer mortality may be lower than 40% [5]. These limitations of current paradigms have led to intense focus on molecular biomarkers to improve detection of prostate cancer and classification of individual prognosis or risk of progression. Coinciding with a burgeoning movement toward application of systems biology approaches to the discovery of clinically relevant cancer biomarkers and pathways there is increasing interest in application of metabolomic profiling in prostate cancer, particularly since the publication of a provocative paper that identified a potential role in progression for the amino acid sarcosine and related elements of the methionine-choline metabolic pathways [6]. Although distinct metabolic characteristics of the prostate have long been known (reviewed in [7]), global metabolomic profiling of prostate cancer is at an early stage. The purpose of this article is to review the current state of prostate cancer metabolomic research.

Metabolomics

One of the goals of systems biology is to define interacting cellular networks in the context of a disease phenotype, tissue-specific functions or reaction to specific stimulus or intervention. Systems biology as applied to cancer research encompasses the “omic” sciences of genomics, transcriptomics, proteomics, and metabolomics. Metabolomics (sometimes known as metabonomics) entails evaluation of the patterns and concentration of low molecular weight metabolites over broad classes of compounds in a tissue or organ. These metabolites are the small molecule intermediates and end products of the biochemical reactions in a cell, and are represented by compounds with mass typically in the range of 80–1000 Daltons. Metabolomic studies range from targeted analysis of one or a small number of metabolites associated with a specific biological pathway to the unbiased profiling or fingerprinting of a large subset of metabolites associated with a specific phenotype or stimulus. Although complementary to genomics, transcriptomics and proteomics, metabolomics may have advantages for defining phenotypes because it is downstream of changes in genes and proteins, and thus may be a better indicator of distinct functional alterations in pathways affected by different pathological states. In this sense, metabolomic profiles represent the integration of genetic regulation, enzyme activity and metabolic reactions in a dynamic profile of the biological state of a tissue [8]. Furthermore, because the total complement of metabolites is likely to be considerably smaller than the number of genes, transcripts, or proteins, metabolomics may be able to more clearly characterize altered cellular networks and activity associated with disease states.

Methods of Metabolomic Analysis

A number of analytic platforms are used for metabolomic analyses; each has advantages and disadvantages and the choice of platform depends on the type of analytical problem to be evaluated. Most analyses employ forms of nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS). NMR spectroscopy exploits the specific magnetic spin or resonance frequency of the protons within atomic nuclei of specific molecules. When nuclei in a magnetic field are exposed to a radiofrequency pulse their protons temporarily move to a higher energy state, and then release a characteristic radiowave when they return to their normal energy state. For a mixture of metabolites in a biological sample the different patterns of energy release are represented as peaks in a chromatogram, and the area of the peaks is indicative of the relative concentration of each type of metabolite. NMR is used for liquids or tissue extracts. Advantages of NMR include its low cost, minimal sample preparation requirements, high reproducibility, ability to quantify metabolites, and identification of unknown metabolites. Proton or ^{1}H-NMR is the most common method and is used to detect hydrogen atoms in a molecule, but ^{31}P-NMR can also be used to measure phospholipid metabolism or high energy phosphates, and ^{13}C-NMR is used to measure carbon fluxes such as those involved in glucose metabolism [9,10]. A variant of NMR called

high resolution magic angle spinning NMR spectroscopy (HR-MAS) was developed to improve spectral resolution in solids such as intact tissue samples. Because vibration of molecules in a solid state is restricted it is difficult to achieve adequate resolution of spectra with NMR. However, by spinning the sample at a precise “magic” angle to the induced magnetic field it is possible to resolve the spectra with high sensitivity. An advantage of HR-MAS is that it preserves the tissue architecture so pathological evaluation is not compromised, particularly if slower spinning speeds are used. [11].

Mass spectrometry (MS) requires an initial separation of metabolites by gas or liquid chromatography (GC, LC), followed by ionization of metabolites and resolution according to mass-to-charge ratio. The advantage of MS methods over NMR is much higher sensitivity and detection of metabolites at much lower concentrations, and it is more suitable for high throughput methods. However, these advantages come at the cost of more extensive sample preparation (particularly for GC-MS), and metabolite detection can be complicated by differences in ionization efficiency, stability, extraction efficiency, and fragmentation behavior. Derivatization is used to optimize these characteristics, but different reagents are used depending on the purpose of the derivatization and where in the GC-MS or LC-MS process it occurs, which can complicate comparisons across studies. Derivatization can also result in metabolite degradation. Other sources of variation include metabolite pK, polarity, processes of extraction and quenching, and type of instrument [8,12].

Magnetic resonance spectroscopic imaging (MRSI) measures metabolite concentrations in vivo, in an analogous fashion to the way conventional magnetic resonance imaging (MRI) measures water. Because the concentration of water and lipids in soft tissues such as the prostate is orders of magnitude greater than the concentration of metabolites, MRSI requires higher field strength than conventional MRI, and water and lipid suppression techniques to allow accurate resolution of metabolite spectra. Potential combined modality applications include combining MRSI and dynamic contrast enhanced MRI for enhanced visualization of suspicious prostate lesions or areas of recurrence, and overlaying MRSI images on transrectal ultrasound images for guiding prostate biopsy [13]. Current limitations to the use of MRSI include relatively high cost and limited availability of higher field strength (3 Tesla or higher) platforms needed for better spectral resolution. Most applications of MRSI in prostate cancer have focused on diagnostic imaging rather than metabolomic profiling of cellular networks so MRSI will not be further discussed in this article; for an excellent review see Sciarra et al. [14].

Metabolic Characteristics of Normal and Malignant Prostate

Tumors have long been known to exhibit altered metabolic profiles and bioenergetic requirements. For example, the Warburg effect represents a shift to increased aerobic glycolysis and increased production of lactate, as well as increased bioenergetic demand [15]. Because some metabolic alterations occur early in the process of neoplastic transformation they may provide not only biomarkers for early detection, but perhaps more importantly, targets for intervention [10,16]. Other alterations include increases in total choline-containing compounds, myo-inositol, taurine, and pyruvate kinase type M2 (glycolytic isoenzyme). However, a generalized tumor metabolic phenotype is not useful because key metabolites vary between tumor types (e.g. alanine, lactate, glycine, citrate), and different metabolic pathways are activated at different stages of neoplastic progression [8,9].

Prostate cells have a distinct metabolic profile reflecting the production of citrate, PSA and polyamines (spermine and myo-inositol) that are major components of prostate fluid. In an informative review Costello and Franklin posit that although genetic alterations are the

drivers of neoplastic transformation in the prostate, the altered cellular activity requires additional metabolic adaptation to accommodate the bioenergetic demands necessary to achieve the full malignant phenotype [7]. These adaptations are briefly summarized here and center around the metabolism of citrate. The normal prostate is unique among human organs by producing extremely high concentrations of citrate. Citrate concentration in prostatic fluid is 200–700 times higher than in blood plasma. Tissues in the peripheral zone exhibit citrate levels an order of magnitude lower than those in prostatic fluid, and these levels in turn are about 30–50 fold higher than for other tissue types. Unlike cells in other organs in which citrate oxidation occurs as part of the intermediary metabolism of glucose during the Krebs cycle, normal prostate peripheral zone cells accumulate and secrete citrate. This is a result of inhibition by zinc of the mitochondrial enzyme m-aconitase, which catalyzes the initial step of citrate oxidation. The prostate has very high intracellular zinc levels; zinc concentration in the peripheral zone is approximately 10–20 fold higher than in most other organs. Because inhibition of citrate oxidation truncates the Krebs cycle, citrate accumulation has a large energy consequence for the cell, which generates approximately 60% less ATP from glucose oxidation than would be produced via the complete Krebs cycle [7].

When prostate cells undergo neoplastic transformation they also experience profound metabolic changes. Cells lose the capacity to accumulate zinc, which leads to restoration of m-aconitase activity and citrate oxidation, with consequent loss of citrate accumulation and increased generation of ATP. Thus, restoration of citrate oxidation results in a large bioenergetic gain for malignant prostate cells. An additional metabolic change associated with malignant transformation is the need for increased lipid biosynthesis for cellular proliferation, membrane formation and intercellular signaling. This requires conversion of citrate to acetyl-coA in the cytosol, which is a precursor for lipogenesis and cholesterogenesis. But to accomplish this conversion another metabolic change is required, an increase in the activity of ATP citrate lyase, which catalyzes the formation of acetyl coA [7]. This is consistent with recent findings that a number of key enzymes involved in fatty acid and cholesterol synthesis are androgen regulated and exhibit increased activity in prostate cancer cells [17,18].

A number of other metabolic changes are found in prostate cancer cells. Pre-operative MRI/3D-MRSI images were used to guide sampling of post-surgical prostatectomy specimens to areas of presumptively healthy tissue (high citrate and polyamines and low choline) or cancer (low citrate and high choline), which were then evaluated with HR-MAS. They found that total choline, and its component metabolites (free choline, phosphocholine, and glycerophosphocholine) were increased in specimens containing ≥ 20% prostate cancer compared to matched healthy epithelial or stromal tissues, but tissues with <20% prostate cancer did not differ from healthy tissue. Prostate cancer specimens also exhibited lower levels of citrate and polyamines than healthy matched epithelial tissue but were not different from healthy stroma [19]. A subsequent study by the same group confirmed higher levels of choline in prostate cancers, and also showed prostate cancer to have increased phosphoethanolamines and decreased ethanolamine compared to benign matched epithelial tissues; stromal tissues also exhibited lower levels of choline-containing compounds. These changes reflect enhanced synthesis and degradation of phospholipid membranes and increased cellular proliferation [20]. Comparison of biopsy tissues from men with and without prostate cancer revealed increased ratios of total choline:citrate, choline:creatine, (glycerophosphocholine+phosphocholine):creatine, and decreased ratio of citrate:creatine. This study only included cancer cases with at least 2 positive cores from one side of the prostate [21]. Concentrations of choline-containing metabolites are higher in metastatic tissues than in primary prostate cancer [10]. ^{1}H-NMR spectroscopy demonstrated that citrate, myo-inositol, spermine, valine-leucine, hydroxybutyrate, and glutamine were all

found in univariate analyses to be significantly lower in expressed prostate secretions obtained by prostate massage from 52 prostate cancer patients compared to 26 controls. In contrast to the study by Swanson et al. [20], phosphocholine was found to be significantly lower in specimens from prostate cancer. It is possible that the different specimen types i.e. tissue vs. expressed prostate secretion could have contributed to the difference. However, in multivariable analysis, only citrate, myo-inositol and spermine remained independently associated with the risk of prostate cancer. Based on the area under the receiver operating characteristic curve (AUROC), each of these three metabolites appeared to have superior performance than PSA. However, PSA data were not available for controls, nor were other characteristics of the participants described, although metabolite differences between the groups were shown to be unrelated to age [22]. Citrate in expressed prostate secretions was also shown in another study to perform better than PSA in detection of prostate cancer [23]. Increased levels of lactate and alanine are seen in tumor compared to benign prostate tissues from prostatectomy specimens [24], and also in tumor vs. benign biopsy tissue, even in biopsies containing <5% tumor (comparable to biopsies in many men with small volume disease suitable for active surveillance) [25]. These are indicative of enhanced glycolytic activity or Warburg effect [24,25]. Comparison of benign-appearing biopsy tissues from men with vs. without prostate cancer did not show differences in lactate or alanine, suggesting lack of a field effect [25]. However, measures of lactate and alanine are subject to artifact and may be overestimated due to the anaerobic depletion of glucose following devascularization during surgical procedures, emphasizing the need for control of this pre-analytical variable by rapid freezing or making comparisons between tissues with similar ischemia times [24]. The ratio of lactate to alanine is also increased in prostate cancer and may be less subject to variability than either metabolite alone [21].

Clinical Correlations with Metabolic Changes

Metabolite patterns in prostate cancer have also been shown to correlate with measures of disease aggressiveness, although sample sizes have been small. HR-MAS analysis of snap frozen biopsy tissue from 18 prostate cancer patients revealed that ratios of free or total choline to creatine were observed to increase with Gleason score, and the ratio of citrate to creatine decreased with Gleason score [21]. An advantage of HR-MAS spectroscopy for studying intact prostastectomy tissues is that the sample architecture is preserved, allowing histologic assessment after spectroscopy. In tissues from 54 prostatectomies, decreased polyamines was significantly correlated with Gleason score ≥ 7 [19]. In a study of 199 tissues from 82 prostatectomies only 20 specimens actually contained malignant glands, while the remaining specimens contained only benign tissue. Principal components analysis was performed on the 36 most intense resonance peaks; these were analyzed with respect to tumor vs. benign tissue, PSA levels, and pathologic stage. When assessed only on benign tissue from the prostatectomies, the principal component dominated by polyamines and citrate was significantly correlated with PSA, and was able to significantly separate T2a/2b, T2c, and T3 tumors, and also discriminated Gleason 6 from Gleason 7 tumors. One other principal component was also significantly correlated with stage and Gleason score but unfortunately, the relevant metabolites were not described [26]. In a later report from the same lab, the ability of metabolomic analysis to predict biochemical recurrence was evaluated with HR-MAS spectroscopy of biopsy tissue [27]. Sixteen men with biochemical recurrence were matched by clinical stage to 16 non-recurrent prostate cancer cases, and matched by pathological stage to a separate set of 16 non-recurrent cases. The 27 most common or intense spectra from analysis of tissues from all 48 patients were then subjected to principal components analysis, resulting in 9 principal components that were able to significantly discriminate the 16 recurrent patients and the clinical stage-matched non-recurrent patients. These 9 principal components were then compared between the recurrent and pathology stage-matched non-recurrent patients, yielding 78% accuracy at predicting the

recurrent patients. Most of the predictive power was found to reside in 4 of the principal components, which yielded predictions with 71% accuracy. The metabolites with the greatest contribution to the relevant principal components were spermine, glutamine, myo-inositol, phosphoryl choline, scylloinositol and glutamate. An acknowledged limitation of the study is that the same set of recurrent patients was used in comparisons to both matched sets of non-recurrent patients. Thus, the principal components developed in the first comparison or "training" set were not validated against an independent "test" set. Similar to the study by Cheng et al. [26], only a minority of tissue samples (11 of 79) contained cancer glands; the rest were benign tissue [27]. A limitation of the reporting of studies employing principal components analyses is that the relevant combinations of metabolites or metabolic processes were not well-described [26,27].

Profiling Metabolomic Networks in Prostate Cancer

Major interest in the potential application of metabolomics in prostate cancer followed a recent report by Sreekumar et al. wherein unbiased metabolomic profiling using LC/GC-MS identified six metabolites whose levels increased with cancer progression from benign prostate tissue adjacent to tumor (n=16) to localized prostate cancer (n=12) to metastatic prostate cancer (n=14) [6]. These included sarcosine, uracil, kynurenine, glycerol-3-phosphate, leucine and proline. Among these sarcosine demonstrated the most pronounced differences, exhibiting increased levels in 79% of metastatic tumor tissues, 42% of localized tumor tissues, and none of the benign specimens. Similar associations were found in an independent set of 89 tissue samples analyzed specifically for sarcosine with isotope dilution GC-MS. In contrast to the the results of metabolomic profiling of prostate tissue, similar unbiased analyses of matched plasma and post-digital rectal exam (DRE) urine did not yield a metabolite profile able to strongly discriminate samples from men with biopsy positive prostate cancer vs. men with negative biopsies.

However, based on the results from tissue, specific evaluation of sarcosine (normalized to alanine) demonstrated significantly increased levels in both urine sediment and (to a somewhat lesser degree) urine supernatant from biopsy positive men. Similar results were observed when sarcosine was normalized to creatinine. In samples from 53 men within the diagnostic "gray zone" of PSA 2–10 ng/ml, urine sarcosine exhibited somewhat better ability than PSA to discriminate biopsy positive and biopsy negative men, AUROC 0.69 and 0.53, respectively [6].

The finding of increased sarcosine in tumor tissues was also consistent with *in silico* analyses that demonstrated increased methyltransferase activity in metastatic tissues. Sarcosine was also found to be increased in several prostate cancer cell lines compared to benign prostate cells, and addition of sarcosine to benign prostate epithelial cells increased their invasiveness as measured in a basement membrane chamber assay [6]. The physiological function of sarcosine is unknown, but it is synthesized by glycine N-methyltransferase (GNMT) as a byproduct of methionine metabolism, a pathway that plays an important role in methylation of DNA, RNA and proteins [28]. A specific test of this pathway revealed that knockdown of GNMT by RNA interference significantly decreased intracellular sarcosine levels and reduced invasiveness of DU145 cells [6]. This potential role of GNMT in prostate carcinogenesis was independently validated in a recent study wherein GNMT knockdown with siRNA resulted in decreased proliferation and increased apoposis in LNCaP and PC-3 prostate cancer cell lines but little effect on RWPE-1 prostate cells. This study also observed higher cytoplasmic staining for GNMT in tumor tissue than matched benign adjacent glands, and significant correlations with Gleason score, pathologic stage, and biochemical recurrence [29]. In contrast, Huang et al. observed lower cytoplasmic GNMT immunostaining in human prostate tumors compared to tissues from non-cancer

controls or BPH patients, and association with lower stage [30]. Possible differences between these two studies include the type of comparison (e.g. separate case and control patient groups vs. matched tumor and benign adjacent tissue), the definition of positive staining (not defined in [30]), clinical characteristics of the patients (not defined in Huang et al. but some prostate cancer tissues were obtained by transurethral resection, suggesting they had metastatic disease).

Despite the fact that the authors themselves described the predictive performance of urine sarcosine as “modest,” and emphasized the potential that a panel of molecules in the sarcosine pathway may provide biomarkers for, and/or increased understanding of the biology of prostate cancer progression [6], the study was widely interpreted as a demonstration of urine sarcosine as a promising early detection or prognostic biomarker. In an attempt to validate these potential roles of urine sarcosine Jentzmik et al. used a commercial amino acid extraction kit and GC-MS to retrospectively evaluate sarcosine in post-DRE urine supernatants from 106 prostate cancer patients and 33 biopsy negative controls; sarcosine was normalized to creatinine. Urine samples were collected from consecutively enrolled patients following a standardized DRE protocol. In contrast to the results of Sreekumar et al., these authors found the sarcosine-creatinine ratio was non-significantly lower in samples from prostate cancer patients compared to biopsy negative controls. There was no correlation of sarcosine concentration with either biopsy or prostatectomy Gleason score, nor with pathological stage. Sarcosine was also uncorrelated with age, PSA, or prostate volume, and did not differ between pre-DRE and post-DRE samples. Finally, among patients with serum total PSA <20 ng/ml, the AUROC was similar for sarcosine (0.63) and PSA (0.64), both of which were significantly lower than the AUROC for percentage free PSA (0.81); similar associations were observed when restricted to patients with PSA <10 ng/ml [31].

A number of studies of sarcosine in body fluids followed, but did not resolve the differences in results observed by [6] compared to [31]. Sreekumar et al. validated their initial findings with an independent set of 40 patients demonstrating significantly higher sarcosine-alanine ratio in urine sediments from biopsy positive vs. biopsy negative patients [32]. Colleselli et al. found non-significantly higher sarcosine-creatinine ratios in post-DRE urine supernatants from controls [33]. Using isotope dilution GC-MS with microwave-assisted derivatization, Wu et al. observed non-significantly higher sarcosine levels in urine supernatants from 20 prostate cancer cases compared to 8 BPH patients or 20 healthy controls. However, in an unbiased profiling effort they did observe prostate cancer cases to have significantly higher levels of dihydroxybutanoic acid and xylonic acid, and significantly lower levels of pyrimidine, ribofuranoside, and xylopyranose [34]. Cao et al. investigated sarcosine in urine supernatants and sediments, and normalized levels separately to creatinine and to alanine. Regardless of the specimen type or normalizing analyte, sarcosine was significantly higher in prostate cancer patients (n=71) than men with elevated PSA or abnormal DRE but no cancer (n=31), or healthy men (n=20) or women (n=20). Creatinine values were not significantly different in cancer and non-cancer groups. There were no differences between sediment and supernatant sarcosine values, and none of the sarcosine algorithms were correlated with biopsy Gleason score or clinical stage. The sarcosine algorithms exhibited AUROCs ranging from 0.647–0.698, all of which were non-significantly higher than that of serum PSA (AUROC 0.537), and non-significantly lower than those of PCA3 (AUROC 0.703) or percent free PSA (AUROC 0.712). However, when any of the sarcosine algorithms were added to a logistic model including PCA3 or percent free PSA the AUROC increased, ranging from 0.720–0.775. Similar results were observed within clinically relevant subgroups [35]. In the only report of serum sarcosine levels, Struys et al. found no significant difference between non-cancer controls, localized prostate cancer or metastatic prostate cancer [36]. It is important to note that several of these reports were letters to the

editor [32,33,36] and provided few details about patient characteristics, specimens, or methods.

The association between tissue sarcosine and prostate cancer histopathologic parameters was explored in a retrospective analysis of prostate tumor and matched benign-appearing tissue from 92 prostatectomies [37]. Sarcosine was measured by GC-MS as a component of a global metabolite profile, and normalized to sample weight and median of reference samples. Seventy-three percent of patients had Gleason score 7 or higher, 41% of tumors were pathological stage T3, and median PSA was 7.5 ng/ml. The normalized sarcosine ratio was slightly but significantly higher in tumor compared to matched benign tissue. However, there was no association between sarcosine and Gleason score or tumor stage, and sarcosine was not correlated with age, PSA, percent free PSA, prostate volume, or biochemical recurrence [37]. These results argued against tissue sarcosine as a stand-alone biomarker of prostate cancer aggressiveness. Possible limitations of this study included a small number of recurrent patients most of whom had pT3 and Gleason 8–10 disease, and variation in tissue storage times (surgery performed 2001–2007).

A very comprehensive metabolomic profiling study recently evaluated metabolites in bone metastases as a means of discovering biomarkers of prostate cancer aggressiveness [38]. Biopsies of bone metastases from 7 hormone naive and 7 castration resistant patients were compared to matched normal appearing bone from 4 and 6 of the same patients, respectively using GC/time of flight MS (GC/TOFMS). Data were analyzed with orthogonal partial least squares discriminant analysis. Seventy one metabolites were found to significantly discriminate the metastatic bone specimens, of which 34 were identifiable. These metabolites were validated using a separate set of bone metastasis biopsies from 6 castration resistant patients with matched normal bone from 4 of the patients. Amino acid metabolism was the most prominent pathway discriminating the metastatic samples. However, cholesterol was the most highly discriminating single metabolite, with significantly increased levels in the metastatic samples. Cholesterol in bone metastases from prostate cancer patients was also found to be significantly higher than in bone metastases from breast, lung, kidney, and esophageal cancer. Other metabolites with prominent increases in the prostate cancer metastatic samples were myo-inositiol-1-phosphate, citric acid, fumarate, glycerol-3-phosphate and fatty acids, several of which were also associated with progression in the study by Sreekumar [6], and which may be indicative of the large bioenergetic demands of cellular proliferation in bone metastases [38]. Additional comparison of primary prostate tumor from men with (n=7) or without (n=6) bone metastases resulted in 8 identifiable metabolites with discriminant ability. Among these, aspargine, threonine, fumaric acid and linoleic acid were common to the set of significant discriminating metabolites in bone metastases. Comparison of plasma samples from 15 patients with and 13 patients without bone metastases revealed increases in glutamic acid, taurine, phenylalanine, and decreases in stearic acid in common with the set of markers for bone metastasis. A concern noted by the authors was that alterations of citrate metabolism were not observed, and choline was not detectable; both metabolites have been shown to be altered in a number of studies of prostate cancer. Finally, specific analysis of sarcosine showed significantly elevated levels in bone metastasis from the prostate cancer patients, but not those from other cancers. However, there was no clear signal associated with benign vs. primary tumor tissue, although the number of specimens was small. No significant differences in sarcosine levels were observed in plasma from the different patient groups [38], similar to the results of Sreekumar [6]. As with most studies to date of clinical correlations with metabolomic profiles, sample sizes were small relative to the number of potential targets, and limited information was provided about the patients or the methods of sample collection and handling.

Table 1 summarizes the clinical associations with prostate cancer that have been identified in metabolomics studies.

Methodologic Considerations for Metabolomic Profiling Studies

In an editorial commenting on the discrepant results from the initial reports by Sreekumar et al. [6] and Jentzmik et al. [31], Schalken noted differences in the methodologies from both studies without speculating on their potential impact. He emphasized the need for greater standardization in biomarker validation studies, with specific attention to assay methodology, the standard (or comparator) to which the biomarker is compared, specimen collection procedures, and the study cohort [39]. The latter is a critical issue which is often overlooked in biomarker studies [40]. Neither of the two studies evaluating urine sarcosine described how individuals were selected into the study cohort, the timing of specimen collection relative to biopsy, or if specimens from some patients were excluded (and if so, comparability of those patients to the analysis cohort). It is important to recognize that study results of an individual biomarker from different researchers are likely to incorporate different sources of bias, particularly in early stage research such as the use of metabolomics in prostate cancer. There is increasing recognition of the need to document potential sources of variability in biomarker studies, and to provide sufficient detail in the published reports about study design and potential sources of variability to facilitate comparison of multiple reports of the same biomarker [41,42].

The importance of potential differences in analytical methods were detailed in a letter by Hewavathirana [43]. Differences in analytical methodology that could confound comparison even across studies using the same type of instrument (e.g. GC-MS) included the amino assay extraction method, the percentage of sarcosine recovered from the urine (extraction efficiency), type of internal standard and whether the sarcosine concentration is calibrated to the internal standard or reported as unprocessed data, the type of derivatization reagent and efficiency of derivatization, and how well sarcosine and alanine (which have the same molecular mass) are separated chromatographically [43]. Studies can also differ with respect to other sources of noise or extraneous variation such as diurnal variation, differences in diet, medications, stress, processing of specimens (timing, temperature, preservatives), and instrument variation [44].

Potential artifacts are also a concern when evaluating intact *ex vivo* tissue samples by HR-MAS. For example, biopsy specimens can be contaminated by echo gel used for ultrasound, or the presence of periprostatic fat in the specimen can artificially increase lipid signals. Traces of ethanol used in tissue handling can also produce aberrant spectra. Polyamines such as spermine can be underestimated if they react with negatively charged molecules released upon tissue degradation [21]. These issues again highlight the need not only for careful attention to and documentation of pre-analytical factors, but sufficient reporting of specimen handling and analytical protocols so that susceptibility to artifact can be evaluated by a reader.

The actual comparison being made also needs to be considered. Some of the studies described herein compared malignant glands with matched adjacent benign glands; in several studies many of the specimens from cancer patients contained no malignant glands at all. If there is a field effect or host response to tumor such comparisons may miss metabolite changes associated with carcinogenesis, and may provide different results than studies that compare tissues from men with and without prostate cancer.

Perspective

Metabolomic profiling shows tremendous promise as one of the important ‘omics methodologies for a systems biology approach to prostate cancer. Changes in cellular metabolism are downstream of genomic, transcriptomic and proteomic alterations and thus are closer to functional characteristics of the malignant phenotype. Because it is likely that a particular disease-associated metabolic profile may arise through a number of alternate pathways of genomic and proteomic alterations, iterative analyses combining these multiple approaches may be able to elucidate critical cellular networks with the potential of identifying “druggable” targets. However, efforts to link these multiple ‘omics approaches are in their infancy, and there is a real need to begin to define the parameters of study designs that will combine these technologies.

Most metabolomic research on prostate cancer has focused on relatively small groups of metabolites that have long been known to be relevant to the prostate, such as citrate, choline and polyamines. Efforts at more global profiling of the prostate metabolome are increasing, and are benefitting from advances in bioinformatics that accompanied high dimensional genomic and proteomic data analyses. However, no single platform is currently able to capture the entire metabolome, and the size of the human metabolome is not known. Additional information is needed to more comprehensively classify the types of metabolite classes that can be detectable with each of the different metabolomic analysis platforms, and to increase the coverage of spectral libraries used for metabolite identification [38]. Furthermore, because of the dynamic nature of the metabolome and its responsiveness to perturbations associated with stimuli such as diet, medications, activity levels, stress, and diurnal variation, greater understanding will be needed of baseline variability in key metabolites or metabolic pathways. In addition, despite the high sensitivity and reproducibility of NMR and MS-based techniques, there are still significant challenges in defining and controlling artifacts and variability introduced by pre-analytical conditions and other batch effects. The ability to make valid comparisons across studies of the same target metabolites or clinical context will depend not only on methodologic advances but also on improving the quality of information provided in published reports. The latter was emphasized in a recent commentary on the problems of artifacts induced by batch effects in high throughput experiments: “Foremost among these challenges is the need for consistent reporting of the most common potential sources of batch effects ….” [45].

A strength of metabolomics as a tool for eventual clinical application is the capability of different platforms to allow analysis of biofluids, tissue extracts, intact tissue specimens and *in vivo* imaging. This provides the potential for enhanced risk classification, diagnostic accuracy, assessment of disease extent and aggressiveness and response to therapy. Furthermore, the integration of metabolomic assessment into clinical practice is feasible because the infrastructure already exists to rapidly, inexpensively and reproducibly measure metabolites in hospital and clinical laboratories, and there are already established quality control standards [46]. Despite this promise, the difficulties should not be underestimated. The potential clinical translation of genomics and proteomics has been slow to materialize, and few biomarkers have made their way from often impressive experimental studies into clinical practice. The task of integrating metabolomics with the other ‘omics approaches to extract clinically useful tools offers tremendous challenges, but will bring us closer to the goal of personalized medicine.

Conclusions

Studies of the metabolic alterations associated with prostate cancer have demonstrated characteristic decreases in citrate and polyamines, and increases in cholines,

glycerophospholipids, lactate, and components of a number of pathways of amino acid metabolism. Results for sarcosine have been prominent but inconsistent. However, it is likely that inconsistent findings are not unique to sarcosine. Rather, the attention given to sarcosine has resulted in reports of validation efforts focused on this molecule, whereas reports from other metabolomic profiling studies have focused on discovery and have not emphasized null associations. Metabolite profiles with potential relevance to prostate cancer biology have been identified in tissue, bone, urine, expressed prostatic fluid and plasma, and have correlated with clinical progression as well as established prognostic attributes. Given the relatively low cost of metabolomic profiling compared to the other 'omics disciplines, and the parallel advances being made in molecular magnetic resonance imaging, metabolomics has great potential for application to detection of clinically significant disease and monitoring disease progression, in both the active surveillance and post-treatment settings. In addition, because of their functional significance, metabolomic biomarkers or profiles hold particular promise for addressing one of the current challenges to personalized medicine: co-development of targeted therapeutics and companion diagnostics.

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Table 1

Summary of prostate cancer associations with individual metabolites

Prostate cancer association	Metabolite association	Specimen types	Analytical methods	References
Increased in PCa vs. matched* benign tissue	total choline, phosphocholine, glycerophosphocholine, phosphoethanolamine glycerophosphoethanolamine, lactate, alanine, sarcosine, uracil, kyurenine, glycerol-3-phosphate, leucine, proline	Prostatectomy, biopsy	HR-MAS, LC/GC-MS, GC-MS	6, 19, 20, 24, 25, 37
Increased in PCa vs. non-cancer specimen (unmatched)	total choline, free choline, sum of glycerophosphocholine +phosphocholine, lactate:alanine ratio, sarcosine, xylonic acid, dihydroxybutanoic acid	Biopsy, urine sediment or supernatant	HR-MAS, ID GC-MS, LC-MS/MS	6, 21, 3, 34, 35
Increased in metastases vs. primary tumor (unmatched)	sarcosine, uracil, kyurenine, glycerol-3-phosphate, leucine, proline	Biopsy of metastatic site, prostatectomy	LC/GC-MS	6
Increased in bone metastases vs. normal bone (matched)	sarcosine, cholesterol, myo-inositol-1-phosphate, citric acid, fumarate, glycerol-3-phosphase	Bone biopsy	GC-TOFMS, LC/MS	38
Increased in primary tumor from metastatic vs. non-metastatic disease	aspargine, threonine, fumaric acid linoleic acid	Biopsy	GC-TOFMS	38
Association with plasma levels from metastatic vs. non-metastatic disease	Increased glutamic acid, taurine, phenylalanine. Decreased stearic acid.	plasma	GC-TOFMS	38
Decreased in PCa vs. matched benign tissue	ethanolamine, citrate, spermine, spermidine, putrescine	Prostatectomy, biopsy	HR-MAS	19, 20, 24, 25
Decreased in PCa vs. non-cancer specimen (unmatched)	citrate, myo-inositol, spermine, valine-leucine, hydroxybutyrate, glutamine, pyrimidine, ribofuranoside, xylopyranose	Biopsy, EPF, urine supernatant	HR-MAS, ^{1}H-NMR, ID GC-MS	21, 22, 23, 34
No association with prostate cancer vs. non-cancer specimen (unmatched)	sarcosine	Urine supernatant, serum	ID GC-MS, LC-MS/MS	31, 33, 34, 36
No association with metastases vs. primary tumor (unmatched)	sarcosine	Serum	LC-MS/MS	36
Association with increasing PSA	Decreased citrate, polyamines. Increased choline, phosphocholine.	Prostatectomy	HR-MAS	26
Association with increasing Gleason score	Increased total choline, free choline, phosphocholine, sum of glycerophosphocholine +phosphocholine. Decreased citrate, polyamines	Prostatectomy, biopsy	HR-MAS	19, 21, 26
Association with increasing stage	Decreased citrate, polyamines Increased choline, phosphocholine.	prostatectomy	HR-MAS	26
Association with increased probability of biochemical recurrence	spermine, glutamine, glutamate, myo-inositol, phosphoryl choline, scylloinositol	Biopsy	HR-MAS	27

Prostate cancer association	Metabolite association	Specimen types	Analytical methods	References
No association with PSA, Gleason score, stage	sarcosine	Urine supernatant and sediment, serum, prostatectomy	ID GC-MS, LC-MS/MS, GC-MS	31, 33, 35, 36, 37

Abbreviations: PCa, prostate cancer; EPF, expressed prostatic fluid; PSA, prostate specific antigen; %fPSA, percent free PSA; HR-MAS, high resolution magic angle spinning magnetic resonance spectroscopy; ^{1}H-NMR, proton nuclear magnetic resonance spectroscopy; GC, gas chromatography; LC, liquid chromatography; MS, mass spectroscopy; ID, isotope dilution; MS/MS, tandem mass spectroscopy; TOFMS, time of flight mass spectrometry

* "Matched" indicates that malignant tissue and benign or normal-appearing tissue came from prostatectomies or biopsies from men with prostate cancer. "Unmatched" indicates that the benign or normal-appearing tissue came from men without known prostate cancer.