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Metabolomics and its application to the development of clinical laboratory tests for prostate cancer

Jonathan E. McDunn, Steven M. Stirdivant, Lisa A. Ford, Robert L. Wolfert

Metabolon Inc.

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Corresponding Author:
Robert L. Wolfert
Metabolon, Inc.
617 Davis Drive, Suite 400
Durham, NC 27713
E-mail: rwolfert@metabolon.com

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ABSTRACT

Introduction: There is a critical need to develop clinical laboratory assays that provide risk assessment for men at elevated risk for prostate cancer, and once diagnosed, could further identify those men with clinically significant disease.

Methods: Recent advancements in analytical instrumentation have enabled mass spectrometry-based metabolomics methodologies. Further advancements in chromatographic techniques have facilitated high throughput, quantitative assays for a broad spectrum of biochemicals.

Results: Screening metabolomics techniques have been applied to biospecimens from large cohorts of men comparing those individuals with prostate cancer to those with no evidence of malignancy. Work beginning in tissues has identified biochemical profiles that correlate with disease and disease severity, including tumor grade and stage. Some of these metabolic abnormalities, such as dramatic elevations in sarcosine, have been found to translate into biological fluids, especially blood and urine, which can be sampled in a minimally invasive manner.

Discussion: The differential abundances of these tumor-associated metabolites have been found to improve the performance of clinical prognostic/diagnostic tools.

Conclusion: The outlook is bright for metabolomic technology to address clinical diagnostic needs for prostate cancer patient management. Early validation of specific clinical tests provides a preview of further successes in this area. Metabolomics has shown its utility to complement and augment traditional clinical approaches as well as emerging genomic, transcriptomic and proteomic methodologies, and is expected to play a key role in the precision medicine-based management of the prostate cancer patient.

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BACKGROUND ON THE HISTORY OF METABOLITES AS BIOMARKERS IN THE CLINICAL LAB

Specific metabolites have been recognized as clinically actionable biomarkers for over a century. While this field did not begin with the linkage of specific biochemicals to inborn errors of metabolism (many of which have profound pathologies, including developmental and neurological defects), Garrod showed that specific clinical presentations exhibited consistent biochemical fingerprints in blood or urine, such as the marked elevation of homogentisic acid in the urine of alkaptonuric subjects [1]. With the advent of tandem mass spectrometric methods, almost all newborns in the United States have blood samples analyzed for a panel of metabolic markers that are diagnostic for more than 30 of these rare but morbid diseases [2, 3].

Beyond newborn screening, standard clinical chemistry panels measure a number of biochemicals to assess human health. Urea and creatinine provide clinically useful information regarding renal function and glucose is frequently monitored as an indicator of diabetes. As described below, recent advances in analytical technologies have enabled the field of metabolomics which in turn has facilitated the discovery of biochemical markers of many diseases (including cardiometabolic disease and numerous malignancies). When developed into clinical assays, these metabolomic biomarkers are expected to play a role in precision medicine-based patient management [4-6].

CLINICAL NEEDS IN PROSTATE CANCER PATIENT MANAGEMENT

Case management uncertainties surrounding patients presenting with intermediate levels of PSA, create an urgent need for metabolite-based diagnostic tests which might identify prostate cancer and further discriminate between indolent and aggressive disease [7, 8]. The ability to measure metabolites which correlate with the grade and stage of prostate cancer, survival rates and frequency of recurrence, in a minimally-invasive biological specimen such as blood or urine would be of great value to optimize the allocation of healthcare resources to manage prostate cancer patients, both in preventing over-diagnosis and over-treatment and in selection of subjects for active surveillance.

Prostate cancer is the most common male malignancy with an estimated 240,000 new cases and more than 28,000 deaths in the United States in 2012 [9]. Clinical detection of prostate cancer increased following the widespread adoption of serum prostate-specific antigen (PSA) screening; however, a significant fraction of prostate cancers detected solely on the basis of an increased serum PSA are indolent. As a result of concerns of over-diagnosis and over-treatment, a new paradigm of active surveillance in patient management has emerged recently [10]. The resulting broad spectrum of treatment options (active

surveillance, focal therapy, radical surgery or radiation) has been developed in response to the increased detection of low-risk prostate cancer [11]; however, the current panel of diagnostic tests provides limited information regarding the progression potential (that is, the aggressiveness) of an individual's cancer. In a recent meta-analysis of eleven large clinical cohorts, Chou et al. concluded that PSA screening had no effect on prostate cancer-specific mortality [12]. Even when prostate cancer is detected, there is a significant clinical need to improve the accuracy of characterizing the biological potential of the tumor. Therefore, new tests are urgently needed to separate those individuals with aggressive prostate cancer from those with indolent disease.

PROSTATE-SPECIFIC METABOLISM IN HEALTH AND UPON MALIGNANT TRANSFORMATION

The healthy prostate is a hormone-sensitive exocrine gland that secretes a complex milieu of biochemicals into seminal fluid, including citrate, polyamines and *myo*-inositol [13]. The prostate's ability to accumulate intracellular citrate is unique amongst human tissues and is facilitated by zinc-mediated inhibition of aconitase, the citrate-metabolizing enzyme of the tricarboxylic acid cycle [14]. Early biochemical studies identified that citrate and other prostate-specific biochemicals are depleted from prostate adenocarcinoma [15, 16]. The loss of prostate-specific metabolic functions is one manifestation of dedifferentiation upon transformation of healthy prostate cells into prostate adenocarcinoma. Prostate cancer cells also accumulate a spectrum of biochemicals that are mechanistically associated with cellular growth and division pathways such as aerobic glycolysis (that is, the Warburg effect) [17] and membrane biosynthesis [18, 19]. Importantly, androgen receptor activity impacts prostate cancer through regulation of metabolism and biosynthesis [20].

Recent studies have identified relationships between many oncogenes and various metabolic pathways, which have led to the concept of metabolic reprogramming in cancer cells [21-23]. This reprogramming primarily reroutes intracellular metabolism to support cell growth and division however, evidence is emerging that tumors may also exploit metabolic pathways in neighboring cells [24, 25]. Together, cancer and stromal cells may operate in tandem; not only do the cancer cells multiply, but the extracellular matrix is remodeled and regional and systemic physiologic responses can be affected by metabolic products (for example, angiogenesis [26], and immunosurveillance [27]). The mechanistic linkages between individual biochemicals and specific pathophysiologic responses suggest that those metabolites (and possibly metabolic waste products, too) are released by clinically significant tumors.

METABOLOMICS IN PROSTATE CANCER

Recent advancements in analytical instrumentation have enabled the discipline of metabolomics, the high throughput, information-rich study of biochemical compounds and pathways. This suite of methodologies is not constrained to measuring metabolites from a single biochemical pathway, but can measure several hundred biochemicals from a single specimen. The application of these technology platforms to prostate cancer have been reviewed elsewhere [28-30].

Sreekumar et al. [31] conducted global metabolic profiling of benign prostate and prostate tumor to identify a more comprehensive catalog of metabolic alterations in prostate cancer and to determine how these metabolic changes relate to tumor development and progression. Their unbiased metabolite profiling study identified

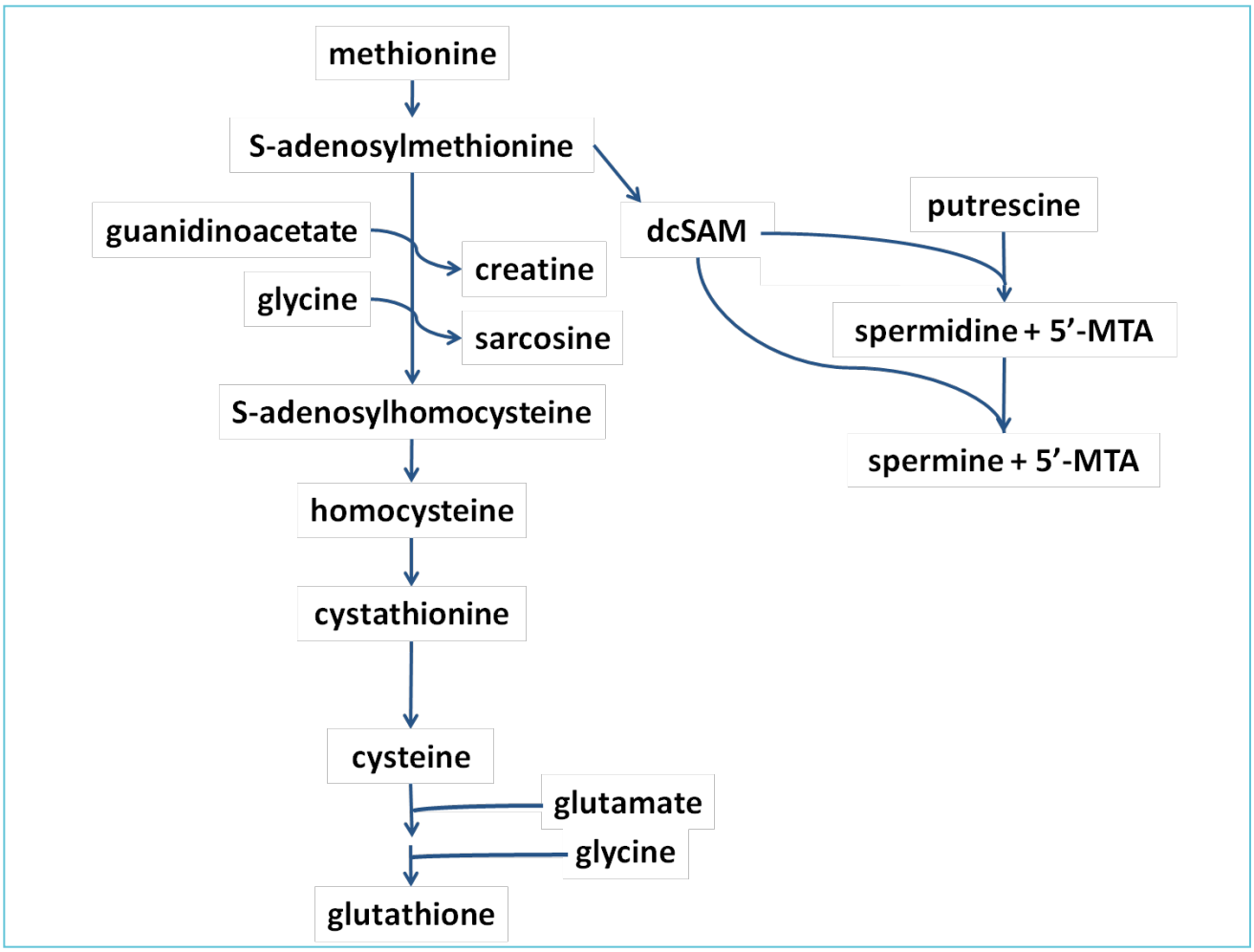
626 biochemicals; over 200 of these compounds exhibited statistically significant changes in pairwise comparisons of either benign tissue to localized prostate cancer or localized prostate cancer to metastatic sites. Six of these metabolites (sarcosine, uracil, kynurenine, glycerol-3-phosphate, leucine and proline) were found to increase across the spectrum of disease progression.

The increasing levels of sarcosine with disease progression were confirmed in an independent cohort of tissue samples using a quantitative gas chromatography-mass spectrometry (GC-MS) assay. Additional support for a role for sarcosine in prostate cancer aggressiveness has come from studies demonstrating the effect of glycine N-methyltransferase (GNMT) and sarcosine dehydrogenase (SARDH) levels on in vitro prostate cancer cell line sarcosine levels and invasive properties [31-33]. Sarcosine can be generated through methylation of glycine by GNMT. Conversely, sarcosine can be converted back to glycine through the action of SARDH. Overexpression of GNMT and knockdown of SARDH increased sarcosine levels and this was correlated with increased cellular invasiveness. An important role for GNMT and SARDH in prostate malignancies is suggested by findings that GNMT protein levels are elevated in tissue biopsies of prostate cancer and metastatic disease [33]. In addition, tumor expression levels of GNMT were found to correlate positively with PSA, stage, and Gleason score, while displaying a negative correlation to PSA-recurrence free survival [33]. The data generated in Sreekumar et al. provided evidence that global metabolomic profiling could identify new biomarker candidates of aggressive prostate cancer.

While the precise role of sarcosine in prostate cancer has yet to be delineated, it is likely related to methylation. Epigenetic silencing through methylation has been identified to play a significant role in the development and progression of many solid tumors including prostate [34, 35]. Glycine N-methyl transferase has recently been shown to harbor a functional androgen response element in its first exon allowing the expression of this gene to be driven by endogenous and synthetic androgens [36]. In histologically normal prostate and low grade prostate cancer, the GNMT protein is expressed in few cells and is primarily localized in the nucleus. Both the abundance and localization of GNMT is dramatically altered in a subset of high grade prostate tumors - GNMT was shown to be present in high abundance and present in the cytoplasm [33]. One interesting possibility is that the increase in sarcosine may be related to the reduction in polyamine abundance in prostate tumors. The metabolic intermediate S-adenosylmethionine (SAM) is used in both biochemical pathways: as a methyl donor in the GNMT reaction of glycine -> sarcosine and as an aminopropyl donor in the reaction of putrescine -> spermidine and spermidine -> spermine. Instead of consuming SAM for polyamine biosynthesis, the prostate cancer cell that can convert SAM to S-adenosylhomocysteine (by using SAM as a methyl donor) would also produce cysteine. This increase in intracellular cysteine abundance could facilitate increased glutathione biosynthesis and confer resistance to oxidative stress (Fig. 1).

A more recent study from our laboratory (McDunn et al. [37]) has extended the utility of global metabolite profiling to identify metabolic signatures of aggressive prostate cancer. Prostatectomy tissues from 331 tumors and 178 cancer-free tissues were subjected to global profiling to identify metabolite signatures associated with malignancy and tumor aggressiveness. Metabolic correlates to Gleason score, extracapsular extension, spread to seminal vesicles and/or lymph nodes and 5-year progression-free survival were identified. For each of the tumor aggressiveness characteristics, a set of metabolites was identified that differentiated between comparator groups. For instance,

Figure 1 Biochemical relationship between methylation and polyamine biosynthesis



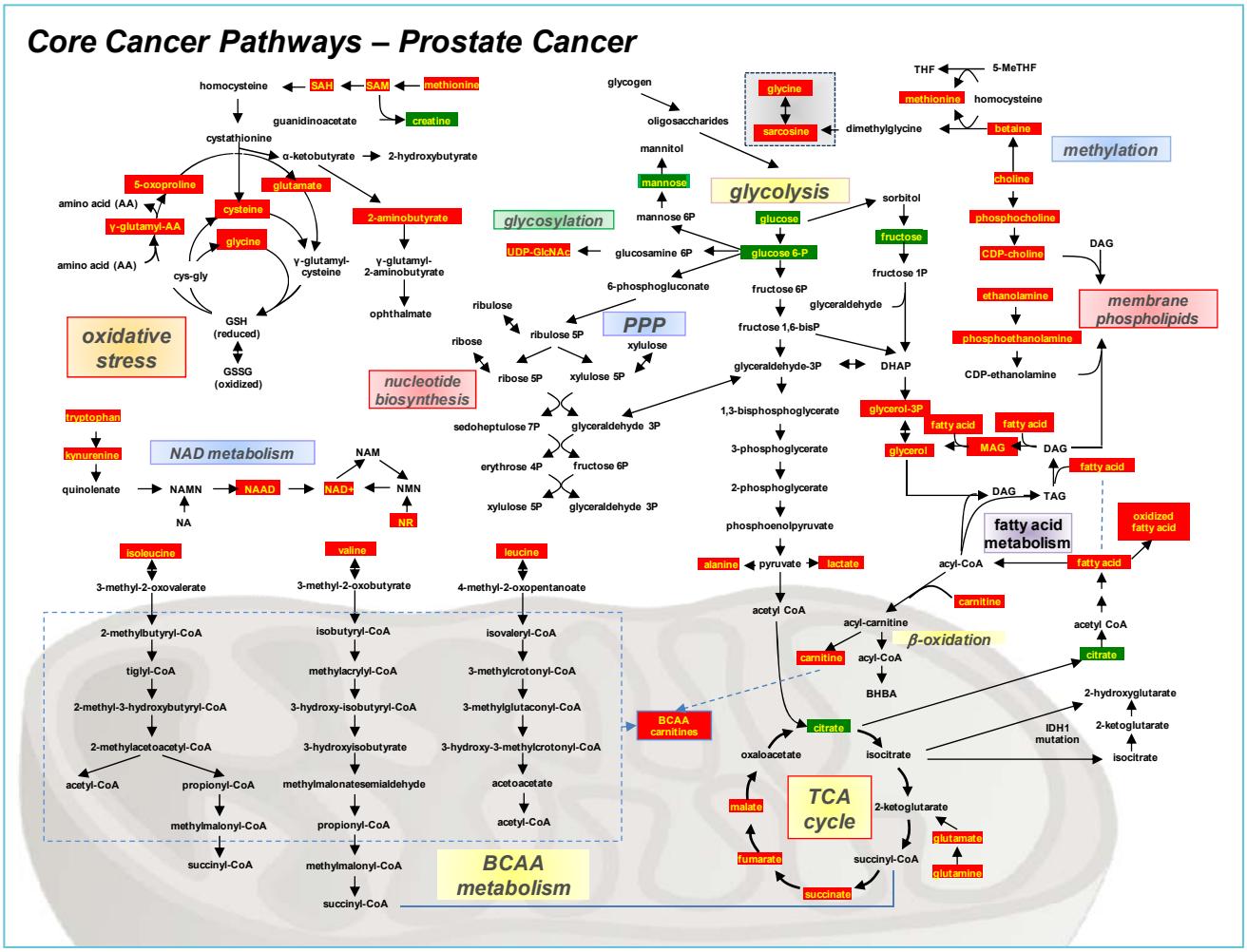
S-adenosylmethionine (SAM) is a key intermediate in both pathways. Loss of polyamine production may result in increased utilization of SAM for methylation, which in turn could result in increased intracellular cysteine and glutathione (dc-SAM, decarboxylated S-adenosylmethionine; 5'-MTA, 5'-methylthioadenosine).

in Gleason score comparisons, 28 metabolites (25 were increased in abundance; 3 were decreased in abundance) were found to correlate to Gleason pattern progression from 6 → (3+4)7 → 7(4+3) → 8, at statistically significant levels. Among the metabolite classes correlating best with Gleason pattern progression were amino acids and their catabolites, energetics related compounds, lipid components and metabolic stress associated compounds (Fig. 2)

All metabolites selected, using clinical criteria that separate clinically significant prostate cancer from indolent disease, were subjected to hierarchical clustering analysis. This approach partitioned the clinical subjects into three groups: one group was enriched for subjects with less aggressive disease and two groups were enriched for subjects with more aggressive disease, each with a specific pattern of dysregulated metabolites (Fig. 3).

Exploratory analysis of the metabolite profiling data was also carried out to determine whether metabolites could augment the performance of clinically useful prediction tools [38, 39]. A 4-metabolite panel was found to augment the performance of the commonly used Partin probability

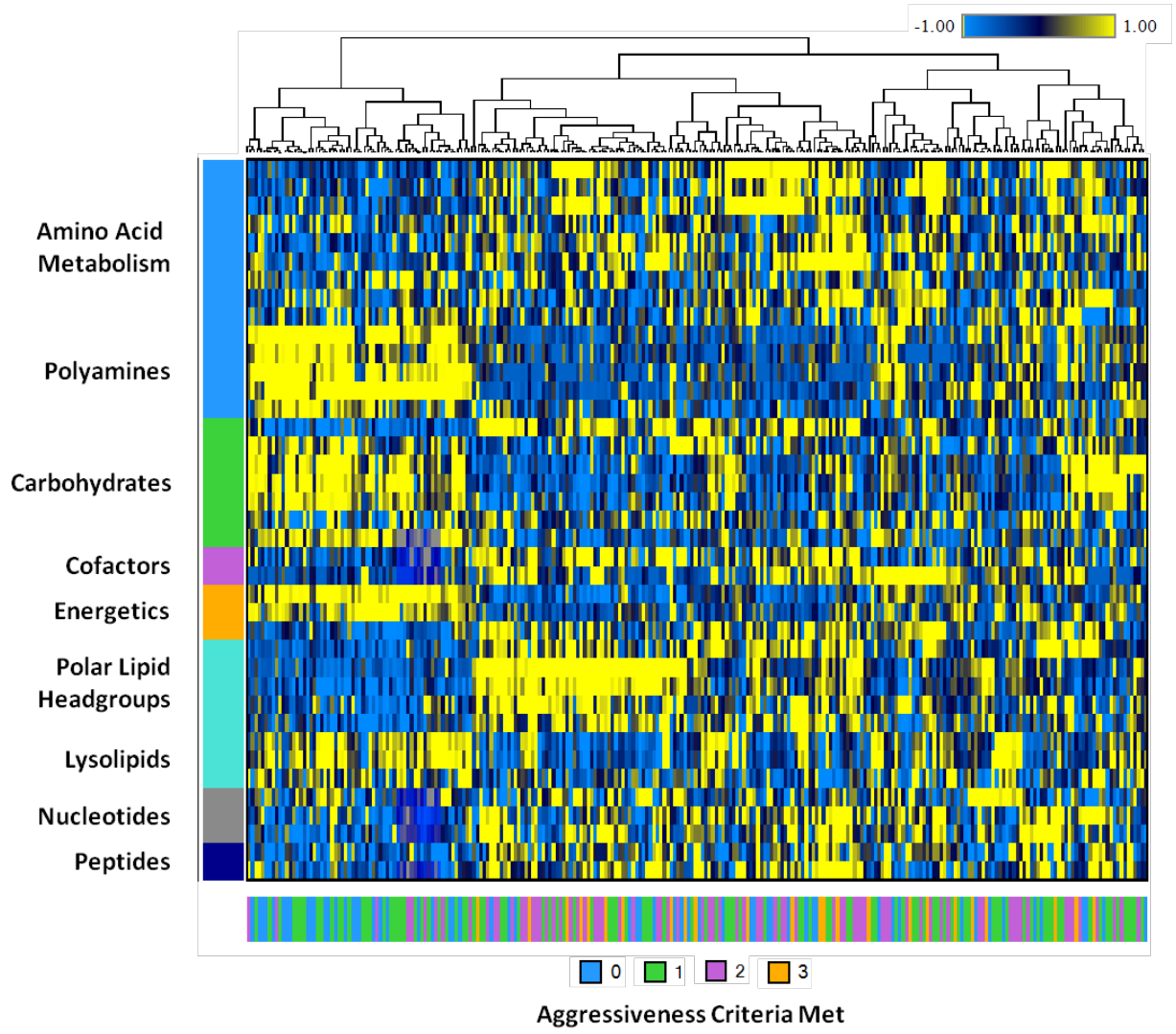
Figure 2 Changes in core metabolic pathways in prostate cancer relative to normal prostate tissue



Core metabolic pathways that are often altered in the malignant tissues are displayed. Metabolites in red boxes have been observed to be increased in prostate tumors relative to cancer-free prostate tissue, while green boxes indicate a decrease (taken from Ref. 28). Clusters of elevated metabolites are seen in pathways related to membrane phospholipid synthesis, methylation and oxidative stress. Increases in branched chain amino acid (BCAA) metabolism are suggested by an increase in BCAA related carnitines and an increase in the three BCAAs. TCA cycle intermediates were elevated along with glutamine and glutamate which can feed the TCA cycle through 2-ketoglutarate. Citrate, which acts as an intermediate in the TCA cycle and is also utilized in fatty acid synthesis, was observed to be lower in prostate cancers. Unlike many tumor types, prostate cancer tissue did not display a large increase in glycolysis intermediates typical of a shift in energy metabolism away from mitochondrial oxidative phosphorylation and toward aerobic glycolysis – although lactate and alanine which can be markers of increased aerobic glycolysis were elevated. The inter-conversion of glycine and sarcosine is highlighted in the dashed box.

nomogram for organ-confined disease, increasing the area under the receiver operator characteristic curve (AUROC) from 0.53 (clinical data alone) to 0.62 (clinical data plus metabolites). At a sensitivity of 90%, the Partin table had a specificity of 11% while the metabolites had a specificity of 17%. The Partin table and metabolites had similar performance at a specificity of 90%, (15% and 17% sensitivity, respectively). A separate 3-metabolite panel was found to enhance the AUROC of a Han 5-year progression-free survival nomogram from 0.53 (clinical data alone) to 0.64 (clinical data plus

Figure 3 Alternative clustering of prostate tissue metabolite data*



*from Ref. [28], showing associations between metabolite classification (Y-axis) and tumor aggressiveness (an aggregate variable containing 1 point for each of the following criteria met: Gleason grade > 3+4; pT2; pT3. In general, less aggressive prostate tumors have higher levels of polyamines, simple sugars and lysolipids while more aggressive prostate tumors are characterized by elevated amino acids, polar lipid head groups, and cofactors.

metabolites). Interestingly, the Han table and the metabolites had similar performance at 90% sensitivity, but the metabolites outperformed the Han table at 90% specificity (with sensitivities of 23% and 11%, respectively).

These results will clearly require validation in additional studies in independent cohorts. However, the findings from McDunn et al. suggest that panels of metabolites may enhance prediction of clinical endpoints and more effectively stratify prostate cancer subtypes, in terms of their aggressiveness and biological potential. Furthermore, these data suggest that there may be more than one metabolic phenotype of clinically significant prostate

cancer, as defined by unique metabolomic signatures. This finding may help map treatment strategies for individual patients, and suggests that metabolomics may play a vital role in the application of precision medicine for prostate cancer patient management.

TRANSLATION OF STUDIES IN TUMOR TISSUES TO CLINICAL LABORATORY TESTS

Defining prostate cancer metabolic changes has most frequently focused on the interrogation and characterization of biochemical changes in tissue samples, but ideally, metabolite markers of prostate cancer could be detected and monitored in more easily accessible specimens, such as blood or urine. Sreekumar et al. [31] found that sarcosine levels were elevated in urine and urine sediments in patients with prostate cancer, relative to specimens from patients with no evidence of malignancy. The increase of sarcosine levels in the urine sediments of men with prostate cancer was recently confirmed in an independent cohort of patients [32]. Additional studies have shown that urine and urine sediment sarcosine are correlated to biopsy findings of prostate cancer and can improve the predictive accuracy of other diagnostic modalities, including the measurements of PCA3 and percent-free PSA [40-42].

It is important to note that there is some inconsistency in the findings of the significance of sarcosine and its association with prostate cancer development and progression, as at least one clinical study has been reported where urine sediment sarcosine was not associated with the biopsy-based diagnosis of prostate cancer [43]. However, these discrepancies may be related to methodological differences in study design (patient populations), specimen preparation and analytical techniques. As Issaq et al. have pointed out, none of these manuscripts have published their methods in sufficient detail for critical comparisons to be made [44].

In addition to the work on sarcosine, a few studies have suggested that blood-borne metabolites may have utility as biomarkers to monitor the development and progression of prostate cancer [45-47].

ANALYTICAL DEVELOPMENT OF QUANTITATIVE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY (LC-MS/MS) ASSAYS FOR SARCOSINE AND OTHER METABOLITES FROM URINE SEDIMENT

The clinical studies that led to the demonstration that sarcosine is a urine marker of prostate cancer demonstrated that sarcosine in urine sediments has equivalent or better performance than intact urine or urine supernatants in separating patients with prostate cancer from patients with benign pathologies. This finding led to the development of a metabolite-based prostate cancer risk stratification tool using urine sediments.

Sediment pellets from urine specimens can be limiting and ultra-sensitive techniques are required for the analysis of metabolites from these specimens. Previously, GC-MS had been used for the metabolomic analysis of urine sediment pellets, due to the enhancement in sensitivity that derivatization can provide for small molecules. However, with the concept of an optimal clinical laboratory test in mind, the techniques required for the preparation of derivatized GC-MS/MS sample extracts and the lengthy run times necessary for GC analysis do not lend themselves to the development of a high-throughput diagnostic test.

In addition to the advances in chromatographic and mass spectrometric technology that enabled the development of a screening metabolomics platform, other advances in analytical

instrumentation have facilitated the use of LC-MS/MS in ultrasensitive, quantitative metabolite measurements. Ultra-high performance LC-MS/MS methods have been developed to measure the metabolites sarcosine, alanine, glutamate, and glycine in their underivatized form, and have demonstrated greater analytical sensitivity and accuracy that is comparable to the traditionally used derivatized GC-MS method, with an analyte correlation of >0.99. (Ford et al, manuscript in preparation). Compared to published GC-MS methods, these changes can increase the throughput of the assay by approximately 10-fold and can significantly decrease sample preparation time and consumables costs, making the development of cost-effective clinical laboratory tests more feasible. The metabolites sarcosine, alanine, glutamate, and glycine can be measured in concentrations as low as 100 picograms per sample, and the 500-fold analytical range of all of the biochemicals is suitable for measurement in typical urine sediment samples. The increase in analytical sensitivity also enables the application of this technique to routine clinical laboratory testing: in contrast to the benchmark GC-MS assay, these changes and improvements in measurement sensitivity decreased the frequency of patient samples reported as "below the detection limit" from 25% to less than 1%.

LABORATORY VALIDATION OF A METABOLOMIC PROSTATE CANCER CLINICAL TEST

The method to quantitatively measure the 4-metabolite panel associated with the presence of prostate cancer was validated and implemented in the Metabolon clinical laboratory as the Prostarix™ clinical test for the assessment of the likelihood that a patient will have a positive finding for a malignancy after a prostatic biopsy. The likelihood score is derived from a logistic regression algorithm of the LC-MS/MS-based metabolomic measurements. Urine sediment quality control samples were prepared from bulk pools of urine to obtain sediments that represent the low, medium, and high levels of analyte measurement. The performance characteristics of the analytical protocol were established by Clinical Laboratory Standards Institute (CLSI) methods, over 23 days, with % CVs in the quality control urine sediments typically ranging from 4-16% and overall Prostarix score % CV less than 6%. The performance of the analytes and the overall Prostarix score are continuously monitored using quality control samples in the clinical laboratory as well as clinical samples from an independent test cohort.

The clinical performance of this assay was determined in a patient cohort with PSA levels between 2 and 15 ng/mL who were being considered for a prostate biopsy. Analyte abundances were associated with biopsy outcomes and these measurements were combined to develop a logistic regression algorithm to generate a Prostarix Risk Score. The test performance and algorithm development were validated in an independent cohort. Results demonstrated that individuals with Prostarix scores >60 were 3.5 times more likely to have prostate cancer detected on biopsy compared to those individuals with Prostarix scores <40. (McDunn et al, manuscript in preparation). The performance of the metabolite panel (AUROC = 0.64) was superior to either PSA alone (AUROC = 0.53) or the clinical parameters that are used to calculate individualized risk of prostate cancer based on the PCPT trial (AUROC = 0.61) [39]. When available, such as in the case of a patient undergoing a repeat biopsy after a previous negative biopsy, the TRUS-measured prostate volume and the patient's most recent PSA measurement can be used along with the metabolite measurements to generate a Prostarix Plus score, which can further improve the stratification into higher risk and lower risk patient groups

(AUROC = 0.78). In the test population, both Prostarix and Prostarix Plus had performance significantly greater than either PSA or the PCPT risk calculator. At 90% sensitivity, the specificity of Prostarix was 28% while Prostarix Plus had a specificity of 41%, and at 90% specificity, the sensitivity of Prostarix and Prostarix Plus were 24% and 47%, respectively.

CONCLUSION AND FUTURE DIRECTIONS

Metabolomics, the measurement of small biochemical compounds in diverse biospecimens, may allow us to better understand the behavior of metabolites, the biochemical byproducts of cellular metabolism. The determination of these molecules is important because it may provide a fingerprint or signature to inform researchers and clinicians of the biochemical cellular activities on-going in healthy and diseased tissues. Both global profiling (the measurement of thousands of small molecules) and targeted analyses (directed, quantitative measurements of a limited panel of biomarkers) may provide a critical opportunity for improving our ability to detect, characterize and potentially manage and treat prostate cancer. Differences in the prostate cancer subtype metabolic behavior may be correlated with characteristic metabolomic biochemical signatures and these signatures could aid in the characterization and classification of tumors. The measurement of small biochemicals reflects a phenotype that should be associated with the development and progression of prostate cancer. These biochemical changes may indicate, and serve to integrate, alterations in the genome, transcriptome and proteome that lead to downstream metabolic dysregulation. Our ability to identify and accurately quantitate the relevant biomarkers will depend on the application of a wide spectrum of analytical and bioinformatic techniques to fully interrogate the entire metabolome. In addition, the ability to reproduce and confirm results from individual laboratories will only be possible with comprehensive published descriptions of methodologies and informatic approaches.

Since the metabolic spectrum should be highly responsive to changes in physiologic status, the development and progression of a prostatic malignancy, conversion to a higher aggressiveness phenotype, and response to treatment, is anticipated to be assessable by baseline measurements and serial monitoring of metabolic biomarkers over the course of the management of the prostate cancer patient. However, there are clearly strengths and limitations to the ultimate objective of developing metabolomic-based clinical diagnostics for prostate cancer. Among the conditions that favor efforts to develop such tests are the high levels of analytical sensitivity and specificity of measurements that are available with the recent advances in instrumentation and bioinformatics, the ability to measure these biomarkers in a variety of biospecimen types (blood, urine, tissue, etc.), and the rapidly expanding application of these techniques into the clinical laboratory.

The challenges to the implementation of this technology remain profound: significant work must be done to establish and characterize potential confounding factors to the accurate measurement of the biomarkers of prostate cancer metabolism, including the influence of diet, medications, supplements, physical activity, sampling times, diurnal variation, specimen preparation, stability, etc. The effects of numerous pre-analytical variables must be assessed and minimized, in the effort to develop robust, reproducible and clinical useful diagnostic methods. In addition, issues of standardization, throughput, and instrument costs will potentially serve as near-term impediments to the broader introduction of the technology into the clinical laboratory.

Since the initial publication identifying sarcosine as a mechanistic biomarker of prostate cancer progression, several researchers have developed analytical methods to measure sarcosine. Early attempts to characterize the clinical utility of sarcosine in different matrices and different clinical populations had mixed results, and the possible reasons for this have been laid out elsewhere [28, 44].

A limited number of clinical studies evaluating the relationship between serum sarcosine and prostate cancer in men have given disparate results (for example, [48, 49]) and more work will be required before the clinical utility of sarcosine in this matrix is fully appreciated.

As described above, there has been considerable effort invested into both the pre-analytical and analytical methods for sarcosine quantitation during the development of Prostarix.

The pre-analytical method was found to be sensitive to contamination and operator-to-operator variability whereas the analytical method required extensive development to achieve an acceptable lower limit of quantitation (methodologic optimization significantly reduced instrument background and minimized matrix suppression and chemical interferences).

Despite these challenges, a number of groups have shown that sarcosine in post-DRE urine specimens can be used to stratify prostate cancer risk (Table 1).

All considerations accounted for, the outlook for the use of metabolomic technology in addressing the clinical diagnostic needs for prostate cancer patient management remains bright. Specific and characteristic metabolic changes have been described that are associated with the prostate and its malignant transformation. Early validation of specific clinical tests provides a preview of further successes in this area. The ability to complement and add important clinical insight to other traditional clinical approaches and emerging techniques applying genomic, transcriptomic and proteomic methodologies makes a strong argument for the potential for metabolomics to improve the practice of precision medicine for prostate cancer patients.

Table 1 **Clinical studies of post-DRE urine sarcosine and its ability to stratify men with regard to subsequent biopsy outcomes**

Number of subjects, N (Biopsy-positive / Biopsy-negative)	Analytical approach	AUC	Reference
131 (86, 45)	LC-MS/MS (derivatized)	0.67	[41]
110 (71, 39)	LC-MS/MS (derivatized)	0.70	[40]
139 (106, 33)	GC-MS (derivatized)	0.63	[43]
93 (49, 44)	GC-MS (derivatized)	0.71	[31]
56 (33, 23)	GC-MS (derivatized)	0.82	[42]
345 (211, 134)	GC-MS (derivatized)	0.71	[32]

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