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Review

Metabolomics for mitochondrial and cancer studies ☆

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ABSTRACT

Metabolomics, a high-throughput global metabolite analysis, is a burgeoning field, and in recent times has shown substantial evidence to support its emerging role in cancer diagnosis, cancer recurrence, and prognosis, as well as its impact in identifying novel cancer biomarkers and developing cancer therapeutics. Newly evolving advances in disease diagnostics and therapy will further facilitate future growth in the field of metabolomics, especially in cancer, where there is a dire need for sensitive and more affordable diagnostic tools and an urgency to develop effective therapies and identify reliable biomarkers to predict accurately the response to a therapy. Here, we review the application of metabolomics in cancer and mitochondrial studies and its role in enabling the understanding of altered metabolism and malignant transformation during cancer growth and metastasis. The recent developments in the area of metabolic flux analysis may help to close the gap between clinical metabolomics research and the development of cancer metabolome. In the era of personalized medicine with more and more patient specific targeted therapies being used, we need reliable, dynamic, faster, and yet sensitive biomarkers both to track the disease and to develop and evolve therapies during the course of treatment. Recent advances in metabolomics along with the novel strategies to analyze, understand, and construct the metabolic pathways opens this window of opportunity in a very cost-effective manner. This article is part of a Special Issue entitled: Bioenergetics of Cancer.

1. Introduction

Metabolomics, the term introduced by Fiehn et al. [1,2], is a technique involved in the global detection of several small molecule metabolites and identification of metabolic preprogramming i.e. changes occurring in metabolic pathways and phenotypes. Metabolomics also involves characterization of metabolic phenotyping, i.e. classification of time varying alterations in metabolism, and discovering metabolite imbalances or stress induced loss of homeostasis. Hence, metabolomics can be defined as the quantification and analysis of dynamic changes in metabolites under either certain culture or biological conditions or in response to the microenvironment and/or agonists/antagonists. In defining metabolomics, it will be prudent to go through the few other terminologies, which provide analogous or metabolic phenotypic information. Metabolomics refers to the detection of all of the metabolites in samples, whereas "Metabonomics" refers to the changes in metabolism due to altered environment. Altered environments include changes in diet, changes in drug consumption and development of diseases. On the other hand, "metabolite footprinting" is a high-throughput technique for analysis of metabolites that are secreted or consumed by cells or tissues in extracellular media, and has been very effective in analyzing microbial metabolism [3]. Metabolite footprinting, or exometabolome, has various advantages over "metabolic fingerprinting," which is a technique for analyzing intracellular metabolites that relies more on classifying the samples in different states. The other commonly used technique, "metabolite profiling," involves characterizing and measuring metabolites. The dynamic analysis of metabolic systems can be achieved by perturbing the extracellular/tissue environment and performing metabolite profiling over time series and further estimating metabolite fluxes (Fig. 1).

Although metabolomics has been in existence for several decades it has not received much attention in clinical research compared to its sister omic sciences such as transcriptomics, genomics and proteomics. This is quite surprising given that metabolomics has been used for at least past 2 decades to decipher congenital disorders and inborn genetic errors of metabolism. However, technological advances in both instrumentation including mass spectrometry (MS), nuclear magnetic resonance (NMR), gas

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and liquid chromatography, and informatics or chemometrics based software has led to a recent surge in utilization of metabolomics in cancer diagnostics, prognosis, and therapeutics and in various other metabolic diseases. Indeed, while it is known that transcriptomics and genomics have played a major role in understanding and solving cancer and other diseases, it is a little known fact that metabolic changes are more sensitive to environmental perturbations and can easily track genetics as well as stress, diet and environment related physiological changes. As compared to transcriptomics and proteomics, metabolomics holds promise because of several reasons: (a) phenotypic changes are reflected as downstream adaptations and alterations in metabolome, thus perturbations are amplified when they are translated to metabolome as compared to transcriptome and genome, thus making metabolomics a highly sensitive technique in comparison with transcriptomics and genomics; (b) profiling of metabolic biomarkers is much cheaper and sample preparation is simpler; (c) it is easier to compare data over several conditions and biochemical derangements; (d) it allows for easy transfer of analysis between organisms [3].

As other -omics fields, metabolomics started within the century and has advanced significantly (Table 1). Metabolomics grew when news of a doctored report that NMR can detect cancer was published in the 1980s [4]. Interestingly, at present, metabolomics is widely being researched in cancer, and it has shown that cancer and normal cells produce altered metabolic profiles that can aid in disease detection, predicted response to therapy, and development of new therapies (Table 2). Metabolomics can be categorized as either one of two main classes: global or specific. Global metabolomics surveys changes in the quantities of a large number of metabolites under certain conditions to find interesting metabolic trends, whereas specific metabolomics surveys changes in a smaller number of metabolites that are hypothesized to be altered under certain conditions. Metabolomics involves specific methodical steps. It involves 1) extraction of metabolites from tissues or cells, 2) methods to quantify the metabolite changes observed due to altered cell culture or tissue conditions, and 3) methods of data analysis. Both global and specific metabolomics involve similar metabolite extraction methods for particular analysis used and similar techniques to analyze the data. The two most used methods for quantifying data are NMR or gas chromatography paired with mass spectrometry (GC/MS). Additionally, a variety of computer programs have been used to analyze data, which will be later discussed.

Significant advances in the field of metabolomics have been made, especially from the disease standpoint. To give an example, it has been widely used in the study of cardiovascular disease and arthritis [5,6]. The interest in the use of metabolomics in the study of cancer began when Otto Warburg proposed the Warburg effect—that cancer cells switch from producing most of their energy in the form of adenosine triphosphate (ATP) through OXPHOS to producing most of their energy through glycolysis. This observation sparked the interest in studying the metabolomics in many types of cancer, including colon [7,8], ovarian [9,10], hepatocellular carcinoma [11], and breast cancers [12]. Metabolomics has been used to study biomarkers for these cancers in order to aid in early detection of disease, especially in cancers where early detection and screening is difficult but results in better prognosis. For example, kidney cancer is one disease that is not normally detected at an early stage because of screening difficulty. Kim et al. showed that

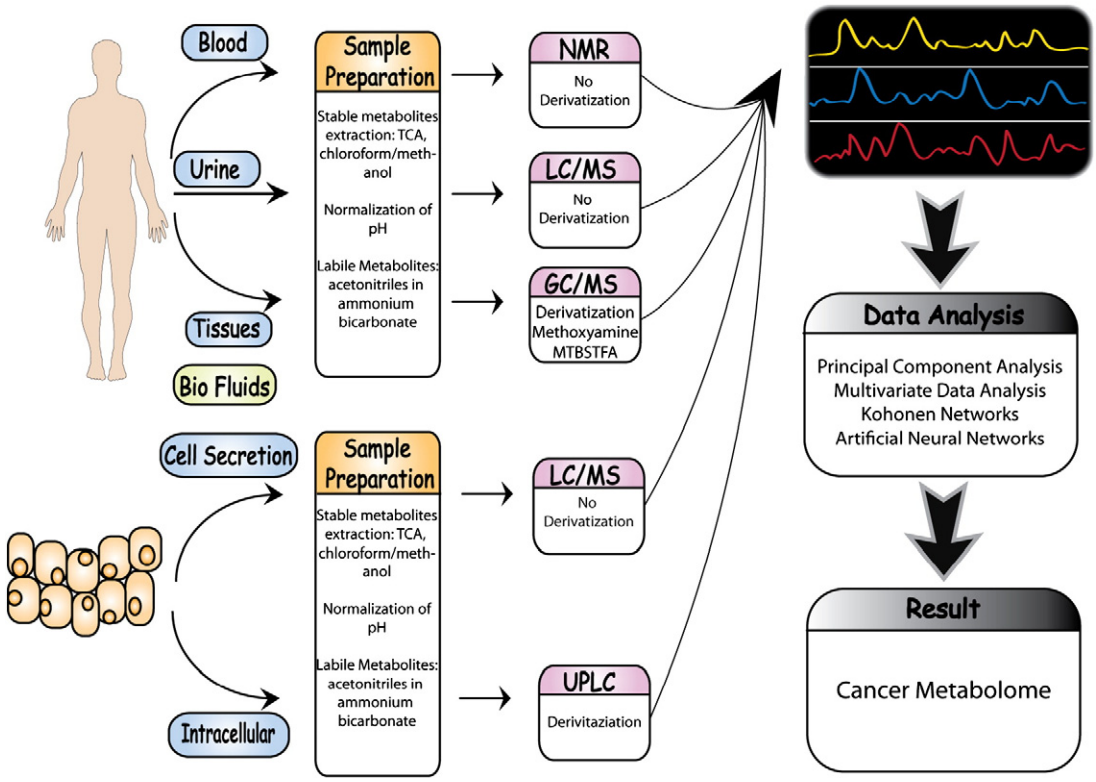

Fig. 1. Schematic of mass spectrometry- (MS) and nuclear magnetic resonance- (NMR) based metabolic strategies for cancer diagnostics. Biofluids such as urine, saliva, tissue, blood are amenable for use in current metabolomics techniques. GC-MS technique derives its high resolution because of derivatization technique. The data obtained are further deconvoluted and then analyzed using supervised and unsupervised chemometrics-based classification techniques. The extracellular media from cells are analyzed in a similar fashion. However, enzymatic activity and UPLC is also suitable for these samples because of reduced complexity.

Table 1
Usage of metabolomics techniques in basic science.

Proteins and pathways	Techniques	Ref.
Metabolism of alcohol through tryptophan build-up due to PPAR-a deletion	Mass Spectrometry	[92]
Pathways responsible for insulin deficiency	H1 NMR, liquid chromatography-tandem mass spectrometry	[86]
Understanding chemotherapy drug toxicity pathways	Ultra-performance chromatography-linked electrospray ionization quadrupole time-of-flight mass spectroscopy	[93]
Understanding of pathways involved in action of chemotherapy drugs bezafibrate (BEZ) and medrosxyprogesterone acetate (MPA)	H1-NMR	[94]
Understand DNA Damage Repair Pathway involved in Cancer	Tandem mass spectrometry	[95]
Identify metabolites and pathways whose levels are dependent on long-chain fatty acid combustion rate, which is associated with insulin resistance	Gas Chromatography/TOF-Mass Spectrometry	[42]
Nutrient Gene Interactions	Isotope tracer based mass spectrometry	[96]
Metabolic adaptation during hypoxia in drosophila flies	1 H NMR spectroscopy	[97]
Eicosanoid metabolism and lipidomics flux analysis	Enzymatic activity	[98]

urine metabolic profiling through the use of hydrophilic interaction chromatography and mass spectrometry (HILIC-MS) can be used to detect biomarkers for kidney cancer [13]. Gao et al. also used metabolic profiling of human serum in order to determine biomarkers alterations specific to renal cell carcinoma [14]. Apart from kidney cancer, ovarian cancer metabolomics is also being widely studied. Odunsi et al. have used H-NMR on serum in order to create a metabolic fingerprint for ovarian cancer as compared to normal postmenopausal women and were able to see significant metabolic differences using a variety of analysis techniques [15]. Metabolomics of prostate cancer is also being studied for a screening sensitivity and specificity that is higher than the gold standard sensitivity and specificity using serum prostate specific antigen (PSA) alone as a measure. Teahan et al. have conducted metabonomics (NMR metabolic profiling) on a non-tumorigenic prostate epithelial cell line and other tumorigenic prostate cell lines, analyzed alterations in intracellular and extracellular metabolites. Results showed that phosphatidyl choline amounts increased in the transformed cells, whereas branched amino acid amounts decreased in transformed cells [16].

Metabolomics can also be useful from the therapeutic perspective, since biomarkers can also be readily identified that can distinguish people who will respond to certain therapies from those that do not respond to therapy. Metabolomics has also proven useful in determining the effect of drugs on cancer. It was shown through metabolomic analysis that bezafibrate and medroxyprogesterone when administered together act to increase reactive oxygen species in Acute Myeloid Leukemia (AML) cells therefore uncovering the mechanism behind cell death due to the drugs [17]. Furthermore, metabolomics can also be applied in evaluating the efficacy of radiotherapy. For example, Lyng et al. have employed a special type of NMR in order to determine the metabolic profile associated with apoptosis of cervical carcinoma cells in response to radiotherapy [18]. All these studies show that there are a number of significant metabolomic studies underway which could potentially yield much needed outcomes, not only determine the effect of existing therapies but also could create new therapies.

Due to the versatility of its use in disease diagnostics and therapy, it can be predicted that research in metabolomics will grow in the future, especially in the cancer field where there is a great need for more sensitive and specific cost-effective diagnostic tools, and a great urgency for effective therapies and biomarkers to predict more accurately the response to a therapy in order to avoid unnecessary drug administration and the accompanied side effects. This paper will give a detailed and comprehensive overview of metabolomics: metabolomics and its uses, relation to cancer, and the future of the field.

2. Metabolomics techniques

Metabolomics is a technique of analyzing the cellular metabolites (~2000 metabolites) and most common techniques used for profiling metabolites are NMR and MS. MS mostly requires chromatographic separation before MS to increase resolution. Commonly used chromatographic systems are high performance

Table 2
Usage of metabolomics techniques in clinical cancer research.

Purpose	Techniques	Ref.
Metabolic analysis of biomarkers in renal cell carcinoma	H1 NMR	[14]
Detection epithelial ovarian cancer in tissue specimens	H1 NMR	[15]
Detection of oesophageal cancer	Gas Chromatography-Mass Spectroscopy	[99]
Create hepatocellular carcinoma metabolic profiles to use for potential early diagnosis	NMR	[100]
Create metabolic profile for potential early diagnosis of prostate cancer	NMR	[16]
Create metabolic profile for early oral cancer detection	H1 NMR	[101]
Investigate potential biomarkers for hepatocellular carcinoma	Gas Chromatography-Mass Spectrometry	[11]
Molecular signature for kidney cancer detection	Hydrophilic interaction chromatography-mass spectrometry	[13]
Use metabolic signatures to differentiate between liver cancer and hepatitis and hepatocirrhosis	HPLC	[102]
Use Metabolomics for detecting drug resistance in cancer	NMR	[103]
Metabolic analysis of invasive and borderline ovarian Cancers	Gas Chromatography/time-of-flight mass spectrometry	[10]
Altered metabolism in human lung cancer patients *in situ*	NMR and GC-MS used for ^{13}C-isotopomer-based metabolic analysis	[23]

liquid chromatography/mass spectrometry (HPLC/MS and LC/MS/MS) and gas chromatography (GC/MS). Table 3 compares both of these techniques. Although GC-MS relies heavily on derivatization chemistry it has been more commonly used because of high resolution and sensitivity over NMR. LC-MS can overcome limitations of derivatization chemistry in GC/MS and is well suited for samples or metabolites that cannot bear volatile treatment conditions of GC/MS. Recent technological advances has led to the development of Fourier-transform-ion cyclotron resonance mass spectrometry (FT-ICR) which allows direct infusion and has high resolution [18,19].

To clearly associate the spectra to compounds, deconvolution of NMR spectra is required and current technologies still suffer from unclear signal discrimination. Ludwig et al. have developed a fast targeted multidimensional Hadamard-encoded NMR spectroscopy method that can not only deconvolute NMR spectra of blood obtained from colorectal cancer patients but also have fast acquisition property [20]. Wen et al. applied NMR-based metabolomics on bile collected from individuals with biliary tract cancer or benign biliary tract disease and included pattern recognition and multivariate data analysis to achieve significant differences between cancer and benign individuals compared to other conventional approaches [21].

LC/MS and GC/MS rely on chemometrics tool, data mining and multivariate data analysis for classification of metabolite peaks. Often, these tools are built-in and programmed in the instrument. Recent advances have led to the usage of nonlinear unsupervised classification techniques like Kohonen networks for structure identification and artificial neural networks for classification of unknown spectra against spectral libraries available from National Institute for Standards and Technology (NIST).

The tracer techniques are useful for obtaining mechanistic understanding of metabolic maps during disease progression, malignant transformations, and epigenetic modifications leading to altered metabolism. The isotopomer techniques work on the principle of isotopic enrichment at different atomic positions in a desired metabolic product, i.e. a labeled metabolite after undergoing a metabolic reaction leads to an isotope-labeled product whose enrichment allows uncovering of the source of alternate pathways in generating that product. Using labeled isotopes, tracing can be done back to the precursor molecules and thus reveal unique distribution of fluxes, information about flux ratios, and directionality of enzymatic reactions. Both NMR and MS are commonly used for isotopomer analysis and resolution of metabolites occurs because of shifts in frequency. However, FT-ICR is gaining popularity because of its ultra high mass resolution and significant advantage over other MS techniques due to non-requirement of prior chromatographic separation [18,19,22]. The advantage of stable isotope labeled metabolomics approach in cancer is the presence of a large library of ^{13}C-labeled isotopes, including ^{13}C-labeled glucose, ^{13}C-labeled glutamine, serine, glycine, other amino acids, ^{13}C-labeled saturated fatty acids, and ^{13}C-labeled acetate, thus allowing determination of metabolic pathways in cancer related to protein and fatty acid synthesis, nucleic acid synthesis, ribose moieties of the nucleotides, citric acid cycle and glycolytic cleavage of carbohydrates. Generally, in isotopomer techniques, both stable and labile metabolites extraction techniques are similar to non-isotope techniques; however, they both differ in terms of structure elucidation techniques.

3. Metabolomics in cancer

A flurry of recent work has established that metabolomics approaches can serve as an important tool for detection, prognosis, biomarker discovery and design of therapeutics. Metabolomics involves collection of biological samples to be analyzed for metabolic changes associated with certain disease conditions. Biological samples commonly utilized include blood, urine, and tumor tissue. Denkert et al. used gas chromatography/time of flight mass spectrometry (GC-TOF MS) to analyze ovarian cancer metabolism in both invasive carcinomas and borderline tumors (noninvasive and have proliferation rate similar to benign tumors) [10]. In their study authors found 51 metabolites (out of a total of 291 different metabolites) to be different between invasive carcinoma and borderline tumors. They connected these metabolites using Kyoto Encyclopedia of Genes and Genomes (KEGG) to different pathways and enzymes and detected differences in glycerolipid (glycerolphosphate alpha, inositol-phosphate and glucose-1-phosphate), free fatty acid (nonadecanoic, stearic, heptadecanoic acid), amino acid metabolism (proline, glutamic acid, glycine, γ-aminobutyric acid, glutamine, threonine, and asparagine) and energy metabolism (malic and fumaric acid). Interestingly, malignant tumors had higher metabolic turnover rates, nitrogen donor amino acids (glutamate, glutamine and asparagine), and amino acids serving as building blocks in protein biosynthesis (cysteine, glycine and threonine). Denkert et al. performed metabolite profiling of normal and colon cancer tissue and found 82 metabolites out of 206 to be significantly different in two groups [8]. Metabolites involved in citrate cycle were lower in cancer tissues compared to normal colon samples. There was an upregulation of precursors needed for DNA synthesis (purines) and amino acids (protein synthesis). Interestingly, authors found that the most important metabolite for alteration of metabolism in colon cancer is β-alanine, which was highly upregulated in carcinoma tissues. Fan et al. infused ^{13}C-glucose into human lung cancer patients and analyzed resected tissues and blood plasma by ^{13}C-isotopmer-based metabolomic studies using NMR and GC/MS. In their study, authors found that in lung tumors there was increased incorporation of carbon from

Table 3
Comparison of various metabolomic techniques.

Characteristics	NMR	MS	Ref.
Sensitivity	Medium	High	
Sample preparation	Easy. Non-destructive sample preparation.	Medium—often need separation methods to increase peak capacity and derivitization	[104,105]
Limitations	Not widely used in clinic, not high throughput for analysis of metabolites, need special training to use equipment	High cost, not widely used in clinics, need special training to use equipment	[104]
Reproducibility	High	Low for biofluids	
Resolution	High	High because of liquid chromatography or gas chromatography	[67]
Quantification	Easy	Difficult	[104]
Throughput	Low	High	[18,19]

glucose into lactate, alanine, citrate, glutamate, succinate, ribosyl moiety of nucleotides, and aspartate relative to noncancerous lung tissue [23].

As mentioned earlier, Warburg effect indicates that cancer cells have altered or dysfunctional mitochondrial metabolism. Metabolomics is a promising technique to unravel differences in mitochondrial pathways between invasive carcinomas with border line tumors and/or normal tissues. Hirayama et al. used capillary electrophoresis time-of-flight mass spectrometry for 16 colon and 12 stomach cancer patients and performed quantification of 94 metabolites in colon and 95 metabolites in stomach involved in glycolysis, the pentose phosphate pathway, the TCA and urea cycles, and amino acid and nucleotide metabolisms [24]. Authors found significant organ level differences in TCA cycle metabolites indicating the dependence of tissue on oxygen and nutrition availability. Most importantly, authors show in their work that metabolomics could be efficiently used to understand altered energy metabolites in cancer.

Below we will review the current and potential applications of metabolomics using various biofluids, its use in cancer diagnosis and prognosis, and for studying bioenergetics of cancer.

3.1. Metabolomic profiling of urine

Urine is often collected and analyzed in order to identify cancer biomarkers. The ability to analyze urine for biomarkers of disease is advantageous when compared to many other screening techniques since it is completely non-invasive and does not cause harm to patients. It has been shown that analyzing urine of individuals with breast cancer and screening for multiple biomarkers can have a similar sensitivity to traditional mammography without exposing the patient to x-rays [25]. Analyzing urine for biomarkers could also provide a convenient way to screen individuals for cancer, especially when cancer is difficult to screen and detect, such as ovarian cancer [26] and hepatocellular carcinoma [11].

Utilizing urine for metabolic screening and biomarker identification employs similar methods that are used for analyzing other biological fluids of cells. After urine is collected from both diseased and healthy individuals, chromatography techniques, including gas chromatography and liquid chromatography, followed by mass spectrometry techniques are used in order to separate and identify metabolites for both the diseased and normal cases. Analysis techniques used to cluster or separate the diseased and normal cases are employed to determine whether metabolites detected can be used to discriminate normal individuals from diseased patients.

Urine has been analyzed for biomarkers for various cancers, including hepatocellular carcinoma [11], breast cancer [25], lung cancer [27], and colorectal cancer [28]. Analysis of collected urine has revealed metabolic changes that can be potentially used to discriminate between cancer patients and non-diseased individuals. Since RNA metabolism is altered in cancer, it was hypothesized by Hsu et al. that metabolomic studies on colorectal cancer patient urine using high performance liquid chromatography, liquid chromatography, and mass spectrometry can show that nucleosides are altered in cancer compared to normal cases. The group showed that higher amounts of 5 different nucleosides were present in urine from colorectal cancer patients in comparison to normal individuals [29]. Urine has also been shown to distinguish patients with lung cancer from normal patients, as opposed to breath tests that are more difficult to perform than urine collection and have low specificity. More specifically, volatile components were shown to be of lower levels in cancer patients as opposed to normal individuals, which is speculated to be linked to metabolic changes associated with cancer [28]. In a study conducted by Woo et al., urine was collected and analyzed for biomarkers of three women's cancers: breast, ovarian, and cervical cancer. Using both LC/MS and GC/MS, the group found potential biomarkers for each cancer, some in relation to oxidative DNA damage, a possible contributor to cancer development [26]. In addition to cancer biomarker discovery, urine analysis has been used to determine biomarkers for highly aggressive forms of cancer as opposed to less aggressive forms. For example, increased levels of sarcosine, an N-methyl derivative of glycine, were shown to be associated with more aggressive and invasive forms of prostate cancer through urine metabolomics [30]. Studies that link a biomarker with a stage and aggressiveness of disease are important since it can potentially increase the likelihood of giving a patient the appropriate therapy, thus reducing that patient's side effects and increasing one's quality of life.

Although the use of urine in metabolomics has led to the identification of potentially useful biomarkers for cancer, it still faces numerous challenges, among which the key hurdles are: (i) some metabolites are non-volatile in urine and need to be derivatized before injection when using gas chromatography [11]; (ii) it is also difficult to determine whether samples were collected after a specified period of time, and proper procedures must be adopted while performing the analysis to compensate for this uncertainty [29]. As with other biological samples, care must be taken when handling and preserving urine specimens to avoid variable results. Urine, for example, cannot be thawed and refrozen many times, which can change the composition, and thus affect the results of the experiment [31]. Lastly, urine composition tends to vary with diet more than with other biological fluids and thus could increase variability in metabolomic profiling [32]. Despite these complicating factors, urine collection and analysis still provides a convenient way to study cancer metabolism and detect cancer biomarkers [13,30,33].

3.2. Metabolomic profiling of blood/serum

DeSilva et al. applied NMR spectroscopy for metabolic footprinting of lipid metabolites in human serum using ^{31}P containing derivatization reagent 2-chloro-4,4,5,5-tetramethyldioxaphospholane (CTMDP) and obtained high sensitivity and spectral resolution for lipophilic components [34]. Their approach holds promise because it is highly quantitative and thus will aid in analyzing lipodsytrophies in human serum. In an interesting study, Hartmann et al. (2008) performed metabolic profiling of colon cancer cells using solid phase microextraction (SPME) followed by GC/MS and found that type and amount of serum supplementation plays a major role in inducing metabolic differences in these groups [35,36].

3.3. Metabolomics profiling of saliva

Although saliva is an important biofluid, it has not been widely used for studying cancer diagnosis. Recently, Sugimoto et al. [37] performed metabolic footprinting of saliva from patients with oral, breast and pancreatic cancer using capillary electrophoresis mass spectrometry, and the study revealed significant differences between pancreatic cancer patients and healthy controls in eight metabolites (leucine, isolecuine, tryptophan, valine, glutamic acid, phenylalanine, glutamine, and aspartic acid) and taurine and piperidine in oral cancers.

3.4. Clinical factors

3.4.1. Prognosis

Metabolomics guided prognosis holds promise for cancer prognosis and is gaining hold in cancer recurrence, metastatic potential, and post surgery analysis. Metabolomics based methods

can fill the gap where clinical stages are undefined because pre-surgery clinical or pathological criteria may not hold well. In prostate cancer, although the patients' prognosis is done based on the serum PSA levels before surgery, after prostectomy, extracapsular extensions, lymph node metastasis are combined to predict recurrence after prostatectomy. Furthermore, after surgery the information obtained from examination of prostate is currently the only viable way to know about malignancy. Maxeiner et al. used *ex vivo* tissue magnetic-resonance-spectroscopy based metabolomics profiles to investigate the recurrence of prostate cancer and using principal component analysis predicted the recurrence with 78% accuracy [38]. Denkert et al. [10] found that differences in metabolic profiles in invasive and borderline ovarian tumors were linked to prognostic factors such as dihydropyrimidine dehydrogenase and thymidine phosphorylase [39]. Sitter et al. used ERETIC (Electronic reference To access in vivo concentrations) an advanced technique for quantifying metabolites in breast cancer patients using high resolution magic angle spinning MR spectroscopy (HR MAS MRS) with different prognosis [40]. Interestingly, authors found lower concentrations of glycine in patients with good prognosis and higher concentrations in patients with poor prognosis. In metastatic patients or patients who had recurrence, the authors found lower values of taurine/glycine and GPC/glycine compared to patients who were healthy 5 years after surgery. Their results show that these metabolite ratios can play an important role in prognosis and recurrence.

3.4.2. Diagnosis

Many cancers go undetected or patients present themselves at late stage when cancer has already metastasized and surgery remains the only intervention. Moreover, surgery may not often lead to cure because of either cancer recurrence after surgery or due to multi-organ syndrome. Hence, development of early detection strategies is key to success in developing medical treatment in cancer. Sreekumar et al. used metabolomic profiles obtained by highly sensitive and specific isotope dilution GC/MS to distinguish benign prostate, clinically localized prostate cancer and metastatic disease [30]. Their results showed that level of sarcosine, an N-methyl derivative of the amino acid glycine, was increased during prostate cancer progression and was highly upregulated in invasive prostate cancer cell lines (VCaP, DU145, 22RV1) relative to benign prostate epithelial cells (PrEC) and immortalized benign RWPE prostate cells. The comparative metabolomics analysis between benign and clinically localized prostate cancer revealed differential expression in 87 out of 518 metabolites, 50 of which were upregulated and 37 of which were found to be downregulated. Additionally, when metastatic samples were compared to clinically localized prostate cancer samples, 124 out of 518 metabolites were found to increase in metastatic samples and 102 metabolites were downregulated. In general, sarcosine, kynurenine, glycerol-3-phosphate, leucine and proline were significantly increased in prostate cancer. They concluded that sarcosine levels correlated well with cell invasiveness and the exogenous addition of sarcosine and its precursor glycine transformed the phenotype of benign prostate epithelial cells into a more invasive one. To further test the specificity of sarcosine with respect to invasiveness, knockdown of sarcosine dehydrogenase—the enzyme that degrades sarcosine—was done. The authors found that invasive phenotype in benign cells increased in the knockdown cells. Interestingly, master transcriptional regulator of prostate cancer, androgen receptor along with the ETS gene fusions was found to transcriptionally regulate sarcosine levels. Urayama et al. used mass spectrometry based metabolite profiling to develop biomarkers for detecting pancreatic cancer from blood plasma and suggest that plasma concentration of lipids, amino and bile acids may play an important role in early detection of pancreatic ductal adenocarcinoma (PDAC) [36].

3.5. Metabolomics of mitochondrial disorders in cancer-perspective applications

Altered mitochondrial electron transport systems and respiratory chain disorders have been known to induce significant changes in metabolic pathways in cancer. However, metabolomics has still not found foothold to study these aberrant changes in cancer. High prevalence of mitochondrial disorders in cancer and unavailability of current approaches in providing genome scale or systems biology based understanding of mitochondrial dysfunction demands novel tools to study metabolic alterations induced by these disorders. Recent applications of metabolomics to study mitochondrial disorders indicate that it could potentially serve as a promising tool to investigate the link between cancer and mitochondrial disorders. Shaham et al. metabolically profiled the cultured media of a respiratory chain disease (RCD) induced in differentiated muscle cells using inhibitors of enzymatic complex I (rotenone) and complex III (antimycin) embedded in the inner mitochondrial membrane using targeted mass spectrometry [41]. The authors found that cultures treated with respiratory chain (RC) inhibitors had higher glucose uptake, lactate and alanine secretion, and lower biogenesis of proteins and phospholipids. Further, they combined the cellular model with clinical investigation involving patients having either pathogenic mutations or an abnormally low respiratory chain enzyme activity in muscle. Interestingly, metabolite profiling of plasma obtained from RCD patients (16 patients with 25 healthy controls) revealed the similar pattern and both lactate and alanine levels were elevated. Moreover, in plasma, they found creatine and uridine levels to be altered in similar way as in cell culture.

A recent study using mitochondrial metabolomics has linked long chain fatty acid oxidation with metabolite signatures of TCA cycle intermediates in skeletal muscle [42]. The authors used GC-TOF/MS to identify intra- and extra-mitochondrial metabolites and pathway fluxes and associated palmitate oxidation fluxes with mitochondrial metabolites. Interestingly, Mervaala et al. used tissue-based metabolomics using GCxGC-TOF/MS to understand angiotensin-induced mitochondrial dysfunction and usage of cardiac substrate [43]. Angiotensin contributes greatly to stroke, congestive heart failure in patients with hypertension-induced ventricular hypertrophy, and using metabolic profile data authors linked angiotensin-induced changes to alterations in mitochondrial biogenesis, respiratory chain enzyme activities, and suggested upregulation of SIRT1 to counteract mitochondrial dysfunction. In an effort to pin down the role of fatty acids in mitochondrial dysfunction in skeletal muscle insulin resistance, Koves et al. used targeted metabolomics and showed that a high fat diet results in excessive β-oxidation [44]. This is in contrast with current theory that muscle insulin resistance is due to impaired mitochondrial uptake and oxidation fatty acids and thus changes the paradigm of the need to upregulate β-oxidation during insulin resistance.

Genomic mutations are known to initiate cancer. The application of metabolomics to study metabolic alterations during DNA damage and link them with tumorigenesis could provide fundamental understanding of neoplasia and cancer progression. In a recent study, a novel application of metabolomics was presented to screen mice for the desired diseased phenotype after mutagenesis. Authors used tandem MS/MS to screen mice with defect in mitochondrial trifunctional protein (MTP) complex that catalyzes β-oxidation of long chain fatty acids. Metabolomics generated mouse models will allow development of treatment strategies for human mitochondrial disorders. The same group earlier used a similar approach to develop mice for abnormalities in mitochondrial-branched chain aminotransferase deficiency resembling human maple syrup disease [45]. This approach could be easily extended to study cancer metabolism.

4. Metabolic engineering and metabolomics

Most of the current metabolomics approaches provide useful information about the metabolic profiles and are a good aid in cancer and disease diagnostic studies. However, they are restricted in providing only metabolic pattern information and thus a static metabolic map. The metabolic pathway or metabolic network-based approaches commonly used for analyzing microbial metabolism are now gaining recognition in mammalian studies, and are versatile enough to capture not only dynamic metabolite perturbations due to environment, diet, and epigenetic changes but also can provide information about source of the specific alterations in metabolism or dysregulated metabolism [46–49]. Either the metabolic pathway approaches could be used as a standalone metabolite profiling technique or the information obtained could be integrated with the existing metabolomics data. The pathway-based approaches are based on topological and stoichiometric construction of the metabolic map and further determination of metabolic fluxes. The enzyme-mediated reactions are first collected from annotated genome and then later stoichiometric information is added for active enzyme using biochemistry data and certain imposed assumptions based on the knowledge of certainty of reaction directions. The next step involves estimation of intracellular fluxes using metabolic flux analysis (MFA) framework, which relies on steady-state assumptions, stoichiometric and pathway constraints of a metabolic pathway network, and on constrained nonlinear optimization technique. MFA has been used extensively for studying the metabolism of microorganisms [50–54], and has been recently applied to compare different physiological states in mammalian systems [55–63].

Hiller et al. [64] have recently developed a nontargeted tracer fate detection technique using stable isotope tracers for quantitative detection of labeled metabolites and pathways involved in the metabolism of used tracer. In their approach, authors used lung carcinoma cell line to obtain relative flux magnitudes of each metabolite pool and found increased flux through glutaminolysis pathway using [U-$^{13}C_5$] labeled glutamine. Interestingly, authors found increased branched chain amino (BCA) acid flux for cells labeled with [a-^{15}N] glutamine, which is remarkable because these amino acids cannot be produced by the body and implications of them in cancer suggests involvement of other essential amino acids in meeting the cellular demand of nitrogen. The findings of the authors are significant because their methodology is nontargeted, requires no *a priori* knowledge of metabolic pathway and metabolites, and allows for obtaining information of all labeled metabolites beforehand and identification of perturbed metabolome.

Alternatively, stable isotope labeling based tracer methods can be integrated with metabolic footprinting framework and used for quantifying in vivo fluxes validation of the intracellular and extracellular MFA obtained fluxes [47,65–68]. In order to obtain a unique solution for the intracellular flux distribution in a cell or tissue system, a minimum number of measurements of rates of uptake and release of extracellular metabolites by the system is required. However, if there are insufficient flux measurements available, then pathway fluxes are predicted using constrained optimization techniques [49,69–72]. However, optimization problem formulation in mammalian systems needs to take into account several cellular objectives. This is based on the hypothesis that mammalian cells at any time, whether they are in diseased or healthy state, need to maintain homeostasis of several different metabolites. Thus, homeostasis conditions, thermodynamic constraints, physical constraints, stoichiometric constraints, are incorporated as equality and inequality constraints along with the inferred cellular objectives in an optimization problem to solve for intermediary metabolism and intracellular fluxes. It is to be noted that inferring cellular objectives is a complex problem and in diseases such as cancer and metabolic diseases such as diabetes may

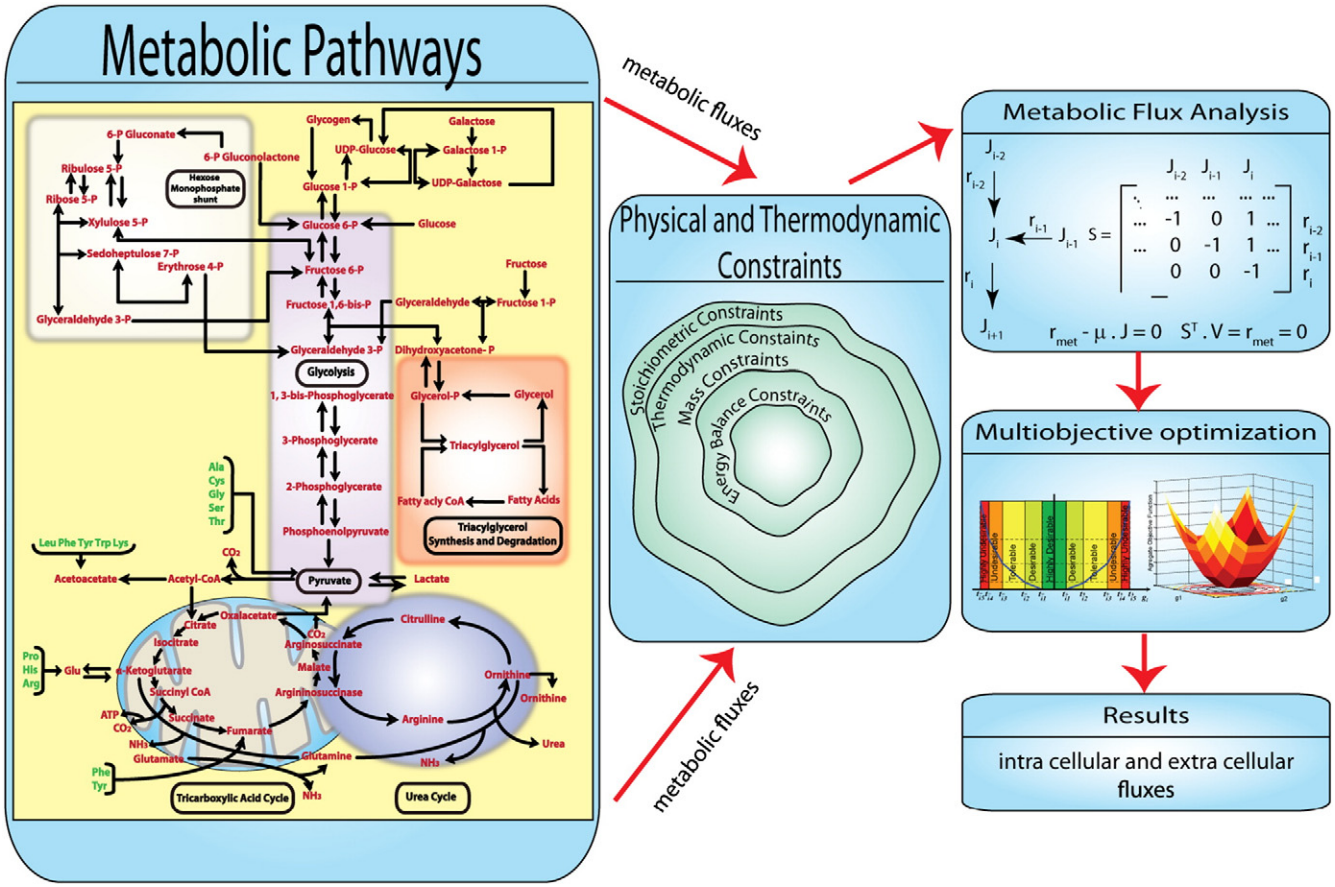

Fig. 2. Metabolic flux analysis based pathway tracing of cancer. MFA approaches can serve as validation and mechanistic tool. This can be done using either isotopomer-based *in vivo* flux analysis or using metabolomic footprinting approaches. For determination of intracellular and pathway fluxes measured fluxes are combined with physical and thermodynamic constraints and solved using nonlinear multiobjective optimization methods that take into account cellular objectives.

need an iterative or a multilevel optimization approach [73–76]. Cancer cells typically not only undergo cell proliferation, but also perform an array of metabolic functions (protein secretion, detoxification, energy production and lipid synthesis), and therefore different and multiple objectives need to be taken into account. Their interaction with stroma, their altered mitochondrial metabolism, and maintenance of nutrition supply makes them possess multiple functions including efficient uptake and subsequent metabolic conversion of amino acids, carbohydrates, lipids, and vitamins. Subsequently, these nutrients are either stored or released after biochemical transformations. These biochemical processes make the tumor microenvironment the epicenter of the metabolic modulation of intermediary metabolism and thus plays an important role in therapeutic strategies targeting tumor metabolism. Several techniques have been developed for analyzing multiple cellular objectives including Bayesian and optimization-based approaches [60,72,77,78]. Recently we have developed a combined energy and flux balance based large-scale nonlinear multiobjective framework for mammalian systems, which can be easily integrated with cancer metabolomics. Our multiobjective optimization based flux balance analysis approach, LPPFBA (Linear Physical Programming-Based Flux Balance Analysis), employs Linear Physical Programming (LPP), and enables the formulation of the optimization problem in terms of physically meaningful terms and parameters, by employing "soft constraints." Fig. 2 shows the detailed framework for estimation of intracellular pathway fluxes.

4.1. Metabolic flux analysis

The stoichiometric coefficients of the metabolic reactions are collected into a stoichiometric matrix $\boldsymbol{S}$, where each element s_{ij} is the coefficient of metabolite i in reaction j. S has dimensions of $M \times N$, where M is the number of metabolites and N is the number of reactions. In matrix form, the mass balance is written as:

$$\frac{dx}{dt} = SJ \tag{1}$$

where each element x_i of x is the intracellular concentration of metabolite i and element J_i of J is the net rate of conversion in reaction j. External metabolite fluxes are generally measured (e.g., uptake/secretion of glucose, pyruvate, lactate, amino acids, and fatty acids). The very high turnover of the intracellular pools of most intracellular metabolites allows the pseudo steady state assumption to the metabolite mass balances and thus

$$SJ = 0 \tag{2}$$

In most of the human metabolome systems, quantitative measurement of all the metabolites is challenging and in reality the number of measured quantities is always less than the number of measurements required for the system to be determined (which is approximately close to the number of metabolic reactions in metabolic pathways). Hence, the computation of unknown intracellular fluxes in human metabolome will require linear optimization (since infinite number of solutions can exist) with linear bound constraints. Mathematically, this is be expressed as:

$$\max_{J_u} \quad c^T J_u \tag{3}$$

subject to

$$S_u J_u = -S_m J_m \tag{4}$$

$$J_{low} \leq J_u \leq J_{high} \tag{5}$$

where the vector $\boldsymbol{c}$ specifies unknown flux vector elements to be maximized (or minimized); vectors J_{low} and J_{high} provide the lower and upper bounds for the unknown fluxes; J_m and J_u indicate measured and unmeasured fluxes, respectively; and S_m and S_u contain the stoichiometric coefficients of measured and unknown reactions, respectively.

4.2. Multiobjective optimal metabolic fluxes

A multiobjective optimization is a problem involving several competing objectives and constraints. A Pareto optimal solution is one where any improvement in one objective can only take place at the cost of another objective. A Pareto set is a set of Pareto-optimal solutions. Often several Pareto optimal points are available in cellular metabolic systems, representing alternative objectives scenario, from which cells select the one that offers the best trade-off among multiple objectives based on still unknown criteria. This optimization generally involves forming an Aggregate Objective Function (AOF) (or, some functional aggregation of the many conflicting criteria). Implicit in this process is the assumption that this AOF has the ability to indeed yield all the potentially useful/desirable optimal solutions. The most common AOF structure is the *weighted-sum* approach, which involves forming a linear combination of objectives-minimized subject to the problem constraints. We have recently developed Linear Physical Programming (LPP) based multiobjective FBA in which we express the preferences of cellular objectives using different *classes* [49]. Each *class* comprises two cases, *soft* and *hard* and all *soft* class functions are integrated in the AOF (that is minimized). Constraints that have to be met are classified as *hard*. All the *soft* class functions become part of the AOF to be minimized, and all of the *hard* class functions simply appear as constraints in the LPP model.

The desired behavior of an objective function, during optimization, is described by sub-classes, *soft* (S) and *hard* (H). These classes are defined as follows:

Soft

- Class-1S Smaller-Is-Better, i.e. minimization
- Class-2S Larger-Is-Better, i.e. maximization
- Class-3S Range-Is-Better
- Class-IVS Value-Is-Better

Hard

- Class-1H Must be smaller, i.e., $g_i \leq t_{i,\max}$
- Class-2H Must be larger, i.e., $g_i \geq t_{i,\min}$

In LPP model, we assign cellular objectives or the desired values of fluxes in terms of targets. We can assign up to ten targets depending upon subclasses. For an example, if we know a priori from experimental data or literature that cells of a certain disease will be either minimizing a particular cellular function, then we will choose Class-1S and assign five desirability preference values for this function (highly desirable, desirable, tolerable, undesirable, and highly undesirable). These preference values are used to form an aggregate objective function, which is then used to compute unknown intracellular metabolic fluxes. Fig. 3 shows the target values which are assigned in LPP for Class-IV problem where the desired objective is to maintain homeostasis of the cellular function. For Class-IV cellular objective, we would assign desired preferences or targets in terms of their desired and undesired ranges (highly undesirable, desirable, tolerable, undesirable and highly undesirable). The proposed LPP approach is advantageous over currently used techniques for optimization and estimation of these unknown fluxes because LPP requires target values which are in physical units of cellular functions, whereas commonly used techniques require meaningless weights which do not have physical meanings and may not provide correct estimation of intracellular fluxes for large-scale networks such as cellular networks.

4.2.1. Constrained multiobjective metabolic flux analysis framework

The following linear programming problem is then solved:

Piecewise Archimedean aggregate function:

$$z_i = \min_{d_{is}^-, d_{is}^+} \sum_{s=2}^{5} \left(\tilde{w}_{is}^- d_{is}^- + \tilde{w}_{is}^+ d_{is}^+ \right) \tag{6}$$

subject to System constraints:

$$S_u J_u = -S_m J_m \tag{7}$$

$$J_{low} \leq J_u \leq J_{high} \tag{8}$$

$$x_{min} \leq x \leq x_{max} \tag{9}$$

Goal Constraints:

$$g_i - d_{is}^+ \leq t_{i(s-1)}^+, d_{is}^+ \geq 0, g_i \leq t_{i5}^+ (s = 2, \ldots, 5) \tag{10}$$

$$g_i + d_{is}^- \geq t_{i(s-1)}^-, d_{is}^- \geq 0, g_i \geq t_{i5}^- (s = 2, \ldots, 5) \tag{11}$$

$$g_j \leq t_{j,max} (\text{for all } j \text{ in class 1H}, j = 1, 2 \ldots n_{hc}) \tag{12}$$

$$g_j \geq t_{j,min} (\text{for all } j \text{ in class 2H}, j = 1, 2 \ldots n_{hc}) \tag{13}$$

$$g_j = t_{j,val} (\text{for all } j \text{ in class 3H}, j = 1, 2 \ldots n_{hc}) \tag{14}$$

$$t_{j,min} \leq g_j \leq t_{j,max} (\text{for all } j \text{ in class 4H}, j = 1, 2 \ldots n_{hc}) \tag{15}$$

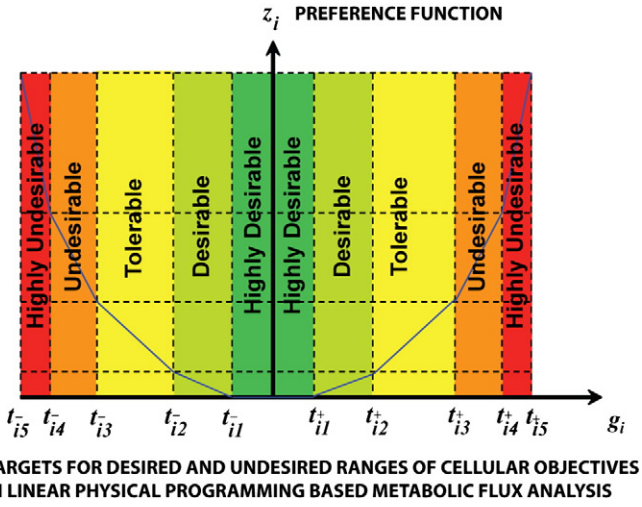

Fig. 3. Description of Linear Physical Programming. LPP approach involves assigning preferences or desirability and undesirability ranges instead of meaningless weights for the cellular objectives. These targets for ranges are used in optimization during metabolic flux analysis to estimate unknown intracellular metabolic fluxes. In most of the genome-scale metabolic networks, optimization is necessary to estimate unknown fluxes. This is because stoichiometric map is hard to be fully determined using experimental measurements. Hence, optimization is used to determine these unknown intracellular fluxes. Current techniques estimate these fluxes using weights of cellular objectives (for example, maximization of ATP, redox balance, nucleotide production). However, LPP assigns targets to these objectives and hence avoid ill-conditioning and accurately estimates unknown metabolic fluxes [49].

where J_m and J_u indicate measured and unmeasured fluxes, respectively; and S_m and S_u contain the stoichiometric coefficients of measured and unknown reactions, respectively.

As discussed previously, the use of preferences for different degrees of desirability aids in simultaneous visualization of a large number of objectives. This facilitates the assessment of the effect of preference specifications on the objectives as well as the complex interplay of these objectives. Each section is color coded according to the desirability level and labeled.

4.2.2. Metabolic thermodynamic constraints

Energy Balance Analysis (EBA) developed to ensure the thermodynamic feasibility of the computed fluxes imposes the thermodynamic constraints on metabolic reaction fluxes [76,79–82]. Essentially, EBA integration in MFA involves the computation of the reaction potentials based on the chemical potentials. Further, thermodynamic constraints based on the first and second laws of thermodynamics are obtained by reaction potentials, and these constraints further reduce the feasible solution space based on stoichiometric constraints. It is important to note that environmental constraints such as cell culture conditions, medium supplements, oxidative stress and extracellular matrices, typically limit the feasible solution space even further. For any reaction set, if stoichiometry is represented by matrix S, μ denotes an M-dimensional vector of chemical potentials, $\Delta\mu$ denotes the N-dimensional vector of reaction potentials, then these potentials can be computed as $\Delta\mu = S^T\mu$. The first law of thermodynamics necessitates energy conservation, which then leads to an equality constraint as

$$K^{\mathrm{T}}\Delta\mu = K^{\mathrm{T}}S^{\mathrm{T}}\mu = 0 \text{ (First Law of Thermodynamics – Based Energy Equality Constraint)} \tag{16}$$

The second law of thermodynamics takes the form of an inequality constraint for each flux as $-J_i\Delta\mu_i \geq 0$. The vector of net flux distribution through the reaction network can be then computed as $J = J_+ - J_-$, which then leads directly to the second law of thermodynamics, i.e.,

$$-J^j\Delta\mu^j = -RT\left(J_+^j - J_-^j\right)\ln\left(\frac{J_-^j}{J_+^j}\right) \geq 0 \text{ (Second Law of Thermodynamics Based Energy In Equality Constraint)} \tag{17}$$

which says that the system must dissipate heat, and entropy must increase as a result of the work being done on the system through the external fluxes.

The other inequality constraint is by ensuring that the total heat dissipation rate of the living system is always positive as indicated by

$$hdr = -J^T\Delta\mu > 0 \text{ (Inequality Heat Dissipation Constraint)} \tag{18}$$

Since $hdr \to 0$ in the limit as $J_- / J_+ \to 1$ component-wise while maintaining $J = J_+ - J_-$ so to prevent this physically unrealistic possibility, an additional inequality constraint

$$(hdr)_{lb} \leq hdr \leq (hdr)_{ub} \text{ (Inequality Heat Dissipation Constraint)} \tag{19}$$

is also imposed as part of Energy Balance Analysis. The procedure for estimation of intracellular fluxes using thermodynamic constraints is illustrated below for a toy problem of two cycle metabolic network.

4.2.3. Two-cycle metabolic network

The steady state mass balances for metabolites *A*, *B*, *C* and *D* in terms of reaction fluxes are given by the following equations used in this metabolic network (Fig. 4):

$$\frac{d[A]}{dt} = J_A - J_1 - J_3 = 0$$
$$\frac{d[B]}{dt} = J_1 + J_2 - J_5 - J_B = 0$$
$$\frac{d[C]}{dt} = J_3 - J_2 - J_4 - J_C = 0$$
$$\frac{d[D]}{dt} = J_4 + J_5 - J_D = 0$$

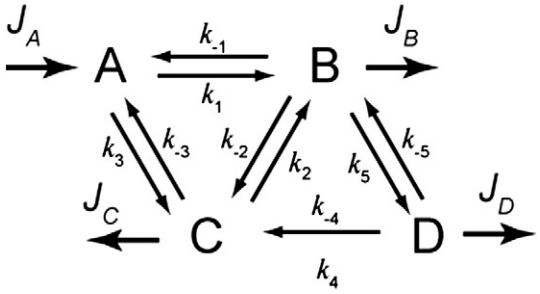

Fig. 4. Two cycle and four node metabolic network.

where the internal fluxes for J_1, J_2 and J_3 are $J_1 = k_1[A] - k_{-1}[B]$, $J_2 = k_2[C] - k_{-2}[B]$ and $J_3 = k_3[A] - k_{-3}[C]$, and $J_4 = k_4[C] - k_{-4}[D]$ and $J_5 = k_5[B] - k_{-5}[D]$. Above equations can be cast into a stoichiometric matrix form as:

$$A_{eq} = \begin{bmatrix} -(k_1 + k_3) & k_{-1} & k_{-3} & 0 & 1 & 0 & 0 & 0 \\ k_1 & -(k_{-1} + k_{-2} + k_5) & k_2 & k_{-5} & 0 & -1 & 0 & 0 \\ k_3 & k_{-2} & -(k_{-3} + k_2 + k_4) & k_{-4} & 0 & 0 & -1 & 0 \\ 0 & k_5 & k_4 & -(k_{-4} + k_{-5}) & 0 & 0 & 0 & -1 \end{bmatrix}$$

and $\vec{x} = \begin{bmatrix} [A] & [B] & [C] & [D] & J_A & J_B & J_C & J_D \end{bmatrix}^T$ and $b_{eq} = \begin{bmatrix} 0 & 0 & 0 & 0 \end{bmatrix}^T$. The chemical potential associated to reactions 1, 2 and 3 are given by equations

$$\Delta\mu_1 = \Re T \ln\left(\frac{[B]}{K_1[A]}\right)$$

$$\Delta\mu_2 = \Re T \ln\left(\frac{[B]}{K_2[C]}\right)$$

$$\Delta\mu_3 = \Re T \ln\left(\frac{[C]}{K_3[A]}\right)$$

For Reactions (4) and (5), it can be expressed as follows:

$$\Delta\mu_4 = \Re T \ln\left(\frac{[D]}{K_4[C]}\right)$$

and

$$\Delta\mu_5 = \Re T \ln\left(\frac{[D]}{K_5[B]}\right)$$

In order to have null chemical potential of the cycles, these relationships must satisfy: $\Delta\mu_1 - \Delta\mu_2 - \Delta\mu_3 = 0$ and $\Delta\mu_4 - \Delta\mu_2 - \Delta\mu_5 = 0$. These equations are equivalent to $K_1 = K_2K_3$ and $K_4 = K_2K_5$. For this metabolic network, the cellular objective could be maximization of a cellular function J_l. The unknown intracellular fluxes are estimated by maximizing cellular objectives as follows:

$$\underset{\vec{x}}{\text{Maximize}} \; (J_l)$$

subject to: $A_{eq}\vec{x} = \vec{b}_{eq}$

$$HDR_{lb} \leq HDR \leq HDR_{ub}$$

$$\vec{x}_{lb} \leq \vec{x} \leq \vec{x}_{ub}$$

4.3. Metabolic flux analysis and bioenergetics of cancer metabolism

The preceding methodology of metabolic flux analysis (MFA) can be utilized to estimate the unknown intracellular fluxes in the cancer metabolic network. The advantage of using multiobjective metabolic flux analysis is that it easily allows incorporation of cellular objectives in tumorigenesis, such as maximization of glycolysis (Warburg effect) and minimization of mitochondrial TCA cycle fluxes in a straightforward manner. Additionally, metabolic fluxes of mitochondrial and glycolytic pathways in cancer can reveal metabolic pathways upregulated and downregulated in cancer. Notably, the stoichiometric analysis can be combined with GC-HPLC-MS and/or NMR techniques to confirm the increase and decrease of metabolic cycle flux and thus can be used to integrate qualitative data (presence or absence of metabolic fluxes) with quantitative metabolic fluxes. Using separate compartments for mitochondrial and cytoplasmic metabolites, MFA can reveal altered pathway fluxes linked to mitochondrial biogenesis, respiratory chain enzyme activities and uptake and oxidation of fatty acids during tumor progression and growth [49,72]. The development of metabolic network analysis map for diseases such as cancer could reveal the dysregulated complex cancer pathways and metabolic interaction networks that regulate the disorders in mitochondrial metabolism. Using novel systems tools such as optimality and thermodynamics based metabolic network we could obtain nutrition based therapies for regulating cancer metabolism at all levels of complexity and thus modulate carcinogenesis.

5. Metabolomics in diseases

Bain et al. describe various advantages of metabolomics over other "omics" technologies in diabetes research: (a) it requires significantly less small molecule metabolites; (b) it provides a measure of integrated phenotypic changes in genome, transcriptome and proteome [83]. In the aim toward developing targeted strategies for pancreatic islet cell transplantation NMR-based mass isotopomer techniques have been utilized for studying glucose metabolism in insulin secreting cells and determining mitochondrial pathways that are responsible in generating coupling factors using glucose responsiveness and unresponsive [84]. In these approaches, cells are cultured with [U-^{13}C] glucose followed by NMR-based isotopomer analysis of secreted glutamate. This allows the determination of glucose to pyruvate and its entry into TCA cycle through catalytic conversion by pyruvate dehydrogenase (PDH) into acetyl COA. Alternatively, pyruvate can enter TCA cycle through catalytic conversion by pyruvate carboxylase (PC) which converts it into oxaloacetate. The isotopomer peak analysis allows analysis of the relative rates of each flux by observing the secreted cycle components. Recently, non-targeted metabonomic profiling of plasma and urine using UPLC-qTOF mass spectrometry revealed metabolic fingerprinting differences occurring in normal glucose tolerant and impaired glucose tolerant (IGT) individuals affected by the pre-diabetic metabolic state [85]. In their study, major metabolic alterations were found in fatty acid, tryptophan, uric acid, bile acid, and lysophosphatidylcholine, as well as the TCA cycle metabolites. In a similar study [86], using a combination of ^{1}H nuclear magnetic resonance spectroscopy and liquid chromatography-tandem mass spectrometry, insulin deficiency induced altered metabolic pathways were determined in plasma obtained from type 1 diabetic humans during insulin treatment and acute insulin deprivation and non-diabetic participants. The metabolic fingerprinting revealed in type 1 diabetes perturbations of pathways involved in protein synthesis and breakdown, gluconeogenesis, ketogenesis, amino acid oxidation, mitochondrial bioenergetics, and oxidative stress; and elevation of lactate, acetate, allantoin, ketones during insulin deprivation. In an interesting study, Zyromski et al. [87] analyzed pancreatic fat content in fatty pancreas and compared various conventional approaches such as thin-layer and gas chromatography with NMR-based metabolomics. Their results correlated well with conventional approaches in the quantification of fat, triglycerides and phospholipids.

Recently findings began to elucidate the role of metabolomics for biomarker discovery in arthritis as well [5,88]. Zhai et al. analyzed serum samples of 123 osteoarthritis cases and 299 healthy controls using targeted metabolite fingerprinting by electrospray ionization tandem mass spectrometry and found ratios of valine to histidine and leucine to histidine correlated with osteoarthritis [88]. The study confirms that the usage of metabolite concentrations instead of enzymatic reaction rates could serve as a biomarker and sets the stage for using this concept in a clinical setting. In another study [5], using ^{1}H NMR spectroscopy based metabolomics approach, authors found that uracil, xanthine, and glycine are the likely biomarkers of a systemic inflammatory joint disease rheumatoid arthritis.

Although in infancy, another area where metabolomics has sparked a strong interest is the area of reproductive biology that involves assessment of oocyte quality by metabolic phenotyping of follicular fluid in order to identify the most viable embryo that will result in pregnancy [89–91].

6. Future directions

Metabolomics although is at its infancy could serve as a key tool in cancer and disease diagnosis. Further developments in the area of advanced metabolite detection technologies and data analysis will provide impetus for wide usage in clinical medicine. However, there are some challenges that need to overcome before recent metabolomics studies can lead to the development of "cancer metabolome" and biomarkers derived from metabolite profiling approaches could facilitate personalized therapy. On metabolomics technological front, to harness the power of various metabolomic techniques it will be necessary to use both NMR and MS based approaches. This will widen the range of analytes and compounds that could be detected for comprehensive metabolomics studies. Current metabolomics technology faces tough uphill battles in developing structural identification databases, spectral libraries of vast compounds. The combination of chemometrics approaches such as multivariate data analysis, principle component analysis with systems biology approaches such as nonlinear multiobjective optimization and time-series models developed for many years in adaptive control community can provide a structural identification of analytes from complex spectra.

Studying metabolic profiling of biofluids is critical not only from biomarker perspective but also because the integration of metabolic markers for cancer phenotype obtained from tissue, cells and biofluids may elucidate the novel role of metabolic markers and could provide better understanding of metabolic adaptations and alterations because of diet and environment. Furthermore, a combined approach involving metabolomics of various biofluids may allow development of metabolic markers necessary to understand resistant forms of cancer in the era of personalized medicine.

There is a pressing need to further investigate metabolomics application in clinical cancer research using *in vivo* metabolomics such as PET imaging and ^{13}C-glucose infusion into human cancer patients. This will demonstrate predictive value of metabolomics derived biomarkers and enable study of drug resistances and cancer recurrence. Notably, chemotherapy and radiation induced cell death is another important area where metabolite profiling could assist in identifying dysfunctional pathways during drugs induced apoptosis. In future, metabolic and nutrition based approaches could address induced and altered metabolic changes by diet and metabolic activators.

Lastly, it will be important to validate metabolomics data using fluxomics and metabolic flux analysis approaches. The integration of pathway-based dynamic metabolic profiling approaches that are based on topological and stoichiometric construction of the metabolic map with static metabolome may provide much needed mechanistic understanding of cancer metastasis and malignant transformations during tumor growth. Moreover, these approaches could allow inference of cellular and tissue metabolic objectives, and contribute to identification of metabolic factors that trigger the onset of cancer. Given that cancer cells and its interaction with tumor microenvironment is quite complex, complete understanding of biochemical pathways in cancer necessitates the use of multiobjective optimization approach to determine metabolic fluxes and/or infer cellular objectives in cancer. Together, studies in metabolomics and fluxomics could reveal the complex cancer pathway and interaction network that regulates the dysregulation of metabolism and aid in development of new personalized cancer therapies, cancer diagnosis, prognosis, and early detection.

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