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Review

# Metabolomics in cancer biomarker discovery: Current trends and future perspectives

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ABSTRACT

Cancer is one of the most devastating human diseases that causes a vast number of mortalities worldwide each year. Cancer research is one of the largest fields in the life sciences and despite many astounding breakthroughs and contributions over the past few decades, there is still a considerable amount to unveil on the function of cancer. It is well known that cancer metabolism differs from that of normal tissue and an important hypothesis published in the 1950s by Otto Warburg proposed that cancer cells rely on anaerobic metabolism as the source for energy, even under physiological oxygen levels. Following this, cancer central carbon metabolism has been researched extensively and beyond respiration, cancer has been found to involve a wide range of metabolic processes, and many more are still to be unveiled. Studying cancer through metabolomics could reveal new biomarkers for cancer that could be useful for its future prognosis, diagnosis and therapy. Metabolomics is becoming an increasingly popular tool in the life sciences since it is a relatively fast and accurate technique that can be applied with either a particular focus or in a global manner to reveal new knowledge about biological systems. There have been many examples of its application to reveal potential biomarkers in different cancers that have employed a range of different analytical platforms. In this review, approaches in metabolomics that have been employed in cancer biomarker discovery are discussed and some of the most noteworthy research in the field is highlighted.



**Contents**



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## 1. Introduction

For decades, cancer research has involved studying the molecular features that are different between cancer cells and their healthy counterparts, with the aim of revealing biomarkers representative of the cancer phenotype as well as possible therapeutic targets. This has led to the identification of many molecular features involved in cancer that function in signal transduction [1], cell senescence [2] and other hallmarks of cancer cells [3]. Although the functional levels of a biological system include the genome, transcriptome, proteome and metabolome, the latter is considered most representative of the phenotype [4]. Exploring the cancer metabolome may be the best way to reveal the phenotypic changes relative to biological function, especially where subtle changes in metabolite concentrations can be tractable. For these reasons metabolomics is considered as one of the fastest developing disciplines in cancer research as well as many other aspects of life science. It is hoped that for a range of cancers, specific biomarkers will be revealed that could be used in screening for diagnostic and prognostic purposes. A reliable biomarker should be reproducibly detected in samples and collection of samples containing the biomarker should be performed with uniformity involving minimal invasion to the patient or subject. The application of metabolomics to cancer is increasing year by year in the search for candidate biomarkers that define a particular cancer, whose directional variation is significantly higher than all other endogenous metabolites that comprise the often complex sample for analysis.

Although metabolomics is still an emerging field, particularly in cancer research, aspects of cancer metabolism have long been a focus to understand central mechanisms in tumours. The best studied feature of cancer metabolism is central carbon metabolism and the relationship between glycolysis, the tricarboxylic acid (TCA) cycle and oxidative phosphorylation. One key hypothesis is that cancer cells preferentially convert pyruvate to lactate rather than fuelling the TCA cycle even in aerobic conditions [5,6]. More recently it has been shown that this is not the exclusive rule for cancer metabolism, however it is a common understanding that tumours display enhanced glycolytic activity along with a down regulation of the TCA cycle and oxidative phosphorylation [7–9]. This is known as the Warburg effect. Hypothesised by Otto Warburg in 1956, the Warburg effect suggests that tumour cells originate from healthy cells in two phases: an irreversible injuring of respiration followed by a replacement of the lost respiration energy with fermentation energy [8]. Furthermore, the Warburg effect implies that cancer cells show elevated uptake of glucose. This is the main feature of the highly sensitive and accurate positron emission tomography (PET) currently employed in solid tumour diagnostics [10].

Using a metabolomics approach, it is possible to detect a range of metabolites in a single assay and therefore metabolomics can be defined as a holistic and data-driven study of the low molecular weight metabolites present in biological systems [4]. This allows further investigation into central carbon metabolism but also the revelation of other biochemical pathways that contribute to cancer function. The metabolome consists of both endogenous and exogenous components: those catabolised or anabolised by the biological system itself, or those that are extra-organism or extracellular respectively. It is inclusive of metabolites present in a biological sample that represent metabolic activity required for growth, maintenance and function, as well as metabolites consumed from the external environment [4]. Fig. 1 highlights the main steps of a metabolomics experiment that can be characterised by three main stages: data collection, processing and analysis.

Metabolomics can be performed on a range of different sample types including tissue, cells, bio-fluids such as serum, plasma, urine and saliva, and recently it has been shown using ion mobility that cancer biomarkers may be detected in breath odour from volatile organic compounds exhaled [11]. Likewise, a range of different analytical platforms and appropriate methodologies for sample preparation can be used in metabolomics, many of which are discussed in the proceeding section. Finally, data are analysed in different ways depending on the experimental design, but commonly involve univariate and/or multivariate analyses that assign statistical significance to the difference in individual metabolite concentrations between experimental groups or determine the multi-variation between groups collectively from all metabolites identified respectively. Practical applications of metabolomics software with particular reference to cancer metabolomics has been reviewed previously, providing an explanation of the different methodologies employed for data processing and analysis [12].

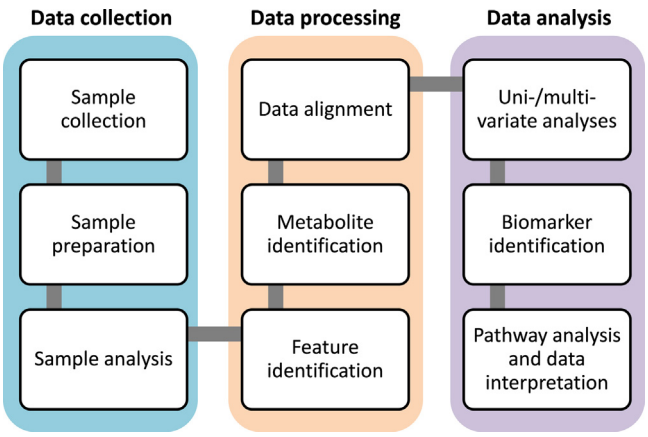


**Fig. 1.** The pipeline for metabolomics in biomarker discovery. The experiment is defined by three main categories: data collection, data processing and data analysis. Sample preparation is dependant both on the sample and on the analytical platform employed, and sample analysis is designed to suit the type of sample collected. Features are identified in the spectra and metabolite identifications are assigned where possible, commonly employing publically available metabolite databases. Data from different experimental groups are aligned and pre-treated for their comparison by univariate or multivariate analysis. From this possible biomarkers are suggested and can be further analysed to interpret the origin of their control through pathway analysis. If a certain subset of metabolites are exposed these can be further investigated in a more focused version of this pipeline.

## 2. Methodologies for metabolomics based biomarker discovery

### 2.1. Analytical platforms

The key pathways that behave differently between tumour and normal cells include glycolysis and the pentose phosphate pathway, nucleotide and protein biosynthesis, lipid and phospholipid turnover, the TCA cycle and redox stress pathways. No single analytical platform can detect all the compounds that can be altered in cancer. Metabolomics experiments can employ one or more different analytical platforms depending on the application; where the pipeline for a metabolomics experiment is similar for each. In general, metabolomics based biomarker discovery typically employs either nuclear magnetic resonance spectroscopy (NMR) [13–15] or mass spectrometry [16–19]; where the latter can be coupled with a separation technique such as gas chromatography (GC–MS) [20–23], liquid chromatography (LC–MS) [24–27] or capillary electrophoresis (CE-MS) [28–30]. Also, mass spectrometry can be performed using a range of different mass analysers depending on the type of experiment.

NMR benefits from being highly reproducible (>98% [31]) and offers the potential to quantify compounds in complex mixtures precisely due to the direct relationship between peak area and the

concentration of specific nuclei [32]. The structure of the molecules from which peaks arise can be determined using this technique and it is particularly advantageous since samples can be analysed in their native state; including the detection of metabolites in bio-fluids or tissues and even *in vivo*. The main limitation associated with NMR spectroscopy is sensitivity. Despite technological advances, the sensitivity is orders of magnitude lower than MS [32].

GC–MS and LC–MS are perhaps the most commonly used platforms in metabolomics of cancer samples. GC–MS is applied to the analysis of low molecular weight metabolites (in the approximate range $m/z$ 650–1000 [33]). Its specific application in cancer biomarker discovery has been reviewed previously [34]. To broaden the range of chemicals detected in the samples, chemical derivatisation is commonly performed prior to analysis. This method facilitates the separation of chemicals, reduces the polarity of the functional groups in chemicals and enhances their volatility and thermal stability prior to GC separation [33]. Hydroxyl, carboxyl and amino functional groups readily react with the chemical derivatisation agents that are added to convert these polar groups into derivatives with increased volatility. In the process of GC–MS, chemical samples are fractioned in the interaction with the stationary phase (analytical column) and mobile phase (carrier gas). The sample is injected at high temperature and pressure in order to vaporise the liquid. The carrier gas then deposits the sample onto the analytical column through which the sample migrates and interacts with the stationary phase. This process separates components of the sample mixture that are eluted and recorded as peaks on the chromatogram. The eluents are commonly ionised by the electron ionisation (EI) source before entering a mass analyser. Fragments are detected based on $m/z$, producing a spectrum for every eluent peak of the chromatogram. GC–MS is highly sensitive, quantitative and reproducible and the latter has made possible more advanced compound identification compared to other techniques in the field [35]. Although derivatisation offers many advantages for GC–MS analyses, the main drawbacks of this technique also arise from this process. For example, sample handling is laborious and the appearance of several derivatives for compounds with different functional groups makes interpretation of spectra more challenging.

LC–MS, which can also be prefixed with ultra or ultra-high performance, is particularly useful for non-volatile chemicals, therefore it is complementary to GC–MS [33]. Various mass analysers can be used in LC–MS and it is possible to analyse in both positive and negative ion mode; thus a wide range of metabolites can be analysed using this platform.

CE-MS is ideally suited to the separation and detection of polar or ionic compounds in low sample volumes, is associated with high resolution and requires no pre-column derivatisation as with GC–MS [36]. The potential for CE-MS in metabolomics has recently been reviewed by Ramautar et al. [37]. Although the sensitivity is lower and the variability can be higher than that of GC–MS or LC–MS, CE-MS has the capacity to analyse complex mixtures and has been recently reported in a cancer related study that assessed the effect of dietary polyphenols on colon cancer cells [36].

With any mass spectrometry based platform, advances in high resolution mass analysers have improved accurate mass measurements of metabolites in complex samples. Examples include state of the art Q-TOF, and Orbitrap® mass analysers that are typically used to determine the elemental composition of samples and to allow tentative metabolite identification using databases, or Q-TOF, TOF–TOF and LTQ-Orbitrap® mass analysers that are excellent choices for MS/MS or $MS^n$ experiments [36].

As discussed, different analytical platforms are suited to different types of samples as well as experiments with respect to the metabolic information required from the sample. In addition to choosing the right analytical platform for the application, choosing the correct way to prepare a sample based on its type and the instrument to be employed for the experiment can enhance the results. Bio-fluids such as urine, serum and plasma can be directly injected or pre-treated using methods such as liquid–liquid extraction, solid phase extraction or protein precipitation. Different options for sample treatment to aid detection of metabolites in bio-fluids are reviewed in Dettmer et al. [38]. For the treatment of *in vitro* cell samples the following procedure is usually performed: cells are washed and quenched with ice cold methanol and adherent cells are removed from the growth surface using trypsin or cell scrapers. The subsequent solution is collected and subjected to a series of freeze-thaw cycles for metabolite extraction. Supernatant is collected for analysis following high speed centrifugation to pellet the cell debris [39]. A similar protocol can be applied to the preparation of tissue samples, where freeze thaw cycles are used in the process of tissue homogenisation. This and the preparation of other biological samples are also reviewed in Dettmer et al. [38].

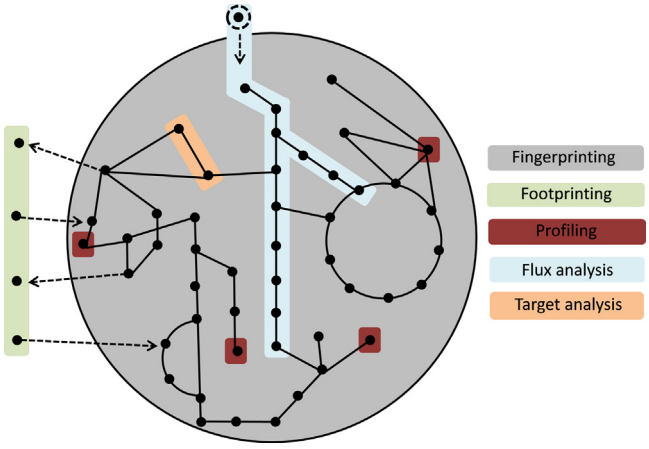


**Fig. 2.** Different approaches in metabolomics. Fingerprinting involves the global screening for all detectable metabolites from within the system under investigation. Footprinting (mainly referred to in *in vitro* cell systems) is the analysis of metabolites from the environment around the system under investigation and reveals information about metabolic exchange. Profiling is the screening of a particular class of chemicals, *e.g.* amino acids, for which standards are also analysed. Flux analysis is the tracing of one compound, usually isotope labelled carbon, through a particular pathway or set of pathways to determine the fate of the compound. Target analysis is the comparison of one or a few closely related target metabolites whose concentrations may change depending on the experimental conditions.

### 2.2. Metabolomics approach

Metabolomics is an 'umbrella term' for many types of experiments that can be applied. It includes, but is not restricted to metabolic fingerprinting and metabolic profiling which are most commonly performed in cancer biomarker discovery. For this reason these techniques are the main topic reviewed in the present article. Other branches that are occasionally used in cancer biomarker discovery include metabolic target analysis and metabolic flux analysis; key examples for which are detailed below. Fig. 2 depicts the different types of metabolomics that can reveal different information about metabolites in the system under investigation (the sample).

Metabolic target analysis involves the quantification of a particular metabolite or small group of related metabolites of interest. For example in one study, LC–MS was used to quantify glycolytic metabolites to investigate whether or not an accumulation of glycolytic metabolites and a diversion in glycolytic intermediates to serine metabolism occurs in cells expressing an M2 isoform of pyruvate kinase (PK) [9]. PK is the final enzyme in glycolysis that catalyses the reaction to yield pyruvate and ATP. It is thought to be

one of the most important regulators in cancer and exists in several isoforms including M1 that is expressed in most adult tissues and M2 that is usually expressed in embryos during development [9]. With reference to cancer metabolism, it has been shown that tumour cells have the ability to switch from an M1 isoform to an M2 isoform of PK and that this switch is observed in conditions that are particularly hypoxic [9]. Tumour hypoxia, a feature of solid tumours usually associated with oxygen partial pressures below 10 mmHg [40], occurs in cells located further than 100–180 μm from the closest capillary [41]. It was revealed that tumour cells exhibited higher concentrations of phosphoenolpyruvate, 3-phosphoglycerate and serine compared to cells expressing the M1 isoform of PK. Since the former is the substrate of PK and the latter two are involved in serine synthesis, it was concluded that expression of the M2 isoform of PK causes both an accumulation of glycolytic intermediates and diverts them towards serine metabolism [42]. Therefore these glycolytic intermediates can be considered as biomarkers for this function in cancer.

Metabolism can be characterised by variation in metabolite concentrations and fluxes and it is thought that phenotyping may only be possible with knowledge of enzyme controlled intracellular fluxes [43]. One way to estimate this is through metabolic flux analysis: the method of recording the rate of movement of atoms, usually carbon or nitrogen, through metabolic pathways that can highlight biological functions in a system. Cells can be fed labelled metabolites (*e.g.* $^{13}$C glucose) and the fate of $^{13}$C atoms can be traced over time to reveal which metabolic pathways are being employed under the environmental conditions being tested. This method is commonly used to study cancer metabolism *in vitro* and following a certain set of rules (metabolic intermediates must be in isotopic steady state; metabolites are homogeneously distributed within a cellular compartment; the stoichiometry of all reactions is known; enzymes are known not to discriminate between labelled and unlabelled metabolites), quantitative data interpretation can be achieved through mathematical isotopomer modelling [44].

One of the most recent examples of the use of metabolic flux analysis in cancer research came from the Gottlieb laboratory in the Beatson Institute for Cancer Research, Glasgow. Metabolic flux analysis using $^{13}$C glucose and $^{13}$C glutamine was employed to determine the effects of a fumarate hydratase (FH) knockout on the carbon supply to the TCA cycle in a modified kidney mouse cell model [45]. FH is an enzyme of the TCA cycle that is mutated in patients with hereditary leiomyomatosis and renal-cell cancer and causes fumarate accumulation that appears to promote cell survival despite a dysfunctional TCA cycle [46,47]. Tracing the fate of $^{13}$C allowed the identification of a key pathway involved in cells with the FH knockout. It was subsequently shown that targeting this pathway provides a new target for treatment in patients with hereditary leiomyomatosis and renal-cell cancer [45]. From another similar experiment, cancer cell survival has been associated with the maintenance of an imbalance between metabolite fluxes in the oxidative and non-oxidative pentose phosphate pathway [43].

While the latter example utilised LC–MS, many flux experiments in metabolomics employ GC–MS since its application is often used to trace compounds through central carbon metabolism that are readily detected using this technique. In one study, GC–MS was used in combination with two-dimensional NMR spectroscopy to determine the fate of uniformly labelled $^{13}$C glucose in breast cancer cell lines. Data were used in a $^{13}$C flux model of all known central carbon metabolism reactions which made possible the determination of metabolic fluxes and flux ratios in these pathways [44]. From this, it was possible to achieve information such as (i) the relative contribution of glycolysis and the pentose phosphate pathway to pyruvate synthesis; (ii) the importance of oxidative *vs.* non-oxidative ribose synthesis; (iii) the comparison of relative velocities of interconversions between pentose-5-phosphates and oxidation of glucose-6-phosphate; (iv) the synthesis, elongation and desaturation of fatty acids [44].

Metabolic profiling and fingerprinting are widely used non-targeted approaches in metabolomics and there are a wide range of examples in the literature for their application in cancer biomarker discovery. The two terms are sometimes used interchangeably, although they are mostly defined as the screening of metabolites in a relatively broad class (*e.g.* amino acids) or the complete global screening for any compounds with no predetermination of chemical class, other than the limits posed by the analytical technique used, respectively. Non-targeted metabolomics can be performed in many different ways from the comprehensive studies of all detectable metabolites in biological samples to the investigation of the fate or effect of an exogenous metabolite on an entire system. It is generally not considered quantitative; however it is useful for making relative comparisons between biological systems. The presence, absence or relative difference in concentration of the metabolites detected can be compared between experimental groups. These metabolites can be representative of the entire metabolic network and as such the metabolome-wide effects of an environmental or experimental perturbation, including a disease, can be tested. The applications of different platforms for metabolic fingerprinting and profiling have been described for a range of cancers and some of the most notable examples are summarised in the proceeding sections.

## 3. Applications of metabolomics in cancer biomarker discovery

### 3.1. Colorectal cancer

As with many cancers, until very recently there have been few accurate markers for the early detection of colorectal cancer. Colorectal cancer has a high cure rate if the disease is treated in early stages [48]. Discovering biomarkers could lead to improving diagnosis and prognosis of many cancers, while also offering the potential ability to predict cancer onset so that in the future cancer treatment could be more preventative rather than curative. In an example sampling cross continental populations of patients with colorectal cancer, Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) was employed in biomarker discovery followed by the structural characterisation of each using tandem LC–MS/MS and NMR. The clinical utility of the biomarkers was evaluated for further independent populations employing a targeted monitoring approach [49]. The authors report the discovery of ultra-long-chain fatty acids: $m/z$ 446, $m/z$ 448 and $m/z$ 450 as being significantly reduced in the serum of colorectal cancer patients relative to healthy controls. This poses the susceptibility of colorectal cancer risk to lifestyle and diet and further research is being conducted to connect colorectal cancer progression with these influencing risk factors [49].

In another serum profiling experiment to reveal potential biomarkers of colorectal cancer, electrospray ionisation (ESI) tandem mass spectrometry with no prior separation technique was employed to quantitatively profile the concentrations of 26 amino acids, 11 of which were found to be significantly different between cancer and control subjects [17]. All except one had been previously reported as potential colorectal cancer biomarkers in previous literature covering a range of different analytical platforms.

Urine is a commonly analysed bio-fluid in biomarker discovery by metabolomic approaches. In screening for urinary biomarkers of colorectal cancer as a larger scale follow-up to previous research by the same group [50,51], Cheng et al. [48] have shown that colorectal metabolic profiles include gut microbial-host

co-metabolism and that the combined panel of citrate, hippurate, p-cresol, 2-aminobutyrate, myristate, putrescine and kynurenate are able to discriminate cancer from healthy colorectal profiles. It has also been suggested that colon mucosa is a useful sample in which potential colorectal cancer biomarkers can be searched for. Using high-resolution, magic angle spinning NMR as well as GC–MS, metabolic profiles for colorectal tumour biopsies and matched colon mucosae samples were collected to reveal potential biomarkers indicative of elevated tissue hypoxia, glycolysis, nucleotide biosynthesis, lipid metabolism inflammation and steroid metabolism in cancer [52].

Although most commonly NMR involves $^{1}H$, it is possible to use other isotopes such as $^{13}C$ and $^{31}P$. Both $^{1}H$ and $^{31}P$ have been engaged in profiling mainly lipophilic metabolites to reveal biomarkers associated with the response of HT-29 colon carcinoma tumour xenografts following treatment with PX-478; a drug known to reduce tumour growth by targeting hypoxia inducible factor-1α (HIF-1α) [53]. The latter is a component of the HIF heterodimer that promotes tumour survival and growth in hypoxic conditions [54]. A significant reduction in total choline as well as phosphomonoesters and phosphodiesters were associated with treatment with PX-478 as measured using $^{1}H$ and $^{31}P$ NMR respectively. HIF has been a current hot topic of cancer research especially in the field of metabolomics for over a decade [55] and its involvement in cancer survival has been reviewed extensively [56,57].

CE-MS has been used to detect charged metabolites from glycolysis, the pentose phosphate pathway, TCA and urea cycles and amino acid and nucleotide metabolisms, and has revealed that these cancers are associated with low glucose and high lactate concentrations (indicative of the Warburg effect) along with accumulation of all amino acids except glutamine which suggests evidence of glutaminolysis [58]. This is an interesting revelation from the analysis of the tumour microenvironment rather than a biofluid that is more commonly analysed using a metabolomics approach for cancer biomarker discovery.

Analytical platforms are often simultaneously applied to biomarker discovery in cancer research. This enables detection of a wider range of metabolites, or in the case that one suspected biomarker can be identified from the analysis with more than one technique; more confidence can be assigned to the identification. GC–MS and UPLC–MS have been utilised in the metabolic analysis of serum from 64 colorectal cancer patients and 65 control subjects to test the hypothesis that bio-fluids contain relevant biomarkers of tumour malignancy [50]. Using both analytical platforms, metabolites were identified to be statistically significantly different between the test and control groups. Pyruvate, lactate, tryptophan, tyrosine and uridine were analysed using both analytical techniques and pyruvate and lactate were found to be significantly increased while tryptophan, tyrosine and uridine were decreased in the cancer patients [50]. From the study it was concluded that the combination of these analytical platforms was useful in the characterisation of colon cancer metabolism to broaden the range of metabolites that could be detected and to increase the confidence of conclusion determined for metabolites detected using both techniques [50].

### 3.2. Gastric, pancreatic and liver cancer

Pancreatic cancer has been described as having the worst prognosis amongst all cancers [14,59]. Pancreatic ductal adenocarcinoma is usually associated with uncontrolled proliferation, being highly metastatic and resistant to therapy [60]. Previously, protein biomarkers have been described for pancreatic ductal adenocarcinoma that include carbohydrate antigen 19-9 (CA 19-9), a cell surface associated mucin that protects against infection through binding to pathogens (MUC1), the immunoglobulin carcinoembryonic antigen-related cell adhesion protein 1 (CEACAM1) and protein marker MIC-1. However pancreatic ductal adenocarcinoma can often not be distinguished from chronic pancreatitis using these markers and there are issues with the specificity and sensitivity for their detection. Thus the search for metabolic biomarkers using GC–MS, LC–MS and hydrophobic interaction chromatography (HILIC)-LC–MS analysis of plasma has been performed in the hope that they could be screened for in the early detection of pancreatic cancer [60]. Candidate biomarkers such as N-methylalanine, lysine, glutamine, phenylalanine, arachidonic acid, tauro(ursodeoxy)cholic acid, and (deoxy)cholylglycine were revealed due to their significant fold change between patients and healthy controls.

In another search for candidate biomarkers in pancreatic cancer, NMR spectroscopy was employed in the analysis of serum from pancreatic cancer patients revealing that in comparison to healthy controls, pancreatic cancer causes a combined significant decrease in 3-hydroxybutyrate, 3-hydroxyisovalerate, lactate, and trimethylamine-N-oxide as well as significant increases in isoleucine, triglyceride, leucine, and creatinine [59]. In another NMR study, elevated plasma levels of N-acetyl glycoprotein, dimethylamine, citrate, alanine, glutamate, glutamine, histidine, lysine and valine among others were described as a biomarker pattern that differentiates pancreatic cancer patients from patients with chronic pancreatitis or healthy controls [14].

Gastric cancer biomarkers can be screened for in gastric mucosae and if successful markers are revealed that could be clinically relevant, it may offer an alternative for diagnosis compared to currently used methods of endoscopy and biopsy. Using a combination of univariate and multivariate analyses of GC–MS metabolic profiling data from gastric mucosae including tumorous and non-tumorous tissue, one study has described 18 potential biomarkers, 5 of which could also mark the difference between invasive and non-invasive tumours [61]. The latter included elevated L-cysteine and L-tyrosine, which were connected to enhanced glycolytic activity and its effect on TCA cycling, elevated hypoxanthine, which was attributed to its necessity as a substrate and nitrogen source in the highly propagating tumour cells, and decreased phenanthrenol and butanoic acid in the invasive tumours [61].

Hepatocellular carcinoma is one of the most prevalent malignant tumours worldwide and the prognosis for patients with this disease is heavily dependent on the stage of the cancer [62]. Therefore, as with other cancers a method for early detection is vital also for the liver. GC–MS profiling has revealed prospective biomarkers for this cancer that include fatty acids, organic and inorganic acids and amino acids in addition to xylitol, arabinofuranose, hypoxanthine, and urea [62].

In another metabolomics application for characterising hepatocellular carcinoma, its progression from liver cirrhosis has been assessed [63]. A comparison of metabolite levels in serum was made between hepatocellular carcinoma patients and cirrhosis controls revealing peaks of significant interest between spectra from each group. Following mass-based database searching for putative identifications, potential biomarkers for hepatocellular carcinoma were verified by comparison of LC–MS/MS fragmentation with authentic standards. Subsequent quantification using isotope dilution by selected reaction monitoring (SRM) LC–MS/MS confirmed sphingosine-1-phosphate and lysophosphatidylcholine 17:0 as being significantly increased in hepatocellular carcinoma patients; while glycohenodexycholic acid 3-sulfate, glycoholic acid, glycodeoxycholic acid, taurocholic acid and taurochenodeoxycholate were significantly decreased [63]. The importance of spingosine-1-phosphate and its associated biochemical pathway is of current interest in a range of diseases. It is responsible for a wide variety of bioprocesses and therefore its (dys)regulation in disease is

studied to understand the pathway, in particular its potential as a therapeutic target in the future [64].

### 3.3. Breast and ovarian cancers

By far the most studied women's cancer is breast cancer, for which there is a wide range of literature describing biomarker discovery through metabolic profiling. The lipidome has been described as a composite biomarker for breast cancer [65]. It has been suggested that an elevation in monounsaturates as well as a low ratio between $n-6$ and $n-3$ fatty acids can be associated with lowering the risk of breast cancer and that the lipidome could be used as a template to design a dietary way to reduce breast cancer risk [65]. In a related study it has been suggested that dietary energy restriction in pre-menopausal women can reduce the risk of breast cancer post-menopause [66].

In the analysis of serum from patients with malignant or benign breast tumours as well as controls using GC–MS followed by multivariate data analysis; palmitic acid, stearic acid and linoleic acid differentiated malignancy from other groups along with the total free fatty acid profile being elevated due to malignancy [20]. In another study, it was shown that lysophospholipids increase with aggressiveness in breast cancer cells and a switch from unsaturated to saturated fatty acids in phosphatidylinositols occurs in breast cancer relative to controls [67]. It was concluded from this study that the lipid profile can be useful to determine the stage of breast cancer. Aside from lipids, other potential biomarkers for breast cancer as determined in urine by GC–MS include homovanillate, 4-hydroxyphenylacetate, 5-hydroxyindoleacetate and urea [68].

UPLC–MS has been useful in the analysis of plasma metabolic profiles for malignant and benign ovarian tumours through successful validation of four candidate biomarkers (L-tryptophan, LysoPC(18:3), LysoPC(14:0), and 2-piperidinone) that were significantly lower due to malignancy from the 535 metabolites found to classify malignancy from benignity in ovarian cancer [69].

In another investigation of ovarian cancer, a combination of GC–MS and LC–MS/MS was used to compare primary epithelial ovarian cancer and metastatic tumours resulting from primary ovarian cancer. Upon transformation to metastasis, ovarian tumours were shown to significantly alter energy utilisation through metabolites associated with glycolysis and beta-oxidation of fatty acids (such as carnitine) as well as significant changes in phenylalanine metabolism marked by the change in phenylpyruvate and phenyllactate [70]. In urinary analyses of a range of women's cancers using GC–MS and LC–MS, previously suggested biomarkers for breast cancer: 5-hydroxymethyl-2-deoxyuridine and 8-hydroxy-2-deoxyguanosine were confirmed for breast cancer and related markers for oxidative DNA damage and DNA methylation were observed to significantly increase in ovarian cancer compared to controls [71].

### 3.4. Urinary cancers

Urinary cancers include cancers of the bladder, prostate, kidney and testicle. In a study of kidney and bladder cancer, the analysis of serum with LC–MS using both HILIC and reverse-phase (RP) separation of metabolites has been useful to reveal potential biomarkers that include eicosatrienol, azaprostanoic acid, docosatrienol, retinol and 14′-apo-bet-carotenal for bladder cancer and glycerophosphorylcholine, ganglioside GM3 (d18:1/22:1) and C17 sphinganine amongst other lipids for kidney cancer [72].

Urine can be screened in the hope to reveal biomarker patterns for the early detection of bladder cancer and one of the most reported findings is the difference in carnitines that are involved in transport of fatty acids across mitochondrial membranes [73]. It has been reported that bladder cancer can lower the concentration of C9:1 carnitine, that urinary acylcarnitine deficiency is prevalent in bladder cancer and that acetylcarnitines increase in cancer whereas they stay in equilibrium in controls [73]. In the non-targeted analysis of urine, acylcarnitines have also been exposed as features of kidney cancer and for cells it has been reported that acylcarnitines increase which could be indicative of enhanced fatty acid metabolism in kidney cancer [74].

Renal cell carcinoma is usually associated with good prognosis if detected early, however when detected after symptoms have arisen, prognosis can be poor [75]. HILIC column technology has recently advanced and when coupled to ESI MS it has had useful applications in renal cell carcinoma biomarker discovery, particularly in detecting highly polar compounds from bio-fluids [75,76]. When applied in parallel to LC–MS, it has been shown that HILIC-MS can be used in diagnosis and potential staging of this cancer. A total of 30 metabolites were exposed as potential biomarkers of malignancy that could be specific to renal cell carcinoma. Upon metabolic pathway analysis of these potential biomarkers it was suggested that renal cell carcinoma may be associated with disruption in phospholipid catabolism, sphingolipid metabolism, fatty acid beta-oxidation, cholesterol metabolism and arachidonic acid metabolism in addition to phenylalanine and tryptophan metabolism [76].

The profiling of sarcosine and related metabolites as potential prostate cancer biomarkers has been described in the CE-MS analysis of urine published by Soliman et al. [77]. In this case, it was necessary to develop a specific protocol for the profiling of this specific group of metabolites. The addition of standards allowed calibration and approximation of the endogenous concentrations for metabolites of interest in the urine samples [77]. Aggressive prostate cancer that eventually spreads to the bone is a fatal disease [78]. Prostate cancer bone metastases have been compared to bone metastases from other sources and to normal bone to reveal that cholesterol is significantly higher and could be a biomarker for this origin of cancer [78].

### 3.5. Cancers of oesophagus and lung

One of the most notable findings in metabolic differences associated with cancers of the oesophagus is the change in carbohydrate metabolism suspected from the promotion of glycolysis and disruption of the TCA cycle. This was particularly observed in a study by Wu et al. [23], where 20 central carbon metabolites were observed to be significantly different in tumorous tissue compared to control tissue from similar origin in a metabolic profiling analysis performed using GC–MS. In another example where the combination of analytical platforms has enhanced the discovery of potential biomarkers, NMR has been simultaneously employed with LC–MS in the analysis of oesophageal cancer metabolism. Similar potential biomarkers of central carbon metabolism were highlighted by each technique, however when combining metabolites in multivariate analysis, 12 metabolites varied consistently in diseased patients compared to healthy controls and the significance was increased upon the inclusion of all data in one model [79]. Since a range of techniques have described central carbon metabolism and carbohydrate metabolism for key features of cancer of the oesophagus, more confidence is provided for these biochemical pathways in that metabolites were not highlighted due to their enhanced detectability in one type of instrument.

Similar candidate biomarkers have been suggested for lung cancer as other cancers. For example, fatty acid derivations, sphingomyelin and lysophosphatidylcholine have been associated with lung cancer progression [80]. In fact theses metabolites have been

reported with 100% sensitivity and 91% specificity in the comparison of serum from lung cancer patients and healthy controls [80].

### 3.6. Biomarker discovery with no particular attributed cancer

Although most research in cancer biomarker discovery is based upon the analysis of samples from specific cancers, it is possible to focus on potential biomarkers in different cancer types. Thus, research is not attributed to a single cancer. Metabolites may be considered as potential biomarkers for a range of cancers. For example, CE-MS analysis of cancer patient saliva has revealed potential biomarkers for oral, pancreatic and breast cancer. It was found that 57 metabolites were common biomarkers to all three cancer types since their concentration was significantly elevated in the saliva of cancer patients relative to healthy controls [28]. In the CE-MS spectra collected for this study, an average of 90 peaks could be attributed to metabolites, of which two metabolites were specific identified markers of oral cancer (taurine and piperidine) and eight metabolites were specific identified markers of pancreatic cancer (isoleucine, leucine, tryptophan, valine, glutamic acid, phenylalanine, glutamine, and aspartic acid). There were 28 metabolites identified as being significantly increased in breast cancer compared to other cancers or controls; however none of these were uniquely identified in breast cancer and were therefore not considered as biomarkers [28].

A summary of metabolite biomarkers discovered to distinguish between patients and controls for each type of cancer reviewed is given in Table 1.

In some instances certain metabolites or chemically related groups of metabolites have been studied as important biomarkers of cancer with no particular localisation in the body. For example, nucleosides and related metabolites with *cis*-diol structures are thought to be an important class of metabolites that are potential cancer biomarkers [81]. Screening bio-fluids to reveal metabolic changes with respect to nucleosides in cancer is becoming increasingly popular and the success of nucleoside profiling in cancer has been published in recent years [82,83]. Of course, screening of nucleosides can also be useful to study specific cancers through a targeted approach, for example the recently published targeted analysis of 12 specific nucleosides involved in urogenital cancer [84].

It is possible to assess the carcinogenic effects of particular entities on metabolism using a metabolomics biomarker discovery approach. Many studies have attempted to link tobacco and cancer through revealing urinary carcinogens as potential cancer biomarkers; a topic that is well reviewed in Hecht [85]. Included in these biomarkers are metabolites of benzo[α]pyrene, *N*-nitrosoproline, 8-oxodeoxguanosine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol and its glucuronides that have been highlighted for their particular importance as biomarkers since they are derived from a carcinogen that is specific to tobacco products [85].

Another approach in cancer biomarker discovery is to assess the metabolic effects of oncogenes. For example, one published example includes the fingerprinting of cancer cells with UPLC–MS to assess the bio-energetic and anabolic changes induced by c-Myc, KLF4 and Oct1, revealing important information regarding amino acid uptake and secretion as well as mitochondrial metabolism [86]. In this example, stimulation of glycolysis and reduced mitochondrial respiration was attributed to c-Myc deficiency and glycolysis was also stimulated by KLF4 deficiency. Conversely, Oct1 deficiency appeared to reduce glycolysis but stimulate oxidative phosphorylation. Specific patterns in amino acids were observed for each deficiency, revealing an important relationship between isoleucine, α-aminoadipic acid and γ-aminoisobutyric acid [86].

## 4. Current challenges and future directions

The challenges associated with metabolomics are true for its application to any biological field, not just cancer, but are particularly relevant in biomarker discovery since the latter is limited to the bounds possible with the technique used. For example, one challenge is that the volume of metabolites can be too large to analyse and some metabolites cannot be detected through current experimental methods. As instrumentation, sample preparation and data analysis continue to advance, in the future it is hoped that the number of features it is possible to identify will increase.

Other limitations are largely associated with experimental design and the information that can be obtained from different samples. One consideration when interpreting the results of metabolomics studies on human samples is whether or not the data come from matched and truly comparable cases. It is extremely difficult to perform a scientifically accurate 'fair test' between human subjects, but effort made to match diseased and control groups with respect to sample size and epidemiology of subjects provides the possibility for a more reliable comparison between groups. This can be difficult if availability of samples is limited due to small numbers of healthy volunteers to act as control subjects or small numbers of diseased subjects, particularly for rare conditions. Unbalanced sample sizes can severely affect the validity of a study, particularly when statistical procedures are applied that depend on balanced groups. Usefully, an increasing number of studies are comparing equal numbers of samples and include age and sexed matched controls, allowing more confidence in the biomarkers revealed [87]. Other approaches undertaken to remove inter-subject variation compare diseased and non-diseased samples from the same human. For example, in an attempt to reveal gastric cancer biomarkers, tumorous tissue and non-malignant tissue from the same gastric mucosa were extracted for pairwise comparison between subjects [61]. A similar experimental method was applied in the elucidation of biomarkers for cancer of the oesophagus, whereby pairwise samples were compared for tumorous and non-malignant mucosae of oesophageal cancer patients [61].

Metabolomics on human samples offers an advantage over *in vitro* cell studies or studies on model organisms, in that the metabolic profiles are likely to be truer representatives of the system under investigation. *Ex vivo* samples are typically tissue extracts [88,89] or bio-fluids [90–93]. Bio-fluids such as blood plasma, urine or saliva involve less invasive sample retrieval and the procedure for extraction is simpler than for tissue which can enable a more representative analysis or a 'snap shot' of metabolism [94]. However, the limits to what metabolites will be present in bio-fluids compared to tissue extracts may offer a disadvantage. Urine for example is largely composed of waste products that are difficult to connect with biological function. Also, there is a much higher rate of variability associated with *ex vivo* samples. For example, Variations in urinary profiles have been shown to be more pronounced after meals compared to those samples taken at random times or after periods of fasting [75]. Therefore, depending on the application and the information required, proper experimental design can lead to optimum information from data.

Aside from choosing the right sample for the application, one of the main challenges in metabolite biomarker discovery for cancer research is the way to classify biomarkers and potential biomarkers. Candidate biomarkers have been suggested for a range of cancers and some of the key examples in recent research have been highlighted in this review. However, often depending on the experiment performed with respect to the choice of samples analysed, the analytical platform used and the data analysis employed, very different biomarkers can be suggested for the same cancer. In the search for gastric cancer biomarkers, a very different list of potential

**Table 1**
Summary of the most significant biomarkers discovered for different cancers from metabolomics experiments.

| Tumour | Sample | Analytical platform | Metabolite biomarker | Reference |
|---|---|---|---|---|
| Colorectal | Serum | FTICR-MS<br>NMR<br>LC–MS/MS | Ultra long-chain fatty acids 446, 448, 450 | Ritchie et al. [49] |
| | | ESI-MS/MS | Lysine, alanine, aspartic acid, glycine, histidine, (iso)leucine, methionine, sarcosine, threonine, tyrosine, valine | Leichtle et al. [17] |
| | | GC–MS<br>UPLC–MS | Pyruvate, lactate, tryptophan, tyrosine, uridine | Qiu et al. [50] |
| | Urine | GC–MS | Citrate, hippurate, p-cresol, 2-aminobutyrate, myristate, putrescine, kynurenate | Cheng et al. [48] |
| | Mucosa | NMR<br>GC–MS | 31 markers including amino acids; metabolites from glycolysis, TCA cycle, lipid synthesis | Chan et al. [52] |
| | Tissue | CE-MS | 22 markers including hydroxyproline, nucleotides, amino acids; metabolites from glycolysis, TCA cycle and pentose phosphate pathway | Hirayama et al. [58] |
| Pancreatic | Plasma | GC–MS<br>LC–MS | N-methylalanine, lysine, glutamine, phenylalanine, arachidonic acid, tauro(ursodeoxy)cholic acid, (deoxy)cholylglycine | Urayama et al. [60] |
| | | NMR | 29 markers including N-acetyl glycoprotein, hydroxybutyrate, formate, acetone, creatine, citrate, lipids, amino acids, glycolytic metabolites | Zhang et al. [14] |
| | Serum | NMR | 3-Hydroxybutyrate, 3-hydroxyisovalerate, lactate, trimethylamine-N-oxide, triglyceride, (iso)leucine, creatinine | OuYang et al. [59] |
| | Saliva | CE-MS | Iso(leucine), tryptophan, valine, glutamic acid, phenylalanine, glutamine, aspartic acid | Sugimoto et al. [28] |
| Gastric | Mucosa | GC–MS | 18 markers including 5 distinguishing invasive from non-invasive tumours (cysteine, tyrosine, hypoxanthine, phenanthrenol, butanoic acid) | Wu et al. [61] |
| Liver | Serum | LC–MS/MS | Sphingosine-1-phosphate, lysophosphatidylcholine 17:0, glycohenodexycholic acid 3-sulfate, glycoholic acid, glycodeoxycholic acid, taurocholic acid, taurochenodeoxycholate | Ressom et al. [63] |
| | Urine | GC–MS | 18 markers including fatty acids, organic and inorganic acids, amino acids, xylitol, arabinofuranose, hypoxanthine, urea | Wu et al. [62] |
| Breast | Serum | GC–MS | Free fatty acids, palmitic acid, stearic acid, linoleic acid | Lv et al. [20] |
| | Cultured cells | ESI-MS | Lysophospholipids, saturated fatty acids in phosphatidylinositols | Luisa Doria et al. [67] |
| | Urine | GC–MS | Homovanillate, 4-hydroxyphenylacetate, 5-hydroxyindoleacetate, urea | Nam et al. [68] |
| | | GC–MS<br>LC–MS | 5-Hydroxymethyl-2-deoxyuridine, 8-hydroxy-2-deoxyguanosine | Woo et al. [71] |
| Ovarian | Plasma | UPLC–MS | L-tryptophan, LysoPC(18:3), LysoPC(14:0), 2-Piperidinone | Zhang et al. [69] |
| | Tissue | GC–MS<br>LC–MS/MS | Carnitine, acetylcarnitine, butyrylcarnitine, phenylpyruvate, phenyllactate, 2-aminobutyrate, tocopherols, N-acetylaspartate, N-acetyl-aspartyl-glutamate | Fong et al. [70] |
| | Urine | GC–MS<br>LC–MS | 1-Methyladenosine, 3-methyluridine, 4-androstene-3,17-dione | Woo et al. [71] |
| Kidney | Serum | LC–MS | PE (P-16:0e/0:0), glycerophosphorylcholine, ganglioside GM3(d18:1/22:1), C17 sphinganine, SM (d18:0/16:1(9Z)) | Lin et al. [72] |
| | | | 30 lipids | Lin et al. [76] |
| | Cultured cells | | 26 carnitines | Ganti et al. [74] |
| Bladder | Serum | LC–MS | Eicosatrienol, azaprostanoic acid, docosatrienol, retinol,14′-apo-bet-carotenal | Lin et al. [72] |
| | Urine | LC–MS | Acetylcarinitine, carnitine C8:1, carnitine C9:1, carnitine C10:1, 2, 6-dimethylheptanoyl carnitine, hippuric acid, leucylproline, phosphorylcholine, trigonelline | Huang et al. [73] |
| Prostate | PlasmaTissue | GC–MS | Cholesterol | Thysell et al. [78] |
| | Urine | CE-MS/MS | Sarcosine | Soliman et al. [77] |
| Oesophageal | Serum | NMR<br>LC–MS | Lactic acid, valine, (iso)leucine, methionine, tyrosine, tryptophan, myristic acid, linoleic acid, beta-hydroxybutyrate, lysine, glutamine, citrate | Zhang et al. [79] |
| | Tissue | GC–MS | 20 markers of central carbon metabolism | Wu et al. [23] |
| Lung | Serum | FTICR-MS | 7 markers including fatty acid derivations, lysophosphatidylcholine, sphingomyelin | Guo et al. [80] |
| Oral | Saliva | CE-MS | Taurine, piperidine | Sugimoto et al. [28] |

biomarkers were suggested in research articles published by Wu et al. [61] and Hu et al. [95]. These examples both employed GC–MS but of different samples. It could be that these potential biomarkers could be real biomarkers for the specific sample type from which they were obtained and that they could be reliably screened for in those respective samples in the clinic. Before they can be used medically, clinicians will need to be sure that potential biomarkers are reliable for detecting a particular cancer and it needs to be decided whether or not a biomarker can be detected as significantly elevated or decreased and whether this should be unique to the cancer of interest and cannot arise from any other phenotype [28]. Answering such questions will be vital for bridging the gap between metabolomics research and clinical utility of biomarkers for the screening of various cancers in the future.

Despite the challenges in the field, there are many areas under continual enhancement that are leading us into the future direction for the field. For example, progress is currently being made towards creating a combined repository of methodologies and data from the analysis of a wide selection of biological samples. This is to include data from a number of biological species using a range of analytical techniques and will be made available for the whole metabolomics community to use. Such a repository has proven successful in genomics through GenBank, which provides nucleotide sequences for over 380,000 organisms and laboratories worldwide are continually enhancing it through daily data exchanges [96]. There remains a requirement for a comprehensive and publically available database that would be useful in data interpretation and standardisation for metabolomics. Cancer researchers employing metabolomics techniques in biomarker discovery would then be able to access information and build upon the current knowledge about the metabolism of the cancer of interest.

Another aspect of metabolomics in cancer biomarker discovery under rapid development is the use of mass spectrometry imaging, the potential application of which in metabolomics has been previously reviewed [97]. Instrumentation such as matrix assisted laser desorption ionisation (MALDI)-MS and secondary ion mass spectrometry (SIMS) have the option of imaging samples to reveal significant chemical differences between regions in a sample's surface. Initial progress has been made through the initiation of a database of metabolite standards that can be used for metabolomics experiments conducted through SIMS [98]. Two very recent examples of the application of SIMS in cancer research have been described that show differences in the localisation of metabolites within tumours dependent on oxygen availability [99,100]. Although this is a very new application in cancer research, with further development it could be possible to profile the metabolism and spatially localise biomarkers in tumours, where their location could be useful in diagnosis and even for suggesting prognoses.

Another direction that is becoming increasingly popular is the incorporation of a systems biology approach in the interpretation of results. Cancer as a system is hugely complex and requires to some extent detailed knowledge of its components. Although the biochemistry is by no means fully understood to the detail required to prevent or cure cancer, systems biology can be employed to link components together to reveal their system properties. This could help highlight the most important aspects of the cancer phenotype that should be further investigated, perhaps even using traditional reductionist methods. Considering the system from a top-down perspective could lead to revealing new biomarkers or potential targets for future therapy.

Systems biology is likely to lead to a better understanding of a metabolite biomarker's role in cancer phenotype, especially if combined with other 'omics data' to reveal a global picture of the system. The fluxes and concentrations of metabolites can originate from more than one hierarchical route, such that changes observed in the metabolic phenotype of a biological system can be ambiguous with respect to their origin. Therefore, although it can reveal biomarkers of the disease, it may not provide definitive information of the underlying biological processes. Using a combination of 'omic techniques' to assess entities such as gene regulation and expression, as well as protein synthesis and expression could provide one way to elucidate bioprocesses that control the metabolome and why certain metabolites appear to be biomarkers in cancer.

## 5. Conclusion

Advancements in instrumentation and computational methods in metabolomics so far have been adequate enough to make biomarker discovery possible. Furthermore, combining the use of different analytical platforms extends the number of metabolites it is possible to detect in biological samples. Biomarkers are valuable identifications, regardless of their hierarchical origin, for revealing phenotypic properties in a biological system and therefore metabolomics provides an appropriate level at which to study cancer phenotype. Moreover, in the identification of key metabolic pathways from which significant metabolites are highlighted, it is possible that metabolomics alone could reveal potential targets for cancer therapy.

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