Review

Liam M. Heaney*

Applying mass spectrometry-based assays to explore gut microbial metabolism and associations with disease

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Abstract: The workings of the gut microbiome have gained increasing interest in recent years through the mounting evidence that the microbiota plays an influential role in human health and disease. A principal focus of this research seeks to further understand the production of metabolic by-products produced by bacteria resident in the gut, and the subsequent interaction of these metabolites on host physiology and pathophysiology of disease. Gut bacterial metabolites of interest are predominately formed via metabolic breakdown of dietary compounds including choline and L-carnitine (trimethylamine N-oxide), amino acids (phenol- and indole-containing uremic toxins) and non-digestible dietary fibers (short-chain fatty acids). Investigations have been accelerated through the application of mass spectrometry (MS)-based assays to quantitatively assess the concentration of these metabolites in laboratory- and animal-based experiments, as well as for direct circulating measurements in clinical research populations. This review seeks to explore the impact of these metabolites on disease, as well as to introduce the application of MS for those less accustomed to its use as a clinical tool, highlighting pertinent research related to its use for measurements of gut bacteria-mediated metabolites to further understand their associations with disease.

Keywords: biomarker; gut microbiome; mass spectrometry; short-chain fatty acids; TMAO; uremic toxins.

Introduction

The average human body plays host to an estimated 4×10^{13} bacteria, a quantity which is approximately equal to the total number of host human cells [1, 2]. These commensal bacteria are present both on and within the human biological system and can be found in multiple sites including, but not limited to, the oral cavity, respiratory tract, skin, genital regions and gastrointestinal tract [3, 4]. The latter, commonly referred to as the gut microbiome, has been perhaps the most well-studied when investigating the impact of the microbiota on health and disease [5]. Although the co-existence of bacteria and host generally leads to a symbiotic relationship that maintains a mutual status of biochemical homeostasis, incidences where the genetic or metabolic landscape are altered have been associated with ill-health and disease [6]. The change in genetic diversity of the microbiome is often termed as ‘dysbiosis’ and is related to an alteration in the numbers or species of bacteria present within the ecosystem [5]. These changes have been associated with the development and progression of multiple conditions including cardiovascular disease, chronic kidney disease (CKD), non-alcoholic fatty liver disease and diabetes, inflammatory bowel disease, amongst many others [7–10]. Metabolic changes, of which will be the focus of this discussion, relate to shifts in bacterial metabolism that either increase/decrease or alter the metabolite by-products released as part of their routine cellular workings. These molecules are released into the gut lumen and are capable of crossing the gut epithelial barrier to enter the systemic circulation of the host [11]. As many of these metabolites are categorized as small molecule metabolites, they are particularly well-suited to subsequent analysis by hyphenated mass spectrometry (MS) techniques such as gas chromatography-MS (GC-MS) and liquid chromatography-MS (LC-MS). This review aims to provide a brief introduction to MS for readers who are not familiar with the technology, and to present an overview of how MS-based assays are providing novel and informative data into the metabolism of the gut microbiota.

*Corresponding author: Dr. Liam M. Heaney, School of Sport, Exercise and Health Sciences, Loughborough University, Loughborough, LE11 3TU, UK, E-mail: L.M.Heaney2@Lboro.ac.uk.
<https://orcid.org/0000-0002-8791-0167>

through the discussion of selected small molecule gut metabolites. Details are given on how these metabolites are being measured by MS for clinical research with the view to understand their utility as clinical biomarker measurements and lead the way to warrant future translation into routine laboratory medicine.

Basics of MS and its benefits to clinical chemistry

MS is an analytical technology based on the measurement of the molecular mass of a charged molecule, referred to as an ion. The MS instrument can manipulate and/or monitor the flight path of an ion, subsequently assigning a value which is a calculation of the molecular mass divided by the charge state of the ion. This is termed the mass-to-charge ratio and is symbolized by m/z . For small molecule MS, the charge state of the ion is commonly at +1 or -1 (often through the addition [+1] or loss [-1] of a proton [H^+ ion]), and therefore the m/z value relates to the approximate molecular mass of the measured analyte. However, in some circumstances (predominantly for measurement of larger molecules such as peptides), the charge state can be increased (e.g. +2, +3, etc.) and so the m/z value will reflect a value that is lower than the molecular mass of the analyte of interest. There are multiple approaches in which to ionize a molecule and they are typically referred to as soft ionization (where the molecule remains predominantly intact) and hard ionization (where the molecule is deconstructed into smaller fragments), with the chosen method based on the instrumentation and suitability to the analyte(s) of interest; see an excellent summary by Gary Siuzdak for more technical information on these processes [12]. The response (intensity) of the measured ion(s) is then plotted against m/z to produce a mass spectrum which can be investigated to understand the presence and intensity for an analyte(s) of interest.

As clinical biochemical analyses are performed on complex matrices such as urine, plasma and serum, in most circumstances the application of MS alone is not adequate to provide a definitive answer to a clinical question. If all molecules present in the mixture enter the mass spectrometer at the same time (known as direct infusion), the instrument will return a response for all ions that are produced which makes it difficult to isolate the analyte of interest and obtain reproducible data (albeit a technique that was traditionally applied for newborn screening [13]). Furthermore, for soft ionization techniques such as electrospray (commonly known as ESI) there is a competition

for charge in the ionization source. This means that molecules with greater affinity to achieve a charged state will 'swamp' the analysis by suppressing the ionization capability of other molecules. One major issue with this sequestering of charge is that molecules that dominate the ionization space will not be of equal concentration across samples. Therefore, the ion suppression present for one patient sample will not equal that of another, further complicating the analysis and producing what is known as the matrix effect. One way to overcome this phenomenon is to manipulate the molecules so that they enter the mass spectrometer at different times across the analytical run. This is predominantly achieved using a separation technique in which the sample is initially passed through prior to entry into the mass spectrometer. For quantitative bioanalysis, this is typically achieved using chromatographic techniques such as GC and LC. Comprehensive development and optimization of the separation techniques allows the analyte(s) of interest to elute within a given time window with partial or full separation from molecules that are competing for ionization. Moreover, the analyte(s) elutes from the column over a short time period following a Gaussian distribution profile, allowing the area under the curve to be calculated and compared back to calibration curves to provide accurate quantitative data. In order to further adjust for ion suppression, stable isotope labeled (SIL) versions of the analyte(s) (employing multiple incorporation of 2D , ^{13}C or ^{15}N atoms) are included at a known concentration. These SIL compounds behave identically within the chromatographic space to enter the mass spectrometer at the same point as the unlabeled molecule but have an increased molecular mass and can be identified in isolation. Any ion suppression that occurs will do so to both the unlabeled and SIL compound and thus the unlabeled data can be adjusted accordingly against the known SIL concentration.

A further strength of MS for biochemical analyses comes from the application of multiple stage analysis referred to as tandem-MS, or MS/MS. MS can be performed in an open (full) scan process where no prioritization of ions is implemented which can lead to elevated levels of background noise and subsequently a reduction in analytical sensitivity. However, applying a multiple stage selection and filter of ions of interest helps ensure that a specific biomolecule is analyzed. This multiple stage selection, commonly termed as selected or multiple reaction monitoring (S/MS), employs a filtering process to select an ion(s) of interest, performs collision induced dissociation of that ion(s) to produce smaller fragment molecules, and further selects the product fragments that are specifically associated to the targeted precursor molecule; see Figure 1

for a basic overview of the advantages of MS/MS. These approaches are inherently able to diminish background noise to remove almost all interference while simultaneously improving overall selectivity. S/MRM assays can be performed on different formats of mass spectrometers but are most suited to a triple quadrupole instrument due to its capabilities for high-speed scanning and polarity switching (the ability to change between the measurement of different ions of interest and between positive and negative ion analyses, respectively), analysis over multiple orders of magnitude, two-stage ion filtering providing enhanced selectivity and sensitivity (see Table 1 and Figure 1 for more detail on this process), and an innate robustness for high-throughput workflows. In addition, triple quadrupole instruments are able to perform multiple S/MRM assays simultaneously, providing quantitative data for tens to hundreds of molecules from a single multiplexed analysis [14–16]. See Table 1 for a brief description on the triple quadrupole and alternative mass analyzers commonly employed for mass spectrometry measurement of biological samples.

Translation of MS into the clinical laboratory has been less rapid than one might have expected given the advantages and benefits of MS for biomarker measurements. While there is no one definitive reason for this slow uptake, a number of contributing factors exist such as: the large initial cost for instrument purchase, the requirement for employment of highly skilled/trained personnel and the often laborious sample preparation required which can be troublesome to automate [13]. There are also limitations for the translation of MS assays from research

laboratories into the clinical laboratory, and from one hospital laboratory to another [17]. This difficulty stems from the fact that there are multiple MS set-ups available that do not necessarily match up on specifications such as levels of sensitivity. This means that where an assay is perfectly suited to one laboratory, it may not be transferable to another, requiring a whole new set of experiments to be performed to optimize and validate for that laboratory. Vogeser et al. [18] recently proposed guidelines for clinically focused MS assay reporting, emphasizing the need to include more extensive information on instrument/assay parameters to allow the translation to an alternative laboratory to be more accessible. The authors further suggested the fundamental necessity to test the assay in multiple independent laboratories in order to compare results. This would allow for comparison of data obtained using different MS set-ups and laboratory personnel. If more analytical teams were to follow suit with these ideas, it would likely improve the overall uptake of MS into routine clinical measurements.

Despite the relatively restricted translation to everyday hospital analysis, research experiments completed using MS-based assays are demonstrating excellent applicability for clinical biomarker science. Over recent years, investigations into the understanding of gut microbial metabolism, via the measurement of gut microbiota-mediated metabolites, have provided novel information on disease pathogenesis, progression and risk prediction. These works have been spearheaded through the application of MS-based analyses and are building a knowledge base that has the capacity to improve diagnostic,

Figure 1: A simplified schematic diagram to demonstrate the advantages of MS/MS (using a triple quadrupole mass spectrometer, bottom) over a single mass analyzer (top) for selected/multiple reaction monitoring applications providing highly sensitive, selective and reproducible analyses.

Table 1: A brief description of the mass analyzers commonly employed for MS measurement of biological samples.

Mass analyzer	Common written and verbal abbreviations	Basic mechanism of measurement	High mass accuracy? ^a	Relative cost
Quadrupole	Quad Single quad	Consists of two pairs of parallel rods with one pair having a static DC potential applied across it. The second pair has a superimposed, and variable, RF frequency which is set to allow ions with a specific m/z to pass through to the detector, with all other ions either colliding with the rods or being ejected from the ion flight path. This RF frequency can be rapidly altered across a single scan point when multiple ions of interest are present (this relates to the instruments scanning speed), or continuous scanning across a m/z range can be performed (i.e. continuously ramping from the lower to upper m/z value across the analysis – this is an open, or full, scan)	No	Low
Triple quadrupole	Triple quad TQ QqQ/QQQ	An instrument with three quadrupoles positioned in series. Quadrupole 1 (Q1) is used to select a specific ion or ions of interest (precursor ion); Q2 contains an inert gas (usually N ₂ or Ar) and has an applied voltage to cause excitation of the ions that leads to collision induced dissociation (fragmentation); and Q3 allows specific selection of an ion or ions that are related to the fragmentation of the precursor ion (product ion) Note: modern instruments have exchanged Q2 for a smaller non-quadrupole collision cell or have used a curved quadrupole analyzer to help reduce the laboratory footprint of the instrument	No	Low to medium
Time-of-flight	ToF/TOF	Ions are accelerated by an electric field across a flight tube that is held under vacuum. The time taken for the ion to travel from the ‘pusher’ to the detector is related to its m/z . Therefore, ions with a greater m/z value require more time to reach the detector. Increases in the ion flight path distance provide increased mass accuracy and therefore modern instruments employ a V or W wave analysis where the ions are reflected by one (V) or more (W) ion mirrors to increase the total distance travelled before hitting the detector	Yes	Medium
Orbitrap	Orbi	The orbitrap mass analyzer works by trapping ions and orbiting them around an inner spindle-like electrode. This process produces a current which can be interpreted by the instrument to generate a m/z value, with ions of different m/z orbiting the central electrode at different frequencies. The orbitrap analyzer is capable of highly sensitive and highly accurate analyses and provides a unique ability to perform experiments applying a parallel reaction monitoring (PRM) approach. This allows precursor ions to be measured, ejected from the analyzer and fragmented in a separate collision cell, and then returned to the analyzer where the product ions can be measured. This means both precursor and product ions can be measured simultaneously and without loss from a single sample analysis	Yes	High

^aMass accuracy relates to the ability of the analyzer to distinguish between very similar but different mass-to-charge (m/z) ratios (e.g. differences in 0.01 Da or less) and is sometimes referred to as high resolution. Instruments that are not considered as having high mass accuracy are capable of confidently measuring to the nearest Da (nearest m/z integer) and are often referred to as having nominal mass resolution.

prognostic and therapeutic monitoring of diseases that display associations with gut microbiome-mediated metabolism. A selection of the most pertinent examples of current research into gut microbiota-mediated metabolites is provided below.

Trimethylamine N-oxide (TMAO)

TMAO is a metabolite produced within the liver following gut bacterial release of its precursor, TMA. TMA is produced by bacteria as a by-product following the metabolic

breakdown of quaternary ammonium-containing dietary compounds such as choline (and choline-containing lipids), L-carnitine and betaine [19, 20]. The released TMA is then able to cross into the hepatic portal vein where it is transported to the liver and converted to TMAO by flavin-containing monooxygenases (e.g. FMO3) via N-oxidation [21]. TMA and TMAO have been considered important in medicine for many years through the metabolic disorder trimethylaminuria, referred to as ‘fish-odor syndrome’. This condition occurs through a genetic defect in FMO3 production, meaning that the TMA produced by gut bacteria cannot be converted to TMAO and therefore builds up within the body [22]. TMA has a strong ‘fish-like’ smell, owing to the high quantities of TMAO that are degraded to TMA in spoiled fish [23], which is then released in the sweat, urine and breath of the patient causing a strong and unpleasant body odor [24].

TMAO was first associated with alternative diseases through the finding that circulating levels were higher in cardiovascular disease patients when compared to matched control participants [19]. This discovery was only possible through the use of MS, applying a technique known as non-targeted metabolomics. Metabolomics studies take samples (often biofluids such as plasma, serum and urine) and apply GC/LC coupled to high-resolution MS (HRMS) using a full-scan approach in an attempt to measure as many compounds present in the matrix as possible [25]. HRMS instruments, including time-of-flight and orbitrap mass analyzers, are capable of measuring m/z values with extremely high accuracy (<0.001% error), providing an improved ability to identify an unknown compound by accurately isolating the response of a selected ion from its co-eluting compounds [26]. This, in turn, provides a snapshot indication of the current metabolic status of the individual, with the view to isolate evidence for associations with a (patho)physiological state of interest [26]. TMAO was further investigated in a large cohort of patients undergoing elective diagnostic cardiac catheterization, where it was observed that individuals with increased circulating levels of the metabolite were at a higher risk of a poor outcome (myocardial infarction, stroke or death), therefore demonstrating a prognostic quality of TMAO as an independent biomarker for disease risk stratification [27].

These initial studies signified the discovery and characterization of TMAO as a potential clinical biomarker and there has since been a wealth of data published on both its measurement and application. The majority of validated TMAO assays are performed by LC-MS/MS using a triple quadrupole mass spectrometer [28–31]. However, additional MS-based methods have been developed to

include the use of HRMS [32–34] and GC-MS-based protocols [35, 36], alongside non-MS approaches using nuclear magnetic resonance spectroscopy [37]. These MS-based assays have afforded a high-throughput (e.g. 2.5 min per sample [34]), quantitative approach to TMAO measurement and have even allowed for the added development of multiplexed assays where TMAO is measured in parallel with its precursor molecules including TMA, choline, L-carnitine and betaine [38–40]. Owing to this abundance of high-quality assays within the literature, many researchers have been capable of furthering the scientific and clinical understanding of TMAO and its gut microbial-mediated origins. For example, animal models have demonstrated that the increased presence of circulating TMAO causes an acceleration in atherosclerotic plaque formation [20] and renal fibrosis [41], enhances platelet hyperactivity [42], exacerbates pressure-overload induced heart failure [43], and promotes inflammatory-mediated endothelial dysfunction [44]. Although these mechanistic implications on TMAO and disease causality have yet to be proven in the human biological model, many researchers have found corroborating evidence to show that the severity/risk of numerous diseases is associated with elevated blood TMAO levels. Owing to the fact that TMAO was first identified in cardiovascular disease, investigations in cardiology have been the most intensely studied and published within the literature. Building on the initial data for the prognostic quality of TMAO in heart disease patients, the utility of TMAO as clinical a biomarker has been further observed for patients diagnosed with chronic and acute heart failure [45–48], acute coronary syndromes including myocardial infarction [49, 50], as well as other cardiovascular related conditions such as renal disease [41, 51] and cerebral ischemic stroke [52, 53]. Further evidence has been proposed to suggest that TMAO may also provide prognostic quality for hepatic diseases [54, 55], colorectal cancer [56] and pneumonia [57].

In all, these findings indicate that the elevated production/build-up of TMAO has negative associations with a range of diseases and therefore offers a potential therapeutic target to improve overall patient health. As a promising clinical biomarker, TMAO measurements would directly benefit from increased accessibility of mass spectrometry within the clinical laboratory.

Uremic toxins

Uremia, or uremic syndrome, is a progressive condition where a reduction in renal capacity causes a range

of molecules to be retained that would otherwise be effectively removed from the systemic circulation by the healthy kidney [58]. These retention solutes can include middle molecules, defined as molecules with a molecular mass of 500–60,000 Da (e.g. β 2-microglobulin, parathyroid hormone) [59], and small molecules (<500 Da) that are either free water-soluble compounds (e.g. uric acid, malonaldehyde) or protein-bound molecules (e.g. indole- and phenol-based compounds) [60]. These compounds can exhibit a range of physicochemical characteristics and actions within the body, and those that are associated with pathophysiological actions are collectively termed as uremic toxins (UTs) [61]. While many of these UTs are associated with host metabolism followed by a build-up in concentration, the production of some molecules is entirely dependent on gut microbial metabolic pathways. The most prominent of the microbiota-mediated compounds are based on the metabolism of tryptophan to indole by tryptophanase and tyrosine to p-cresol (pC) by hydroxyphenylacetate decarboxylase, which are then further converted to their sulfated forms through hepatic metabolism (i.e. indoxyl sulfate [IS] and p-cresol sulfate [pCS]) [62]. These molecules have long been associated with disease and have more recently been demonstrated to exhibit a varied array of pathophysiological actions within the cardiovascular and renal systems. These actions include, but are not limited to, the promotion of cardiovascular disease risk factors including thrombotic [63, 64] and atherogenic [65] potential, mediation of blood pressure [66, 67], vascular calcification [68], cardiac fibrosis [69] and impairment of endothelial progenitor cell-mediated neovascularization [70]; as well the induction of renal fibrosis [71], inflammation [72, 73] and cellular senescence [74, 75]. Interestingly, IS has also been shown to induce endothelial cell-derived release of microvesicles that contain upregulated levels of miRNAs that are associated with the downstream promotion of inflammation, cellular senescence and apoptosis [76].

There has been an invested interest in the measurement of IS and pC in biosamples, with original analyses being pioneered with the application of high-performance liquid chromatography (HPLC) coupled to ultraviolet light or fluorescence detectors [77–80]. These methods predominantly assessed a single analyte or a pair of structurally related molecules (e.g. phenol and pC), with more modern applications of HPLC allowing simultaneous measurement of phenolic (phenol, pC and pCS) and indolic (IS and indole-3-acetic acid) compounds [81]. Similarly to the TMAO story, the desire to understand gut microbial metabolism in a broader scope has driven the translation of analysis to hyphenated

MS techniques to perform multiplexed approaches to UT measurements. Initial uptake of MS-based methods included the analysis of pC, pCS and p-cresol glucuronide by GC-MS [82], with measurements being more commonly performed using S/MRM assays employing LC-MS/MS. Similarly to HPLC-based assays, LC-MS/MS methods have been developed to specifically measure a single or pair of analytes [83–86] or provide a wider focus on gut-derived UTs [87], as well as more complex multiplexed methods to assess additional metabolites outside of the indole and phenol classes (e.g. uric acid, TMAO, hippuric acid, kynurenic acid) [88, 89]. These analyses primarily focus on the measurement of UTs within the systemic circulation (i.e. serum, plasma), owing to the reduced ability to filter out into the urine. This has been demonstrated by the observation that less than 35% of circulating IS/pCS reduction occurs during hemodialysis [90], and that kidney transplant patients exhibit reduced circulating levels of pCS and IS that are comparable to healthy individuals [91, 92]. However, additional MS-based methods have been developed that are capable of measuring IS and pCS in saliva [93], as well as the presence of the IS precursor indole in exhaled breath gases [94–96]. However to date, the links between less traditional sample mediums and blood concentrations have yet to be explored in diseased populations where circulating UTs are of particular interest.

Renal disease has understandably received the major interest for clinical measurement and monitoring of UTs. This is due to the presence of an increased UT blood content in line with a progressive decline in renal function, as indicated by step increases in circulating levels of IS and pCS across CKD stages 1 to 5 [86]. Aside from renal disease, increased levels of IS have also been associated with more severe diastolic cardiac dysfunction (measured by E/e'), although circulating levels did not correlate with measurements of systolic dysfunction (left ventricular ejection fraction) nor with the established heart disease biomarker B-type natriuretic peptide [97]. As prognostic markers of renal disease IS and pCS have been able to demonstrate risk predictive qualities for renal decline [98], cardiovascular events [98–104] and mortality [100–102, 105–107] in CKD and hemodialysis patients. However, conflicting results are present in some investigations that demonstrate only one of the metabolites to be prognostic, or for prediction across event type (e.g. hospitalization, death, etc.) to be variable. For example, in hemodialysis studies IS has been reported as an independent predictor of outcomes where pCS was not [107], and vice-versa where pCS was predictive in place of IS [102]. These trends were reinforced in a recent meta-analysis of 11 publications

investigating UTs as prognostic biomarkers in CKD. The authors observed that pCS was retained as an independent predictor of all-cause mortality and cardiovascular events, where IS was solely capable of independently predicting all-cause mortality [108]. These contrasting results provide a level of uncertainty for the true quality of clinical laboratory measurements of gut microbial-derived UTs, a point that was further reinforced by a recent large-scale study that showed that neither IS nor pCS were prognostic in hemodialysis patients [109]. Outside of renal disease, IS and pCS have been less well studied. However, initial trials have demonstrated potential utility as prognostic biomarkers in dilated cardiomyopathy [97] and heart failure [110], representing cardiovascular disease as an area that could benefit from further research into circulating UTs.

Altogether, in-depth multiplexed assays offer an excellent opportunity to more adequately understand the dynamics of UT production and build-up, with the capacity to measure multiple metabolites outweighing the downfalls where a single metabolite measurement has not shown to be reliable across patient cohorts. Current data showcase the beneficial prospects for implementation of UT measurements into routine clinical laboratory-based MS services for use in prognostic and therapeutic monitoring in conjunction with current biomarker measurements.

Short-chain fatty acids

Short-chain fatty acids (SCFAs) are volatile aliphatic carboxylic acids with a low carbon number (containing no more than five carbon atoms) and are common metabolic by-products of bacterial metabolism [111]. A list of SCFAs and their chemical information is provided in Table 2. Much like TMA, concentrated SCFAs present with an unpleasant odor and are commonly produced by bacteria on human body surfaces and thus are often described as redolent of ‘cheesy feet’ [112]. SCFAs produced by bacteria within the gastrointestinal system are metabolic by-products of non-digestible fiber fermentation, producing straight chain species (e.g. acetic, propionic, butyric acid), or protein fermentation creating branch-chained species including isobutyric, 2-methylbutyric and isovaleric acid specifically from the branch-chained amino acids valine, isoleucine and leucine [111]. Like other bacterially-produced metabolites, SCFAs can cross the gut epithelial barrier to enter the systemic circulation, albeit a large proportion remain within the gut

ecosystem and are excreted within the feces [113, 114]. As SCFAs are carboxylic acids they exist in solution as dissociated forms and are therefore regularly referred to by their carboxylate anion, with acetate (C2), propionate (C3) and butyrate (C4) the principal components of total SCFAs present [115] (see Table 2 for more information on these anions).

The measurement of SCFAs using MS has been of interest for multiple decades, with early reports of GC/GC-MS assays as far back as the 1970s [116, 117]. More recently, there has been a flourish of published protocols, owing predominantly to the increasing interest of the impact of SCFAs in physiology and, importantly, pathophysiology. The majority of methods utilize GC-MS analysis of SCFAs derivatized with a range of different adducts and are principally applied to fecal collections [118–120]. Clinically, the measurement of fecal content of SCFAs may provide information on current or dynamic status of gut microbial metabolism, but it is not possible to provide a more accurate insight into the absorption of SCFAs into the systemic circulation, and therefore a potential impact on host cellular/tissue activity. A small but increasing number of protocols are emerging in the literature which assess circulating SCFA content, with methods including the traditional derivatized GC-MS approach [120–122], but also with further application of LC-MS systems [123–125]. Whilst derivatization of acidic metabolites generally offers greatly improved sensitivity and chromatographic reproducibility, the ability to exclude derivatization steps is of benefit to the clinical laboratory as it decreases the handling of hazardous chemicals whilst simultaneously increasing throughput and capacity. To this end, researchers have successfully developed assays that require only basic sample preparation prior to analysis. This has been afforded by use of wax-based GC columns (suitable for retention of acidic compounds) [118] as well as post-LC neutralization with ammonia, causing ammonia-adduct SCFA ions to form which can be easily measured using ESI-MS [126]. Interestingly, non-invasive sampling of SCFA levels has been proposed through the absorption of SCFAs excreted onto the surface of the skin [127]. This approach applies a chemically absorbent patch onto the skin which is later measured by MS following thermal desorption of molecules off the patch and into the analytical system. While this could provide a method to non-invasively measure circulating SCFAs in a clinical context, there are currently no data available to demonstrate the comparability of these measurements to direct quantitation in more routinely collected clinical sample types (i.e. blood and urine).

Table 2: A list of short-chain fatty acids containing between one and five carbon atoms.

Carbon number	Nomenclature		Anion		Chemical structure
	Common	IUPAC	Common	IUPAC	
1	Formic acid	Methanoic acid	Formate	Methanoate
2	Acetic acid	Ethanoic acid	Acetate	Ethanoate
3	Propionic acid	Propanoic acid	Propionate	Propanoate
4	Butyric acid	Butanoic acid	Butyrate	Butanoate
4	Isobutyric acid	2-Methylpropanoic acid	Isobutyrate	2-Methylpropanoate
5	Valeric acid	Pentanoic acid	Valerate	Pentanoate
5	Isovaleric acid	3-Methylbutanoic acid	Isovalerate	3-Methylbutanoate
5	2-Methylbutyric acid	2-Methylbutanoic acid	2-Methylbutyrate	2-Methylbutanoate

IUPAC, International Union of Pure and Applied Chemistry: a standardized system for the naming of chemical compounds; Anion, the conjugate base of the acid that is present when in solution (i.e. in a biofluid).

Contrary to the growing evidence of the negative impact of gut microbial metabolites on health and disease, there is an overwhelming indication that SCFAs

offer protective effects. These beneficial aspects of gut metabolism have been repeatedly shown for *in vitro* and *in vivo* investigations, however, the overall impact of

changes in SCFA levels in human participants are not currently well known. A strong focus of the research to date has been performed in models of renal disease, notably with the development and progression of CKD. Multiple studies have shown an interaction of SCFAs with G-protein coupled receptors on the cell surface that have shown beneficial modulations on blood pressure, inflammation, induced-tissue damage and overall immune response [114, 128–131]. In particular for CKD, butyrate supplementation has demonstrated a blunting of fibrotic activity through the amelioration of TGF- β 1 production in renal epithelial cells [132, 133] and via moderation of pro-inflammatory and pro-oxidative stress pathways [133, 134]. In addition to butyrate, protective effects of SCFA supplementation have been demonstrated for acetate and propionate, with suppression of TNF- α stimulated MCP-1 expression [135] and tempering of histone deacetylase activity in T cells following sepsis-induced kidney injury [136]. An *in vivo* model of ischemia-reperfusion of the kidneys demonstrated that supplementation of SCFAs could reduce indices of renal injury, helping to maintain renal function at a level that was comparable to pre-ischemia and reduce necrotic tissue formation by approximately 50% [129]. Fascinatingly, these positive outcomes were also achieved by supplementing mice with bacteria known to be acetate-producers, demonstrating a probiotic-like pre-treatment strategy that could reduce complications where ischemic kidney injury is common (e.g. cardiac surgery). Outside of renal disease, further beneficial effects of SCFA supplementation has been demonstrated for cardiovascular risk factors such as blood glucose homeostasis [137], reductions in atherogenic potential [138, 139], and amelioration of cardiac fibrosis and left ventricular hypertrophy in a murine model of hypertension [140]. Contrastingly, Tirosh et al. [141] demonstrated that supplementation of mice with propionate initiated an increased production of glucagon, fatty acid-binding protein 4 and norepinephrine which led to increased insulin resistance and compensatory hyperinsulinemia. This was mirrored, to some extent, in human participants who showed a reduction in circulating propionate levels that correlated with improved insulin sensitivity and overall weight loss. Whilst some investigations have started to look at the associations of circulating SCFAs with indicators of disease, few studies have looked at the use of SCFA profiling for application as diagnostic or prognostic biomarkers. One study reported that elevated plasma levels of valerate and propionate were independent predictors of coronary artery disease (CAD) in CKD patients, however, the absolute quantitative differences between those with and without CAD for these SCFAs were only around +1 $\mu\text{mol/L}$ with standard

deviations for both $>1 \mu\text{mol/L}$ [142]. Whilst statistically these data may have shown differences, in a practical sense this separation of values between positive and negative CAD presence is far too narrow to be reliable as a clinical test. A note of interest in this study which was not emphasized by the authors was that decreased acetate levels (approximately $-10 \mu\text{mol/L}$ but again with large standard deviations) showed an increased likelihood of self-reported CAD, stroke, peripheral arterial disease, congestive heart failure, or arrhythmia. Although these data are too premature to assign confidence, these results serve as preliminary evidence that increased acetate levels may be associated with reduced disease risk. For the use of SCFAs in prediction of outcomes in hospitalized patients, Weng et al. [143] observed that propionate levels were increased in sepsis patients and further elevated in patients with septic shock. Furthermore, elevated propionate was an independent predictor of mortality both in-hospital (during ICU stay) and at 28- and 90-days post-diagnosis in these patients.

Unlike TMAO and uremic toxins, accumulating evidence shows that SCFAs are beneficial in protecting from disease progression and could therefore provide a targeted therapeutic approach to improve patient outcomes. In the small number of studies performed to date on clinical samples, there is contrasting evidence that shows both increased and decreased circulating SCFAs levels could be predictive of negative outcomes and therefore it is important that further research is done in this area to include investigations to look across a range of diseases. It is apparent that the use of circulating SCFA measurements are well away from clinical translation at this stage, but accelerated research in this area could provide an interesting avenue for future clinical biomarker applications.

Outlook

MS offers numerous opportunities to improve and expand current clinical biomarker measurements, with the measurement of gut bacteria-mediated metabolites an excellent example of this future potential. The metabolites discussed in this review are by no means an exhaustive list, but act to highlight a growing interest in gut microbiome metabolites as potential clinical targets to provide an excellent resource to improve patient care, diagnosis, prognosis and therapeutic interventions/monitoring. The translation and uptake of these measurements would be facilitated through the acceleration of MS instrumentation into routine clinical laboratories, alongside the continued specialist training of laboratory scientists within this field. This focus,

coupled with a continued effort in basic research of novel and potentially informative biomarkers, will allow the enhanced understanding of gut microbial metabolism to benefit patient care in the era of personalized medicine.

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