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Metabolomic Analysis in Inflammatory Bowel Disease: A Systematic Review

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Abstract

Background and aims: The inflammatory bowel diseases (IBD), Crohn's disease and ulcerative colitis, are chronic, idiopathic gastrointestinal (GI) diseases. Whilst their precise etiology is unknown, it is thought to involve a complex interaction between genetic predisposition and an abnormal host immune response to environmental exposures, likely microbial. Microbial dysbiosis has frequently been documented in IBD. Metabolomics (the study of small molecular intermediates and end products of metabolism in biological samples) provides a unique opportunity to characterize disease-associated metabolic changes and may be of particular use in quantifying gut microbial metabolism. Numerous metabolomic studies have been undertaken in inflammatory bowel disease populations, identifying consistent alterations in a range of molecules across several biological matrices. This systematic review aims to summarize these findings.

Methods: A comprehensive, systematic search was carried out using Medline and EMBASE. All studies were reviewed by two authors independently using predefined exclusion criteria. A total of sixty-four relevant papers were quality assessed and included in the review.

Results: Consistent metabolic perturbations were identified, including increases in levels of branched chain amino acids and lipid classes across stool, serum, plasma and tissue biopsy samples, and reduced levels of microbially modified metabolites in both urine (such as hippurate) and stool (such as secondary bile acids).

Conclusions: This review provides a summary of metabolomic research in IBD to date, highlighting underlying themes of perturbed gut microbial metabolism and mammalian-microbial co-metabolism associated with disease status.

Key words: Inflammatory Bowel Disease, Metabolomics, Microbiome

Introduction

Metabolomics and inflammatory bowel disease

The inflammatory bowel diseases (IBD), ulcerative colitis (UC) and Crohn's disease (CD), are chronic idiopathic conditions characterized by gastrointestinal inflammation[1]. While the pathogenesis of the conditions remains uncertain, it is widely believed to involve a complex interaction between genetic predisposition, and an abnormal host immune response to environmental exposures, likely microbial[2,3]. IBD is characterized by dysregulation of the gut immune system and by unfavorable shifts in the gut microbiome, termed dysbiosis, including a decrease in diversity of the microbiome[3]. Diagnosis typically relies on clinical, endoscopic, histological and radiological findings[1].

'Metabolomics' (a term now used interchangeably with 'metabonomics' or 'metabolic profiling') refers to the analysis of patterns of small molecular metabolites in biological samples[4,5]. This approach allows the generation of a 'metabolic barcode' of the current metabolic status of an individual. Such analysis is undertaken via the use of analytical chemistry techniques such as proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy or mass spectrometry (MS)[6] to qualitatively and quantitatively characterize the metabolite complement of the tissue, biofluid or cell extract. Due to the complexity of the high throughput data produced by such techniques, multivariate pattern-recognition data processing methods are frequently used for data interpretation[7]. These approaches allow the elucidation of compositional trends and variation between samples in relation to a pathological or physiological endpoint, which can be probed further through metabolite annotation and pathway analysis.

Metabolomic techniques have been widely used to study IBD, providing insights into disease pathogenesis, and with potential applications to disease subtyping and therapeutic response assessment. Many different biosamples including urine, stool, blood, breath and tissue biopsies have been studied, in differing clinical contexts.

We present a comprehensive systematic review of metabolomics in IBD.

Metabolomic methodologies and analysis

Metabolomic techniques allow the identification and quantification of hundreds (or potentially thousands across assays) of small molecules[6]. Changes in concentrations of these metabolites, their intermediates and byproducts reflect host metabolism at the time of sampling, or in the case of urine and stool, offer a time-averaged window of the preceding few hours.

Depending on the analytical platform and biosample analyzed, different metabolites can be detected at varying concentrations. Each biosample provides different biochemical information. For example, blood components may provide a snapshot of systemic metabolism, while urine provides an endpoint profile of exogenous (e.g. drugs and dietary compounds) and microbial or host-microbial co-metabolites, as well as endogenous metabolism; stool profiles are more indicative of digestive metabolism, including microbial transformation of dietary components.

The sample type used may also influence the technique chosen to carry out the analysis. The most popular platforms for metabolomic analysis include liquid chromatography (LC)-MS, gas-chromatography (GC)-MS, and ^{1}H-NMR spectroscopy[6]. Both types of MS allow the identification of thousands of biological features that can be quantified using targeted

analysis once identified. Untargeted analysis provides an opportunity to broadly assess metabolic changes and identify potential biomarkers in health and disease. Many protocols are already established for rapid analysis of biosamples. However, the data analysis stage can be complex, involving lengthy annotation and identification of thousands of features. In untargeted datasets a relatively small numbers of metabolites are fully identified, leaving larger numbers unannotated, often referred to as the 'dark matter' of metabolomics[8]. In contrast, targeted assays focus on preselected compounds, reducing time spent in the analysis stage and improving accuracy due to the use of more sensitive equipment. Additionally, accurate concentrations of compounds can be obtained, as opposed to relative abundances or simply fold changes that are often reported in untargeted analysis. Disadvantages, however, include the significant time it takes to develop and validate a targeted method, and requirement for a predefined biological hypothesis[9].

With regards to their application, GC-MS is frequently used for the analysis of short chain fatty acids (SCFA) and other volatile compounds, whereas LC-MS captures a wide array of molecules including lipids and a range of polar molecules[10]. For MS (particularly GC-MS) extensive sample preparation and extraction is often required prior to analysis. MS may offer greater sensitivity than NMR spectroscopy, but the relatively simpler sample preparation, speed and reproducibility make NMR a popular platform for high throughput analysis[11].

After the preprocessing of metabolomic data, both traditional univariate and multivariate statistical approaches may be employed for interpretation of the data. Popular multivariate pattern recognition approaches include principal component analysis (PCA) and partial least

squares discriminant analysis (PLS-DA)[7]. PCA is an unsupervised approach that reduces the high dimensionality of the data by sequentially describing the largest sources of variation in the dataset[7,12]. Multivariate analysis, often in combination with graphical visualization tools, allow for the initial identification of patterns in the data relating to outliers, disease/controls or sub-phenotypes and facilitates identification of metabolic features responsible for differences between groups[12]. Unsupervised analyses such as PCA, whereby information relating to the sample class is not used to model the data, are commonly followed by supervised approaches such as PLS-DA or related techniques. Supervised approaches such as PLS-DA relate class membership (e.g. disease versus control) to metabolic features; the models generated undergo several stages of cross validation and permutation testing to assess the quality and significance of the model produced. Multivariate analysis often precedes metabolite annotation and identification, allowing the recognition of features or biomarkers related to the biological question or outcome of interest. A wide range of alternative multivariate methods, including machine learning methods and Bayesian approaches, have also been used extensively and the approach taken to processing metabolic data is dictated by the study design and data collection techniques used[13].

The microbiota and the metabolome

'The microbiota' refers collectively to the bacterial, viral, fungal and archaeal species found on and within the human body in a number of distinct environmental niches, such as the gastrointestinal (GI) tract. Techniques such as 16S rRNA analysis, and shotgun metagenomics, a more expensive but higher resolution technique allowing for strain level identification as opposed to genus level, have detected of over 1000 species in the human GI tract alone[14]. The microbiota has been shown to function symbiotically with human

metabolism and its dysregulation has been implicated in several metabolic conditions such as obesity, diabetes and cancer[15]. The gut microbiome has been thought to play a role in IBD pathogenesis for many years through the presentation of gut microbial antigens or loss of tolerance to commensal species, potentially triggering an inappropriate response, contributing to the development of IBD in genetically predisposed individuals. Overall, studies characterizing the microbiome in IBD have revealed an unfavorable shift in the microbiota often referred to as 'dysbiosis' which is reflected in reduced alpha diversity (species richness). This taxonomic shift associated with IBD corresponds to a lower proportion of the Firmicutes phyla and larger number of Proteobacteria, a phylum associated with gastrointestinal inflammation, containing relatively more pathogenic species than other phyla found in the human microbiota[3,16]. The role of Proteobacterium *Escherichia coli* has been extensively investigated in IBD, as adherent strains with cell damaging properties have been identified in both UC and CD[17]. Within the Firmicutes phylum, *Faecalibacterium prausnitzii* and *Roseburia homonis*, have frequently been found to decrease in IBD - these are both butyrate producing bacteria[3,18–22]. It is postulated that this may contribute to inflammation and loss of barrier integrity, as butyrate serves as an energy source for colonocytes and can inhibit inflammation[23,24]: butyrate is typically found at lower levels in IBD metabolomic studies, compared to controls. Also, in the Firmicutes phyla, *Ruminococcus gnavus* has been observed as enriched in IBD (specifically CD[22,25,26]) - it is known to have mucin degrading capacities which may contribute to loss of barrier function and inflammation in IBD[25]. Paneth cells, key modulators of intestinal homeostasis, actively regulate the gut microbiome and influence immune responses via the release of antimicrobial peptides, such as lysozyme and α-defensins, through degranulation and

autophagy. There is evidence that, in IBD, Paneth cell dysfunction may contribute to unfavorable shifts in the gut microbiome and ineffective regulation of the microbiome, particularly in CD[27,28].

It is known that biofluid and tissue metabolomes are strongly influenced by the gut microbiota due to their critical role in the production of microbial metabolites, and mammalian-microbial 'co-metabolites'. This is summarized in figure 1. However, the expression of the effect of the microbiome on the metabolome differs between biosamples. Examples of these variations include those in SCFA and bile acids, which are commonly analyzed in both stool and blood samples. SCFA are a product of the bacterial fermentation of dietary fiber, whereas bile acid pools are directly influenced by bacteria, which carry out deconjugation, dehydroxylation and reconjugation transformations. Multiple bacteria (such as *Enterococci, Clostridia, Bacteroides* and *Lactobacillaceae*) demonstrate bile salt hydrolase activity, the enzyme responsible for deconjugation of glycine and taurine from bile acids [29]. Therefore, changes in concentration of these metabolites in stool and blood can be attributed to gut microbes both directly and indirectly. The influence of microbial metabolism is also reflected in other common biosamples used in metabolomic studies, such as urine. Urine contains endogenous host metabolites, exogenous products of diet and drug intake and metabolites of microbial origin, as well as host-microbial 'co-metabolites' such as uremic toxin 4-cresyl sulfate, derived from the bacterial conversion of tyrosine to 4-cresol, which subsequently undergoes phase 2 conjugation in the liver thereby involving both bacterial and host metabolism[30]. The intestinal tissue metabolome also relates to the gut microbiome: SCFA, for example, such as butyrate, are utilized by colonocytes. Targeted intestinal biopsy analysis may allow investigation of the spatial functionality of the intestine,

which is heavily influenced by bacterial communities present. The role of the oral microbiome has recently been investigated in IBD: changes in salivary *Streptococcus* and *Prevotella* species contributed to oral dysbiosis in IBD[31–33]. These studies showed significant correlations between oral dysbiosis and several metabolic pathways and inflammatory markers, such as fecal calprotectin and interleukins[31,32].

Metabolomic techniques have been widely used in the investigation of altered metabolism associated with health and disease: conditions such as type I and type II diabetes, liver disease, neurodegenerative diseases, cardiovascular disease, and many cancers have been recently investigated[34–37]. Examples of significantly discriminatory metabolites include panels of long chain fatty acids in colorectal cancer, bile acids in hepatic diseases, and tryptophan metabolites in neurodegenerative diseases[38,39]. Other major influences on physiology (and host-microbiome metabolism), such as ageing and obesity have been studied, as has the influence of bariatric surgery on the microbiome and hence systemic health, notably in the transformation of dietary components, such as choline, phenols, and various amino acids[40,41]. Shifts in the gut microbiome associated with ageing include reductions in both diversity and commensal species. Associated changes in functionality, such as lower levels of SCFA, may influence overall intestinal health[42]. With regards to obesity, changes in the abundance of Firmicutes and Bacteroidetes have been identified, associated with enrichment in carbohydrate fermentation and accompanying increases in fecal SCFA[43,44]. Following, bariatric surgery, long term shifts in the microbiome and its functionality have been observed, including decreases in Firmicutes, decreases in fecal and blood SCFA concentrations and alterations in bile acids[45–47]. Metabolomics, therefore, provides insights into the close relationship between health and host-microbiome

metabolism.

This systematic review summarizes metabolomic investigations in IBD, highlighting the functional metabolic importance of the gut microbiota. The integration of microbiome sequencing and metabolomic data will be discussed, focusing on contributions to our current understanding of disease pathogenesis, development and treatment.

Methods

A comprehensive literature search was carried out in January 2020 using databases Medline and Embase which were searched in their entirety. Studies investigating the human metabolome in inflammatory bowel disease were identified using specific search terms (see supplementary information S1): a total of 1532 articles were identified, before excluding duplicates (n=228). For the exclusion process, title and abstracts of 1304 studies were screened. A full text review was carried out on the remaining papers (n=130), and a further 66 studies were excluded, reasons for which include 'wrong outcomes', such as the analysis of glycans or the proteome, 'wrong patient population', where the study did not investigate IBD patients (for example irritable bowel syndrome, diabetes or coeliac disease), and 'wrong study design', where techniques such as PCR were used in place of metabolomics. The exclusion criteria used for both screening stages are documented in the supplementary information (supplementary information S2). As a result, 64 full text articles were included in the main body of the review. The PRISMA flow diagram[48] associated with this search can be seen in figure 2. The retained studies with details regarding study design are provided in table 1. All retained studies were additionally quality assessed using the QUADOMICS tool[49], adapted from the QUADAS tool[50], specifically for '-omics' studies: a table summarizing these

results can be found in table 2. Throughout this review, only metabolomic studies involving adult human participants were included. Two authors (KG and AC) undertook the literature review.

Results and Discussion

The metabolome in IBD

All studies identified for this review of the IBD metabolome are summarized in table 1. For the purposes of this review, studies have been stratified according to the biosample investigated (urine, stool, blood plasma/serum, tissue and breath).

Urinary metabolomic studies

Urine has been extensively studied in metabolomic experiments due to ease of availability and collection, and the breadth of metabolic information that it provides. The urinary metabolome contains in the region of 3000 metabolites including endogenous metabolites (such as Tricarboxylic acid (TCA) constituents and amino acids) reflecting energy metabolism and homeostasis, exogenous metabolites (such as those from diet and drugs), and metabolites from microbial metabolism. Mammalian-microbial co-metabolites, such as hippurate, trimethylamine-*N*-oxide and 4-cresyl sulfate provide insights into the systemic effects of microbial metabolism[113].

In IBD research, studies have approached the analysis of urine via multiple analytical techniques, finding significant differences in metabolic profiles of CD and UC patients when compared to control groups. Where NMR spectroscopy was used, the host-microbial co-metabolite hippurate has been consistently found at significantly lower concentrations in IBD relative to controls[52,62,95,101,107,108]. Hippurate is mainly derived from microbial

conversion of dietary phenols to benzoate, followed by phase 2 glycine conjugation in the liver and is thus a product of both microbial and mammalian metabolism[114]. It has been positively correlated to microbiome diversity, suggesting a link between the metabolite and gut health[115], and inversely associated with BMI[116] and blood pressure[117]. In addition to its role as host-microbial co-metabolite, changes in hippurate may also reflect perturbations in endogenous mitochondrial metabolism, where glycine conjugation also occurs via hepatic and renal mitochondria[114]. To further investigate changes in hippurate in IBD, Williams *et al,* (2010)[108] administered sodium benzoate to CD patients and healthy controls, with known benzoate-containing foods excluded from participant diets, demonstrating that CD patients had no underlying deficiency in benzoate conjugation, thus strongly supporting dysbiosis as the cause of decreased urinary hippurate in IBD. Similarly to hippurate, formate has been found at lower concentrations in IBD cohorts than in controls[62,95,107]. Formate is the oxidation product of formaldehyde which is mainly derived from microbial production of methanol. It not only plays a key role in one carbon metabolism, but may also influence microbial communities present, as it is utilized by bacteria with aerobic metabolic capacities[118].

Metabolites including trigonelline and SCFA are also frequently identified as significantly altered in IBD when compared to control samples. Trigonelline is an important component of niacin metabolism, necessary for numerous physiological functions and is biosynthesized by gut bacteria[119]. It has consistently been observed at lower levels in IBD patients compared to controls[52,62,95,101]; the SCFA acetate[53,101], 2-hydroxyisobutyrate[62] and butyrate[53] also seem to be lower in IBD patients than controls, further implicating the microbiome in altered IBD metabolism.

Further consistent findings across studies include lower urinary excretion of the TCA cycle components, citrate and succinate, in IBD[52,62,95,101] and overall decreases in amino acid profiles in IBD compared to controls, most notably alanine[52,62], asparagine[91,101], glycine[91,101], and taurine[62,95,101]. Covariation in excreted levels of hippurate and TCA cycle intermediates has been identified in several metabolomic studies and as initial benzoate metabolism takes place in the mitochondrial matrix, requiring ATP, impaired mitochondrial function has been posited as an explanation for this covariation[114]. Lower concentrations of these metabolically interconnected biochemical classes may reflect changes in energy metabolism and amino acid demands in response to inflammation (due to the role of amino acids as anaplerotic TCA cycle intermediates), or decreased uptake of these metabolites as a direct result of inflammation[120,121]. These alterations may also be the result of changes in the IBD-associated microbiome, for example succinate can be produced by the microbiota, or can undergo metabolism to propionate by Bacteroidetes or Firmicutes[122].

Urinary metabolomics has also been evaluated in regard to the assessment of patient treatment outcomes and clinical management. In patients who had undergone surgical resection, Keshteli *et al* (2018) reported that increases in urinary levoglucosan, a breakdown product of dietary carbohydrates, were related to postoperative recurrence of CD up to 12 months post-procedure[79]. This study also investigated the microbial profiles of CD patients, reporting a distinct gut microbial profile associated with post-surgical recurrence including enrichment in Fusobacteria and Proteobacteria, and a reduction in Bacteroidetes. In particular, levoglucosan was correlated positively with Gammaproteobacteria (a class containing several pathogenic bacteria), and negatively with Bacteroidales. The study proposed that diet and microbiome composition are implicated in the risk of surgical

recurrence. As diet is a major determinant microbiome composition[123] further research will be required to confirm the causes of these taxonomic changes and their correlation with metabolomic findings.

Stool metabolomics studies

Stool has long been a favored substrate for metabolomic studies in IBD. This is due to the range of bioactive metabolites found in fecal samples, such as bile acids and SCFA, as well as its utility in the investigation of gut microbiome functionality.

Bile acids are an important class of bioactive molecules that are subject to microbial modification through bacterial enzyme activity, such as bile salt hydrolases, which transform primary bile acids into secondary bile acids through deconjugation[124]. Two recent studies, carried out by Weng *et al* (2019) and Franzosa *et al* (2019), identified lower concentrations of primary and secondary bile acids, and their glycine and taurine conjugates, in IBD patients compared to controls[22,105]. In another study, focusing on CD in twins, glycolate, taurocholate and chenodeoxycholate were significantly enriched in ileal and colonic CD compared to control samples[77]. Lloyd-Price *et al* (2019) compared dysbiotic samples to non-dysbiotic samples for each IBD subtype[26]. Primary bile acids, glycholate and taurocholate, were noted to be significantly enriched in dysbiotic CD compared to non-dysbiotic samples, while lithocholate and deoxycholate were significantly reduced - changes that may be ascribed to differences in microbial composition, which included changes in bacteria known to modify bile acids[26]. Murakami *et al* (2018) also highlighted the link between the microbiome and bile acids, identifying a strong correlation between the ratio of microbially modified deoxycholic acid to the sum of deoxycholic and cholic acid with increased proportions of

Clostridium sub-clusters in both serum and fecal samples in a cohort including IBD patients[87]. *Clostridium* spp. are known to have the enzymatic capacities required for bile acid modification[125]. Further analysis found that the serum ratio of these bile acids was useful for discriminating between disease remission states in both UC and CD (decreased) compared to controls. Bile acid pools are not only important to host health but also have considerable influence on the composition of the gut microbiome, as bile acids display varying antimicrobial properties, which may contribute to dysbiosis[125]. Altered bile acid profiles may also play a role in the pathogenesis of IBD and associated conditions[29], given their role as signaling molecules and in maintaining intestinal health[124] through the farnesoid X receptor (FXR). FXR is a nuclear receptor that plays a key role in bile acid homeostasis, through controlling hepatic reabsorption and both intestinal absorption and secretion[126]. In addition to this, FXR has several roles that may contribute to IBD pathogenesis, including maintaining mucosal barrier integrity, thus preventing bacterial translocation, and in immune modulation and inflammatory responses within the intestine through interactions with macrophages[126].

Changes in stool SCFA composition have also been documented in a number of studies. Acetate[20,85], propionate[58,83] and butyrate[58,64,85] have been identified at lower concentrations in IBD patients compared to controls. SCFA are a well-studied category of metabolites in the context of the microbiome as they are the saccharolytic byproducts of gut microbial fermentation[127]. These metabolites are then utilized by host cells. For example, butyrate is the main source of energy for colonocytes, propionate is used in hepatic metabolism and acetate is used as a primary substrate for cholesterol synthesis[127]. In addition to its role as an energy source, butyrate is also thought to be protective against colorectal cancer (CRC),

which IBD patients are at higher risk of developing[128], via several epigenetic mechanisms such as inhibiting CRC cell proliferation through decreasing Neuropilin-1; an overexpressed receptor in CRC[129].

In a recent study, when comparing dysbiotic IBD samples to non-dysbiotic samples, lower butyrate and propionate levels were specific to dysbiotic CD, a finding that complemented microbial analysis which showed known butyrate producers, F. *prausnitzii* and R. *homonis,* to be depleted in CD[26]. Machiels *et al* (2014) identified a depletion of these same species, with corresponding lower concentrations of propionate and acetate, but not butyrate[20]. This discrepancy may relate to differences in cohort demographics.

In addition to characterizing IBD, De Preter *et al* (2013), through the use of novel prebiotics, sought to encourage normalization of microbial metabolic profiles in CD patients by targeting the microbes producing such metabolites[63]. The study found that compared to baseline samples, CD patients taking Oligofructose Enriched Inulin had increased levels of multiple stool volatile compounds including butyrate. Changes in the microbiome were also favorable, with decreases in *R. gnavus* and an increase in *Bifidobacterium longum,* which was specifically associated with an improvement in disease activity. Higher levels of *R. gnavus* have been implicated as a key contributor to dysbiosis in IBD[26].

Recent stool lipid profiling has identified several classes of lipids present at higher concentrations in IBD patients than controls. Lloyd-Price *et al* (2019) characterized several lipid species associated with IBD. In particular, acylcarnitines were highly correlated with dysbiosis-related communities such as *R. homonis*[26]. Several other recent studies also implicate carnitines in IBD[22,105]. Acylcarnitines are fatty acyl esters of L-carnitine, a

mammalian metabolite known to be utilized by gut bacteria, therefore may be influenced microbial composition[130]. Circulating levels of acylcarnitines, as a result of incomplete oxidation of fatty acids, have been previously associated with type 2 diabetes and insulin resistance, and have the potential to activate inflammation[131]. Other lipid classes, such as energy storage- related long chain triacyl glycerides, and lysophosphocholines, with well-studied roles in immune cell function, were also found in higher concentrations in IBD groups compared to controls[22,26]. Lloyd-Price *et al* (2019) also reported higher concentrations of arachidonate (a precursor to immune and inflammatory-related molecules such as eicosanoids and prostaglandins[132]) specifically in dysbiotic IBD, corroborating the findings of earlier studies[22,77,86].

Stool samples of IBD patients consistently contain higher levels of amino acids (AA) compared to controls, notably the branched chain amino acids (BCAA)[26,58,85], taurine[22,26,85], glycine[58], lysine and alanine[58,85]. Higher fecal phenylalanine and tyrosine have also been shown to be particularly discriminatory between IBD and control groups[58,77,105]. Higher fecal AA concentrations may be due to malabsorption[85], a result of compromised intestinal stability and persistent inflammation of the gastrointestinal tract. However, several bacterial species are known to utilize AA for bioconversion to other biologically active compounds and changing AA levels may also relate to the composition of the gut microbiome and the dysbiosis seen in IBD[133,134].

Serum and plasma metabolomic studies

Serum and plasma are the most frequently studied biofluids in metabolomic analysis as they require minimal sample preparation, have well-characterized biological profiles, and reflect

systemic metabolism[135].

Changes In BCAA, isoleucine, leucine and valine have been consistently observed. In both CD[62,72,95] and UC[62,92,95,102] isoleucine was elevated when compared to controls; valine levels appear to be lower in CD [59,72,88,95,96] and UC[59,92,95] patients (although one recent study found higher levels of valine in UC patients compared to controls[102]), as was leucine[66,95,96]. Whilst recent research has shown changes in BCAA relating to insulin resistance, their role in IBD is less well understood. Given their involvement in protein synthesis and energy production[136], it has been suggested that these changes relate to the chronic immune response seen in IBD, although dietary influences must also be considered.

3-hydroxybutyrate, a downstream product of BCAA degradation, has consistently been reported in higher concentrations in UC patients than controls[62,78,96,109,112], with only one paper reporting increases in CD patients[96]. There is some evidence that it may serve as a marker of disease activity: in a small cohort (13 UC patients in remission and 7 UC relapsed patients), Keshteli *et al* (2017) observed increases in serum ketones 3-hydroxybutyrate, acetone and acetoacetate in relapsed patients compared to those in remission[78]. Increases in ketone bodies are often the result of glucose depletion and have been strongly associated with diabetes. However, elevated ketones have also been seen in several studies to contribute to cellular injury through oxidative stress and can contribute to complications in other pathologies[137].

Other AAs such as glutamine have consistently been observed at lower concentrations in both CD and UC patients compared to controls[59,80,90,95,96], and notably lower in CD than UC patients[90,96]. Glutamine has well established roles in intestinal health and integrity through

tight junction protein regulation, thereby decreasing bacterial translocation which can contribute to immune activation in the GI tract[138]. Decreased plasma glutamine has also been previously associated with increased immune activation[138]. Likewise, histidine has been frequently reported at lower concentrations in IBD[59,62,80,90,92] and was identified as a prognostic marker for relapse in UC in six month[92] and one year[75] follow up studies. Histidine is known to be involved in the mediation of oxidative stress, potentially influencing intestinal inflammation[120].

The AA tryptophan and its metabolites have been identified as significantly altered in the blood of IBD patients compared to controls. Whiley *et al* (2019) developed a method for the targeted analysis of tryptophan metabolites, validated in a cohort of UC patients[106]. This study observed a higher concentration of kynurenine and a lower concentration of xanthurenic and picolinic acid in the plasma of UC patients compared to healthy controls[106]. These findings are similar to previous studies investigating tryptophan and its metabolites, as tryptophan has been detected at lower concentrations in IBD blood samples in several studies[70,80,89,90,105]. Metabolites that make up the kynurenine pathway, the major route of tryptophan degradation in mammals, were also altered in IBD compared to control groups[105]. Kynurenine, the first metabolite in this pathway, was observed in higher levels in IBD groups compared to controls[73,80,106] - the ratio of kynurenine to tryptophan was also increased[89,99]. Other metabolites reported include quinolinic acid, observed in higher concentrations in UC patients compared to controls[111], and picolinic and xanthurenic acid, reported to be lower in IBD[89,106]. Moreover, tryptophan also proved to be a potential indicator of response to the commonly used biologic treatment Infliximab in CD patients[89]. It has been postulated that the tryptophan pathway may play a role in IBD pathogenesis,

potentially through the rate limiting enzyme involved in its catabolism (Indoleamine 2, 3-dioxygenase 1) that is directly involved in immune responses[89].

The gut microbiota can also directly influence host tryptophan availability through the metabolism of tryptophan into molecules such as indoles, for example indole acetic acid[96,102] and indole propionic acid[81], which were both significantly altered in IBD groups. Several species have the enzymatic capacity to do this, including *Lactobacillus* sp, *Clostridium* sp, and *Bacteroides* sp, all of which have been seen to decrease in IBD[3,139]. It is thought that indoles can contribute to intestinal immune system regulation through the induction of IL22, which Nikolaus *et al* (2017) found to be concomitantly lower with tryptophan levels in IBD patients[89].

The availability of tryptophan and its metabolites are also thought to contribute to the gut-brain-axis, the relationship between the gut microbiome and neurological health[140]. Changes in tryptophan availability may affect the production of neuroactive metabolites serotonin and melatonin from tryptophan, as well as kynurenine and indole derivatives which are also thought to contribute to brain health[140,141]. Further investigation into the possible role of tryptophan and its catabolites in IBD is warranted, given its links to both host physiology and microbial metabolism.

Changes in primary[81,96] and microbially-modified secondary bile acids[87,96,111], have also been documented: Roda *et al* (2019) observed that secondary bile acids significantly increased following anti-TNFα treatment in a prospective cohort of CD patients[94], a finding that compliments previous microbiota focused studies finding shifts in the gut microbiome of anti-TNFα treated patients, towards that of healthy controls[142].

With regards to the role of lipids in IBD, arachidonic acid and arachidonate, precursors to several immune mediators, are found in lower concentrations in the blood of CD patients[81,84,96] and are increased in stool samples[22,26,77]. Levels of urinary leukotriene E4, a proinflammatory derivative of arachidonic acid were also higher in IBD patients compared to controls[100]. Arachidonic acid is a precursor to eicosanoids that have well established roles in largely pro-inflammatory pathways[143]. Long chain carnitines also appear to be lower in the CD metabolome relative to control groups[56,81,96] and have been proposed to contribute to IBD through increased eicosanoid production[144]. Significant changes in other lipid classes such as glycerol-based and lysophospholipids[81,103] and triglycerides[88,103] have also been identified. Several studies have suggested that changes in lipids, particularly phospholipids may be the result of damage to the intestinal mucosal membranes as a result of prolonged inflammation[88,103]. This is supported by other studies identifying changes in the lipid profiles of tissue biopsy samples from IBD patients[55,65].

The complexity of lipid species and their roles make lipid profiling data challenging to interpret, relative to other metabolite classes. Nonetheless, the metabolic information available through 'lipidomics' may offer valuable biological insights into IBD.

Tissue metabolomic studies

Biopsy samples are routinely taken for both diagnosis and monitoring of IBD, as histological analysis is required for confirmation of disease type and activity. The metabolic profile of tissue reflects, *inter alia,* lipid classes, amino acids and TCA cycle components[145].

Studies of colonic biopsies have frequently shown lower choline[54,57,97] and glycerophosphocholine[54,57,93,97] levels in IBD when compared to controls. Levels of choline

(as well as carnitine, also found to be lower in tissue samples of UC patients compared to controls[66]) can be influenced by the gut microbiome as both metabolites are converted to trimethylamine by gut bacteria with choline TMA-lyase, and carnitine oxygenase enzymes[146]. These enzymes have been associated with *Clostridium XIVa* and *E. coli* respectively, bacteria which both contribute to IBD associated dysbiosis[3,22,146]. TMA is subsequently metabolized to trimethylamine-*N*-oxide (TMAO) through hepatic conjugation[147]. TMAO has also been found in lower concentrations in the plasma of IBD patients compared to controls[110]. Shifts in gut microbial communities may contribute to changes in concentrations of circulating choline metabolites including TMAO, both of which have been associated with impaired cardiovascular and renal health when increased[148].

Myoinositol has consistently been identified at lower levels in IBD samples compared to controls[54,57,93,97], and is also decreased in active versus inactive UC[93]. Myoinositol is an important osmolyte, inhibiting intestinal glucose reabsorption, and when phosphorylated is associated with cell growth and signaling. Experimental models have demonstrated its potential use in the treatment of several diseases, including cancer[149].

Differences in the amino acid profiles of colonic biopsy samples in IBD compared to controls have also been frequently reported[54,57,66,90,93,97], including alanine and aspartate which are closely linked to central energy metabolism through liver amino transferases[150]. Changes in these amino acids, and other TCA cycle components, such as succinate, citrate, fumarate and malate may reflect alterations in cellular metabolism[54,66,90]. Tissue BCAA are also present in lower concentrations in IBD groups compared to controls[54,66,90,93], which may be related to changes in rate of tissue repair in the gastrointestinal tract as a result of

inflammatory-mediated tissue damage.

Levels of glutamate, and its downstream metabolite glutamine, were also frequently altered in IBD tissue compared to control groups. In a comparison of normal and diseased tissue, both glutamine and glutamic acid were decreased in inflamed tissue from UC patients[66,90]. In studies comparing UC tissue to control tissue, glutamate was higher in UC samples in two studies[57,93] and lower in one[54]. These two compounds are important for the maintenance of intestinal structural integrity[151]. In addition to their individual contributions to metabolism, collectively, the BCAA, alanine, glutamine and glutamate have all been associated with both insulin resistance and metabolic syndrome, potentially mediated by the liver through hepatic transamination[150].

Differences in tissue lipid profiles have been identified in UC, such as ceramides and sphingosines[55,65], and inflammatory-associated arachidonic acid, carnitine and eicosanoid metabolites[65,66,86]. Many of these have been previously associated with IBD in stool and blood analysis[22,26,81,84,102].

Breath metabolomic studies

Breath analysis for metabolomics presents challenges with sample collection and storage, and as a result only a small number of studies have been undertaken. However, it offers a number of benefits including its minimally invasive nature in terms of sampling. Through the use of mass spectrometry techniques, GC-MS and selected-ion flow-tube mass spectrometry (SIFT-MS), volatile organic compounds in breath can be analyzed.

Hicks *et al* (2015), using SIFT-MS, found that hydrogen sulfide was lower in CD patients than in controls or UC patients[74]. Exhaled hydrogen sulfide is largely produced by gut microbes

with sulfate reducing capacities, such as *Desulfovibrio*, which has been found at increased levels in studies of the IBD microbiome compared to controls[152,153]. The gut microbiota can also influence breath ammonia levels, through its use in microbial amino acid metabolism[153]. Changes in levels of exhaled ammonia were identified in IBD compared to controls[74,104].

In two studies by Dryahina *et al* (2013, 2018), pentane (which is associated with lipid peroxidation) was seen in higher concentrations in IBD groups compared to controls[68,69]: this is also an indicator of oxidative stress[154].

Two further studies have investigated the ability of breath analysis to discriminate between active and inactive disease states. Breath acetic acid was lower in active UC compared to inactive[98], although higher in active CD compared to inactive[60], possibly the result of physiological differences in disease phenotype.

Breath metabolomic studies appear to demonstrate potentially useful discriminatory metabolites: this is a promising platform for metabolomic analysis. Potential limitations of this approach include a high variation in profiles according to sampling method and dietary variation. The investigation of volatile organic compounds associated with the gut microbiome is a promising area for future studies, particularly with concomitant analysis of breath and the microbiome.

Conclusion

Metabolomic studies in IBD not only enable the elucidation of disease-associated metabolic perturbations but may also inform our understanding of changes in host metabolism over time, and as a result of therapeutic intervention. Metabolomic data allow relative

quantitation of the significant functional influence of the gut microbiota in health and disease, whether studying microbial metabolites or host-microbial co-metabolites.

NMR spectroscopy and MS have been used effectively in IBD research, both through global profiling and targeted analytical approaches. Whilst standardization in both sample analysis and interpretation in metabolomic studies has improved, consideration of dietary influence on the metabolome is also important given the close link between diet and the microbiome. The impact of diet on the metabolome varies depending on the sample: stool and urine will be more susceptible to dietary influence than blood and tissue samples, a factor that may influence which biosample is most appropriate for the physiological question to be answered. Further metabolomic investigation of other, less used biosamples such as breath and saliva is also warranted.

Limitations of many IBD metabolomic studies include small cohort sizes; however, where multiple studies of variable size report a consensus in metabolite differences, this reinforces the involvement of specific metabolic pathways in IBD. Indeed, this systematic review has demonstrated that there are clear, appreciable patterns of metabolites that differ consistently in the biosamples of IBD patients.

The characteristic metabolic profile associated with IBD, established from numerous studies, is consistent with general inflammation in addition to more specific processes, many involving host-microbiome interactions. Increases in arachidonic acid metabolites and acylcarnitines reflect inflammation; changes in TCA cycle intermediates reflect alterations in energy metabolism. Dysregulation of gut microbial function is typified by reduced TMAO levels (downregulated bacterial degradation of choline), reduced SCFA levels such as

butyrate (in accordance with changes in butyrate producing bacterial species), reduced hippurate levels (host-microbial co-metabolism) and changes in secondary bile acid profiles. A schematic representation of consistent changes in the IBD metabolome is provided in figure 3.

Whilst the integration of metabolomic and gut microbiome data has enabled a greater depth of understanding of microbiome functionality in IBD[22,26], many of the reported associations are based on statistical correlations and there are few examples of subsequent validation in culture-based models or direct evidence of association between specific bacterial species or strains and metabolites, a promising area for further research. Well-documented microbial species play vital roles in SCFA and bile acid production and so the combination of the two approaches allows greater mechanistic understanding of IBD. Future metabolomic and microbial studies, including large inception and longitudinal cohorts, will continue to inform our understanding of the complex pathogenesis of IBD, and may allow enhanced patient stratification with regards to the clinical management of the disease, whilst facilitating the discovery of novel therapeutic targets.

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Conflicts of interest

The authors have no conflicts of interest, financial or otherwise, to disclose.

Data Availability

All data underlying this article are available in the article and in its online supplementary material. The method used to collect this data is also described in the article.

Author contributions

Specific author contributions: K.G, H.RT.W and E.H were involved in the article conception and design; K.G and A.C performed the search, included articles, interpreted the results and prepared the manuscript; K.G, A.C, J.L.G, H.RT.W and E.H. contributed to the interpretation of results and prepared or revised the manuscript. All authors approved the final version of the article and author list before submission.

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Figure Legends

Figure 1.

A summary of host-microbial co-metabolic transformations, displaying physiologically important metabolites produced via the microbial transformation of endogenous and dietary compounds. *SCFA: Short Chain Fatty Acids*.

Figure 2.

Flow chart summarizing the search and reviewing process for this systematic review. Adapted from Moher, *et al* (2009)[48].

Figure 3.

Schematic representation, summarizing consistent changes found in the IBD metabolome. *SCFA: Short Chain Fatty Acids, BCAA: Branched Chain Amino Acids, GPC: Glycerophosphocholine.*

Table 1.

Characteristics of Studies Included in the Systematic Review (n = 64).

Characteristics of studies included

Study	Cohort	Patients with CD	Patients with UC	Controls	Biosample	Sample extraction	Analytical technique
Ahmed *et al*, (2016)[51]	UK	117	100	109	Stool	Yes	GC-MS
Alonso *et al*, (2016)[52]	Spain	203	213	100	Urine	No	^{1}H-NMR
Alothaim *et al*, (2016)[53]	UK	-	40	17	Urine	Yes	LC-MS
Balasubramanian *et al*, (2009)[54]	India	26	31	26	Colonic Biopsy	Yes	^{1}H-MRS
Bazarganipour *et al*, (2019)[55]	Germany	-	98	25	Colon Biopsy and Plasma	Yes	LC-MS
Bene *et al*, (2007)[56]	USA	85	-	48	Plasma	Yes	ESI-MS-MS
Bjerrum *et al*, (2010)[57]	Denmark	-	74	25	Colonic Biopsy	Yes	^{1}HR-MAS
Bjerrum *et al*, (2015)[58]	Denmark	44	48	21	Stool	Yes	^{1}H-NMR
Bjerrum *et al*, (2017)[59]	Denmark	38	48	37	Serum	No	^{1}H-NMR
Bodelier *et al*, (2015)[60]	Netherlands	275	-	110	Breath	No	GC-MS
Crackowski *et al*, (2002)[61]	France	23	-	23	Urine	Yes	GC-MS
Dawiskiba *et al*, (2014)[62]	Poland	19	24	17	Serum and Urine	No	^{1}H-NMR
De Preter *et al*, (2013)[63]	Belgium	67	-	40	Stool	Yes	GC-MS
De Preter *et al*, (2015)[64]	Belgium	83	68	40	Stool	Yes	GC-MS
Diab *et al*, (2019a)[65]	Norway	-	33	14	Colonic Biopsy	Yes	LC-MS
Diab *et al*, (2019b)[66]	Norway	-	28	14	Colonic Biopsy	Yes	LC-MS & GC-MS
Di Giovanni *et al*, (2019)[67]	Belgium	35	-	33	Serum	Yes	GCxGCMS
Dryahina *et al*, (2013)[68]	Prague	20	28	140	Breath	No	SIFT- MS
Dryahina *et al*, (2017)[69]	Prague	149	54	14	Breath	Yes	SIFT-MS & GC-MS

Dudzinska *et al*, (2019)[70]	Poland	27	28	50	Serum	Unclear	LC-MS
Fan *et al*, (2015)[71]	Australia	24	16	84	Plasma	Yes	LC-MS
Fathi *et al*, (2014)[72]	Iran	26	-	29	Serum	No	^{1}H-NMR
Forrest *et al*, (2002)[73]	UK	5	7	12	Serum	Yes	LC-MS
Franzosa *et al*, (2019)[22]	USA/Netherlands	68	53	34	Stool	Yes	LC-MS
Hicks et *al*, (2015)[74]	UK	18	20	18	Breath	No	SIFT-MS
Hisamatsu *et al*, (2015)[75]	Japan	-	369	-	Plasma	Yes	LC-MS
Iwamoto *et al*, (2013)[76]	Japan	21	10	26	Serum	Yes	LC-MS
Jansson *et al*, (2009)[77]	Germany	8 Twin Pairs	-	7 Twin Pairs	Stool	Yes	FT-ICR-MS
Keshteli *et al*, (2017)[78]	Canada	-	20	-	Serum and Urine	Yes	LC-MS, DI-MS & ^{1}H-NMR
Keshteli *et al*, (2018)[79]	Canada	38	-	-	Urine	Yes	LC-MS, DI-MS & ^{1}H-NMR
Kohashi *et al*, (2014)[80]	Japan	39	120	120	Serum	Yes	GC-MS
Lai *et al*, (2019)[81]	USA	20	-	10	Serum	Yes	LC-MS
Le Gall *et al*, (2011)[82]	UK	-	13	22	Stool	No	^{1}H-NMR
Lloyd-Price *et al*, (2019)[26]	USA	67	38	27	Stool	Yes	LC-MS
Machiels *et al*, (2014)[83]	Belgium	-	127	87	Stool	Yes	GC-MS
Manfredi et *al*, (2019)[84]	Italy	15	13	17	Serum	Yes	GC-MS
Marchesi *et al*, (2007)[85]	UK	10	10	16	Stool	No	^{1}H-NMR
Masoodi *et al*, (2013)[86]	UK	-	69	-	Colonic Biopsy	Yes	LC-MS
Murakami *et al*, (2018)[87]	Japan	6	6	26	Serum	Yes	LC-MS
Murgia *et al*, (2018)[88]	Italy	50	78	60	Plasma	Yes	LC-MS & LC-DTIM-QTOF
Nikolaus *et al*, (2017)[89]	Germany	81	67	100	Serum	Yes	LC-MS
Ooi *et al*, (2011)[90]	Japan	21	13	17	Colonic Biopsy and Serum	Yes	GC-MS
Piestansky *et al*, (2019)[91]	Slovak Republic	13	-	10	Urine	Yes	CE-MS-MS &

							LC-MS
Probert, *et al*, (2018)[92]		-	40	-	Plasma	No	^{1}H-NMR
Rantalainen *et al*, (2014)[93]	Denmark	-	48	15	Colonic Biopsy	No	^{1}HR-MAS-NMR
Roda *et al*, (2019)[94]	Greece	40	40	20	Serum	Yes	LC-MS
Schicho *et al*, (2012)[95]	Canada	20	20	40	Plasma	No	^{1}H-NMR
Scoville *et al*, (2018)[96]	USA	20	20	20	Serum	Yes	LC-MS
Sharma *et al*, (2010)[97]	India	9	12	26	Colonic Biopsy	Yes	^{1}H-NMR
Smolinska *et al*, (2017)[98]	Netherlands	-	72	22	Breath	No	GC-MS
Sofia *et al*, (2018)[99]	USA	-	99	-	Serum	Yes	LC-MS & GC-MS
Stanke-Labesque *et al*, (2008)[100]	France	32	28	30	Urine	Yes	LC-MS
Stephens *et al*, (2013)[101]	USA	30	30	60	Urine	No	^{1}H-NMR
Sun *et al*, (2019)[102]	China	-	48	30	Plasma	Yes	LC-MS
Tefas *et al*, (2019)[103]	Romania	5	17	24	Serum	Yes	LC-MS
Tiele *et al*, (2019)[104]	UK	14	16	9	Breath	No	GC-IMS
Weng *et al*, (2019)[105]	China	173	107	42	Stool	Yes	LC-MS
Whiley *et al*, (2019)[106]	UK	-	19	10	Plasma	Yes	LC-MS
Williams *et al*, (2009)[107]	UK	86	60	60	Urine	No	^{1}H-NMR
Williams *et al*, (2010)[108]	UK	86	60	60	Urine	No	^{1}H-NMR
Williams *et al*, (2012)[109]	UK	24	20	23	Serum	No	^{1}H-NMR
Wilson *et al*, (2015)[110]	USA	73	33	373	Plasma	Yes	LC-MS
Yau *et al*, (2014)[111]	Australia	25	19	9	Plasma	Yes	LC-MS & GC-MS
Zhang *et al*, (2013)[112]	China	-	20	19	Serum	No	^{1}H-NMR

Table 2: Quality assessment of included studies using the QUADOMICS tool

Results of the quality assessment of studies included in the review (n = 64) using the QUADOMICS tool[49].

Study	Were selection criteria clearly described?	Was the spectrum of patients representative of patients who will receive the test in practice	Was the type of sample fully described?	Were the procedures and timing of biological sample collection with respect to clinical factors described with enough detail?	Were handling and pre-analytical procedures reported in sufficient detail and similar for the whole sample? And, if differences in procedures were reported, was their effect on the results assessed?	Is the time period between the reference standard and the index test short enough to reasonably guarantee that the target condition did not change	Is the reference standard likely to correctly classify the target condition?	Did the whole sample or a random selection of the sample receive verification using a reference standard of diagnosis?	Did patients receive the same reference standard regardless of the result of the index test?	Was the execution of the index test described in sufficient detail to permit replication of the test?	Was the execution of the reference standard described in sufficient detail to permit its replication?	Were the index test results interpreted without knowledge of the results of the reference standard?	Were the reference standard results interpreted without knowledge of the results of the index test?	Were the same clinical data available when test results were interpreted as would be available when the test is used in practice?	Were uninterpretable/intermediate test results reported?	Is it likely that the presence of overfitting was avoided?
Ahmed *et al*, (2016)[51]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	?	Y
Alonso *et al*, (2016)[52]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	Y
Alothaim *et al*, (2016)[53]	N	NA	N	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	NA
Balasubramanian *et al*, (2009)[54]	N	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	NA
Bazarganipour *et al*, (2019)[55]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	NA
Bene *et al*, (2007)[56]	N	NA	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	NA
Bjerrum *et al*, (2010)[57]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	N

Bjerrum *et al*, (2015)[58]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	Y
Bjerrum *et al*, (2017)[59]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	Y
Bodelier *et al*, (2015)[60]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	Y
Crackowski *et al*, (2002)[61]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	NA
Dawiskiba *et al*, (2014)[62]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	Y
De Preter *et al*, (2013)[63]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	Y
De Preter *et al*, (2015)[64]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	Y
Diab *et al*, (2019a)[65]	N	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	?
Diab *et al*, (2019b)[66]	N	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	Y
Di *et al*, (2019)[67]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	Y
Dryahina *et al*, (2013)[68]	N	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	NA
Dryahina *et al*, (2017)[69]	N	NA	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	NA	Y	?
Dudzinska *et al*, (2019)[70]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	NA
Fan *et al*, (2019)[71]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	Y
Fathi *et al*, (2014)[72]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	N	Y
Forrest *et al*, (2002)[73]	N	NA	N	N	N	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	NA
Franzosa *et al*, (2019)[22]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	Y
Hicks *et al*, (2015)[74]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	Y
Hisamatsu *et al*, (2015)[75]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	NA
Iwamoto *et al*, (2013)[76]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	NA
Jansson *et al*, (2009)[77]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	N
Keshteli *et al*, (2017)[78]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	N	Y
Keshteli *et al*, (2018)[79]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	N	Y

Kohashi *et al*, (2014)[80]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	Y
Lai *et al*, (2019)[81]	Y	NA	Y	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	N
Le Gall *et al*, (2011)[82]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	Y
Lloyd-Price *et al*, (2019)[26]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	Y
Machiels *et al*, (2014)[83]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	NA
Manfredi *et al*, (2019)[84]	N	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	?	Y
Marchesi *et al*, (2007)[85]	N	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	Y
Masoodi *et al*, (2013)[86]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	N
Murakami *et al*, (2018)[87]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	NA
Murgia *et al*, (2018)[88]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	Y
Nikolaus *et al*, (2017)[89]	Y	NA	?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	NA
Ooi *et al*, (2011)[90]	N	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	N
Piestansky *et al*, (2019)[91]	N	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	NA
Probert *et al*, (2018)[92]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	Y
Raintalainen *et al*, (2014)[93]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	NA
Roda *et al*, (2019)[94]	N	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	N
Schicho *et al*, (2012)[95]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	Y
Scoville *et al*, (2018)[96]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	NA
Sharma *et al*, (2010)[97]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	NA
Smolinska *et al*, (2017)[98]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	NA
Sofia *et al*, (2018)[99]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	NA
Stanke-Labesque *et al*, (2008)[100]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	NA
Stephens *et al*, (2013)[101]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	N

Sun *et al*, (2019)[102]	Y	NA	Y	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	NA	N	N
Tefas *et al*, (2019)[103]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	N	Y
Thiele *et al*, (2019)[104]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	Y
Weng *et al*, (2019)[105]	Y	NA	Y	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	Y
Whiley *et al*, (2019)[106]	N	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	Y
Williams *et al*, (2009)[107]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	Y
Williams *et al*, (2010)[108]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	NA
Williams *et al*, (2012)[109]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	?	Y
Wilson *et al*, (2015)[110]	Y	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	NA
Yau *et al*, (2014)[111]	Y	NA	Y	Y	?	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	Y
Zhang *et al*, (2013)[112]	Y	NA	Y	Y	?	Y	Y	Y	Y	Y	Y	Y	Y	NA	N	Y

Y = Yes, N = No, ? = Unclear, NA = Not Applicable

Figure 1

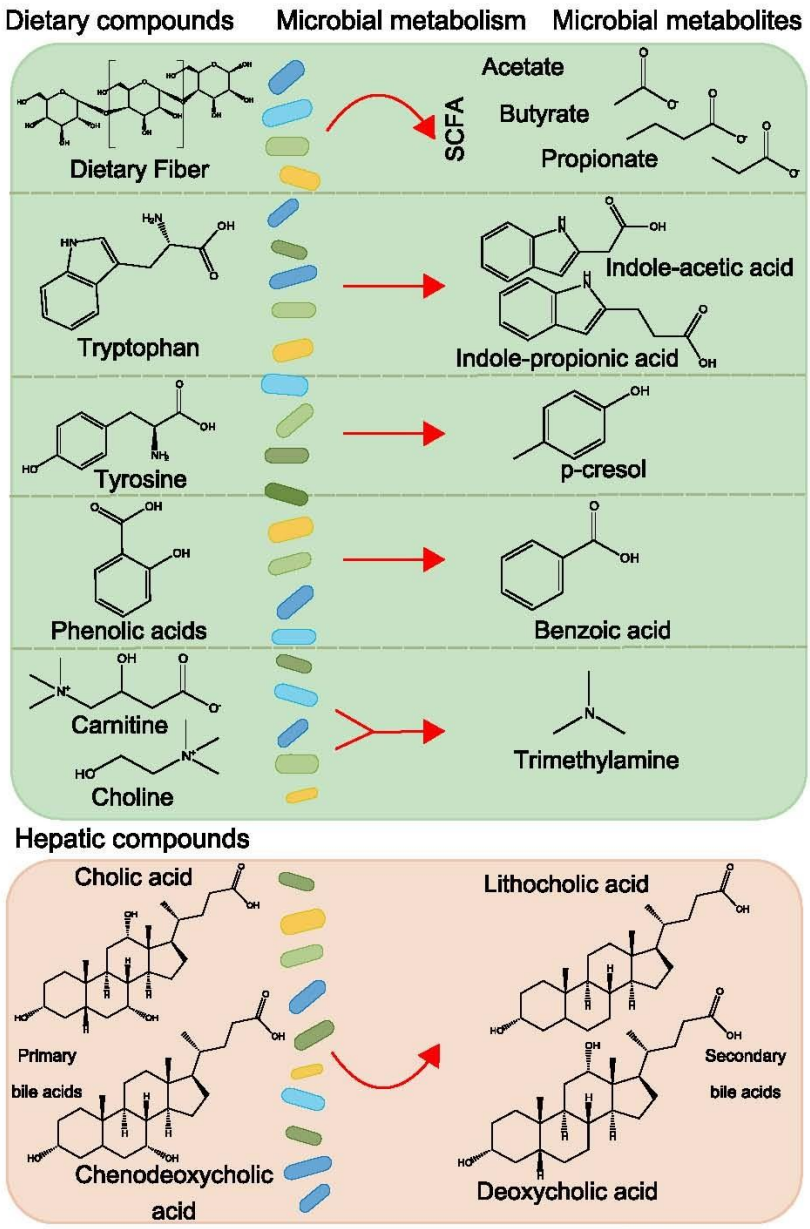

Figure 2

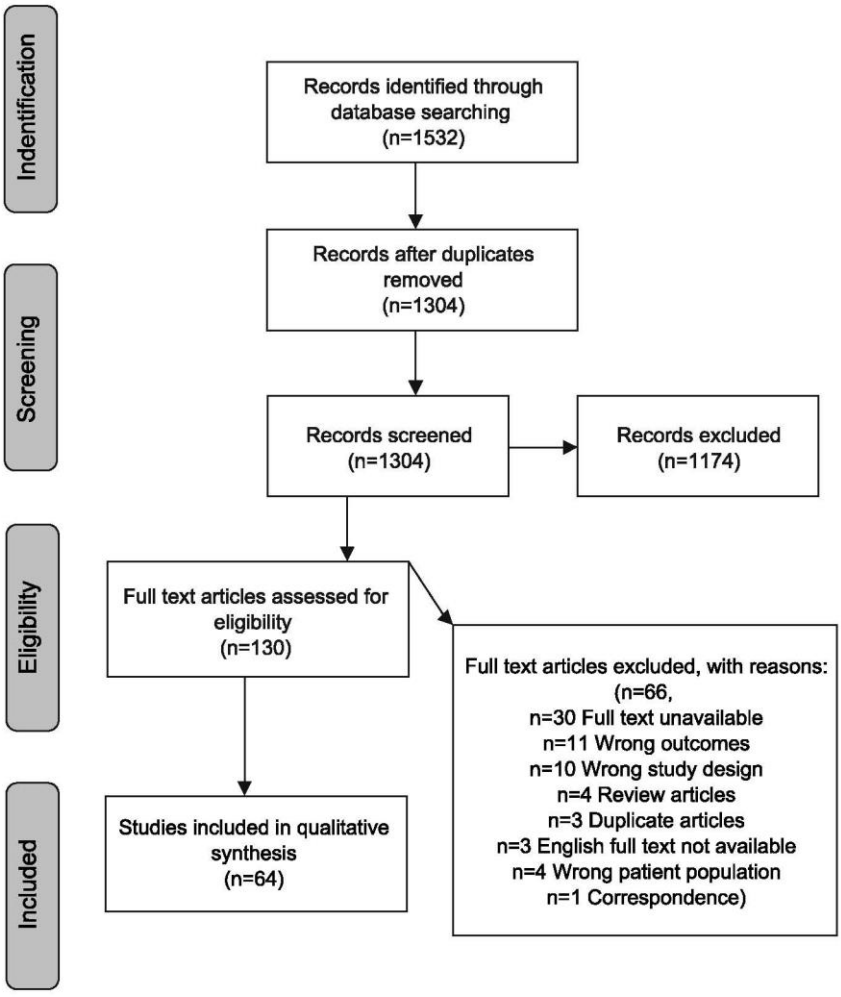

Figure 3