Faecal Metabolomics in Paediatric Inflammatory Bowel Disease: A Systematic Review

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Abstract

Background and Aims: Paediatric inflammatory bowel disease [IBD] is characterized by altered immunological and metabolic pathways. Metabolomics may therefore increase pathophysiological understanding and could develop into characterization of biomarkers for diagnosis and IBD treatment response. However, no uniform metabolomic profiles have been identified to date. This systematic review aimed to identify faecal metabolomic signatures in paediatric IBD vs controls, and to describe metabolites associated with disease activity and treatment response.

Methods: A literature search was performed in Embase, Medline, Web of Science and Cochrane Library. Studies assessing faecal metabolomics in paediatric patients < 18 years with IBD [*de novo*, active, inactive] with comparative groups [IBD vs non-IBD; responders vs non-responders] were included. The quality of included studies was assessed according to the Newcastle–Ottawa Scale.

Results: Nineteen studies were included [540 patients with IBD, 386 controls], assessing faecal short-chain fatty acids [SCFA] [five studies], amino acids [AA] [ten studies], bile acids [BA] [eight studies] and other metabolites [nine studies] using various methodologies. Significantly increased levels of AA [particularly phenylalanine], primary BA and lower levels of secondary BA were described in paediatric IBD compared to controls. Faecal SCFA results varied across studies. Additionally, responders and non-responders to exclusive enteral nutrition and infliximab showed differences in baseline faecal metabolites [based on BA, AA].

Conclusions: This systematic review provides evidence for distinct faecal metabolomic profiles in paediatric IBD. However, results varied across studies, possibly due to differences in study design and applied analytical techniques. Faecal metabolomics could provide more insight into host–microbial interactions in IBD, but further studies with standardized methodologies and reporting are needed.

Key Words: Inflammatory bowel disease; paediatrics; metabolomics

1. Introduction

Inflammatory bowel disease [IBD], consisting of the two main entities Crohn's disease [CD] and ulcerative colitis [UC], is a chronic relapsing–remitting disorder of the gastrointestinal [GI] tract.[1] Over the past few decades, a steady increase in incidence of paediatric IBD has been observed[2,3] and a further rise is expected.[4] Although the pathogenesis of IBD is not completely understood, the most widely accepted hypothesis is that IBD results from an inappropriate immune response following the interplay between host and the gut microbiota, in a genetically susceptible host.[5] Changes in the gut microbiota composition have been reported extensively in IBD,[6] commonly characterized by depletion of Firmicutes such as *Faecalibacterium prausnitzii* and *Roseburia hominis* and an increase in Proteobacteria.[7] These shifts in gut microbiota composition may provoke changes in metabolic pathways.[8]

Metabolomics is a relatively novel omics technique that can shed light on the complex interplay between the host, gut microbiota and environmental factors such as diet. Metabolomic analysis allows for quantification of small

molecules in biological samples such as urine, serum or faeces.[9] Discriminatory metabolites in different conditions such as diabetes, liver disease and different cancers have been identified using metabolomics.[10] Differences in several metabolite classes have also been found in patients with IBD compared to healthy individuals.[5,11–13] The main metabolite classes that have been described are short-chain fatty acids [SCFA], amino acids [AA] and bile acids [BA]. These findings have improved our understanding of the metabolic pathways involved and host–microbial interactions in IBD, and indicate a potential role for metabolomic analysis to serve as a [adjuvant] diagnostic biomarker.

The faecal metabolome in particular is considered to provide a functional readout of gut microbial activity.[14] Several longitudinal studies have observed alterations in faecal metabolites linked to clinical remission and response to therapy.[15–17] This illustrates that faecal metabolomic analysis also has the potential to serve as a [adjuvant] biomarker to monitor disease activity in IBD, and potentially even to predict treatment response. Consequently, more personalized, tailored disease management strategies could be developed with identification of clear metabolomic signatures.

Although the growing interest in host–microbial interactions has resulted in an expansion of metabolomics studies, previous studies on the faecal metabolome in children with IBD are characterized by mostly small cohorts. So far, no disease-specific metabolomic signature has been found. Therefore, the aim of this systematic review was to summarize the available evidence on the differences in faecal metabolomics between children with IBD vs controls, with the aim of finding an IBD signature or identifying general characteristics of the metabolome. Secondly, we reviewed the role of metabolomic analysis to predict treatment response and monitor disease activity in paediatric IBD.

2. Materials and Methods

2.1 Search strategy

A literature search was performed based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses [PRISMA] statement.[18] To identify all relevant publications, systematic searches in the bibliographic databases Medline [PubMed], Embase [Ovid], Web of Science [Core Collection] and Wiley/Cochrane Library were conducted from inception to November 24, 2021, by a medical information specialist. The following terms were used [including synonyms and closely related words] as index terms or free-text words: 'Inflammatory Bowel Diseases', 'Metabolomics', 'Multiomics', 'Children', 'Adolescent'. The references of the identified articles were searched for relevant publications. Duplicate articles were excluded. The full search strategies for all databases can be found in the Supplementary material.

2.2 Study selection and patient population

Studies were included if they met the following criteria: [1] studies consisting of patients with [newly diagnosed, active and inactive] IBD (including the phenotypes CD, UC and IBD-unclassified [IBD-U]); [2] paediatric patients < 18 years old; [3] studies on faecal metabolomic analysis; and [4] inclusion of a comparative group [i.e. IBD vs non-IBD; responders vs non-responders].

Studies were excluded if they were systematic reviews, guidelines, editorials, or case series and case reports; studies

comprising adults with IBD and studies with no comparative group; transcriptomic and proteomics studies; and in languages other than Dutch, English, Spanish or French. Animal studies and conference proceeding abstracts were also excluded.

Two reviewers [J.Z.J. and C.M.V.] independently screened all potentially relevant titles and abstracts for eligibility. If necessary, the full-text article was checked for the eligibility criteria. Differences in judgement were resolved through a consensus procedure.

2.3 Outcome assessment

The primary outcome was the difference in faecal metabolites [including metabolite classes SCFA, AA and BA] between children with IBD as compared to controls. Secondary outcomes were faecal metabolites associated with disease activity, and faecal metabolites associated with response or non-response to therapy in paediatric patients with IBD. We accepted all measurement methods for metabolomic analysis including liquid chromatography [LC], high-pressure LC [HPLC], gas chromatography [GC] coupled with mass spectrometry [MS] or nuclear magnetic resonance [NMR] spectroscopy.

2.4 Eligibility assessment and data extraction

Two reviewers [J.Z.J. and C.M.V.] independently screened abstracts for eligibility of each article. Resulting full-text articles were independently reviewed for complete analysis. Relevant data from papers as well as any available supplements were extracted independently by the two reviewers [J.Z.J. and C.M.V.] using predefined data extraction forms. Disagreements were resolved by discussion between the reviewers and remaining discordance was resolved by consulting a third author.

The following data were extracted from the selected studies: author names, the year of study, country, number of participants, IBD subtype [CD, UC or IBD-U] and control type, disease activity, disease localization, use of concomitant medication, metabolomics approach and analytical technique, and details regarding treatment response [definition and type of treatment]. Data on faecal metabolomics including all metabolites assessed and concentrations of unique metabolites were collected.

2.5 Methodological quality appraisal

The Newcastle–Ottawa Scale [NOS] was used to assess the quality of included studies. This scale consists of three domains with a maximum of nine stars and is applied to case-control and cohort studies. For case-control studies the domains include selection of cases and controls, comparability of cases and controls, and ascertainment of exposure, and for cohort studies they include selection of cohorts, comparability of cohorts and assessment of outcome. Two reviewers [J.Z.J. and C.M.V.] applied the NOS independently and differences were resolved by consensus.

3. Results

3.1 Study selection

The literature search generated a total of 7600 citations: 2946 in Medline [PubMed], 2751 in Embase [Ovid], 1638 in Web of Science and 265 in Cochrane Library. After removing duplicates of citations that were selected from more than one database, 5309 references remained. In total, 5219 citations

were excluded based on title and abstract relevance. The remaining 90 articles were reviewed, of which 19 were included for final analysis. The flow chart of the search and selection process is depicted in Figure 1. Reference lists of the included articles were searched for relevant publications. However, no additional relevant publications were identified. Fifteen relevant conference abstracts were identified for which the authors were contacted for additional data or possible full-text publication. This did not lead to additional inclusion of studies [no published full manuscript $n = 8$, abstract not found $n = 4$, contact details not found $n = 1$, no reply from author $n = 2$].
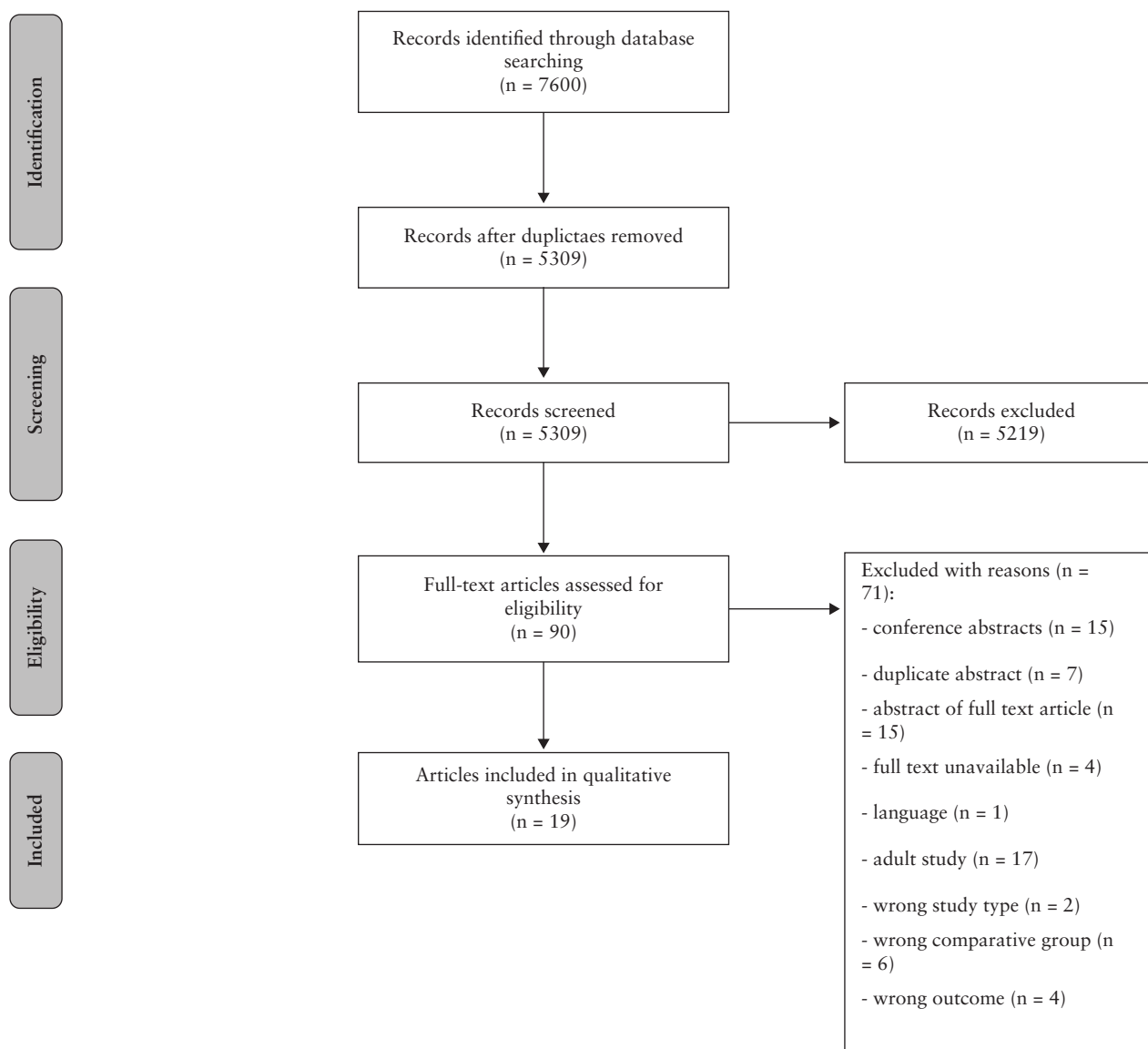
3.2 Study characteristics

This review included 19 studies comprising 540 unique children with IBD and 386 controls. Amongst these studies, nine studies assessed the faecal metabolome in both CD and UC, nine studies in children with CD and one study in children with UC only. Thirteen studies recruited healthy children as a control group, two studies recruited healthy unaffected siblings, and two studies recruited intention-to-diagnose controls who underwent endoscopy to exclude IBD. Regarding disease activity, 14 studies included patients with active IBD, two with patients in remission, and three with both active and inactive disease. Seven studies conducted metabolomic analysis in a cohort of newly diagnosed, treatment-naïve patients, while in the other 12 studies the IBD patients used medication. The characteristics of each individual study are depicted in Table 1. Only three studies[19–21] explicitly reported on sample collection before bowel preparation.

3.2.1 Metabolomic analysis and techniques

Of the 19 included studies, ten reported on AA, five on SCFA and eight on BA. Nine studies reported on metabolites outside of these categories. Included studies varied in their analytical approach, with 11 studies using a targeted approach,[19,21–30] six using an untargeted approach[16,20,31–34] and two that performed both.[35,36] Analytical techniques varied across the different studies. Methods that were used were chromatography ([ultra] high-performance, gas or liquid) optionally coupled

Figure 1. Flowchart of the search and selection procedure of studies.

Table 1. The characteristics of the included studies [n = 19]

First author [publication year]	Country	Disease [n]	Type	Disease localization	Medication	Approach	Analytical technique	Multiple comparison correction
Alghamdi [2018]	UK	CD [11]	Active [PCDAI > 10] Newly diagnosed, treatment-naïve [n = 7]	NR	EEN, AZA, 5-ASA	Untargeted	LC-MS	Yes
		Controls [11]	Healthy	—	—			
Ashton [2017]	UK	CD [4]	Newly diagnosed, treatment-naïve	L3 [n = 4], L4 [n = 1]	None	Targeted [SCFA]	GC	No
		UC [1]	Newly diagnosed, treatment-naïve	E4 [n = 1]	None			
		IBD-U [1]	Newly diagnosed, treatment-naïve	NR	None			
		Controls [3]	Healthy siblings	—	—			
Bosch [2018]	Netherlands	CD [15]	Newly diagnosed, treatment-naïve	L2 [n = 7], L3 [n = 8], L4 [n = 2], P [n = 3]	None	Targeted [AA]	HPLC	Yes
		UC [15]	Newly diagnosed, treatment-naïve	E1 [n = 3], E2 [n = 2], E3 [n = 10]	None			
		Controls [15]	Healthy	—	—			
Bosch [2020]	Netherlands	CD [5]	Newly diagnosed, treatment-naïve	L1 [n = 2], L3 [n = 3], L4 [n = 1], P [n = 2]	None	Targeted [AA]	HPLC	Yes
		UC [6]	Newly diagnosed, treatment-naïve	NR	None			
		Controls [8]	Intention-to-diagnose	—	—			
Bushman [2020]	USA	CD [20]	Mean PCDAI 18.3 [12.1 SD]	L1 [n = 2], L2 [n = 3], L3 [n = 14], L4 [n = 12]	NR	Untargeted	UPLC-MS	Yes
		UC [3] IBD-U [4]	Mean PUCAI 19.3 [18.1 SD]	E1 [n = 1], E2 [n = 4], E4 [n = 2]	NR			
		Controls [38]	Healthy	—	—			
Connors [2019]	Canada	CD [17]	Active	L1 [n = 3], L3 [n = 14]	AZA, MTX	Targeted [BA]	LC-MS-MS	No
Diederen [2020]	Netherlands	CD [43]	Newly diagnosed treatment-naïve	L1 [n = 5], L2 [n = 9], L3 [n = 29]	None	Untargeted and targeted [AA, BA]	1H NMR spectroscopy and HPLC	Yes
		Controls [18]	Healthy	—	—			
Ejderhamn [1991]	Sweden	CD [2]	Clinical remission [not specified]	Colonic [n = 1], Ileocolonic [n = 1]	NR	Targeted [BA]	GLC-MS	No
		UC [16]	Clinical remission [not specified]	Distal colon only [n = 4], Pancolitis [n = 12]	Sulphasalazine			

Table 1. Continued

First author [publication year]	Country	Disease [n]	Type	Disease localization	Medication	Approach	Analytical technique	Multiple comparison correction
Gerasimidis [2014]	UK	Controls [5]	Healthy	—	—	Targeted [SCFA]	GC	No
		CD [15]	Active, newly diagnosed, treatment-naïve [n = 11]	L1 [n = 1], L2 [n = 5], L3 [n = 9], L4 [n = 12]	Background medication [not specified], none			
Jacobs [2016]	USA	Controls [21]	Healthy	—	—	Untargeted	UPLC/ToFMS	Yes
		CD [17]	Clinical remission [HBI < 5]	Small intestine [n = 15], Colon involvement [n = 10], upper GI involvement [n = 6], perianal [n = 7], colon only [n = 2]	Anti-TNF, MTX, thiopurines, 5-ASA			
		UC [4]	Clinical remission [Mayo score < 2]	NR				
		Controls [21]	Healthy siblings	—	—			
Jagt [2021]	Netherlands	CD [40]	Newly diagnosed, treatment-naïve	L1 [n = 4], L2 [n = 11], L3 [n = 25], L4 [n = 15], P [n = 6]	None	Targeted [AA]	HPLC	Yes
		UC [38]	Newly diagnosed, treatment-naïve	E1 [n = 2], E2 [n = 6], E3 [n = 2], E4 [n = 28]	None			
		Controls [105]	Healthy [n = 84] and GI symptoms [n = 21]	—	—			
Kolho [2017]	Finland	CD [13]	Newly diagnosed, treatment-naïve	NR	None	Untargeted	UPLC-MS	No
		UC/IBD-U [10]	Newly diagnosed, treatment-naïve	NR	None			
		Controls [14]	Intention-to-diagnose	—	—			
Ni [2017]	USA	CD [90]†	Active [PCDAI > 10]	Upper [n = 51], Ileum [n = 80], Colon [n = 84], Perianal [n = 15]	Antibiotics, 5-ASA, thiopurines, MTX, systemic steroids, rectal steroids	Untargeted and targeted [AA]	LC-MS and HPLC	Yes
Rotondo-Trivette [2021]	USA	Controls [26]	Healthy	—	—	Targeted [BA]	LC-MS	No
		UC [23]	Active [mild-moderate]	NR	Biological, immunomodulator, 5-ASA, corticosteroids			
		Controls [29]	Healthy	—	—			

Table 1. Continued

First author [publication year]	Country	Disease [n]	Type	Disease localization	Medication	Approach	Analytical technique	Multiple comparison correction
Sundqvist [2019]	Sweden	CD [28]	Active [PCDAI > 15]	Small intestine [n = 5], small intestine/colon [n = 15], colon [n = 2], small intestine/colon/anal [n = 6]	EEN, 5-ASA, other [not specified]	Targeted [faecal tryptic activity, coprostanol, urobilinogen]	Spectrophotometry [faecal tryptic activity, urobilinogen], GLC [coprostanol]	Yes
		Controls [28]‡	Healthy	—	—			
Taylor [2020]	UK	CD [22]	Disease duration > 6 months	L1 [n = 1], L2 [n = 9] L3 [n = 10], L4 [n = 13], Perianal [n = 4]	Biological [infliximab/vedolizumab], thiopurines, MTX	Untargeted	1H NMR spectroscopy	No
Tjellström [2012]	Sweden	CD [14]	Active [clinical index ≥ 10]§	Small bowel/colon, perianal region	5-ASA, AZA	Targeted [SCFA]	GLC	No
		Controls [12]	Healthy	—				
Treem [1994]	USA	CD [22]	Active [mild–moderate, PCDAI ≥ 11] [n = 10], Inactive [PCDAI ≤ 10] [n = 12]	Patchy colonic, terminal ileum involvement	5-ASA	Targeted [SCFA]	GLC	No
		UC [17]	Inactive and mild [n = 12], moderate–severe [n = 5]¶	Pancolitis	5-ASA			
		Controls [12]	Healthy	—				
Wang [2021]	China	CD [24]	Newly diagnosed, treatment-naïve	L2, L3, L4	None	Untargeted	UPLC-MS	Yes
		Controls [20]	Healthy	—	—			

†Age < 20 years old.

‡Healthy controls aged 5–22 years.

§Clinical index: consists of the three history items [abdominal pain, number of liquid stools and general well-being], three physical examination items [weight loss, abdominal examination and perirectal disease] of the PCDAI score, and no laboratory tests.

¶Based on Truelove–Witts criteria.

5-ASA, 5-aminosalicylic acid; AA, amino acids; AZA, azathioprine; BA, bile acids; CD, Crohn's disease; E1, proctitis; E2, left-sided; E3, extensive; E4, pancolitis; EEN, enteral exclusive nutrition; GI, gastrointestinal; GLC, gas–liquid chromatography; HBI, Harvey-Bradshaw index; 1H NMR, proton nuclear magnetic resonance; HPLC, high-performance liquid chromatography; IBD-U, inflammatory bowel disease unclassified; L1, ileal; L2, colonic; L3, ileocolonic; L4, upper GI; LC-MS, liquid chromatography–mass spectrometry; MTX, methotrexate; NR, not reported; P, perianal; PCDAI, paediatric Crohn's disease activity index; PUCAI, paediatric ulcerative colitis activity index; SCFA, short-chain fatty acids; ToFMS, time-of-flight mass spectrometry; UC, ulcerative colitis; UPLC-MS, ultra-performance liquid chromatography–mass spectrometry.

with MS, or proton NMR [H-NMR].[37] An overview can be found in Table 2.

The metabolomics workflow after sample acquisition consists of different analytical steps.[38] Untargeted analysis allows broad assessment of metabolic changes across different metabolite classes, resulting in very high data output but allowing for discovery of unexpected molecules. In contrast, targeted analysis focuses on specific predefined metabolic classes leading to more accurate findings within that limited class.

Two main detection methods are commonly used in the metabolomics workflow: MS and NMR. MS is used to identify and quantify metabolites after separation with GC or LC. In MS, ions are accelerated by attraction and deflected by a magnetic field, after which they are detected and provide a spectrum.[39] Samples detected by MS require separation prior to detection. The separation method used depends on the objected metabolite to be measured. GC is typically used for volatile chemicals such as SCFA. LC separates molecules in the liquid mobile phase, giving the advantage that a much wider range of analytes can be measured.[38] NMR is a detection method that does not require prior separation of metabolites, and can measure different kinds of metabolites simultaneously. In NMR, an external magnetic field is applied, causing an energy transfer derived from nuclear spins of certain metabolites. The signal matching the energy transfer can be measured and processed, leading to a spectrum.[40] However, NMR has a very low sensitivity, which leads to low-abundance metabolites being missed. Therefore, MS is nowadays more frequently used.

After this step, different analytical platforms are used for data interpretation due to the high-throughput data.[41] This leads to varying annotation, interpretation and reporting across studies investigating the faecal metabolome.

3.3 Quality assessment

The NOS was applied to 16 case-control and three cohort studies. The quality assessment of included studies according to this scale is depicted in Supplementary Tables 1 and 2. The majority of the case-control studies gave a clear description of the selection [13/16 studies] and definition of paediatric IBD

Table 2. Analytical techniques used in selected metabolomics studies and their targets

Method	Target	References
GC	SCFA	22,25
GLC	SCFA	27,28
GLC-MS	BA	24
1H NMR	Untargeted	16,35
HPLC	Untargeted, AA, BA	19,21,35,36
LC-MS	Untargeted	31,36
LC-MS-MS	BA	23
Spectrophotometry	Other	26
UPLC-MS	Untargeted	20,32–34

AA, amino acids; BA, bile acids; GC, gas chromatography; GLC, gas–liquid chromatography; 1H NMR, proton nuclear magnetic resonance; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography–mass spectrometry; SCFA, short-chain fatty acids; UPLC-MS, ultra-performance liquid chromatography–mass spectrometry.

[11/16 studies] but this was less often the case for controls [selection 7/16 studies; definition 10/16 studies]. In eight out of the 19 included studies [42%], cases and controls were age-matched, while sex-matching was performed in 5/19 studies [26%]. Only three out of 16 case-control studies [19%] described if the metabolomics assessment was blinded.

3.4 Faecal metabolomics in IBD vs controls
3.4.1 *Short-chain fatty acids*

Five studies reported on faecal SCFA in 120 children with IBD [101 CD, 18 UC, one IBD-U] and 68 controls [65 healthy, three unaffected siblings] using different methodologies [GC, GLC, UPLC-MS].[22,25,27,28,34] Overall, studies reporting on faecal SCFA levels in children with IBD [with concomitant medication] showed no statistically significant differences in cumulative [absolute] levels compared to healthy controls.[25,28] Acetic, butyric and propionic acid levels were most prevalent in samples of children with IBD, similar to controls.[22,25,27,28]

Absolute acetic, isovaleric and valeric acid levels were generally significantly lower or showed no difference in children with CD compared to healthy controls.[22,25,27,28,34] Gerasimidis *et al*.[25] found that absolute values for isovaleric acid were similar, but the percentage contribution of isovaleric acid to the total SCFA pool was significantly higher in newly diagnosed children compared to healthy controls. Results for propionic, butyric and isobutyric acid were inconsistent across studies, reporting on significantly higher, lower or no difference in abundance, as shown in Figure 2.[22,25,27,28,34] Tjellström *et al*.[27] found both decreased and similar levels of propionic acid compared to controls depending on the primary disease location, while Diederen *et al*.[35] found significantly higher concentrations in newly diagnosed CD irrespective of disease location. Concentrations of SCFA from individual studies are depicted in Supplementary Table 3.

Only one study reported on specific SCFA in relation to disease localization in children with active CD with ongoing concomitant treatment [azathioprine, 5-aminosalicylate]. In primary small bowel and colonic localization, decreased levels of isobutyric acid, isovaleric acid and valeric acid were observed, while decreased amounts of propionic acid, isobutyric acid, isovaleric acid and valeric acid were found in a small subgroup of patients with primary perianal disease. No differences in acetic and butyric acid levels were observed between disease locations.[27]

Treem *et al*. [28] reported on the only study that specifically assessed SCFA levels in children with UC with pancolitis compared to controls. This study showed increased butyric acid levels while isobutyric acid and acetic acid were decreased compared to healthy controls [Figure 2]. This study also compared patients with CD to UC in terms of SCFA: patients with CD had significantly higher isobutyric and isovaleric acid than patients with UC, while all other SCFA were not significantly different between the two groups.[28]

A summary of the SCFA results is displayed in Figure 2.

3.4.2 *Amino acids and their derivatives*

Ten studies assessed faecal AA comprising 354 children with IBD [278 CD, 72 UC, four IBD-U] and 276 controls [233 healthy, 43 intention-to-diagnose cases], by using different analytical approaches and techniques.[19–21,29,31–36]
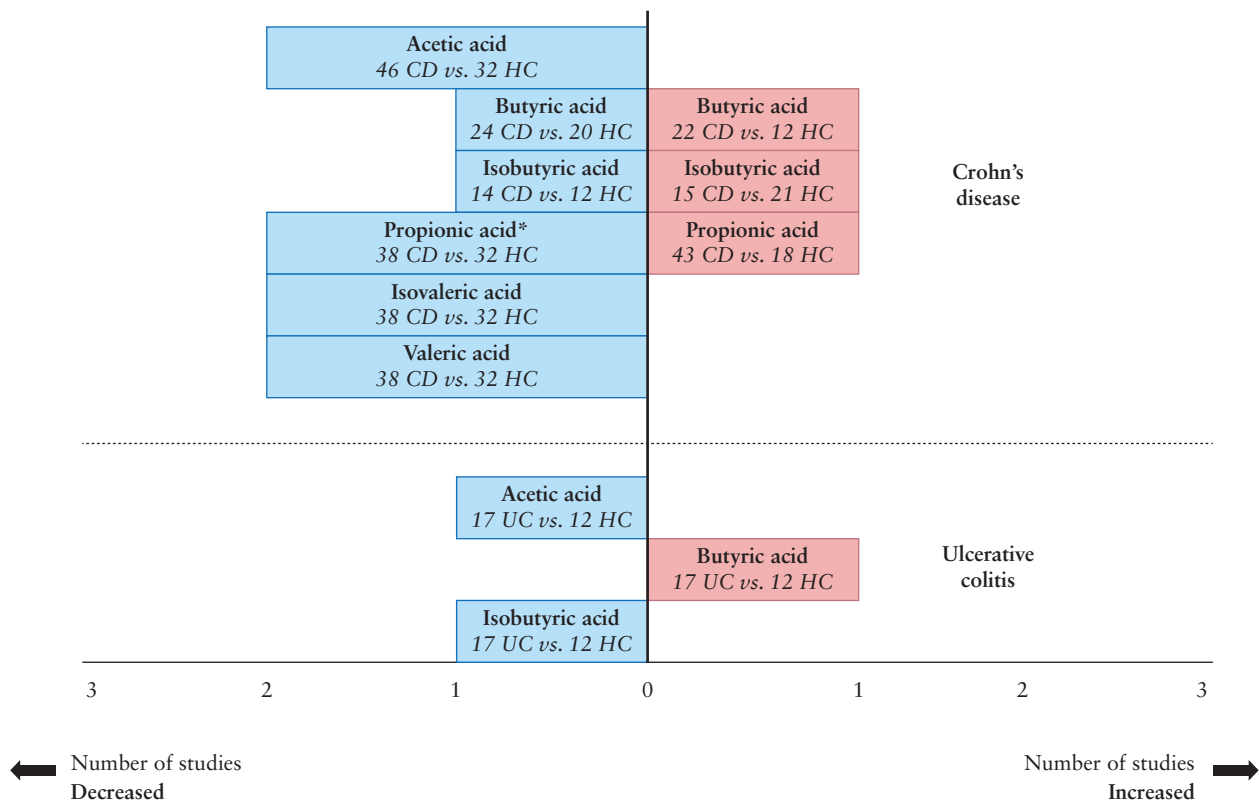
Figure 2. Findings on faecal SCFA in children with IBD vs controls [six studies in Crohn's disease[22,25,27,28,34,35] and one study in ulcerative colitis[28]. All described SCFA were included in this figure. The *x*-axis represents the number of studies in which the SCFA were increased or decreased, in the IBD phenotype compared to controls. The *y*-axis represents the unique SCFA.*The study by Tjellström *et al.* is included, in which propionic acid was increased in Crohn's disease patients with perianal disease but not in children with only small bowel involvement.

Four studies reported the AA in IBD [*n* = 146] vs controls [*n* = 166].[19,21,29,32] In IBD, most consistently increased AA concentrations included leucine,[19,21,29,32] phenylalanine,[19,21,29,32] valine,[19,21,29,32] glutamine,[19,29,32] tryptophan[21,29,32] and tyrosine,[19,21,29] measured by HPLC[19,21,29] or UPLC-MS.[32] Two studies[19,21] reported on areas under the ROC curve [AUCs] of multiple AA for the discrimination between newly diagnosed, treatment-naïve IBD and controls [AUCs varying from 0.78 to 0.99] as shown in Supplementary Table 4.

In CD vs controls, phenylalanine levels were significantly increased in six studies,[21,29,31,34–36] whereas another study demonstrated no difference[19] [Figure 3]. Other faecal AA that were observed at higher levels in CD are shown in Figure 3. Conflicting findings were reported for tyrosine,[29,31,35,36] threonine,[20,29,35,36] taurine[20,29,31] and aspartic acid.[20,29,35,36] Differences in absolute AA concentrations between patients with CD and healthy controls are depicted in Supplementary Table 5.

Compared with controls, faecal samples of patients with UC contained increased AA levels. The most consistently increased AA are depicted in Figure 3. Jagt and colleagues[29] showed that children with newly diagnosed UC could be correctly discriminated from controls based on 29 unique faecal AA, with an accuracy of 90%. Furthermore, levels of tryptophan, valine and histidine were significantly positively associated with more extended disease [pancolitis] in children with UC. Predictive values of faecal AA for diagnosis of UC are shown in Supplementary Table 4.

Regarding phenotype description, three studies[19,21,29] found no significant differences in faecal AA concentrations between children with newly diagnosed UC and CD. Kolho *et al.*[20] observed increased AA including asparagine, aspartate and 5-hydroxytryptophan [tryptophan derivative] in UC as compared to CD, based on a partial least squares discriminant analysis [PLS-DA].

3.4.3 Bile acids

Seven studies including 179 children with IBD [119 CD, 46 UC, four IBD-U, ten UC/IBD-U] and 145 controls [110 healthy, 21 siblings, 14 intention-to-diagnose cases] reported on faecal BA levels.[20,24,30,32–35] Most studies used LC-MS for BA quantification but often did not report on BA levels separately.

For IBD regardless of phenotype, two studies reported on significantly higher concentrations of primary BA in children with IBD compared to healthy controls[24,32] although it should be noted that one study comprised predominantly patients with CD[32] [20 CD, three UC, four IBD-U] and one predominantly patients with UC[24] [two CD, 16 UC] in their cohort. Bushman and colleagues[32] did not report on other differences in BA composition [outside the increased primary BA] in children with IBD compared to controls.

When focusing on CD phenotype in treatment-naïve children, overall total BA levels were comparable to those of healthy controls, according to two studies.[34,35] Primary BA levels [not specified] were, however, reported to be increased in three studies focusing on CD,[33–35] in agreement with data from studies focusing on IBD in general,[24,32] reported in children with both newly diagnosed and longer existing disease. One study found lower levels of secondary BA in children
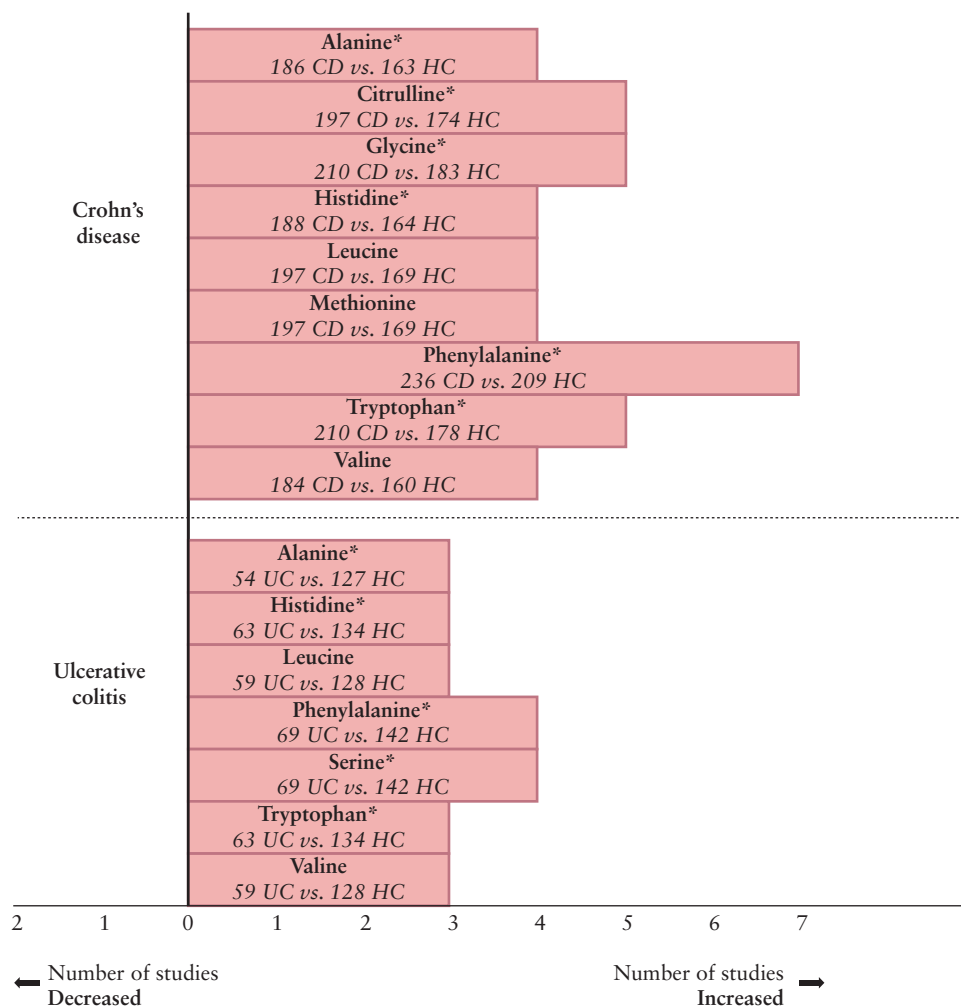
Figure 3. Findings on faecal AA in children with IBD vs controls [nine studies in Crohn's disease[19–21,29,31,33–36] and four studies in ulcerative colitis[19–21,29]. The *x*-axis represents the number of studies in which the AA were increased, in the IBD phenotype compared to controls. The *y*-axis represents the unique AA. Only AA that were increased in three or more studies were included in this figure.*The study by Kolho *et al.* did not test for significance.

with newly diagnosed CD, corresponding to the earlier reported higher primary BA, resulting in a lower secondary/primary BA ratio.[34] This study also showed higher levels of conjugated and lower levels of unconjugated BA in newly diagnosed, treatment-naïve children with CD compared to healthy controls.[34] Jacobs and colleagues[33] additionally found higher levels of sulphated derivatives of chenodeoxycholic as well as deoxycholic acid in children with CD in remission compared to their healthy siblings. In a study by Kolho *et al.*[20] using a PLS-DA variable importance plot for distinguishing disease vs healthy controls, BA levels were evaluated but were not distinctive for disease presence when compared to other metabolites.

Ejderhamn *et al.*[24] reported on an IBD cohort with predominantly patients with UC [16/18 UC; with 12/16 pancolitis and 4/16 distal colon involvement only], and found significantly increased concentrations of total BA in faeces of children with disease in remission compared to healthy controls. They also found significantly higher concentrations of unconjugated as well as glycine/taurine-conjugated BA compared to healthy controls.[24] Similar results were described by Rotondo *et al.*[30] in paediatric patients with UC and active disease vs controls. They found similar total BA levels, with increased primary and conjugated BA compared to healthy

controls, similar to the differences found between active CD and healthy controls.[30]

3.4.4 Other faecal metabolites

Nine articles reported on other metabolites in patients with IBD and controls,[20,25,26,31–36] which will be summarized below. Two studies[32,35] consisting of 70 children with IBD [63 CD, three UC, four IBD-U] and 56 healthy children demonstrated higher concentrations of cadaverine in faecal samples of patients with IBD. Cadaverine is a polyamine produced by decarboxylation of lysine. None of the other studies, however, reported on this individual metabolite. Diederen *et al.*[35] also found significantly higher levels of putrescine in *de novo*, treatment-naïve CD, putrescine being another polyamine related to cadaverine.

Amongst lipids, sphingomyelin and related metabolites, and a breakdown product named ceramide were found in higher concentrations in children with IBD[31,32] compared with healthy controls. In the study by Bushman *et al.*,[32] increased ceramide levels had the greatest impact on the random forest classifier for discriminating IBD from controls. Furthermore, Jacobs *et al.*[33] observed that stercobilin [a haem degradation product] and boldione [product of steroid catabolism] were decreased in children with IBD vs non-IBD controls. Kolho and

colleagues[20] observed higher faecal levels of glyceraldehyde in both *de novo* CD and UC compared to controls.

Regarding children with CD, L-lactate levels were significantly higher compared with controls in one study,[25] while the other study showed no difference.[34] Moreover, Wang *et al.*[34] observed that two organic acids—methylmalonic acid and succinic acid—were included in the ten most discriminative metabolites for prediction of CD from healthy children. The study by Sundqvist *et al.*,[26] consisting of children with active CD and healthy controls, was the only study that investigated the metabolites coprostanol and urobilinogen. These metabolites were decreased in patients with CD.

Kolho *et al.*[20] observed metabolites that could discriminate UC from CD, including increased concentrations of guanosine and γ-glutamylcysteine in patients with UC based on PLS-DA. By contrast, levels of pyridoxine, orotate and 4-pyridoxate were decreased in children with UC as compared to CD.

3.5 Faecal metabolomics related to disease activity

3.5.1 Short-chain fatty acids

Taylor *et al.*[16] found the branched SCFA valeric acid to be one of the principal discriminators in a machine-learning model when comparing high to low faecal calprotectin [FC] samples of children with CD [regardless of clinical disease activity], and correlated to 'mucosal healing', i.e. low calprotectin levels. Another study reported increased propionic acid levels at baseline to normalize in responders to exclusive enteral nutrition [EEN] [>50% reduction FC], but this normalization was not seen in non-responders.[35] Treem and colleagues[28] evaluated the differences in active (paediatric Crohn's disease activity index [PCDAI] > 10) and inactive CD but found no significant differences in SCFA levels between the two groups. In patients with moderate to severe UC [Truelove–Witts criteria], total SCFA, acetic, isobutyric and isovaleric acid were significantly reduced compared to healthy controls. In contrast, children with mild or inactive UC had comparable concentrations of total SCFA, acetate and propionic acid compared to healthy controls. By contrast, children with inactive–mild UC had higher faecal *n*-butyric acid concentrations than controls and patients with severe UC.[28]

3.5.2 Amino acids and their derivatives

Bosch *et al.*[21] found no significant correlations between faecal AA levels and inflammatory markers including FC and C-reactive protein [CRP] in children with IBD. Another study by Bosch *et al.*,[19] however, observed that citrulline, leucine, phenylalanine and valine were significant positively correlated with FC.

Regarding CD, Ni *et al.*[36] observed positive correlations between faecal AA and their derivatives, and FC. These correlations were reduced in patients with CD who used antibiotic treatment at the time of sampling. Taylor *et al.*[16] demonstrated that lysine was increased in children with CD [using biologic therapy] with high FC [≥ 100 µg/g] vs low FC [< 100 µg/g]. Three studies[20,21,29] found no significant correlations between FC and AA in children with *de novo*, treatment-naïve CD. Wang *et al.*[34] observed that several AA such as alanine, aspartic acid, glutamic acid, histidine, leucine, proline, phenylalanine and glycine were positively correlated with PCDAI and other serum indexes of inflammation.

In children with UC, one study[20] showed significant positive correlations between aminoadipic acid, taurine and methionine, and FC whereas the other two studies[21,29] showed no significant correlations between AA levels and disease activity.

3.5.3 Bile acids

One study[34] found glycochenodeoxycholic acid [conjugated primary BA] to be positively correlated with PCDAI, erythrocyte sedimentation rate, white blood cells and CRP in children with newly diagnosed CD. Deoxycholic acid, a secondary BA, correlated negatively with these inflammatory markers [measured by Spearman correlations].[34]

3.5.4 Other metabolites

In patients with IBD, the metabolite carnosine [a hybrid peptide] was significantly positively correlated with FC.[20] This metabolite, together with ribose-5-phosphate, choline and glyceraldehyde, was also positively correlated with FC in patients with UC only.[20]

Amongst CD, one study[20] found that allantoin was significant positively correlated with FC. Wang *et al.*[34] observed that two dicarboxylic acids [methylmalonic acid and succinic acid] and four organic acids [*cis*-aconitic acid, *trans*-aconitic acid, citric acid and isocitric acid] were positively correlated with PCDAI and serum inflammatory markers.

3.6 Faecal metabolomics for predicting treatment response

Three studies including 84 children with CD reported on faecal metabolites associated with response or non-response to therapy [EEN and biologic agents].

Faecal AA levels before initiation of EEN could not predict response to this nutritional therapy in children with *de novo*, treatment-naïve CD.[35] However, at the end of EEN, responders [based on reduction in FC of > 50%] had lower concentrations of serine, glycine and alanine as compared to non-responders.

Connors *et al.*[23] found that all children who did not sustain remission [weighted PCDAI < 12.5] from 12 to 24 weeks after EEN therapy had a primary BA dominant profile with a significantly higher percentage of primary BA contribution compared to patients with sustained remission. Interestingly, this primary BA dominance was not seen in patients who did not reach remission at week 12. Total BA concentration differences were not found between groups at each time point.[23] Diederen and colleagues[35] found no differences between BA concentration, BA hydrophobicity and the fraction of secondary BA at baseline between responders [>50% reduction in FC] and non-responders to EEN.

Wang *et al.*[34] investigated the relationship between faecal metabolomic profiles and therapeutic outcomes of infliximab [IFX] therapy in children with CD. They found higher faecal levels of glycine and the metabolites linoleic acid and L-lactic acid at baseline [prior to IFX treatment] in patients who achieved sustained remission as compared to non-sustained remission patients. Enriched metabolites in non-sustained remission patients [at baseline] included *N*-acetylserotonin, methylglutaric acid, adipic acid, 4-aminohippuric acid, citramalic acid, isovaleric acid, nicotinic acid, pentadecanoic acid and *N*-acteylglutamine.

4. Discussion

This systematic review provides a summary of the available evidence on faecal metabolomics in children with IBD vs healthy controls, with the aim of characterizing a metabolomic signature in paediatric IBD, both for disease activity and for treatment response. All included studies observed distinct metabolomic profiles in children with IBD compared to controls. However, based on the available data, no unique faecal metabolomic signature could be defined due to inconsistency of individual study results and lack of standardization of metabolomics assessment methods. The majority of studies reported on higher faecal AA levels in IBD as compared to controls. Additionally, most studies on BA observed higher levels of primary and lower levels of secondary BA in IBD, while contradictory results were found for SCFA. Although studies on metabolomics linked to therapy response were limited, significant differences in baseline faecal metabolites [BA and AA] between responders and non-responders to EEN and IFX have been described.

The most consistent findings were higher levels of faecal AA in children with IBD as compared to controls, yet this also was the most studied metabolite class. Phenylalanine—an essential aromatic amino acid—was significantly increased in CD, in UC and in both phenotypes combined in the majority of studies. Only one study demonstrated no significant difference in phenylalanine concentrations in CD vs controls, which could be explained by their small sample size [five CD, eight controls].[19] These findings correspond with the results of a recent systematic review on metabolomic analysis in adults with IBD,[42] which showed higher levels of AA, including branched-chain AA, phenylalanine, glycine, tyrosine, alanine and taurine in IBD compared with non-IBD controls.

The use of omics-techniques in IBD has been rising in the hope of reaching a more mechanistic and functional understanding of the disease. Lloyd-Price and colleagues[7] reported on their multi-omics analysis of gut microbial ecosystems using stool, biopsies and blood samples in a cohort of 132 subjects with IBD comprising 61 children from different hospitals. They showed characteristic changes of the microbiome, and disruptions in microbial transcription, serum antibody levels and metabolite pools. Gallagher and colleagues[42] conducted a systematic review describing metabolomic analysis in urine, stool and serum from adults with IBD. Reagrding stool metabolomics results, the authors described lower concentrations of SCFA [namely acetate, propionate and butyrate] compared to controls. This observation was similar to the results we found in children, but was not consistent across all studies.[25,28,35] Interestingly, decreased levels of both primary and secondary BA have been described in adult IBD, although primary BA findings were not consistent across studies.[7,43] We found increased levels of primary BA in children with IBD compared to controls, consistent with the pathophysiological between the dysbiotic microbiome and BA.[44,45]

The metabolome is closely related to the microbiome in that it mainly consists of end products of microbial metabolism. Hence, shifts in microbiome composition, i.e. dysbiosis, also affect faecal metabolite composition including AA, SCFA and BA.[13] Aromatic AA such as phenylalanine, tryptophan and tyrosine are substrates for a pathway from the gut symbiont *Clostridium sporogenes* [phylum Firmicutes], generating aromatic amino acid metabolites. These metabolites affect intestinal permeability and systemic immunity.[46] Additionally,

tryptophan is partly metabolized by gut bacteria into indole metabolites which can serve as aryl hydrocarbon receptor [AhR] ligands.[47] This receptor is considered to play a role in IBD pathogenesis, as shown by Lamas *et al.*[48]They observed that the gut microbiota of *Card9*−/− [an IBD susceptibility gene] mice were unable to metabolize tryptophan into AhR ligands. This inability resulted in a defective interleukin 22 [IL-22] activation and consequently led to increased sensitivity to colitis.[48] It is important to note that higher faecal AA concentrations might hypothetically also result from intestinal malabsorption or protein loss through colonic leakage, rather than reflecting microbial changes.[49]

SCFA are another metabolite class known to interact with the gut microbiota. In this review, differences in SCFA concentrations were observed in children with IBD compared to controls. Specifically, decreased levels of acetic and valeric acid were found in CD, comparable to the results of a meta-analysis on SCFA characterization in adults with CD.[50] SCFA are predominantly produced by bacterial anaerobic fermentation of dietary fibres in the intestine, making microbiome composition crucial in the production of SCFA.[51] SCFA have been considered metabolites of interest due to their role as a primary energy source of colonocytes and immune homeostasis. Immunomodulation by SCFA can occur by regulation of regulatory T cells, activation of G protein-coupled receptors, inhibition of histone deacetylase and other described mechanisms.[52–54] Butyrate, one of the predominant SCFA, has also been described to restore epithelial barrier function by activating genes coding for tight junction components.[55] Butyrate oxidation accounts for more than 70% of the oxygen consumed by human colonic tissue.[56] Depletion of SCFA therefore also leads to a decrease in available colonocyte energy and subsequent damage to intestinal epithelial cells. This has also been hypothesized to be the cause of diversion colitis, in which inflammation could be successfully treated with SCFA enemas.[57]

Butyrate production is often mediated by members of the phylum Firmicutes, while Bacteroides mediate propionate and acetate production.[58,59] Dysbiosis in IBD has commonly been characterized by a lower abundance of certain SCFA producers, such as *F. prausnitzii* and *R. hominis*, and therefore lead to altered SCFA levels in IBD patients.[60] However, IBD-specific SCFA signatures have not been defined so far, although conclusions seem to depend on clinical activity of subject samples.[50] Faecal concentrations of SCFA are not only determined by microbiome composition but also by the type and amount of dietary intake. However, increased metabolic capacity of SCFA synthesis and increased butyrate levels at baseline have been shown to be important in maintaining anti-tumour necrosis factor [anti-TNF] and azathioprine remission in adults.[15,61]

Bile acids have been described to have different immunomodulatory functions, mainly by interaction with different host BA receptors.[62,63] Bile acids are synthesized in the liver and further deconjugated and dehydroxylated by microbial species in the intestine.[45] Changes in the gut bacterial community can thus alter BA composition, as has been described in IBD.[44] In this review, we found significantly higher concentrations of primary BA in patients with IBD [specifically CD] as compared to controls in five studies.[24,32–35] Bile acids, in particular secondary BA, have been shown to modulate intestinal immune responses.[44,64] The increased concentrations of primary BA could at least partly be explained by an

impaired BA absorption in the distal ileum due to inflammation in patients with CD.[65] However, this observation could also be explained by the absence of microbial species with the ability for dehydroxylation [rather than deconjugation], which is a key step in primary to secondary BA conversion in the intestine.[23] Duboc *et al*.[44] observed significantly higher faecal-conjugated BA rates and significantly lower secondary BA rates in colonic IBD compared to controls. These authors also showed that dysbiosis in IBD results in BA dysmetabolism characterized by impaired deconjugation, transformation and desulphation.[44]

Amongst lipids, two of the included studies observed higher levels of sphingomyelin and related metabolites, and the breakdown product ceramide in paediatric IBD compared with controls. An enrichment in sphingolipids, particularly in ceramide and sphingomyelin, has been previously identified in stool of adults with IBD compared to controls.[66] Sphingolipids are crucial components of intestinal membranes and are involved in a series of cellular actions such as cell differentiation, proliferation, migration and apoptosis, all contributing to a healthy gut.[67] Additionally, sphingolipids are produced by commensal bacteria belonging to the phylum Bacteroidetes, and it has been observed that bacterial and host sphingolipid pathways are dysregulated during inflammation and IBD.[68]

Regarding treatment response, we found that a faecal primary BA-dominant profile at baseline has been associated with non-responders to EEN treatment. Furthermore, levels of several metabolites including multiple AA were found to be correlated with the response to IFX therapy in children with CD. In adult IBD studies, faecal BA, lipids and SCFA have been demonstrated to predict response to therapy with biologicals.[69] Ding *et al*.[17] observed that lower faecal levels of histidine [AA] and deoxycholic acid [secondary BA] are predictive of primary non-response to anti-TNF therapy in adults with CD. To date, only limited evidence based on small cohorts of paediatric patients with IBD is available and thus external validation in larger cohorts of patients is needed. If these potentially predictive metabolic features could be validated, this would contribute significantly to the development of personalized treatment strategies.

To our knowledge, this is the first systematic review describing faecal metabolomics in paediatric IBD. However, there are several limitations to the identified studies and therefore to this systematic review. The included studies had small patient groups [12 out of 17 had fewer than 50 patients in total] and only a minority had age- and sex-matched controls. Studies differed in subject disease activity, treatment-naivety, stool consistency, sample handling, sample time points, diet and type of controls. Stool consistency [i.e. stool water content] reflects GI transit time and can severely impact the stool microbiome and metabolome.[70,71] However, none of the studies took this into account when reporting their analyses. This is important to note when comparing children with IBD—often presenting with diarrhoea—to healthy controls with 'normal' stool consistency. Nutritional status also influences the stool metabolome, as we know that specific dietary products can influence the gut, target the microbiome, and subsequently alter metabolism and thus its metabolic readout. However, none of the included studies collected detailed information on dietary intake. Ideally, only newly diagnosed, treatment-naïve children would have been included to ensure the most unbiased metabolomic signatures, as different treatment strategies in

IBD have been shown to influence metabolomic measurements of patients with IBD.[72] All these factors inhibit identification of metabolomic signatures, especially for important subgroups such as active disease vs remission and response to therapy over time.

The lack of standardization in metabolomics analysis between studies should be considered. There was heterogeneity in the methods of metabolomics assessment, including the approach [targeted or untargeted] and techniques [NMR vs MS]. The use of NMR is often hampered by a relatively low sensitivity as compared to MS methods,[73] which leads to differences in faecal metabolites that could be detected and quantification of the faecal metabolites across a number of studies. Even when similar methods were used, not all results were reported in the same manner [absolute vs relative, ratios, selective metabolites only]. Sample handling is particularly important when performing microbiome and metabolome analyses.[74] Hence, different sample [handling and analysis] protocols can influence the results, making it impossible to correct for this variation especially when comparing between different studies.

Future studies should therefore comprise larger sample sizes with standardized time points and disease activity markers, using the same methodologies and analytical techniques for metabolomics interpretation in order to obtain consistent results and allow firm conclusions to be drawn. Additionally, further validation in independent cohorts is recommended to develop new metabolic biomarkers for diagnostics, and prediction of disease course and of treatment response in paediatric IBD. Available evidence has shown that faecal metabolomics provide valuable information on the metabolome and inherently the activity of the gut microbiota, which are influenced by host genetics and environment. Multi-omics studies, including microbiome analysis, should therefore be performed to improve knowledge on the host–microbial interactions in the disease pathogenesis of paediatric IBD.

In conclusion, faecal metabolomic profiles in paediatric IBD differ from healthy controls, particularly in AA. Moreover, distinct metabolic profiles for AA and BA were observed between responders and non-responders to EEN and IFX at baseline. However, the inconsistency of metabolic differences identified across studies and selective reporting hamper the determination of a clear metabolomic signature in paediatric IBD compared to controls, and response to treatment. This highlights the importance of further research with standardized methodologies in order to generate more consistent findings and gain more insight into metabolic alterations in IBD. Defining disease-associated signatures might contribute to the development of novel, non-invasive, personalized diagnostic and even therapeutic approaches in paediatric IBD.

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Conflict of Interest

Author Contribtions

Data Availability Statement

The data underlying this article are available in the article and in its online supplementary material.

Supplementary Data

Supplementary data are available online at *ECCO-JCC* online.

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