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Original Article

Systematic Review and Meta-analysis of Peripheral Blood DNA Methylation Studies in Inflammatory Bowel Disease

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Abstract

Background and Aims: Over the past decade, the DNA methylome has been increasingly studied in peripheral blood of inflammatory bowel disease [IBD] patients. However, a comprehensive summary and meta-analysis of peripheral blood leukocyte [PBL] DNA methylation studies has thus far not been conducted. Here, we systematically reviewed all available literature up to February 2022 and summarized the observations by means of meta-analysis.

Methods: We conducted a systematic search and critical appraisal of IBD-associated DNA methylation studies in PBL using the biomarker-based cross-sectional studies [BIOCROSS] tool. Subsequently, we performed meta-analyses on the summary statistics obtained from epigenome-wide association studies [EWAS] that included patients with Crohn's disease [CD], ulcerative colitis [UC] and/or healthy controls [HC].

Results: Altogether, we included 15 studies for systematic review. Critical appraisal revealed large methodological and outcome heterogeneity between studies. Summary statistics were obtained from four studies based on a cumulative 552 samples [177 CD, 132 UC and 243 HC]. Consistent differential methylation was identified for 256 differentially methylated probes [DMPs; Bonferroni-adjusted $p \leq 0.05$] when comparing CD with HC and 103 when comparing UC with HC. Comparing IBD [CD + UC] with HC resulted in 224 DMPs. Importantly, several of the previously identified DMPs, such as VMP1/TMEM49/MIR21 and RPS6KA2, were consistently differentially methylated across all studies.

Conclusion: Methodological homogenization of IBD epigenetic studies is needed to allow for easier aggregation and independent validation. Nonetheless, we were able to confirm previous observations. Our results can serve as the basis for future IBD epigenetic biomarker research in PBL.

Key Words: DNA methylation; epigenetics; inflammatory bowel diseases; biomarkers; systematic review and meta-analysis

1. Introduction

Inflammatory bowel diseases [IBD], including Crohn's disease [CD] and ulcerative colitis [UC] are chronic, relapsing and remitting inflammatory disorders affecting an estimated 3 million patients in the USA and Europe alone.[1,2] While the exact aetiopathogenesis of IBD remains unknown, current consensus suggests a dysregulated immune response to specific alterations in the microbial composition of genetically susceptible patients that either are triggered or caused by environmental factors.[3] While numerous genome-wide association studies [GWAS] have been performed, the results thereof explain only part of the heritability observed in familial studies with high rates of discordance observed in monozygotic twins.[4–10] Moreover, a rapid increase in the incidence of IBD has been observed in newly industrialized countries, which is thought to be the result of the increasing adoption of a western lifestyle.[1] Together, these observations suggest an important role for gene–environment interactions in the aetiopathogenesis of IBD.

Epigenetics represent mitotically heritable mechanisms that affect the readability of the genome without changing the actual genetic sequence itself. Epigenetic modifications can regulate transcription and could therefore mediate gene–environment interactions. Epigenetic mechanisms are particularly interesting in complex multifactorial diseases, such as IBD, due to their dynamic interaction with the environment.[11] One of the most widely studied epigenetic mechanisms is DNA methylation, representing a methyl group covalently

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bound to the cytosine of a cytosine–guanine dinucleotide [CpG]. The presence of DNA methylation in gene promoters is often inversely correlated with gene expression.[12–14] Due to the growing popularity of epigenetics, an increasing number of studies have sought to understand the role of DNA methylation in IBD pathogenesis and aetiology, which was further strengthened by the discovery of an IBD-associated genetic variant associated with *DNMT3A*, a key *de novo* methylation enzyme.[15] Accordingly, numerous differentially methylated positions [DMPs], regions [DMRs] or genes [DMGs] associated with various IBD phenotypes across different tissues have been identified in both paediatric and adult cohorts.[16–24] Together, these studies make a case for using DNA methylation in classifying IBD phenotypes,[20,25,26] thereby supplementing clinical care with potential novel drug and biomarker targets.

Despite the numerous studies available to date, translation to clinical practice has not occurred. All studies report different sets of biomarkers with different levels of statistical power. Moreover, many studies lack information on patient characteristics that are known to be associated with changes in methylation, such as smoking behaviour.[27] To adequately understand the observations thus far, various attempts have been made to summarize the DNA methylation studies on IBD.

The most recent meta-analysis of IBD-associated epigenetic studies focused on DNA methylation differences in mucosal biopsies of both paediatric and adult IBD patients, including six separate datasets, yet a similar approach in peripheral blood was not performed.[28] Additionally, four studies reviewed the available DNA methylation literature in paediatric and adult IBD patients,[29–32] but did not perform a meta-analysis. Since 2014, the available literature on DNA methylation and IBD has further expanded as a result of the dropping costs of both microarray- and sequencing-based technologies, while the latter have become increasingly capable of covering larger numbers of cytosines. With the ever-increasing pool of data in peripheral blood, the opportunity to combine these data, thereby increasing statistical power, provides a potential to guide future methylation studies in IBD. We therefore sought to summarize the available peripheral blood DNA methylation data by systematically reviewing all currently available literature on peripheral blood leukocyte [PBL] DNA methylation in IBD and performing a meta-analysis on the available Illumina HumanMethylation BeadChip 450k data.

2. Methods

The methodological protocol implemented in this study was registered on PROSPERO [ID: CRD42020176655].

2.1. Systematic review

A literature search was performed on February 1, 2022 using the PubMed/MEDLINE, EMBASE [OVID], Cochrane library and CINAHL [ebsco] databases with the help of a scientific librarian at the Amsterdam University Medical Center. Main search criteria were 'Crohn's disease', 'Inflammatory Bowel Diseases', 'colitis' and 'DNA methylation'. *In vitro* and animal studies were excluded from this search. A detailed description of our search strategy can be found in the Supplementary information file. Screening and eligibility-based assessment was conducted separately by V.J. and I.L.H. adhering to pre-defined inclusion and exclusion criteria [Table 1] using the web application RAYYAN.[33] Conflicts were resolved by A.Y.F.L.Y., whereupon eligible studies were read in full. A pre-specified data extraction form was then used to extract data from the included studies for assessment of study quality and a synthesis of evidence. Extracted information included author, year of publication, study population, sample size, patient demographics, type of assay, study design [targeted or whole-genome] and, if applicable, which CpGs of interest were analysed.

To assess the quality of the selected articles, we used a recently developed quality assessment tool specifically designed for biomarker-based cross-sectional studies [BIOCROSS].[34] The BIOCROSS tool originally included ten items covering five domains: 'Study rational', 'Design/Methods', 'Data analysis', 'Data interpretation' and 'Biomarker measurement', aiming to assess different features of biomarker cross-sectional studies. For the purpose of this systematic review, we adapted the BIOCROSS tool for use in studies aimed at assessing DNA methylation [Supplementart information]. An overview of the entire process is presented in Figure 1.

2.2. Meta-analysis

For the meta-analysis, we acquired published data from studies that had been conducted in a genome-wide fashion using the Illumina HumanMethylation 450k or EPIC BeadChip array. As the analytical design could only utilize overlapping CpGs, including the Illumina HumanMethylation 27k BeadChip array would discard ~94% of the 450k probes, which we deemed excessive. Accordingly, we decided against

Table 1. Inclusion and exclusion criteria

Inclusion criteria
1.Any prospective or retrospective study measuring peripheral blood leukocyte DNA methylation in both paediatric or adult IBD patients [cohort, case-control, RCT]
2.Epigenome-wide association studies [EWAS] and targeted DNA methylation study designs
3.DNA methylation analysis performed on peripheral whole blood or specific peripheral blood cell types
4.Studies comparing DNA methylation between Crohn's disease and ulcerative colitis or comparing either disease with healthy controls
Exclusion criteria
1.Studies using intestinal tissue samples
2.Studies investigating DNA methylation markers primarily in patients with cancer or pre-stages of cancer
3.Studies with fewer than five included patients per phenotype
4.Case reports, narrative reviews, *in vitro* studies [e.g. using cell lines], studies of genetic [rather than epigenetic] mutations or animal studies

including studies that had performed analyses using the Illumina HumanMethylation 27k BeadChip array. We specifically sought to acquire summary statistics to stay as close as possible to the interpretation of the original authors, where we sought to mitigate publication bias by acquiring the full summary statistics from both significant and non-significant CpGs. If the full summary statistics were unavailable, even after request, processed data was downloaded from the Gene Expression Omnibus [GEO], imported into the R statistical environment [v4.1] and processed per the methods outlined in the original publication. However, if the self-generated results were inconsistent with the results outlined in the original publication, the data were discarded. This restriction confined the analyses only to data obtained from the HumanMethylation 450k BeadChip array [Table 2]. Annotation of the CpGs to their location and genes was performed using the annotation file provided by Illumina [HumanMethylation450 v1.2 Manifest] as imported using minfi.[35] If no annotated gene was found among the top 20 DMPs per comparison, a manual search was conducted using

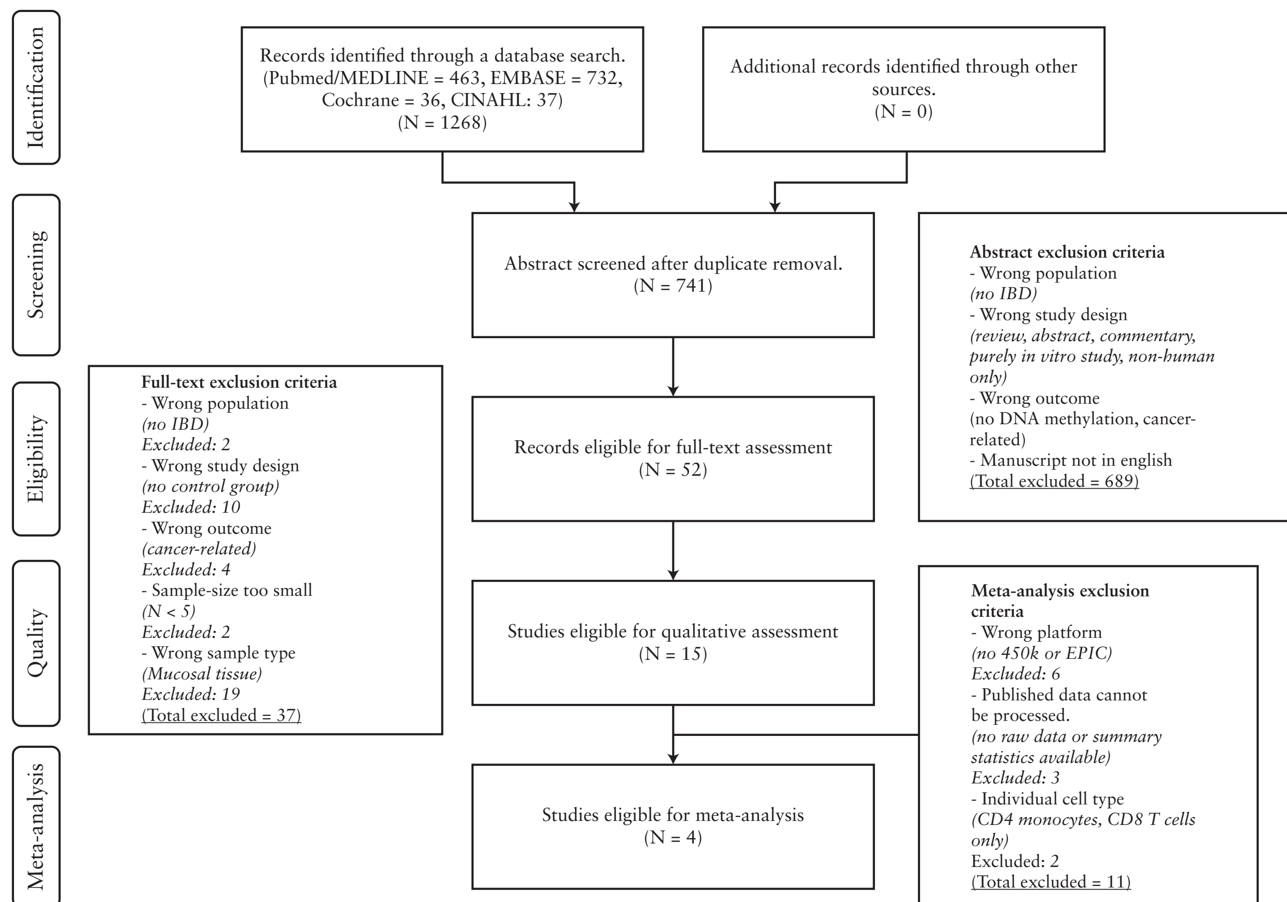

Figure 1. Study selection. Studies obtained through database searches were initially screened based on abstract exclusion criteria, followed by full-text exclusion criteria and final meta-analysis exclusion criteria.

Table 2. Overview of the datasets included in the meta-analysis

	Samples	Array	Accession ID
Harris *et al*. 2012[36]	17 CD—PBL 11 UC—PBL 14 HC—PBL	450k	GSE32148
Adams *et al*. 2014[18]	35 CD—PBL 36 HC—PBL	450k	NA*
Li Yim *et al*. 2016[37]	15 CD—PBL 25 HC—PBL	450k	GSE81961
Ventham *et al*. 2016[20]	121 CD—PBL 119 UC—PBL 191 HC—PBL	450k	GSE87650

CD, Crohn's disease; UC, ulcerative colitis; HC, healthy controls; PBL, peripheral blood leukocytes.
*Data acquired following request.

the UCSC genome browser [hg19] to identify the nearest gene. No specific selection for promoter-bound CpGs was applied. All genes have been annotated using the HUGO nomenclature.

The meta-analysis was subsequently performed by utilizing the framework outlined by Choi *et al.*[38] and the GeneMeta package.[39] In short, we calculated the standardized effect size [SES], as represented by Cohen's *d* and Hedges' *g*, and variance from the *t*-statistic per probe per study. Heterogeneity between the studies in effect size was quantified using the Cochran Q-test and corrected for by including the Cochran Q-derived moments estimator τ^2 as a random effects in the subsequent random-effects model to combine the standardized effect into the unbiased estimator of effect size. Two-sided Z-tests were then performed to calculate the *p*-value per CpG, after which the resultant *p*-values were corrected using the Bonferroni correction where CpGs presenting a Bonferroni-adjusted *p*-value <0.05 were considered to be statistically significantly differentially methylated positions [DMPs].

For the differential methylated gene [DMG] analysis, we sought to investigate whether particular genes were enriched for low *p*-values. To this end, the meta-analysis *p*-values for CpGs annotated to a particular gene per the provided annotation obtained from Illumina's annotation file were combined using Fisher's method for combining *p*-values as implemented in the aggregation package.[40] The resulting combined *p*-values were then corrected using the false discovery rate [FDR] where we defined FDR-adjusted *p*-values <0.05 as statistically significant. The resultant DMGs were then subjected to gene ontology [GO][41,42] overrepresentation analysis using the clusterProfiler package,[43] where we considered an FDR-adjusted *p*-value <0.05 as a statistically significant difference.

3. Results

3.1. Study selection and quality appraisal

A total of 741 records were screened for eligibility according to our pre-defined inclusion criteria [Table 1], after which 15 studies were included for full text systematic review [Figure 1 and Supplementary Table 1] and qualitative assessment using our modified BIOCROSS tool for critical appraisal [Table 3].

Systematically reviewing all 15 studies indicated that 14 studies provided a clear rationale for studying DNA methylation in relation to IBD pathogenesis [domain 1]. Twelve studies presented a comparable case and control group [domain 2] and 13 provided a concise summary of the clinical characteristics [domain 4]. In addition, 11 studies described the laboratory procedures used in their study in detail [domain 8]. Finally, 12 contextualized the observed differences in DNA methylation with respect to existing literature, thereby providing insight into the functional implications [domain 6]. By contrast, we noted that many studies lacked important methodological aspects of a DNA methylation study design. First, key confounding factors [domain 5] were not reported on in five studies, with the most frequently missing factors being smoking behaviour [four studies] and whether or not the data were corrected for the cellular distribution [five studies]. Similarly, sample preservation, quality control and any potential batch effects were not reported on [domain 9]. Second, only five studies justified their sample size [domain 3]. Third, in six studies neither technical nor independent validation had been performed [domain 5]. Lastly, the associative nature of DNA methylation studies limits causal inference, which is only addressed in two studies [domain 7]. A summary of the quality appraisal for each of the nine domains per study is provided in Table 3.

3.2. Comparing Crohn's disease with healthy controls

The CD with HC comparison represents the most investigated comparison in PBL [$N_{studies}$ = 10][18,20,22,26,36,37,46–49] and its constituents, such as peripheral blood mononuclear cells [PBMCs] [$N_{studies}$ = 3],[23,44,45] CD14+ monocytes [$N_{studies}$ = 2],[17,20] CD4+ T-cells [$N_{studies}$ = 1][20] and CD8+ T-cells [$N_{studies}$ = 2].[20,50] Most studies sampled from an adult population,[20,22,23,37,44,46–48] whereas a minority of studies sampled from a paediatric cohort.[18,26,36,49,50] Overall, the studies can be categorized as either epigenome-wide [$N_{studies}$ = 11] or targeted [$N_{studies}$ = 4].

From four epigenome-wide association studies [EWAS] we were able to acquire full summary statistics, which was based on a combined 177 CD patients and 243 HC individuals.[18,20,36,37] Meta-analysis thereon identified 256 consistently differentially methylated positions [DMPs] with a Bonferroni-adjusted *p*-value <0.05 that displayed a difference between CD and HC consistent across all studies [Figure 2a and Supplementary File a]. Among the top ten consistent DMPs, nine were annotated to known genes of which three [*FKBP5*, *BCL3* and *NLCR5*] were hypomethylated, while the remaining six [*TRAF1*, *CDC42BPB*, *BAHCC1*, *LYN*, *TOLLIP* and *KCNAB2*] were hypermethylated among CD patients compared to healthy controls [Figure 2b and Supplementary Table 2]. Expectedly, two of the nine genes [*CDC42BPB*[20] and *TOLLIP*[18]] were discussed in the included studies. While the genes *FKBP5*,[51,52] *BCL3*,[53,54] *TRAF1*,[55] *BAHCC1*,[26] *NLRC5*[56] and *KCNAB2*[20] were not specifically highlighted in the included studies, their association with inflammation or IBD has been described before. For example, DMPs cg25114611, cg26470501, cg15551881 and cg26599989 associate with *FKBP5*, *BCL3*, *TRAF1* and *TOLLIP*, respectively, all of which are involved in NF-κB-signalling, underlining its importance in IBD pathogenesis.[57–63] Furthermore, increased *FKBP5* expression levels correlated with CD disease duration and endoscopic scoring [CDEIS] as well as were able to classify endoscopically active from non-active CD.[51,52] Decreased expression of the transcriptional coactivator BCL3 has been identified as a novel risk factor for CD.[53] In addition, *Bcl3*$^{-/-}$ deficient mice were found to be less sensitive to DSS-induced colitis,[64] and mucosal tissue expression of *BCL3* was strongly elevated in active UC as well as active/inactive CD.[54] Significantly increased *TRAF1* expression has been demonstrated in IBD tissue vs controls, as well as inflamed compared with non-inflamed tissue,[55] while decreased levels of the TOLLIP protein have been found in mucosal tissue when comparing active UC and CD with HC,[54] potentially resulting in a decreased inhibition of the NF-κB/JNK/MAPK signalling pathways. By contrast, *LYN* [cg02508743] has thus far not been associated with IBD. The encoded protein belongs to the family of tyrosine kinases known to function in cell-surface receptor signalling with particular importance in innate/adaptive immune responses, haematopoiesis, response to growth factors and cytokines, and integrin signalling.[65] *Lyn*$^{-/-}$ mice have been shown to be highly susceptible to T-cell (increased interferon-γ [IFNγ] production in CD4+ and CD8+ T-cells) dependent DSS-induced colitis which correlated with dysbiosis.[66] Moreover, the reverse has been observed in Lyn^{up}

Table 3. Quality assessment

Quality assessment	Study rationale	Design/Methods		Data analysis		Data interpretation		Biomarker measurement		Quality score
	1. Hypothesis/ Objective	2. Study population selection	3. Study population representativeness	4. Study population characteristics	5. Statistical analysis	6. Interpretation and evaluation of results	7. Study limitations	8. Specimen characteristics and assay methods	9. Laboratory measurement	
Balasa *et al*. 2010[44]	3	2	0	1	1	0	0	1	0	8
Harris *et al*. 2012[36]	3	2	0	1	3	2	1	1	3	16
Nimmo *et al*. 2012[22]	3	2	1	1	2	2	0	1	2	12
Kim *et al*. 2012[45]	3	1	0	0	2	2	1	1	1	11
Adams *et al*. 2014[18]	3	3	1	1	4	2	2	2	3	21
Karatzas *et al*. 2014[46]	3	2	0	1	2	2	1	1	1	13
Li Yim *et al*. 2016[37]	3	2	0	0	3	2	2	2	2	16
McDermott *et al*. 2016[23]	3	3	0	1	4	2	2	1	3	19
Ventham *et al*. 2016[20]	2	3	1	1	4	2	3	1	3	20
Klasic *et al*. 2018[47]	3	3	1	1	3	2	0	2	1	16
Moret-Tatay *et al*. 2019[48]	3	2	0	1	1	2	1	2	3	15
Somineni *et al*. 2019[26]	3	3	1	1	3	1	2	1	3	18
Li Yim *et al*. 2020[17]	2	1	0	1	2	2	1	2	2	13
Samarani *et al*. 2020[49]	3	2	0	1	2	1	1	1	1	12
Gasparetto *et al*. 2020[50]	3	3	0	1	4	2	2	1	3	19

Quality items were scored as completely present [green], completely absent [red] or partly present [yellow]. The final quality score is an addition of all items present. All nine items were interpreted as defined by the modified version of the BIOCROSS tool [Supplementary Table 2].

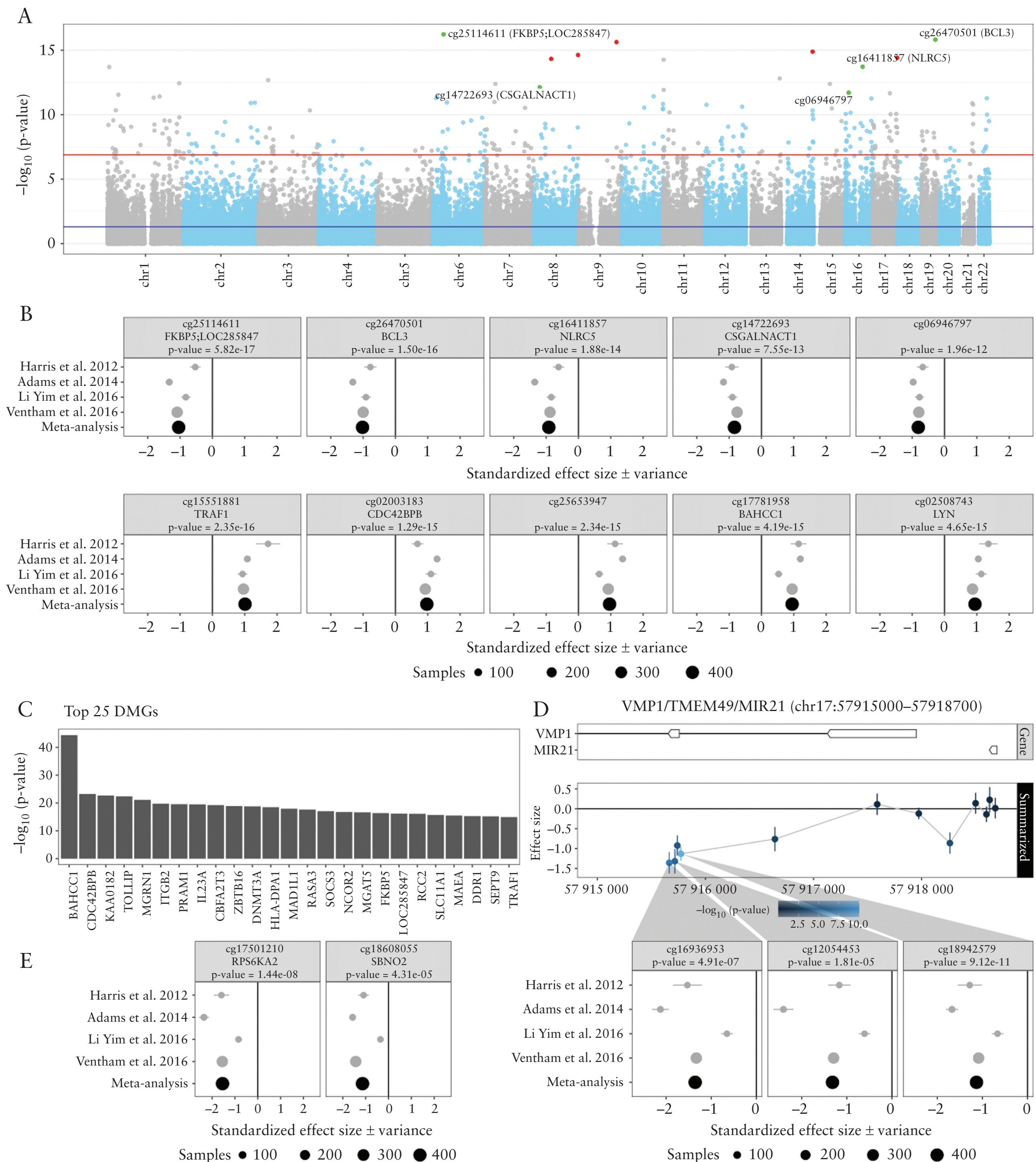

Figure 2. Meta-analysis of the comparison Crohn's disease [CD] with healthy controls [HC] using data from Harris *et al.* 2012, Adams *et al.* 2014, Li Yim *et al.* 2016 and Ventham *et al.* 2016. [A] A Manhattan plot representing the statistical significance depicted as $-\log_{10}$[*p*-value] on the *y*-axis relative to the location across the genome [hg19] on the *x*-axis. Red and green dots represent the five most significantly hyper- and hypomethylated differentially methylated probes [DMPs], respectively. [B] Forest plots representing the standardized effect size [SES] of interest per study of the five most significantly hyper- and hypomethylated DMPs annotated with the associated gene and *p*-value from the meta-analysis. [C] Barchart representing the top 25 most significant differentially methylated genes [DMGs]. The length of the bar is proportional to the $-\log_{10}$[*p*-value] obtained from the DMG analysis. [D] Genomic visualization and forest plots of the estimated difference in methylation for the DMPs cg16936953, cg12054453 and cg18942579, all of which have been associated with VMP1/TMEM49/MIR21. [E] Forest plots of DMPs cg17501210 and cg18608055, associated with RPS6KA2 and SBNO2, respectively, which had been reported on in multiple earlier studies.

mice, which presented a protective response during inflammation.[67] Additional functional information for genes annotated to the top 20 DMPs can be found in Supplementary Table 2.

Turning our attention to genes that were enriched for significant DMPs yielded 326 statistically significant DMGs. From the top 20, ten were previously identified within

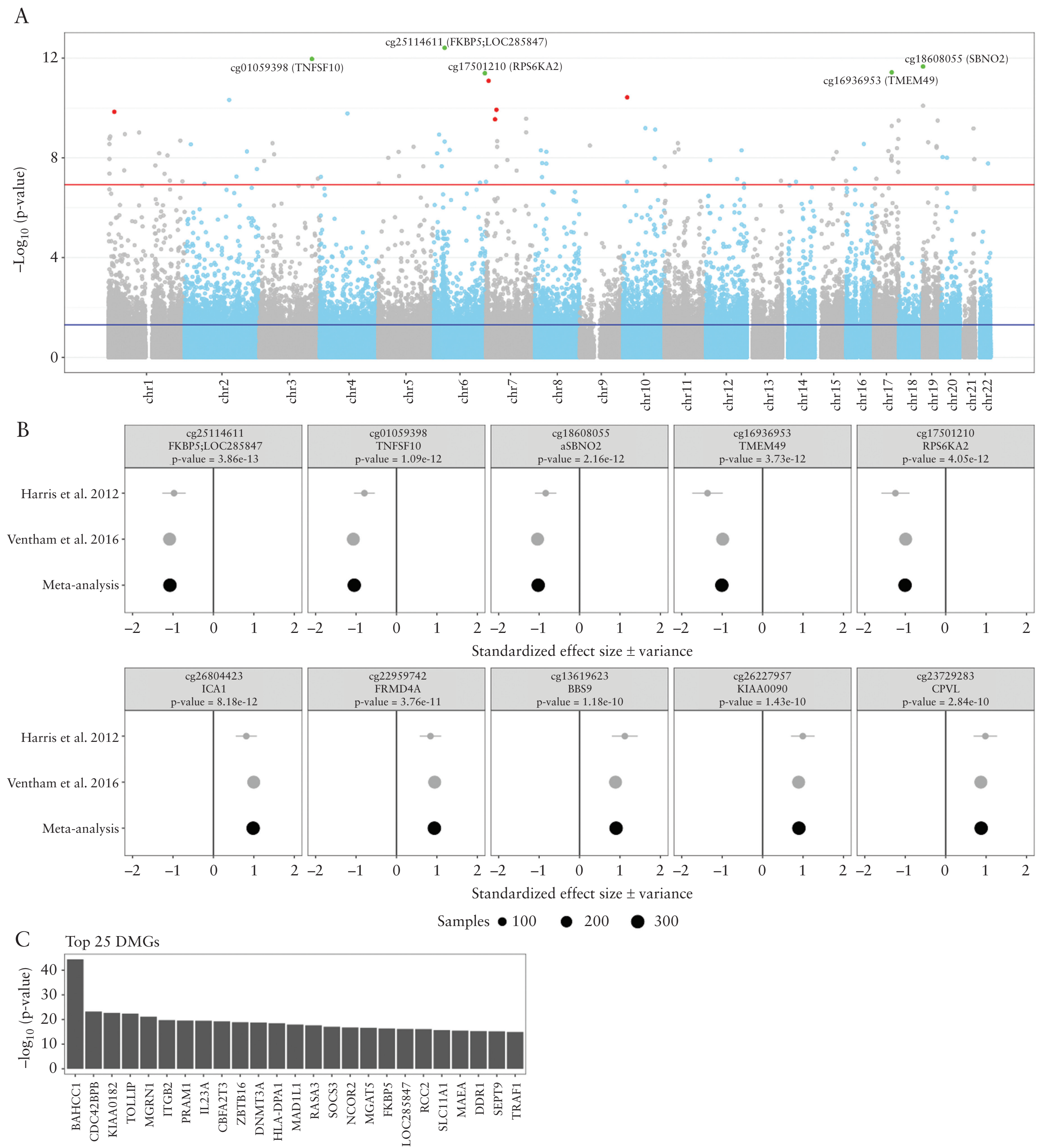

Figure 3. Meta-analysis of the comparison ulcerative colitis [UC] with healthy controls [HC] using data from Harris *et al.* 2012 and Ventham *et al.* 2016. [A] A Manhattan plot representing the statistical significance depicted as $-\log_{10}$[*p*-value] on the *y*-axis relative to the location across the genome [hg19] on the *x*-axis. Red and green dots represent the five most significantly hyper- and hypomethylated differentially methylated probes [DMPs], respectively. [B] Forest plots representing the standardized effect size [SES] of interest per study of the five most hyper- and hypomethylated DMPs annotated with the associated gene and *p*-value from the meta-analysis. [C] Barchart representing the top 25 most significant differentially methylated genes [DMGs]. The length of the bar is proportional to the $-\log_{10}$[*p*-value] obtained from the DMG analysis.

the context of CD [*MGRN1*,[36] *ITGB2*,[20,23,68] *PRAM1*,[26,69] *IL23A*,[70] *CBFA2T3*,[71] *ZBTB16*,[72,73] *DNMT3A*,[15] *HLA-DPA1*,[18] *MAD1L1*,[74] *SOCS3*[73,75]] [Figure 2c, Supplementary Table 3 and File b]. Of particular interest is *RASA3*, a gene that to our knowledge has previously not been associated with IBD. *RASA3* encodes RAS P21 Protein Activator 3, which is a negative regulator of the Ras signalling pathway through stimulation of GTPase.[76] *In vitro RASA3* depletion of cultured endothelial cells was found to increase β1 integrin activation and cell adhesion to extracellular matrix components, decrease cell migration and block tubulogenesis.[77] In addition, *RASA*-depleted cells showed reduced turnover of vascular endothelial cadherin-based adhesions, resulting in more stable endothelial cell–cell adhesion and decreased endothelial

permeability.[77] Differential methylation of *RASA3* could thus potentially alter endothelial–leukocyte adhesions, known to be of major importance for gut homing of inflammatory cells in IBD, targeted by drugs such as vedolizumab.[78] By performing overrepresentation analyses of the identified DMGs against the GO resource, we identified significant overrepresentation for 36 GO terms. Specifically, overrepresentation was observed for immune response [GO:0002683, GO:2000181, GO:0002683, GO:2000181, GO:0002366, GO:0050727, GO:0002861, GO:0006909, GO:0045638, GO:0002862, GO:0002263], cytokine production [GO:0001818, GO:0034341, GO:0032609, GO:0032649, GO:0001819] and cell–cell adhesion [GO:0045785, GO:1903037, GO:1903039, GO:0022409, GO:0022407, GO:0007156, GO:0007159] [Supplementary File h].

We next turned to DMPs that were reported as significant in multiple studies focusing on cg12054453, cg16936953 and cg18942579 [*VMP1/TMEM49*], cg17501210 [*RPS6KA2*] and cg18608055 [*SBNO2*].[18,20,26] Meta-analysis statistics of these loci, expectedly, confirmed the direction of effect across all the individual studies included [Figure 2d and e].

3.3. Comparing ulcerative colitis with healthy controls

The comparison of UC with HC was performed in fewer studies, with studies interrogating the DNA methylome in PBL [$N_{studies}$ = 4][20,36,46,49] or its derivatives PBMCs [$N_{studies}$ = 2],[23,44] CD4+ T-cells [$N_{studies}$ = 1][20] and CD8+ T-cells [$N_{studies}$ = 2].[20,50] Notably, a more balanced distribution of paediatric [$N_{studies}$ = 3] and adult cohorts [$N_{studies}$ = 4] was observed. The majority of these studies adopted an EWAS approach [$N_{studies}$ = 5] using mostly the Illumina HumanMethylation BeadChip 450k array [$N_{studies}$ = 3]. Altogether, we obtained summary statistics from two studies totalling 132 UC patients and 217 HCs.[20,36] We identified 104 DMPs with a Bonferroni-adjusted p-value <0.05 [Figure 3a and Supplementary File c]. All top ten DMPs annotated to known genes, of which seven showed consistent hypomethylation [*FKBP5*, *TNFSF10*, *SBNO2*, *TMEM49/VMP1*, *RPS6KA2*, *ZEB2* and *SBNO2*] with the remaining three [*ICA1*, *FRMD4A* and *BBS9*] presenting consistent hypermethylation when comparing UC with HC [Figure 3b and Supplementary Table 4]. Expectedly, several DMPs among the top 20, namely cg01059398 [*TNFSF10*], cg18608055 [*SBNO2*], cg16936953 [*TMEM49/VMP1/MIR21*], cg17501210 [*RPS6KA2*], cg26804423 [*ICA1*], cg22959742 [*FRMD4A*] and cg20995564 [*ZEB2*], were reported as significant by Ventham *et al.*[20] However, for six DMPs, namely cg02734358 [*GPRIN3*], cg20228731 [*FLJ43663/LINC-PINT*], cg23729283 [*CPVL*], cg16755922 [*FOXK2*], cg23761815 [*SLC29A3*] and cg27243685 [*ABCG1*], neither site nor gene has been associated with UC or IBD previously. For *FLJ43663/LINC-PINT* [Long Intergenic Non Coding RNA, P53 Induced Transcript] recent data in rheumatoid arthritis patients indicate that *LINC-PINT* affects the production of tumour necrosis factor α [TNFα].[79] Moreover, the exact CpG [cg20228731] was found to be significantly hypomethylated in patients with Behçet's disease compared to healthy controls,[80] which was associated with an increased expression of the gene. Other DMP-associated genes, *CPVL*[81] and *ABCG1*,[82] have been associated with myeloid or macrophage function, with *CPVL* encoding a serine carboxypeptidase that is specifically expressed in macrophages and is proposed to function in proteolytic digestion of lysosomal components after phagocytosis.[81] *ABCG1* encodes an ATP-binding cassette sub-family G member, which mediates cholesterol accumulation and efflux in macrophage foam cells and has therefore been associated with atherogenesis.[82,83] Moreover, Abca1$^{-/-}$ mice demonstrated increased secretion of pro-inflammatory cytokines such as TNFα, IL-6, IL-1β and IL-12p70[84] and increased neutrophil and monocyte populations in peripheral blood.[85]

Aggregating the DMPs at the gene level yielded 45 statistically significant DMGs [Figure 3c and Supplementary File d]. Despite the considerable overlap with Ventham *et al.*,[20] we observed novel DMGs of potential interest in UC pathogenesis, namely *LOC285847*, *MZB1* and *FOXK2*. The long non-coding RNA gene *LOC285847* was found to be differentially methylated in women with systemic lupus erythematosus.[86] *MZB1* [Marginal Zone B And B1 Cell Specific Protein] is highly expressed in B-cell lineages and plasmacytoid dendritic cells [pDCs],[87] where a knockout of *Mzb1* in mice was associated with a reduction in immunoglobulin M [IgM]-[88] and IFNα secretion.[87] *FOXK2* is mostly described in cancer pathogenesis,[89] where it is thought to be involved in autophagy,[90] a process that has been associated extensively with IBD.[91] Notably, functional overrepresentation analyses against the GO resource did not identify any significant GO terms that passed the multiple test correction.

3.4. Comparing Crohn's disease with ulcerative colitis

In ~5–15% of all IBD patients, a definitive diagnosis of CD or UC is not always possible, leading to patients being diagnosed as either IBD-unclassified or indeterminate colitis.[92] While the majority of these patients are re-diagnosed as either UC or CD at a later point in time, earlier classification might prevent the delay of an optimal treatment.[93] Therefore, to explore the difference in DNA methylation between CD and UC we reviewed the available literature.

We included three studies for the systematic review that compared CD with UC in PBL[20,46,49] and two studies in PBMCs.[23,36] Three[20,23,36] of these studies adopted an EWAS approach using the Illumina HumanMethylation BeadChip 450k array in an adult population, of which two provided summary statistics that enabled a meta-analysis. Both studies reported a considerable overlap between UC- and CD-associated DMPs with over 66–97% of the UC-associated DMPs overlapping with CD-associated DMPs, while 45% of the CD-associated DMPs overlapped with the UC-associated DMPs.[20,23] Indeed, comparing the separate meta-analyses revealed a strong positive correlation [Figure 4a]. By combining both CD and UC and comparing that with HC, we identified DMPs annotated to *FKBP5*, *TMFSF10*, *SBNO2*, *TMEM29/VMP1*, *RPS6KA2*, *ICA1*, *FRMD4A*, *BBS9*, *KIAA0090*, *CPVL*, *BCL3*, *BAHCC1* and *CALHM1* and DMGs found in *RPS6KA2*, *TMEM49/VMP1*, *TNFSF10/TRAIL*, *FKBP5*, *BAHCC1* and *SOCS3* being identified as significantly different [Figure 4b, Supplementary Tables 7 and 8 and Files f and g]. GO term overrepresentation analysis between IBD patients and HC identified 224 significantly enriched GO terms. Among the top ten most enriched terms we observed significant overrepresentation for cell–cell adhesion [GO:0098742]. Notably, the majority of the remaining significantly associated GO terms were associated with developmental biology focusing on organogenesis [Supplementary File i].

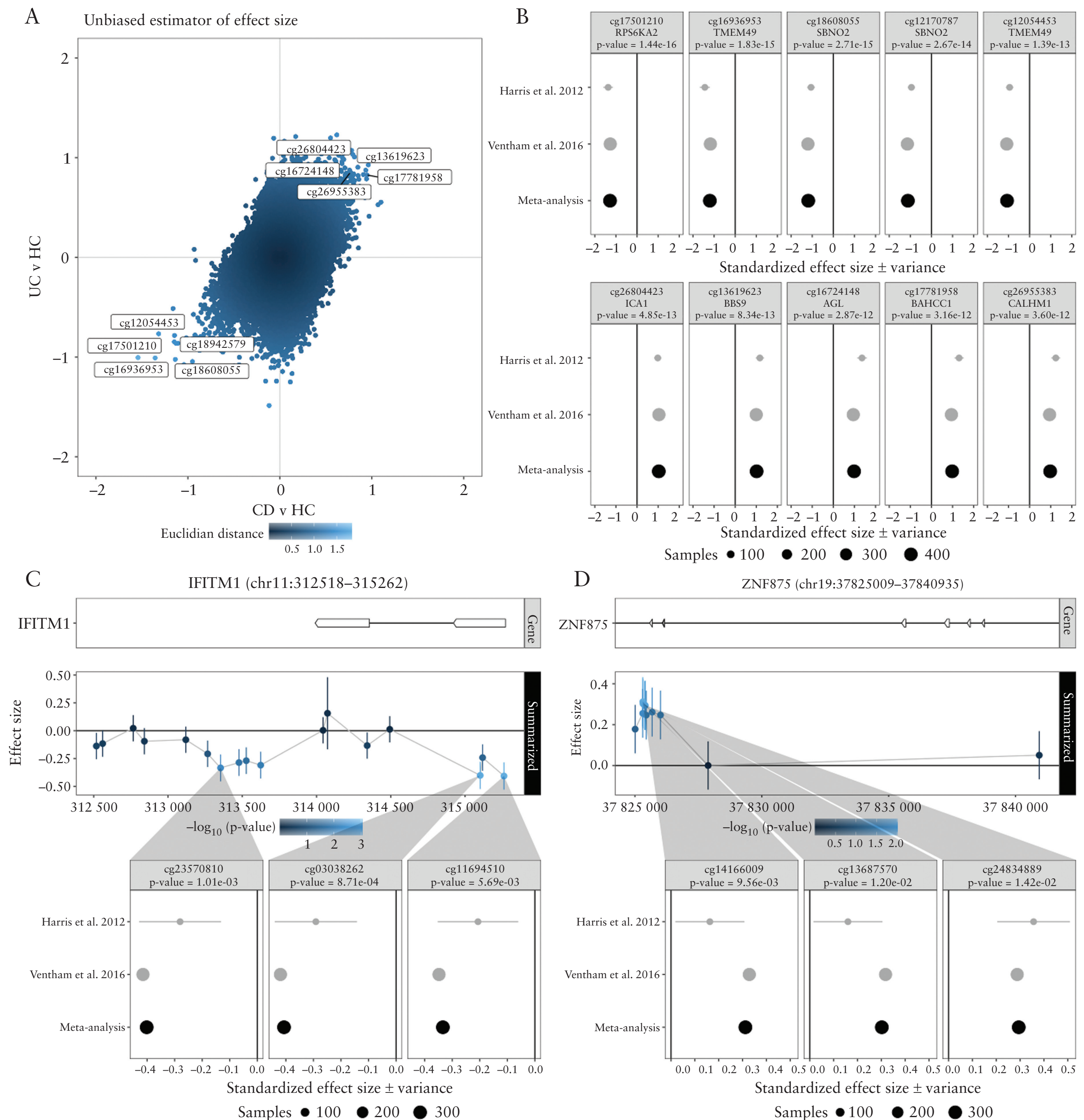

Figure 4. Meta-analysis of the comparisons Crohn's disease [CD] with ulcerative colitis [UC] and inflammatory bowel disease [IBD] with healthy controls [HC] using data from Harris *et al.* 2012 and Ventham *et al.* 2016. [A] A scatter plot of the included CpGs representing aggregated effect size of CD with HC and UC with HC on the *x*- and *y*-axis, respectively. Colour intensity is proportional to the distance of each CpG to the origin [0,0]. Annotated are the CpGs that are concordantly hyper- and hypomethylated in both CD and UC, and hence IBD, relative to HC. [B] Forest plots representing the standardized effect size [SES] of interest per study of the five most hyper- and hypomethylated IBD-associated DMPs annotated with the associated gene and *p*-value from the meta-analysis. Genomic visualization and forest plots of the estimated difference in methylation for the CD/UC discordant genes [C] IFITM1 and [D] ZNF875, alongside for the most significant differentially methylated probes.

Comparing CD [$N = 139$] with UC [$N = 133$][20,36] did not yield any significant DMPs following Bonferroni correction, underlining the previously reported similarities in global DNA methylation between both diseases [Figure 4a]. Notably, a DMG analysis identified two genes enriched for low *p*-values, namely *IFITM1* and *ZNF875* [Figure 4c and d, Supplementary Table 6 and File e]. *IFITM1* encodes Interferon Induced Transmembrane Protein 1, which is part of the interferon-stimulated gene families and is expressed in a variety of cells in response to interferon and viral immunity.[94] *IFITM1* has shown to interact with IFNγ, a key interferon with anti-proliferative function.[95] In IBD, IFNγ is known as a major cytokine involved in the pathogenesis and is found to be highly upregulated in both CD and UC patients.[96] In addition, genetic polymorphisms of *IFITM1* have been associated with UC patients, implicating *IFITM1* as potential risk locus for UC.[97]

ZNF875 [Zinc Finger Protein 875] is part of the Krüppel-associated box zinc finger proteins [KRAB-ZFPs] which act as transcription factors thought to be involved in carcinogenesis[98] and resistance to therapy in lung cancer.[99] However, its relationship with IBD as yet remains unknown.

3.5. Classification of IBD using DNA methylation

While previous analyses were mostly geared at identifying and quantifying differences in methylation, an increasing number of studies have adopted a classification approach to distinguish CD, UC and HC focusing on the diagnostic potential of DNA methylation. Most of the studies that performed classification analyses were done so in PBL[18,20,22,26,49] with a single study in mucosal tissue.[21] Performance-wise, the resulting classification models indicated good distinctive capabilities in discriminating IBD from HC, reporting area under the curve (AUC) scores as high as 0.94 for CD and 0.91 for UC.[49] Distinguishing CD from UC was notably less prominent, with an AUC score ranging between 0.71 and 0.81,[18,20,49] corroborating the findings that both IBDs are similar.[20,23,68] Most classification studies lacked details on the exact implementation of the model as well as the positions used as features for classification,[19,20,26] thereby preventing independent validation. Only two previously reported paired-probe classification models, *RPS6KA2/VMP1* [cg17501210/cg12054453] and *RPS6KA2/TNFSF10* [cg17501210/cg01059398],[18] were validated in a separate study.[20] Besides the diagnostic capabilities of DNA methylation, several classification studies also investigated the use of DNA methylation in predicting disease progression, thereby distinguishing a different risk of treatment escalation over time[100] or time to use of biologics.[21]

4. Discussion

Our study provides the most comprehensive summary of IBD-related DNA methylation studies in PBL to date. We initially performed a systematic review of all available literature using a pre-specified protocol according to the PRISMA guideline,[101] whereupon we critically appraised both strengths and weaknesses using a modified version of the BIOCROSS tool.[34] Despite finding multiple relevant studies, we noted substantial discrepancies in the way sample sizes were selected, clinical characteristics were documented and the level of data availability. Accordingly, we provide a methodological checklist that could aid future researchers with an interest in IBD epigenetics to increase the reproducibility of their work [Table 4].

Through a meta-analysis, we identified consistent IBD-associated differences in DNA methylation between CD, UC and HC in PBL. The different comparisons in our meta-analyses show that there are certainly several notable markers that are indeed consistently differentially methylated across all studies. In addition, we corroborate the reported consistent differential methylation for previously identified probes: cg16936953, cg12054453, and cg18942579 [*VMP1/TMEM49/MIR21*] and cg17501210 [*RPS6KA2*], which demonstrated homogeneity across all studies despite the heterogeneity in clinical characteristics, such as age group, sex, smoking behaviour and inflammatory status. However, to understand the pathogenesis and aetiology of IBD, the results presented in this meta-analysis remain observational at best as we cannot ascertain the source of the signal, nor can we ascertain the causal relationship of the newly identified DMPs with the outcome of interest [IBD, CD or UC] due to reverse causation.[103] That being said, the GO analyses of DMGs identified when comparing CD with HC returned multiple GO terms associated with inflammation, immune response and cell–cell adhesion, largely corroborating the GO analyses performed by the individual studies.[18,20,37] By contrast, GO analyses of the DMGs obtained when comparing UC with HC yielded no significant GO terms. We are unsure why the UC vs HC DMGs present no statistically significant overrepresentations. We hypothesize that a possible cause may be the lack of included studies, with only two studies being eligible. Similarly, it is unclear why the DMGs obtained when comparing IBD with HC are overrepresented for GO terms associated with pathways related to developmental

Table 4. Checklist of key components that should be described in DNA methylation studies

Justify sample size:	• If previous literature has been published: estimate sample size[102] • If no previous literature has been published: perform a pilot study in a small number of patients as an exploration. Report as explorative study design, a stepping-stone for future research
Limit confounding:	• Include detailed cohort description: ◦ Age, sex, smoking behaviour ◦ Disease duration, Montreal classification, surgical history ◦ Inflammatory status at time of sampling [CRP, faecal calprotectin, clinical and or endoscopy scores] ◦ Current and previous IBD-related medication, concomitant medication • If mixed tissue [whole blood, mucosal biopsy]: explore cellular heterogeneity • Describe sample quality control and describe actions taken to limit batch effects
Increase reproducibility:	•Report sample type [tube], sampling protocol, storage and isolation of DNA [kits] • When reporting differences in methylation, specify where the differences are found using genomic coordinates • Publish raw data in .idat or .fastq format in publicly available databases [Gene Expression Omnibus, ArrayExpress, European Genome-phenome Archive]
Increase interpretability:	• Include technical and/or independent validation in your design • Perform functional analysis of observed markers

CRP, C-reactive protein; IBD, inflammatory bowel disease.

biology. Nonetheless, our observations open up novel routes for targeted functional studies to address the potential downstream role of the observed differences in methylation and its relationship to IBD pathogenesis.

Despite our best efforts at providing an overview of the existing literature on PBL-associated DNA methylation in IBD, we are aware that limitations exist due to the differences in sample sizes and clinical phenotypes between the included studies, and advances in analytical methods. We note that the majority of the reported DMPs are in line with the observations by Ventham *et al*.,[20] which is the study with the largest sample size included in our meta-analysis, hence providing the largest weight. Nonetheless, the other studies provided similar direction of effect for the top DMPs, indicating that the observations were not solely restricted to Ventham *et al*.[20] We utilized summary statistics from all the studies to remain as close as possible to the authors' interpretations. This approach unfortunately excluded Somineni *et al*.,[26] as their summary statistics were incomplete, nor were we able to reproduce their summary statistics due to the lack of patient metadata [GWAS-derived principal components and Houseman-estimated cell distribution]. That being said, the analytical approach of the included studies for calculating the summary statistics was not identical. In particular, correcting for the cellular composition was often inconsistent, with some not performing any,[36] whereas others corrected for the measured leukocyte counts,[18] remove unwanted variation-estimated surrogate variables,[37] or used the Houseman algorithm to estimate the proportions.[20] While an alternative would have been to redo the analyses per study using the raw data and a single analytical pipeline, the raw methylation data in .idat format were not provided by every study [Supplementary Table 9].

The studies included in our systematic review and meta-analysis have mostly been used for understanding the manifestations of IBD at the DNA methylation level. However, it remains to be seen whether such differences can be utilized for biomarker purposes. Indeed, the use of machine learning and high-dimensional statistics for classification analyses are becoming more prevalent,[18,20,22,26,49] yet these models have to be translated to clinical practice before they can complement or even replace current diagnostic endoscopies. To this end, such models would need to be extensively validated, both in similar population cohorts as well as in randomized controlled trials. Unfortunately, many of the reported models lack implementation details, such as the weights or the actual predictive loci, complicating reproducibility. Moreover, the majority of the methylation data in IBD have been generated using a cross-sectional design. As such, no assurance on temporal stability of the identified biomarkers can be attributed. While independent validation is a key step towards clinical translation, focusing on markers that show limited intra-individual variability might increase the probability of replicating findings.[104] Although studies in adult healthy individuals[105,106] or patients with systemic lupus erythematosus[107] have demonstrated time-stable methylation markers, no such studies in IBD have been performed. It is therefore questionable if the reported biomarkers in current IBD DNA methylation studies show similar patterns within the same individual across multiple time points, irrespective of inflammatory status. We expect future studies to further address this question.

Besides the diagnostic potential of DNA methylation, additional avenues are currently being investigated, such as risk of disease progression. Therapy response prediction remains an open target as well, as the use of DNA methylation has shown potential in cancer[108,109] and rheumatology.[110,111] To date, no such response-associated DNA methylation studies in IBD have been published. Taken together, we expect future DNA methylation studies in IBD to be more clinically relevant, where the focus will lie on the classification of specific IBD phenotypes, such as early disease progression, therapy response or post-operative recurrence.

5. Conclusion

Our findings demonstrate consistent differential methylation patterns discerning CD, UC and IBD from healthy controls across multiple studies, which provide a basis for future epigenetic biomarker research in IBD. While the utility of DNA methylation in prediction and classification is becoming more apparent, methodological homogenization is needed, allowing easier aggregation and validation of data, greatly enhancing the field.

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Conflicts of Interest

VJ, IH, JS, AA, NV, WdJ, PH, GD and ALY: none to declare.

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Author Contributions

Conceptualization: VJ, IH, ALY, JS, AA, GD. Methodology: VJ, ALY, IH, JS, AA, GD. Formal analysis: ALY. Data acquisition: VJ, IH, ALY. Writing—original draft: VJ, IH, ALY. Writing—review and editing: VJ, IH, JS, AA, NV, WdJ, PH, GD and ALY. Approval of final manuscript: all authors.

Data Availability

The data that support the findings of this study are openly available in Gene Expression Omnibus [GEO] at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE32148, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE81961 and https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE87650 with reference numbers GSE32148, GSE81961 and GSE87650 respectively. Data from Adams *et al* [2014] were acquired after request to the corresponding author.

Supplementary Data

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

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