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Original Article

Characterisation of the Circulating Transcriptomic Landscape in Inflammatory Bowel Disease Provides Evidence for Dysregulation of Multiple Transcription Factors Including NFE2, SPI1, CEBPB, and IRF2

Jan K. Nowak,[a,b,*] Alex T. Adams,[a,*] Rahul Kalla,[c] Jonas C. Lindstrøm,[d,e] Simen Vatn,[e,f] Daniel Bergemalm,[g] Åsa V. Keita,[h] Fernando Gomollón,[i] Jørgen Jahnsen,[e,f] Morten H. Vatn,[e,j] Petr Ricanek,[f] Jerzy Ostrowski,[k,l] Jaroslaw Walkowiak[b]; IBD Character Consortium: Jonas Halfvarson,[g,] Jack Satsangi[a,m,**]**

[a]Translational Gastroenterology Unit, Nuffield Department of Medicine, Experimental Medicine Division, University of Oxford, John Radcliffe Hospital, Oxford, UK
[b]Department of Pediatric Gastroenterology and Metabolic Diseases, Poznan University of Medical Sciences, Poznan, Poland
[c]MRC Centre for Inflammation Research, Queens Medical Research Institute, University of Edinburgh, Edinburgh, UK
[d]Health Services Research Unit, Akershus University Hospital, Lørenskog, Norway
[e]Institute of Clinical Medicine, University of Oslo, Oslo, Norway
[f]Department of Gastroenterology, Akershus University Hospital, Lørenskog, Norway
[g]Department of Gastroenterology, Faculty of Medicine and Health, Örebro University, Örebro, Sweden
[h]Department of Biomedical and Clinical Sciences, Linköping University, Linköping, Sweden
[i]HCU 'Lozano Blesa', IIS Aragón, Zaragoza, Spain
[j]EpiGen Institute, Akershus University Hospital, University of Oslo, Oslo, Norway
[k]Department of Genetics, Maria Skłodowska-Curie National Research Institute of Oncology, Warsaw, Poland
[l]Department of Gastroenterology, Hepatology and Clinical Oncology, Centre for Postgraduate Medical Education, Warsaw, Poland
[m]Institute of Genetics and Molecular Medicine, University of Edinburgh, Edinburgh, UK

Corresponding authors: Dr Jan K. Nowak, Translational Gastroenterology Unit, Experimental Medicine Division, John Radcliffe Hospital, Headley Way, Headington, Oxford OX3 9DU, UK. Email: jan.nowak@ump.edu.pl; Jack Satsangi, Translational Gastroenterology Unit, Experimental Medicine Division, John Radcliffe Hospital, Headley Way, Headington, Oxford, OX3 9DU, UK. Email: jack.satsangi@ndm.ox.ac.uk.
*Shared first authorship
**Shared senior authorship.

Abstract

Aim: To assess the pathobiological and translational importance of whole-blood transcriptomic analysis in inflammatory bowel disease [IBD].

Methods: We analysed whole-blood expression profiles from paired-end sequencing in a discovery cohort of 590 Europeans recruited across six countries in the IBD Character initiative (newly diagnosed patients with Crohn's disease [CD; *n* = 156], ulcerative colitis [UC; *n* = 167], and controls [*n* = 267]), exploring differential expression [DESeq2], co-expression networks [WGCNA], and transcription factor involvement [EPEE, ChEA, DoRothEA]. Findings were validated by analysis of an independent replication cohort [99 CD, 100 UC, 95 controls]. In the discovery cohort, we also defined baseline expression correlates of future treatment escalation using cross-validated elastic-net and random forest modelling, along with a pragmatic ratio detection procedure.

Results: Disease-specific transcriptomes were defined in IBD [8697 transcripts], CD [7152], and UC [8521], with the most highly significant changes in single genes, including *CD177* ($\log_2$-fold change [LFC] = 4.63, $p = 4.05 \times 10^{-118}$), *MCEMP1* [LFC = 2.45, $p = 7.37 \times 10^{-109}$], and *S100A12* [LFC = 2.31, $p = 2.15 \times 10^{-93}$]. Significantly over-represented pathways included IL-1 [$p = 1.58 \times 10^{-11}$], IL-4, and IL-13 [$p = 8.96 \times 10^{-9}$]. Highly concordant results were obtained using multiple regulatory activity inference tools applied to the discovery and replication cohorts. These analyses demonstrated central roles in IBD for the transcription factors NFE2, SPI1 [PU.1], CEBPB, and IRF2, all regulators of cytokine signalling, based on a consistent signal across cohorts and transcription factor ranking methods. A number of simple transcriptome-based models were associated with the need for treatment escalation, including the binary *CLEC5A*/*CDH2* expression ratio in UC (hazard ratio = 23.4, 95% confidence interval [CI] 5.3–102.0).

Conclusions: Transcriptomic analysis has allowed for a detailed characterisation of IBD pathobiology, with important potential translational implications.

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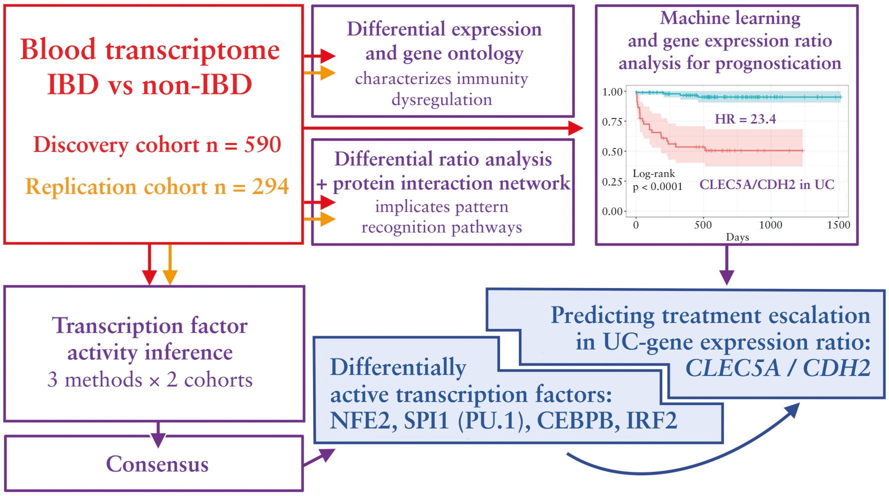
Graphical Abstract



Blood transcriptome IBD vs non-IBD

Discovery cohort n = 590

Replication cohort n = 294

Differential expression and gene ontology characterizes immunity dysregulation

Differential ratio analysis + protein interaction network implicates pattern recognition pathways

Machine learning and gene expression ratio analysis for prognostication

Transcription factor activity inference 3 methods × 2 cohorts

Consensus

Differentially active transcription factors: NFE2, SPI1 (PU.1), CEBPB, IRF2

Predicting treatment escalation in UC-gene expression ratio: *CLEC5A / CDH2*

Key Words: Inflammatory bowel disease; transcriptome; Crohn’s disease; ulcerative colitis; transcription factor

SIGNIFICANCE OF THIS STUDY

What is already known about this subject?

- Transcriptomic studies in IBD have identified susceptibility genes that represent new biological pathways and potential treatment targets, including PAI1.
- Blood expression profiles associate with the need for treatment escalation in IBD. Mucosal transcriptomic signatures of IBD may predict response to biologic agents.
- Dynamic growth of knowledge about regulatory networks has enabled inference of transcription factor activity from transcriptomic data.
- Despite a decade of expression profiling research, whole-blood transcriptome in IBD remains underexplored.

What are the new findings?

- We demonstrate that an IBD-specific whole-blood transcriptomic profile can be defined and replicated with similarities in expression profiles between Crohn’s disease and ulcerative colitis.
- New-onset IBD is characterised by overexpression of bactericidal neutrophil glycoprotein *CD177* and an intestinal stem cell marker *OLFM4*.
- Smoking in IBD is associated with greater expression of *GPR15*, which encodes a T cell colon-homing receptor.
- Dysregulation of IL-1, IL-4, and IL-13 pathways are present at diagnosis.
- Comparative transcription factor activity in the IBD Character and independent replication cohorts revealed consistently increased expression footprints of NFE2, SPI1 [PU.1], CEBPB, and IRF2 in IBD compared with controls.
- A number of relatively simple oligo-marker expression models are associated with treatment escalation in UC and include two-parameter models—notably the proportion of expression of *CLEC5A* to *CDH2* [C-type lectin-like protein to cadherin 2].

How might it impact on clinical practice in the foreseeable future?

- The identified transcription factors may reveal targets for novel therapeutic interventions.
- Whole-blood transcription analysis may allow for the assessment of disease course and outcome: a critical step towards precision medicine in IBD.

1. Introduction

The inflammatory bowel diseases [IBD], Crohn’s disease [CD] and ulcerative colitis [UC], are common causes of chronic illness worldwide, affecting 0.5–1.0% of the population in Europe and North America.[1] IBD is now a global health burden with rising incidence and prevalence, particularly among newly industrialised nations.[1] Although mortality is low, morbidity associated with IBD remains high and health care costs continue to increase.

IBD is characterised by intestinal and circulatory inflammatory changes, and much of the progress in drug development has been fuelled by our improved understanding of the molecular processes involved in disease pathogenesis. Although this has transformed the medical management of IBD,[2,3] the pathogenesis of this disease is still not completely understood. Furthermore, given the heterogeneity within IBD, treatment response differs widely between patients. With the advancements in ‘-omic’ technologies, there has been progress in our understanding of the molecular profiles of disease onset, drug resistance, and disease course.[4]

Among these, transcriptomic analyses have contributed to addressing these challenges by identifying signatures correlated with response to therapy[5,6] and a T cell-derived signature that associates with the need for treatment escalation.[7] The reproducibility of these signals has not been established and the field has been confounded by concerns regarding inconsistencies in analytical techniques and in the criteria for treatment escalation.[8] Most recently, analysis of the whole blood-derived transcriptome has shown promise in a study by Biasci *et al*., and has led to a multicentre clinical trial in the UK to validate this signature as a predictor of treatment escalation.[9]

The applications of '-omic' technologies to define disease pathogenesis and to identify new targets for therapeutic interventions are high on the research agenda for IBD.[10] Here, we provide the first report of the circulating transcriptome in the extended IBD Character inception cohort, presenting with suspected IBD across six European centres. We describe the gene expression profiles and, using a series of recently developed analytical tools, define expression modules and novel transcription factor [TF] involvement. We confirm these key findings in an independent, publicly available dataset, setting the scene for subsequent mechanistic and interventional studies.

2. Materials and Methods

2.1. Discovery cohort

Patients with suspected or established IBD and healthy controls were recruited across six European centres over 2013–2016 [see Supplementary Methods, including a schematic overview of the study]. Whole blood was collected in Paxgene tubes. Ion AmpliSeq Human Gene Expression Core Panels [20 802 amplicons] were run with the Ion AmpliSeq Library Kit Plus in the Wellcome Trust Clinical Research Facility in Edinburgh.

2.2. Replication cohort

The replication cohort[11] from Poland included 96 adult and 103 paediatric patients with IBD [CD, $n = 99$, and UC, $n = 100$] and 95 controls of whom 52 were children. Moderate-to-severe disease was present in 36 participants with CD [29 children] and 38 with UC [31 children]. The controls included adults undergoing cancer screening and children with surgical, orthopaedic, or ophthalmological conditions without inflammation. Details of paired-end sequencing of whole-blood RNA in the replication cohort and clinical characteristics are given by Ostrowski *et al*.[11]

2.3. Transcriptomic analysis

After alignment, filtering, and quality check, differential expression was assessed with DESeq2. Genes expressed differentially with log-fold change [LFC] >1, which also retained significance after Bonferroni correction, were subject to ontology investigations. DESeq2-normalised expression data were used in downstream applications. A weighted gene co-expression network analysis was done with the R package WGCNA. Traits correlated with modules included: diagnosis, gender, age, C-reactive protein [CRP], smoking, Montreal classification, and the need for treatment escalation. Three methods were used for the inference of TF activity from the expression data: Effector and Perturbation Estimation Engine [EPEE],[12] ChIP-X Enrichment Analysis 3 [ChEA3],[13] and Discriminant Regulon Expression Analysis [DoRothEA2] v2.[14] In order to maximise the true-positive rate, the EPEE and ChEA3 results were intersected [Supplementary Methods]. Immunity-focused differential ratio analysis with intermediary inference [DRAIMI] was conducted. Similarities of individual transcription profiles to LM22 cell type signatures were assessed using CIBERSORT. To determine how these results compare with the mucosal expression profiles, we used a meta-analysis of intestinal biopsy microarray studies performed by Granlund *et al*.[15]

2.4. Treatment escalation

This sub-study aimed to differentiate between patients who required an escalation of therapy within the first year following diagnosis and those who remained escalation-free throughout and at the end of the follow-up period lasting not less than 1 year [patients with escalation within the first week following diagnosis were excluded]. Treatment escalation was defined as the need for a biologic, ciclosporin, and/or surgery, instituted for a disease flare after initial remission. In UC, the definition of treatment escalation also included any patient requiring colectomy during the index admission. We built models predicting the need for escalation using analyses of whole-blood transcriptomes and models based on: [a] the elastic-net regression, [b] the random forest, and [c] a custom procedure exploiting differences of unrelated transcript ratios. Additionally, we applied elastic-net and random forest classifications to a set of transcripts matching the ones used by Biasci *et al*.[16] in order to confirm their predictive potential. Details, including escalation criteria from Biasci *et al*., are given in the Supplementary Methods.

3. Results

3.1. Overview of results section

This section starts with the presentation of the differential expression analysis in the discovery and replication cohorts, and the related gene ontology investigations. These data are followed by a description of modules and hub genes demonstrated by weighted gene correlation network analysis [WGCNA]. Next, outcomes from a new type of gene expression ratio-based analysis are presented. Subsequently,TF inference using the three methods across the discovery and replication cohorts is described, and the results are compared and analysed in the contexts of gene expression and deconvoluted cell type abundance. Finally, the transcriptomic data are used to build models predicting treatment escalation. We demonstrate that the best-performing predictive model in UC was related to the key identified TFs. A graphical overview of the study is also presented in Figure 1.

3.2. Clinical characteristics of the discovery cohort [IBD Character]

In total, 590 participants were recruited, including 156 patients with CD, 167 diagnosed with UC, and 267 controls, which included 54 healthy controls and 213 symptomatic

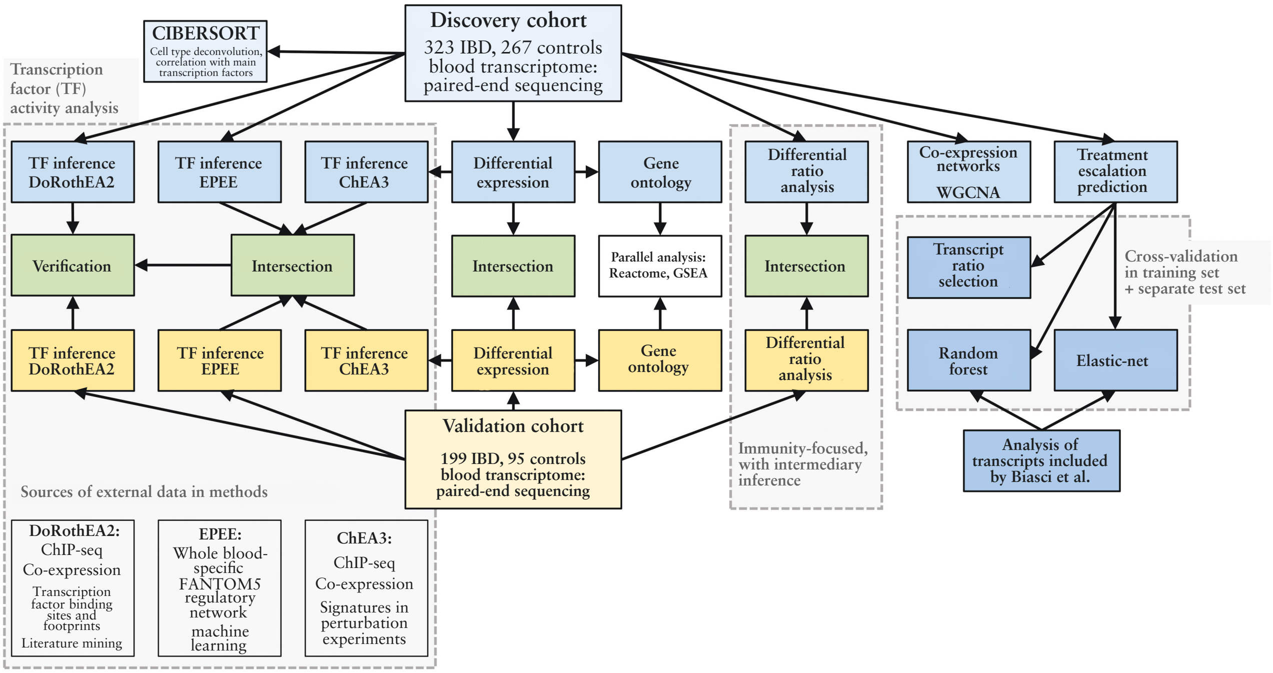

Discovery cohort
323 IBD, 267 controls
blood transcriptome:
paired-end sequencing
CIBERSORT
Cell type deconvolution, correlation with main transcription factors
Transcription factor (TF) activity analysis
TF inference DoRothEA2
TF inference EPEE
TF inference ChEA3
Differential expression
Gene ontology
Differential ratio analysis
Co-expression networks WGCNA
Treatment escalation prediction
Verification
Intersection
Intersection
Parallel analysis: Reactome, GSEA
Intersection
Transcript ratio selection
Cross-validation in training set + separate test set
TF inference DoRothEA2
TF inference EPEE
TF inference ChEA3
Differential expression
Gene ontology
Differential ratio analysis
Random forest
Elastic-net
Validation cohort
199 IBD, 95 controls
blood transcriptome:
paired-end sequencing
Immunity-focused, with intermediary inference
Analysis of transcripts included by Biasci et al.
Sources of external data in methods
DoRothEA2:
ChIP-seq
Co-expression
Transcription factor binding sites and footprints
Literature mining
EPEE:
Whole blood-specific FANTOM5 regulatory network
machine learning
ChEA3:
ChIP-seq
Co-expression
Signatures in perturbation experiments

Figure 1. Overview of the study.

controls with no evidence of IBD at follow-up. The baseline clinical characteristics are summarised in Table 1 in Supplementary Methods by centre. Of note, UC patients were older [$p = 2.4 \times 10^{-5}$] and less likely to smoke [$p = 1.0 \times 10^{-8}$] compared with controls, but no differences in smoking patterns or demographics were observed between CD patients and controls.

3.3. Discovery cohort: differential expression analysis highlights inflammation, neutrophils, and antimicrobial peptides

After filtering, 14 182 whole-blood transcripts were included in the differential expression analysis in the discovery cohort. The number of differentially expressed transcripts was 8697 in IBD vs. controls, 7152 in CD vs. controls, 8521 in UC vs. controls, and 1664 in CD vs. UC [Supplementary Table 1]. The split between the over- and under-expressed genes was almost equal. However, the absolute value of the $\log_2$-fold change [LFC] in comparisons against controls exceeded unity only for over-expression [1.4% for IBD vs. controls]. Colonic CD and UC appeared transcriptomically similar. An increased expression of alpha defensins was found in colonic vs. ileal CD. The genes with the most significant expression differences are presented in volcano plots [Figure 2; Supplementary Table 1]. Results of a subanalysis focusing on smoking, which implicate GPR15 and LRRN3, are presented in Supplementary Table 2.

3.4. Replication cohort: differential expression analysis and integration confirm involvement of neutrophil marker CD177, alpha-defensins, and OLFM4

The results of an analogous DESeq2 analysis of pooled IBD and control data from the replication cohort are presented in Supplementary Table 1 and Supplementary Figure 1. Briefly, 13 264 genes were included and the number of entities with a false-discovery ratio [FDR] <0.05 was 5402 in IBD, 4200 in CD, 5491 in UC, and 385 in an additional CD vs. UC comparison. From the highest results for both datasets [absolute LFC >1], genes with significant Bonferroni-corrected p-values were extracted to provide a consensus shortlist [Figure 3].

3.5. Gene ontology brings focus to neutrophils, antimicrobial peptides, and metalloproteinases

To identify common ontology terms between the discovery and replication cohort, gene ontology analyses were performed. Primary gene ontology results were concordant in CD and UC, providing a high level of significance for IBD. The main ontology terms in the discovery and the replication cohort overlapped [Figure 4]. Myeloid leukocyte function and immune responses best described the differences between IBD and controls. Neutrophil degranulation [$p = 1.1 \times 10^{-16}$], antimicrobial peptides [$p = 9.8 \times 10^{-8}$], and metalloproteinase activity [$p = 5.5 \times 10^{-4}$ Figure 4] were implicated. The complete results of the gene ontology and Reactome analyses can be found in Supplementary Tables 3 and 4.

3.6. Weighted correlation network analysis modules link inflammation and clinical traits, and feature toll-like receptor and neutrophil cytosolic factor genes

WGCNA of the discovery dataset identified 20 modules in IBD, 19 in CD, and 16 in UC. From these, we selected modules that were at least moderately associated with any of the traits [r >0.4, p <0.05]: three in IBD, two in CD, and five in UC. The pro-inflammatory modules were similar in all analyses and comprised genes associated with leukocyte activation and immunity. Conversely, the identified anti-inflammatory modules related to T cell differentiation and activation. The only module to associate with the need for treatment escalation [r = -0.50, p <0.0001] was found in UC and included genes with low module membership scores

Table 1. Basic characteristics of the discovery [IBD Character] transcriptomics cohort. Values are expressed as a median [1st–3rd quartile] or a percentage, and also as mean ± standard deviation for endoscopic scores.

	CD *n* = 156 [27%]	UC *n* = 167 [28%]	Non-IBD *n* = 267 [45%]
Age, years	26 [21–37]	35 [27–46]	30 [23–40]
Sex, % female	77 [49%]	67 [40%]	145 [54%]
CRP, mg/L	7 [2–28]	3 [1–14]	2 [1–5]
Current smoker	43/132 [33%]	10/163 [6%]	47/245 [19%]
CD location			
L1/ L1+L4	49 [31%]/ 1 [1%]		
L2/ L2+L4	41 [26%]/ 5 [3%]		
L3/ L3+L4	47 [30%]/ 10 [6%]		
L4	3 [2%]		
CD behaviour			
B1	127 [84%]		
B2	13 [8%]		
B3	12 [8%]		
Froslie score [*n* = 121]	4 [2–7] mean 5.3 ± 4.6		
Harvey–Bradshaw Index [*n* = 99]	6 [2.5–7.75]		
UC extent			
E1		42 [25%]	
E2		54 [32%]	
E3		72 [43%]	
Mayo endoscopic subscore [*n* = 122]		4 [2-7] mean 4.6 ± 3.0	
Treatment-naive	38 [75%]	104 [63%]	
Most common medications at recruitment	Oral prednisone 16 [10%] Intravenous steroids 11 [7%] Oral budesonide 8 [5%] Oral 5-ASA 6 [4%]	Rectal 5-ASA 26 [16%] Oral 5-ASA 20 [12%] Oral prednisone 15 [9%] Intravenous steroids 13 [8%]	
Froslie score in patients on treatment	3 [2.75–7.0] mean 4.5 ± 3.4		
Mayo endoscopic subscore in patients on treatment		4.0 [2.0–5.0] mean 4.0 ± 2.9	

CD, Crohn's disease; hsCRP. high-sensitivity C-reactive protein; IBD, inflammatory bowel disease; UC, ulcerative colitis; 5-ASA, 5-aminosalicylate.

[hub gene *AMPD2*]. The analysis of modules related to clinical traits of IBD using protein-protein interaction networks revealed the involvement of *TLR4*, *NCF2*, and *SPI1* across IBD, and integrins in UC [*ITGA2B*, *ITGB3*]. A more detailed description of WGCNA results along with a complete list of hub genes are presented in Supplementary File 1. A description of the modules and a full list of genes with module membership scores and tables of module-trait associations are presented in Supplementary File 1 and Supplementary Table 5.

3.7. Immunity-focused differential ratio analysis with intermediary inference implicates regulation in pattern recognition receptors and NfkB pathways

In the discovery dataset, DRAIMI unequivocally implicated pathways linked to pattern recognition receptors. The top result was *RELA* [also known as p65; 67.2% of all protein-protein interactions], followed by *TLR4* [55.3%], and *MYD88* [54.2%]. High involvement was noted also for three dual-specificity phosphatases [*DUSP7*, *DUSP4*, *DUSP6*] and natural cytotoxicity receptors 1 and 3 [*NCR1*, *NCR3*]. In terms of the number of interactions, these were supplemented by *NFKB1*, *TRIM21*, and *MAPK3*. The top results also included *SYK*. Almost all the listed genes [*RELA*, *TLR4*, *MYD88*, *DUSP4*, *DUSP6*, *DUSP7*, *NFKB1*, *TRIM21*, *MAPK3*] belong to pattern recognition pathways and specifically highlight the TLR4-MYD88-RELA[p65]-NfkB axis. Within the 304 implicated genes [Supplementary Table 6], notable results also include *HLA* and *IRF*, *NFATC2*, *IL4* and its receptor, *IL13*, *STAT3*, *ITGAV1*, *ITGAM*, *JAK1*, *IL2* and its receptors, *FYN*, *TYK2*, *TNF*, *VCAM1*, and other genes that fit well with prior knowledge. The list also includes *CEBPB*, which is one of the key genes identified by the TF activity inference analysis.

In the validation cohort, *SYK*, *MAPK1*, and *MAPK9* were among the top results, emphasising the role of pattern-recognition receptor pathways, and *RELA* was replicated, albeit with less significance [Supplementary Table 6]. The two most important results in the validation analysis were *STAT4* and *PTPN6*, and the results' gene ontology was strongly linked to cell activation in the immune context and also included protein degradation. Overall, DRAIMI underscored

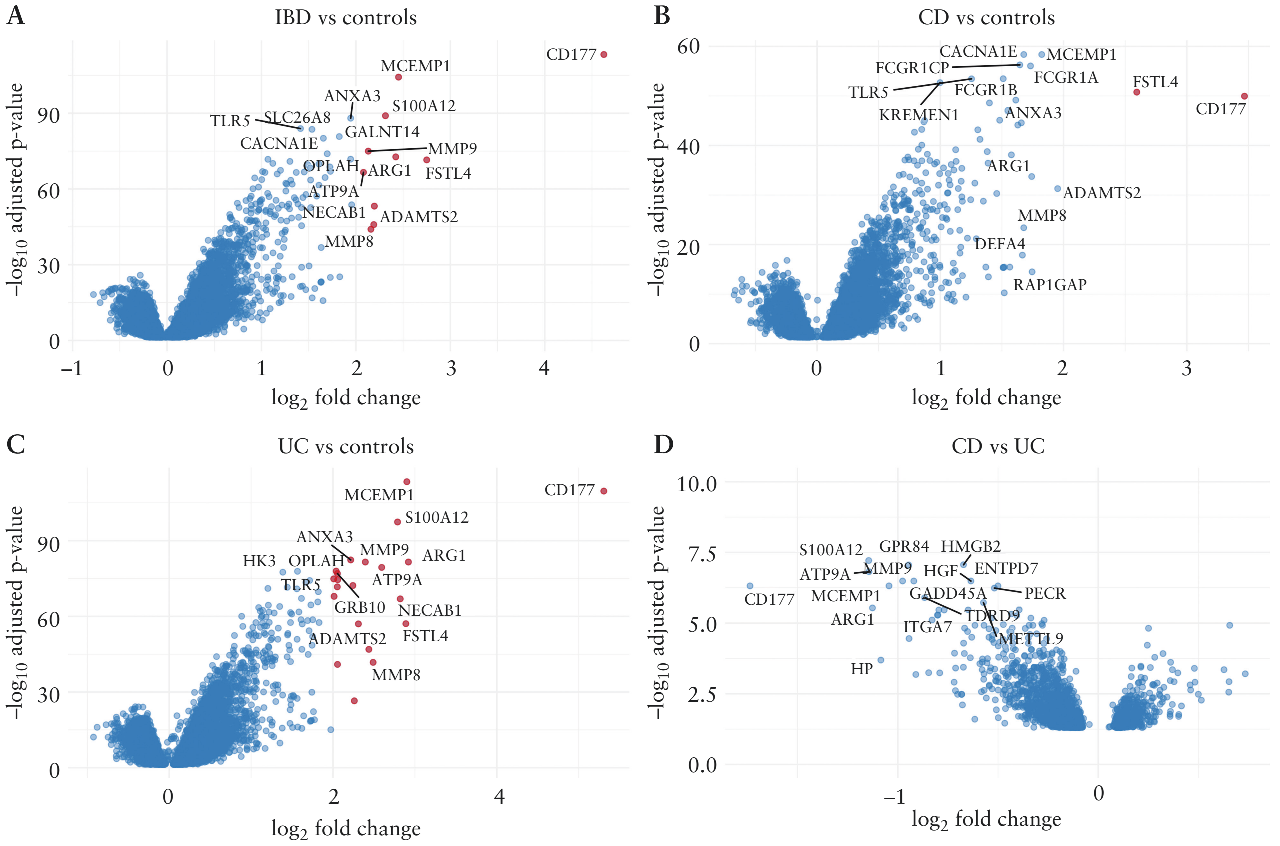


Figure 2. Volcano plots showing differential expression of whole-blood transcripts between inflammatory bowel disease [IBD] and subtypes of IBD vs. controls [A–C] and CD vs. UC [D] in the discovery cohort. Only transcripts with a false-discovery rate <0.05 are shown. Genes with log2-fold change [LFC] >2 are indicated in red. Genes with the top 10 most significant *p*-values and top 10 LFC are labelled [the two lists may overlap]. The genes labelled in panel D may be considered overexpressed in CD relative to UC. CD, Crohn's disease; UC, ulcerative colitis.

the importance of regulation in pattern recognition receptors and NfkB pathways in the whole blood of patients with IBD.

3.8. Transcription factor activity inference across cohorts and methods exposes the relevance of NFE2, SPI1, CEBPB, and IRF2 to IBD

TF activity inference was then conducted, and the obtained results were intersected across methods and cohorts. Both EPEE and ChEA3 identified TFs whose activity differed between patients from the IBD groups and the controls. The number of TFs ordered according to EPEE was 302, and in the case of ChEA3 it equalled 1632. Here, we initially focused on TFs most consistently appearing in the most important EPEE and ChEA3 results from the discovery [IBD Character] and replication [Ostrowski *et al.*] datasets [Table 2]. All the EPEE regulator scores and ChEA3 and DoRothEA2 results are presented in the supplementary data [Supplementary Tables 7–9].

3.9. Intersection of results from TF inference uncovers patterns constant across diverse methods and two IBD cohorts

Four TFs were identified using the intersection procedure: NFE2, SPI1 [PU.1], CEBPB, and IRF2 [Figure 5]. DoRothEA2 predicted the activity of all four TFs to be increased in IBD. Given the transcriptome similarity between CD and UC—and insignificant differences in the Montreal subgroup analyses—attempts at identifying CD- or UC-specific TF activity yielded inconsistent results. EPEE suggested that IRF9, STAT1, and ELF1 activity in CD was lower than in UC. On the other hand, the transcriptional footprints of SPI1 [discovery] and IRF4 [replication] were clearer in CD than in UC, where C-reactive protein [CRP] was lower. In a supplementary analysis, ChEA3 highly ranked NFE2, SPI1, CEBPB, and IRF2 in IBD mucosal transcriptomes.

3.10. Correlation between key TFs and other transcripts unveils inflammatory context

Correlations of *NFE2*, *SPI1* [PU.1], *CEBPB*, and *IRF2* with other transcripts are presented in Supplementary Table 10. *NFE2* correlated strongly with *OSM* [rho = 0.79, $p = 4.75 \times 10^{-69}$], *TLR4*, *NCF4*, and *ITGAM*. *SPI1* [PU.1] correlated with the kinase *RPS6KA1* [rho = 0.86, $p = 3.75 \times 10^{-97}$] and matrix metalloproteinase inhibitor *TIMP2*, as well as with *NCF2* and *NCF4*. *CEBPB* expression associated with *STX3* [rho = 0.74, $p = 1.73 \times 10^{-56}$], whose loss causes microvillus inclusion disease,[17] INFGR2, inflammasome-triggering *NLRC4*, and *TLR5*. Last, *IRF2* correlated with mitochondrial superoxide dismutase *SOD2* [rho = 0.75, $p = 2.1 \times 10^{-60}$], the IL-1 antagonist *ILR1N*, the immune signal transducer *MYD88*, and *IRF9*.

3.11. Analysis of TFs and cellular composition hint at a possible role of NFE2 and SPI1 in macrophage polarisation in IBD

The expression of the four selected TFs in individual subjects was correlated with the predictions of cell type abundance obtained using LM22 signatures in the discovery

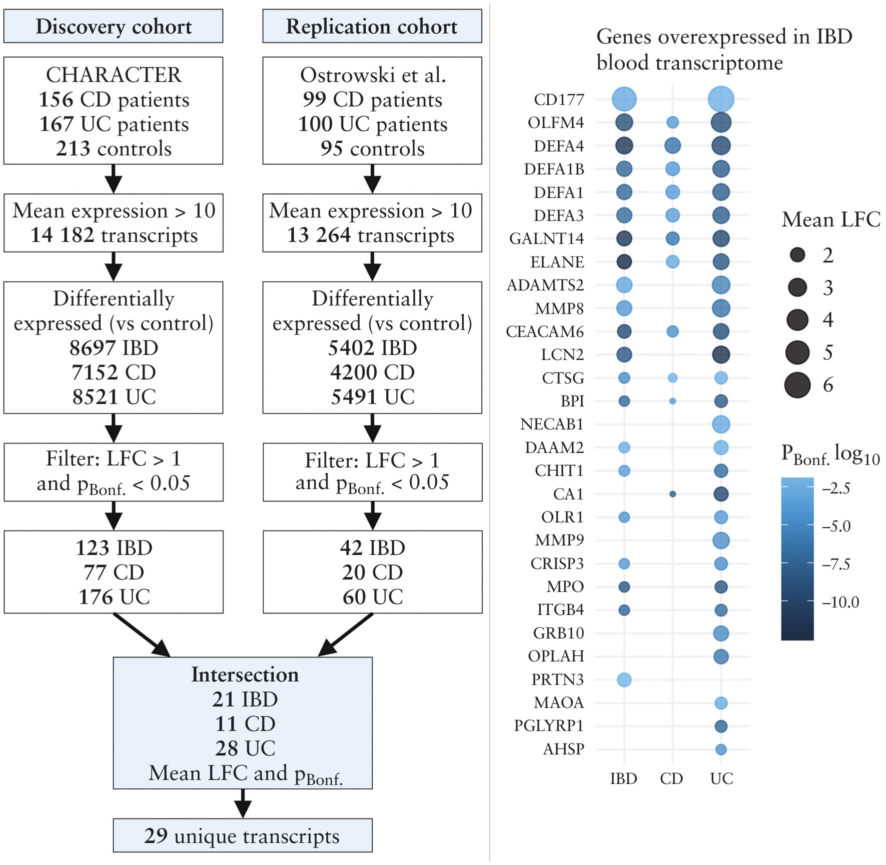


Figure 3. The inflammatory bowel disease [IBD] differential expression consensus shortlist obtained by intersecting data from the discovery [IBD Character] and replication cohorts [Ostrowski *et al.*]. Flowchart illustrating the intersection of differential expressed transcripts in the discovery and the replication data. Mean log2-fold changes [LFC] and Bonferroni-corrected *p*-values [$p_{Bonf.}$] for genes overexpressed in the discovery and replication cohorts. Only genes with absolute LFC >1 and $p_{Bonf.}$ <0.05 in both datasets were selected. This selection was performed separately in the IBD, Crohn's disease [CD], and ulcerative colitis [UC] data.

cohort. Correlations in IBD and controls appeared different with regard to B cells [in IBD, stronger correlation with TFs in naïve and weaker in memory B cells], γδ T cells [stronger correlation with *CEBPB* in IBD], and M0 macrophages [stronger correlation with NFE2 and SPI1/PU.1 in IBD, Figure 6]. Of note, stronger correlations imply a more systematic association between cell type abundance and gene expression, not necessarily higher expression. Similarities to LM22 signatures by group can be accessed in Supplementary Table 11.

3.12. Prediction of treatment escalation using gene expression data

Data on escalation of treatment were available for 204 IBD patients from the discovery cohort [mean follow-up 580 days, 24.0% required escalation], including 89 with CD [mean follow-up length 502 days, escalation in 34.8%] and 115 with UC [mean follow-up length 641 days, escalation in 15.6%]; 151 patients have not been exposed to anti-inflammatory or immunosuppressive medication at baseline [74%; Table 3]. Before escalation in the training cohort, patients with CD most commonly received the following treatments: azathioprine [n = 9], 6-mercaptopurine [n = 4], azathioprine and prednisolone [n = 2], azathioprine and budesonide [n = 2], prednisolone [n = 2], budesonide [n = 2], intravenous methylprednisolone [n = 2]. In UC, the following treatments were most frequently used before escalation: prednisolone and 5-aminosalicylates [n = 6], azathioprine [n = 4], 5-aminosalicylates alone [n = 2].

3.13. Machine learning methods achieve transcriptome-only prognostication

We applied machine learning to build prognostic models differentiating between patients who would require treatment escalation within the first year or remain escalation-free for at least 1 year. Elastic-net models had moderate performance in cross-validation (area under the curve [AUC] 0.70–0.79), which was confirmed in test samples of IBD and UC but not in CD cases [AUC 0.80–0.83 and in CD 0.41; Supplementary File 2]. Random forest classifiers had cross-validation AUCs ranging from 0.68 in IBD to 0.82 in UC [in test sets 0.74–0.76], indicating that the transcriptomic data could serve to predict escalation, albeit not precisely. Analysis of variable importance in random forest models hints at the potential role of *C14orf28* in IBD overall, and of *FZD6* and *ARRDC2* in CD, as well as of *MPZL2* and *CSNK2A1* in UC [Supplementary File 2]. Gene ontology did not reveal specific pathway enrichment.

3.14. CLEC5A-to-CDH2 gene expression ratio enables prognostication in UC and appears related to macrophage polarisation

The custom search for predictive transcript ratios yielded some of the highest AUC values in cross-validation [IBD

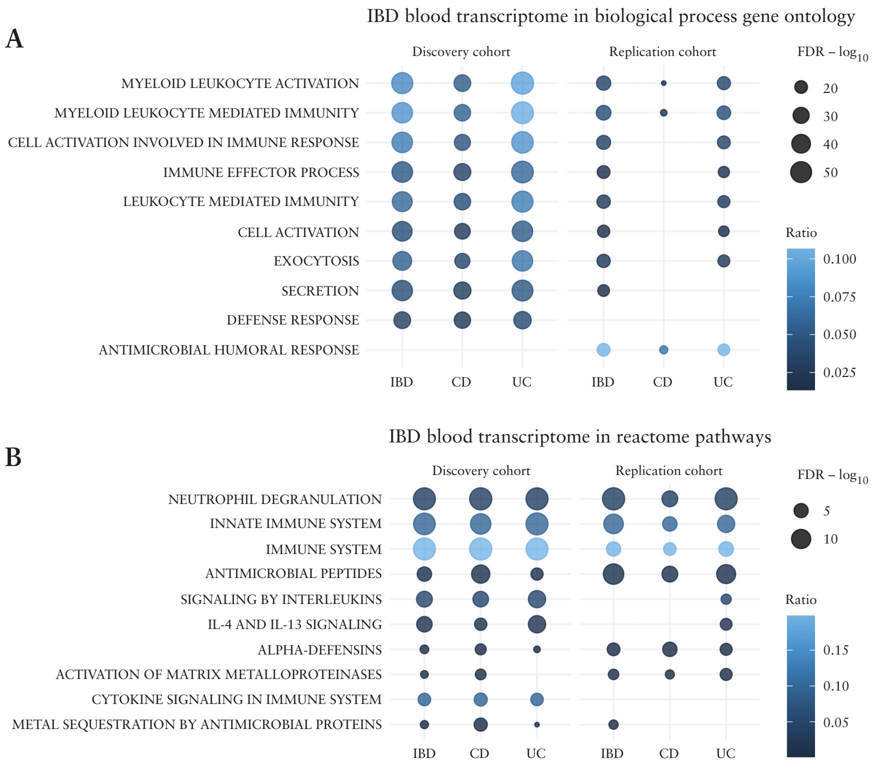


Figure 4. Most significant gene ontology sets [A] and reactome pathways [B] enriched by genes overexpressed in transcriptomes from the discovery [IBD Character] and replication [Ostrowski *et al.*] cohorts. Ratio represents the fraction of genes from the target dataset which were identified among the overexpressed genes. FDR, false-discovery ratio; IBD, inflammatory bowel disease.

0.76, CD 0.87, UC 0.90]. The top-performing ratios discovered by the custom procedure in the training sets were *CLEC5A*/*CDH2* in UC [AUC in test set 0.85], *HIST1H3H*/*GPR162* in CD [AUC 0.74], and *STAB1*/*GPR162* in IBD [AUC 0.69]. The *CLEC5A*/*CDH2* ratio achieved performance superior to complex models, with a hazard ratio for treatment escalation of 23.4 [95% CI 5.3–102.0; Figure 7]. In UC patients with available high-sensitivity [hs] CRP, AUC for the *CLEC5A*/*CDH2* ratio was higher compared with hsCRP: 0.90 [0.84–0.96] vs. 0.69 [0.53–0.85; $p = 0.016$] and not affected by prior exposure to IBD medications. In UC, the ratio with the second-best discriminatory power was *ANPEP*/*SEC61A2*. The top quotients are listed in Supplementary Table 12.

3.15. Genes belonging to a model by Biasci *et al.* are validated to have predictive potential

Of the 15 informative [non-housekeeping] transcripts used in the prediction score proposed by Biasci *et al.*, 12 could be extracted from the IBD Character dataset [Supplementary Methods]. The optimal elastic-net and random forest models performed well despite the limited number of predictors. A cross-validation AUC of 0.65–0.82 was achieved for IBD Character escalation criteria and 0.60–0.79 using the original escalation criteria from Biasci *et al.* The random forest model for UC reached AUC 0.87 in the test set using the discovery cohort [IBD Character] escalation criteria. Variable importance analysis of IBD Character random forest models revealed that the following transcripts from Biasci *et al.* were the most informative: *NUDT7*, *GZMH*, and *IL18RAP* in IBD overall, *GZMK* and *LY96* in CD [dominating the remaining genes], and *IL18RAP* in UC [exceeding other transcripts in UC].

4. Discussion

This combined dataset represents a comprehensive study of the whole-blood transcriptome in IBD, and successfully identifies and replicates genes of interest in differential expression profiles. We also are able to describe co-expression networks and, in a novel detailed analysis, we identify and replicate TFs that may be driving the disease. To strengthen the TF analyses, we employed two complementary methods and examined two independent datasets of IBD cases and controls. New correlates of treatment escalation in UC are proposed. The results highlight underexplored areas of IBD biology, which provide new insights into pathogenesis and possible therapeutic interventions.

4.1. Patients and controls

The IBD Character cohort comprised comparable numbers of patients with CD, UC, and controls across Europe. This was an inception cohort at presentation, which is reflected by a slightly higher upper quartile of CRP in CD. The location of CD and the extent of UC mirror the general structure in the population.[18] The controls included both symptomatic controls undergoing investigations for IBD, with no evidence of IBD at follow-up, and young, healthy Swedish volunteers. The access to and analysis of the dataset described by Ostrowski *et al.* provided replication of the findings from the discovery cohort.

4.2. Differential expression

The overriding finding in this context was that CD and UC expression profiles were remarkably similar. *CD177*, *OLFM4* [olfactomedin 4], *DEFA4* [defensin A4], *GALNT14*, and *ELANE* exhibited the greatest differences in the joint

Table 2. Key transcription factor [TF] activity differences in inflammatory bowel disease [IBD], Crohn's disease [CD], and ulcerative colitis [UC] as measured vs. controls in two datasets using two different approaches [EPEE and ChEA3]. TFs inferred from both datasets [discovery: IBD Character, replication: Ostrowski *et al.*], using both methods, and confirmed by DoRothEA2, are printed in bold and highlighted. Each set of results lists TFs in decreasing order of importance. SPI1 encodes PU.1

	Discovery cohort			Replication cohort		
	IBD	CD	UC	IBD	CD	UC
EPEE – overactive	**SPI1**, **NFE2**, FOXO3, ETS2, ITGB2, RXRA, IRF1, ETV6, **IRF2**, RARA	ARID3A, **CEBPB**, PKNOX1, PLAGL1, MAX, CEBPA, MAFB, CUX1, E2F3, LMO2	ARID3A, **NFE2**, **CEBPB**, CEBPA, ETS2, PLAGL1, RFX2, MAX, E2F3, TAL1	**SPI1**, FOXO3, **NFE2**, ITGB2, STAT6, IRF1, KLF13, EWSR1, **IRF2**, ETV6	**SPI1**, IRF1, **NFE2**, ETV6, FOXO3, FOXO4, STAT6, FLI1, CEBPD, KLF13	**SPI1**, FOXO3, **NFE2**, KLF13, ITGB2, STAT6, ETS2, FOXO4, XBP1, ELF4
EPEE – deficient	LEF1, RUNX3, KLF12, SPIB, IRF8, SP4, FOXP1, TP53, SMAD3, FOXJ3	EWSR1, LEF1, ELK1, STAT6, RUNX3, SP1, IRF1, ELF1, ZFX, YY1	LEF1, IRF1, EWSR1, STAT6, RUNX3, ZFX, SP1, ELK4, ELK1, SP4	SREBF1, POU2F2, GATA3, SPIB, TFCP2, ZNF740, TBP, IRF8, TBX21, ZNF350	TBX21, POU2F1, NFKB2, EWSR1, SPIB, SP1, SMAD3, REST, FOXO1, BHLHE40	ELF1, SP1, CREB1, TBX21, POU2F1, GABPA, NFKB2, REST, BHLHE40, FOXO1
ChEA3 – altered activity	**SPI1**, **NFE2**, NFE4, ZNF746, **CEBPB**, ZNF438, MTF1, BATF2, HLX, GATA1, CEBPE, ELF4, **IRF2**, MXD1, LYL1, ZNF467, SP110, IRF1, BORCS8-MEF2B, TRAFD1	**SPI1**, NFE4, BATF2, **CEBPB**, **NFE2**, HLX, IRF1, MTF1, ZNF438, ZNF746, ZNF467, **IRF2**, PLSCR1, SP110, MXD1, ELF4, CEBPE, TRAFD1, IRF9, TFEC	**SPI1**, **NFE2**, NFE4, ZNF746, ZNF438, **CEBPB**, MTF1, GATA1, HLX, CEBPE, ELF4, MXD1, BORCS8-MEF2B, LYL1, ZNF467, TAL1, SP110, TET2, STAT5B, TFE3	DNTTIP1, **NFE2**, CEBPE, ZNF524, ZNF787, **SPI1**, NFE4, GLMP, TFE3, LTF, BORCS8-MEF2B, ELF4, ZNF581, **CEBPB**, KLF1, RARA, TFEB, ZNF746, ZNF672, LYL1	ZNF524, **NFE2**, CEBPE, HLX, ZNF787, **SPI1**, DNTTIP1, LTF, GLMP, BORCS8-MEF2B, TFE3, ZNF467, FLI1, ZNF581, RARA, CEBPA, KLF1, **CEBPB**, NR1H2, LYL1	ZNF524, ZNF787, DNTTIP1, LTF, GLMP, TFE3, ZNF581, ZNF408, BORCS8-MEF2B, CEBPE, KLF1, **NFE2**, THAP7, **SPI1**, GMEB2, ZBTB45, NFE4, ZNF672, ZBTB17, **CEBPB**

analysis. *CD177* encodes human neutrophil alloantigen 2a, which is present on cells exhibiting bactericidal activity in IBD,[19] and impairs neutrophil migration.[20] Interestingly, CD177⁺ neutrophils producing IL-17 are increased in allergic asthma.[21] Olfactomedin 4 is a marker of intestinal stem cells and is known to be increased in the IBD mucosal proteome.[22,23] The copy number of defensins A1–3 correlates with the diagnosis of CD and its colonic location.[24] The product of *GALNT14*—a glycosylase—is enriched in neutrophils, and ELANE is a neutrophil elastase. Increased expression of alpha defensins in colonic vs. ileal CD is in line with a current hypothesis on defensin deficiency in ileal CD.[25]

Smoking is considered the most significant modifiable risk factor for CD and its differential effects in CD and UC are of particular interest. The only transcript that clearly distinguished itself in the comparisons of current vs. never smokers was the G-protein coupled receptor *GPR15*, which encodes a colon-homing molecule for T cells.[26] GPR15⁺ T cells secrete less IFN-γ, but more IL-17 after stimulation, suggesting a Th17-like phenotype.[27] It has been shown that regulatory T cell GPR15 expression in UC patients is increased[28] and, most recently, the dependence of *GPR15* transcription on aryl hydrocarbon receptor signalling has been demonstrated.[29]

4.3. Weighted gene co-expression network analysis

WGCNA yielded modules correlated with IBD and CRP. Yet, none of the modules associating with the diagnosis were independent of inflammation. Although the resulting gene lists were too extensive to clearly illustrate specific functions, they seem informative with regard to the actors of the anti-inflammatory response: *CD3E*, which is a subunit of the CD3 T cell co-receptor [otelixizumab target], *CARD11*, which induces apoptosis and NF-κB, and *SMAD3*, which is crucial for TGFβ signalling.[30] Some of the genes with high anti-inflammatory module membership cause very early-onset IBD when deficient [*ZAP70*, *LRBA*].[31]

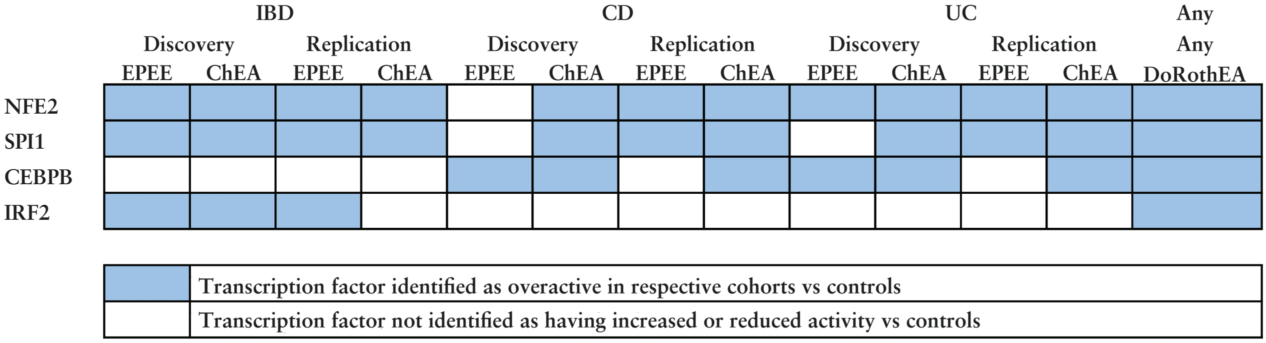
4.4. Transcription factors implicated in analysis

A key novel discovery in our study lies in the replication of multiple TFs that are dysregulated in IBD [Table 4].

NFE2 is underexplored in the context of mucosal inflammation and immunity in general. Both *NFE2* and *SPI1* [PU.1] are overexpressed and undermethylated in systemic sclerosis in comparison with controls.[43]

SPI1 [PU.1] governs Th9 immunity, which in IBD is still insufficiently understood, but appears predominantly pro-inflammatory.[44] In animal models, interleukin-9 deficiency protected mice against trinitrobenzene sulphonic acid [TNBS]- and oxazolone-induced colitis.[45] The IL-9 receptor is also overexpressed in the intestinal epithelium of patients with IBD. Consequently, IL-9 appears as a therapeutic target in UC. Enokizumab [MEDI-528], a humanised anti-IL-9 monoclonal antibody, was well tolerated in asthmatic patients.[46] To the best of our knowledge, there are no current trials examining anti-IL-9 for the treatment of IBD. Moreover, strategies against PU.1 [*SPI1* product] are currently being developed in oncology.[47]

CEBPB was found to lie 150 kb downstream from an IBD risk locus.[48] Interestingly, profiling of transcription start sites in IBD biopsies also highlighted *CEBPB* as potentially involved in the disease.[49] It is overexpressed in the mucosa of patients with UC[50] and strategically positioned at the interface



	IBD				CD				UC				Any
	Discovery		Replication		Discovery		Replication		Discovery		Replication		Any
	EPEE	ChEA	EPEE	ChEA	EPEE	ChEA	EPEE	ChEA	EPEE	ChEA	EPEE	ChEA	DoRothEA
NFE2	🟦	🟦	🟦	🟦	⬜	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦
SPI1	🟦	🟦	🟦	🟦	⬜	🟦	🟦	🟦	⬜	🟦	🟦	🟦	🟦
CEBPB	⬜	⬜	⬜	⬜	🟦	🟦	⬜	🟦	🟦	🟦	⬜	🟦	🟦
IRF2	🟦	🟦	🟦	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	🟦

🟦	Transcription factor identified as overactive in respective cohorts vs controls
⬜	Transcription factor not identified as having increased or reduced activity vs controls

Figure 5. The intersection of EPEE and ChEA3 results in the discovery and replication data. Rectangles filled in blue indicate the presence of a transcription factor among the most significant results in the given analysis. Contrasts between inflammatory bowel disease [IBD], Crohn's disease [CD], ulcerative colitis [UC], and controls were explored. DoRothEA2 was used to confirm the findings [thus filtering out IRF1, which is not shown]. SPI1 encodes PU.1.

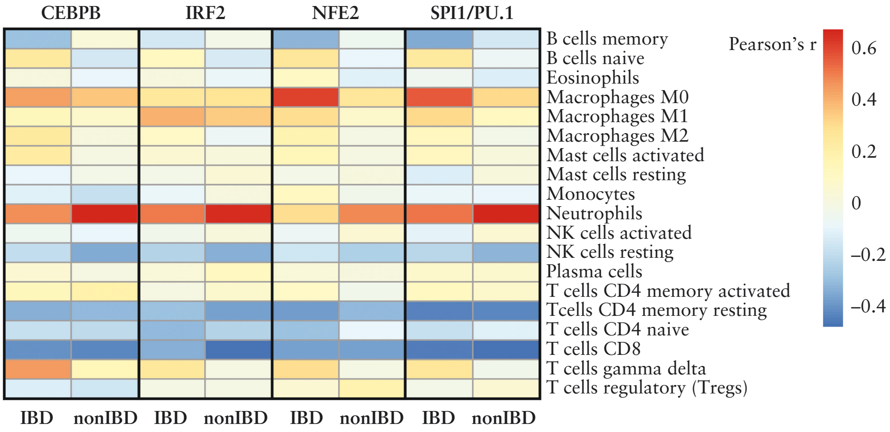


Figure 6. Heatmap illustrating Pearson's correlations between transcription factor expression and LM22-predicted cell type abundance in the whole blood of patients with inflammatory bowel disease [IBD] and in controls, in the discovery cohort. A stronger absolute correlation suggests more consistent expression in the cell type. Apart from B cells, γδ T cells, and M0 macrophages, the absolute differences between the two groups were small [<0.3].

Table 3. Basic characteristics of randomly split subgroups of the discovery cohort [IBD-Character] used for building predictive models and assessing their performance. Values are expressed as a median [1st-3rd quartile] or a percentage.

	CD		UC	
	Training	Testing	Training	Testing
n	71	18	92	23
Sex, % female	38 [53%]	10 [56%]	39 [42%]	9 [39%]
Age, years	27.0 [24.0-34.0]	32.0 [24.0-52.0]	36.5 [27.0-44.0]	32.0 [24.0-46.0]
CRP, mg/L	8.75 [2.2-43.0]	9.4 [2.2-73.0]	3.7 [1.2-14.0]	2.2 [1.5-9.1]
Treatment-naive	56 [79%]	14 [78%]	64 [70%]	17 [74%]

CRP, C-reactive protein.

of myeloid and epithelial inflammation clusters in a network analysis of mucosal IBD transcriptomes.[51] A recent study by Sudhakar *et al.*, employing ChEA3 in transcriptomic modules from CD4+ and CD14+ cells in 33 patients with CD, hinted at the involvement of both SPI1 and CEBPB.[52] Some research has indicated a role for fibrates in reducing CRP [IL-6] in response to IL-1 via CEBPB.[53]

Finally, IRF2 mediates the functions of IFNγ. Fontolizumab, a monoclonal antibody against IFNγ, was not efficacious in CD trials, a result that might have been influenced by a large placebo effect as well as the anti-inflammatory effects of IFNγ through downregulation of IL-23.[54]

4.5. Transcription factor activity inference

In brief, the two main TF inference methods employed in this study—EPEE and ChEA3—are complementary in terms of accepted transcriptomics data, computational approaches, regulon tissue-specificity, and regulatory network sources. Other notable TF inference tools include BART, DoRothEA v2, iRegulon [a Cytoscape application], TFEA.ChIP, and

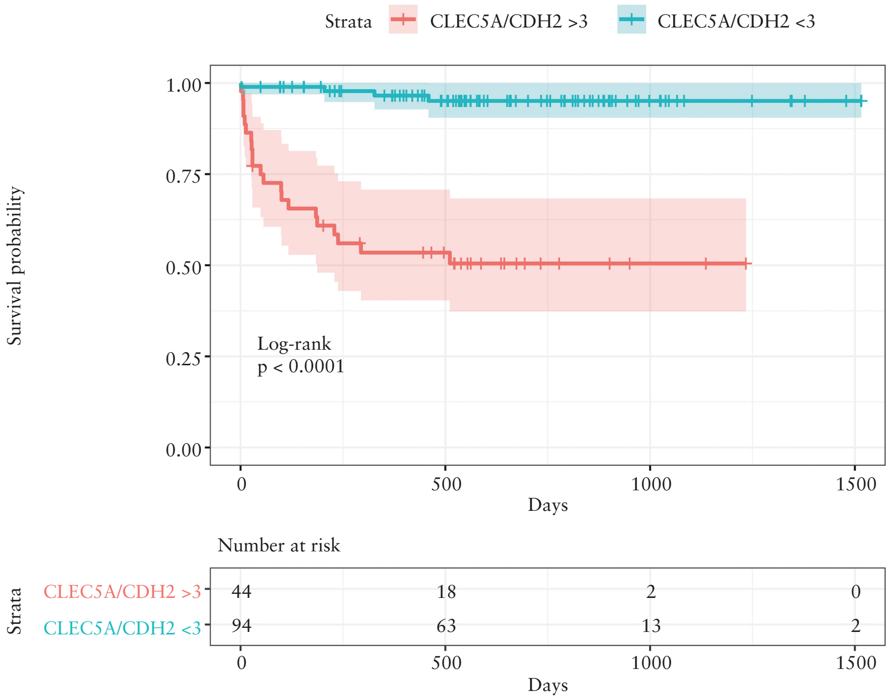


Figure 7. Ulcerative colitis treatment escalation depending on the CLEC5A/CDH2 ratio [low-risk <3]. The log-rank test *p*-value is shown. Hazard ratio: 23.4 (95% confidence interval [CI] 5.3–102.0). Patients with escalation after 1 year and censored within the first year [excluded from modelling] are also included for illustration.

Table 4. Transcription factors implied in inflammatory bowel disease by this study point towards involvement of the Th9 immune response, monocytes/macrophages, and IFN-ϒ.

	Biological significance
NFE2	Implied in megakaryocyte maturation and development of erythroid colonies.[32,33] May bind IL-8 promoter.[34] In late-stage megakaryocytes, NFE2 is upregulated by IRF2 [see below], but suppressed by IFNα[35,36]
SPI1 [PU.1]	Key regulator of Th9 immunity, marker of Th9 cells. Inhibits uncontrolled neutrophil activation.[37] Together with CEBPB [see below] enables *IL1B* transcription[38]
CEBPB	Involved in monocyte survival,[39] expression of IL-17-regulated genes,[40] and development of Th2 cells. May form homo- or heterodimers with other CEBP proteins
IRF2	Induced by IFNϒ, competes with IRF1 to deactivate the expression of IFNα and IFNβ. Activates IL-7 and belongs to non-canonical inflammasome detecting cytosolic lipopolysaccharide[41,42]

NFE2, nuclear factor erythroid 2; SPI1, Spi-1 proto-oncogene; CEBPB. CCAAT enhancer binding protein beta; IRF2, interferon regulatory factor 2.

oPOSSUM. We chose DoRothEA v2 for reasons that included an up-to-date regulon library [Supplementary Methods]. Our concordance-based approach to filtering is arbitrary, but motivated by the way TF inference tools are developed and aimed at indicating only TFs unequivocally supported by diverse methods in independent data sources.

4.6. Predicting treatment escalation

The translational application of transcriptional profiling of IBD in practice is of immediate clinical interest. A whole blood-derived 17-gene classifier proposed by Biasci *et al.* has been shown to be highly predictive of disease course. This forms the basis of the current biomarker-stratified PROFILE trial in CD.[16] By applying machine learning to our dataset using 12 out of 15 informative genes, we provide confirmatory evidence of such a signature predicting treatment escalation with an AUC of up to 0.87. This is important as the first independent confirmation of this signature. Moreover, we report that simpler models, involving two transcripts only, may have comparable utility. In the strongest binary model we derive, the *CLEC5A/CDH2* signature [Box 1] strongly associates with the need for treatment escalation in UC and makes a promising candidate for further external validation [Box 1].

The predictive accuracy of the signature proposed by Biasci and colleagues is worthy of discussion. This was assessed using two sets of criteria: our more stringent criteria, predefined for the discovery cohort, and the broader criteria, originally applied by Biasci *et al.*, which included azathioprine. Both demonstrate that the signature may predict the need for an escalation of therapy. However, there are some caveats. First, we note that our analysis is approximated, as the original equation is not available and could not be assessed. We did not use separate criteria for CD and UC and no data on the number of escalation events were available. Furthermore, not all transcripts used by Biasci *et al.* were expressed in the discovery cohort at sufficient levels. Nevertheless, these favourable results highlight the potential for generalisation

BOX 1. FUNCTIONS OF GENES IN THE ULCERATIVE COLITIS [UC] TREATMENT ESCALATION-ASSOCIATED *CLEC5A/CDH2* RATIO.
CLEC5A [C-type lectin-like protein] is expressed in leukocytes and remains under the control of SPI1 [PU.1] and CEBPB,[55,56] which we show herein to be critical IBD transcription factors. Although CLEC5A is chiefly known for being a dengue virus receptor, it was recently implicated in IBD as a marker for M1 macrophages, which express TNF.[57,58] The CLEC5A signalling cascade includes IBD-related proteins such as Zap-70, TREM-2, and SYK. CDH2 [cadherin 2, N-cadherin] is a calcium-dependent cell adhesion glycoprotein that enables dendritic cell migration[59] and was proposed as a marker for the epithelial-to-mesenchymal transition in circulating tumour cells.[60] CDH2 may associate with immune tolerance as it predicts breast cancer recurrence, shorter survival in multiple myeloma,[61] and Ewing's sarcoma.[62] Increased CDH2 levels are also found in CD stenotic tissue.[63] In mice, CDH1 [but not CDH2] is a marker of M2 macrophages; establishing a similar role for CDH2 would suggest that the CLEC5A/CDH2 ratio is chiefly related to polarisation of these cells.

of this specific gene signature, and the value of prognostic transcriptomic markers in IBD in general.

4.7. Limitations and future directions

The prognostication part of the study, albeit based on a large, international cohort, would require independent validation, and a quantitative polymerase chain reaction [qPCR] assay would need to be developed. Alternatively, flow cytometry or immunohistochemistry could also be helpful in further investigation of the role of CLEC5A in IBD, with the latter potentially bringing the method close to the bedside by prospectively studying blood smears or retrospectively analysing archived UC intestinal biopsy material. A larger sample size of any future investigations in this domain would allow for the study of the relationship between disease activity on treatment and the accuracy of prediction of the disease course.

The presented analyses implicating NFE2, SPI1, CEBPB, IRF2, and *CLEC5A*, *CDH2* would all benefit from functional validation. Further study of the transcription factors' importance could involve chromatin immunoprecipitation-sequencing. With regard to differentially expressed genes, discriminating between IBD-related transcriptomic changes that are protective and those that are pathogenic would also require further mechanistic study. These limitations are balanced by the large sample size, as well as by a significant overlap of the main results between different methods and in an independent replication cohort.

4.8. Conclusion

Detailed whole-blood transcriptomic analyses in two large European cohorts has progressed our understanding of several aspects of IBD. The most overexpressed genes may represent important molecules in the pathogenesis of this disease and are potentially druggable targets—in this context we highlight *CD177*, *OLFM4*, and *GPR15*. Moreover, a number of TFs are implicated in our discovery and validation datasets as having an important role in inflammation. In particular, NFE2, SPI1 [PU.1], CEBPB, and IRF2 are the most involved and warrant further study. Importantly, we also provide the first validation of the predictive signature proposed by Biasci *et al.* to be of translational use, and provide evidence that simpler models, such as the *CLEC5A*/*CDH2* expression ratio, constitute promising candidates for validation in the prediction of treatment escalation in IBD.

Overall, our study provides the foundation for future work focusing on the mechanistic and clinical roles of the TFs implicated in IBD, including single-cell sequencing studies of blood leukocytes in IBD, with the aim of pinpointing targetable pathways and establishing the role of genes overexpressed in IBD. Further scaling up of transcriptomic research in terms of cohort sizes will be necessary to reveal the disease biology in finer detail and to establish practical classifiers with a range of diagnostic, subtyping, and prognostic applications.

Supplementary Data

Supplementary data are available at *ECCO-JCC* online.
The data underlying this article are available in ArrayExpress (accession E-MTAB-11349).

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Conflict of Interest

JKN reports personal fees from Norsa Pharma, a grant from Biocodex Microbiota Foundation, and non-financial support from Nutricia outside of the submitted work. RK has served as a speaker for Ferring and has received support for research from IBD-Character [EU FP7 2858546]. DB has received personal fees as speaker and/or advisory board member for Ferring, Janssen, Pfizer, and Takeda. JW reports personal fees and non-financial support from Biocodex, BGP Products, Chiesi, Hipp, Humana, Mead Johnson Nutrition, Merck Sharp & Dohme, Nestle, Norsa Pharma, Nutricia, Roche, Sequoia Pharmaceuticals, and Vitis Pharma, outside of the submitted work, and also grants, personal fees, and non-financial support from Nutricia Research Foundation Poland, also outside of the submitted work. JJH has received personal fees as speaker, consultant, and/or advisory board member for AbbVie, Aqilion AB, Celgene, Celltrion, Dr Falk Pharma and the Falk Foundation, Ferring, Hospira, Janssen, MEDA, Medivir, MSD, Olink Proteomics, Pfizer, Prometheus Laboratories, Sandoz/Novartis, Shire, Takeda, Thermo Fisher Scientific, Tillotts Pharma, Vifor Pharma, UCB, and has received grant support from Janssen, MSD, and Takeda, outside of the submitted work. JS has served as a speaker, a consultant, and an advisory board member for MSD, Ferring, Abbvie, and Shire, a consultant with Takeda, has received speaking fees from MSD, travel support from Shire, and has received research funding from Abbvie, Wellcome, CSO, MRC, and the EC grant IBDBIOM.

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Author Contributions

Study design: JKN, ATA, RK, MHV, PR, JH, JS. Patient recruitment and sample processing: ATA, RK, JCL, SV, DB, AVK, FG, MHV, PR, JO, JW, JH, JS. Data analysis: JKN, ATA, RK, JS. Drafting the manuscript: JKN, ATA, RK. Study coordination: JH, JS. All authors were involved in critical review, editing, revision and approval of the final manuscript.

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IBD Character Consortium: Erik Andersson, Ian D. Arnott, MD, Monica Bayes, PhD, Ferdinando Bonfiglio, PhD, Ray K. Boyapati, MD, Adam Carstens, MD, Christina Casén, MSc, Ewa Ciemniejewska, MSc, Mauro D'Amato, PhD, Fredrik A. Dahl, PhD, Trond Espen Detlie, MD, Hazel E. Drummond, BSc, Gunn S. Ekeland, MSc, Daniel Ekman, MSc, Anna B. Frengen, PhD, Mats Gullberg, PhD, Ivo G. Gut, PhD, Marta Gut, PhD, Simon C. Heath, PhD, Fredrik Hjelm, PhD, Henrik Hjortswang, MD, PhD, Gwo-Tzer Ho, PhD, Daisy Jonkers, PhD, Nicholas A. Kennedy, MBBS, PhD, FRACP, Charles W. Lees, PhD, Torbjørn Lindahl, MSc, Mårten Lindqvist, PhD, Angelika Merkel, PhD, Eddie Modig, BSc, Aina E. F. Moen, PhD, Hilde Nilsen, PhD, Elaine R. Nimmo, PhD, Colin L. Noble, MD, Niklas Nordberg, PhD, Kate R. O'Leary, MSc, Anette Ocklind, PhD, Christine Olbjørn, MD, Erik Pettersson, PhD, Marieke Pierik, MD, PhD, Dominique Poncelet, PhD, Dirk Repsilber, PhD, Céline Sabatel, PhD, Renaud Schoemans, PhD, Alan G. Shand, MD, Johan D. Söderholm, MD, PhD, Janne Sølvernes, MS, Mikael Sundell, BSc, Tone M. Tannæs, PhD, Leif Törkvist, MD, PhD, Anne-Clémence Veillard, PhD, Nicholas T. Ventham, MRCS[Eng], PhD, MBBS, David C. Wilson, MD, MRCPCH, Panpan You, MS.

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