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

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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 (log2-fold change [LFC] = 4.63, p = 4.05 × 10^-118), MCEMP1 [LFC = 2.45, p = 7.37 × 10^-109], and S100A12 [LFC = 2.31, p = 2.15 × 10^-93]. Significantly over-represented pathways included IL -1 [p = 1.58 × 10^-11], IL -4, and IL -13 [p = 8.96 × 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.
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

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**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.
understanding of the molecular profiles of disease onset, drug resistance, and disease course.  

Among these, transcriptomic analyses have contributed to addressing these challenges by identifying signatures correlated with response to therapy and a T cell-derived signature that associates with the need for treatment escalation. 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. 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.

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. 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 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.

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], ChIP-X Enrichment Analysis 3 [ChEA3], and Discriminant Regulon Expression Analysis [DoRothEA2] v2.4 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 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.

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. 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
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^{-4}\] 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 log₂-fold change [LFC] in comparisons against controls exceeded unity only for over-expression \([1.4\% \text{ 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 implicated GPR15 and LRRN3, are presented in Supplementary Table 3 and 4.

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 \([\text{FDR} <0.05]\) 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^{-4}\], 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.
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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 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

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%]            |
| **L1/L1+L4**   | 49 [31%]/1 [1%]     |                     |                         |
| **L2/L2+L4**   | 41 [26%]/5 [3%]     |                     |                         |
| **L3/L3+L4**   | 47 [30%]/10 [6%]    |                     |                         |
| **L4**         | 3 [2%]              |                     |                         |
| **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] |                     |                         |
| **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%] | Rectal 5-ASA 26 [16%] |                         |
| **Oral budesonide 8 [3%]** | Oral 5-ASA 6 [4%] | Oral prednisone 15 [9%] |                         |
| **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.
In panel D may be considered overexpressed in CD relative to UC. CD, Crohn’s disease; UC, ulcerative colitis.

Genes with the top 10 most significant p-values and top 10 LFC are labelled [the two lists may overlap]. The genes labelled 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 NFE2, SPI1 [PU.1], CEBPB, and IRF2 [Figure 5].

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 IRF2 in IBD mucosal transcriptomes.

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

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 × 10^{-69}], TLR4, NCF4, and ITGAM. SPI1 [PU.1] correlated with the kinase RPS6KA1 [rho = 0.86, p = 3.75 × 10^{-57}] and matrix metalloproteinase inhibitor TIMP2, as well as with NCF2 and NCF4. CEBPB expression associated with STX3 [rho = 0.74, p = 1.73 × 10^{-54}], 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 × 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 replication data. Mean log2-fold changes (LFC) and Bonferroni-corrected p-values [pBonf.] for genes overexpressed in the discovery and replication cohorts. Only genes with absolute LFC >1 and pBonf. < 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 naive 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 and MZ1L2 in IBD overall, and of C14orf28 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
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], HIST1H3HI/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
| Replication cohort | IBD | CD | UC |
|-------------------|-----|----|----|
| **Discovery cohort** | IBD | CD | UC |
| **CEBPB** | ARID3A, NFE2, CEBPB, CEBPA, PLAGL1, MAX, MAFB, CUX1, ETV1, ETV6, CEBP | ARID3A, NFE2, CEBPB, CEBPA, PLAGL1, MAX, MAFB, CUX1, ETV1, ETV6, CEBP | ARID3A, NFE2, CEBPB, CEBPA, PLAGL1, MAX, MAFB, CUX1, ETV1, ETV6, CEBP |
| **EPEE** – overactive | SPI1, NFE2, FOXO3, ETV1, IFI16, IRAK1 | SPI1, NFE2, FOXO3, ETV1, IFI16, IRAK1 | SPI1, NFE2, FOXO3, ETV1, IFI16, IRAK1 |
| **EPEE** – deficient | SPI1, NFE2, FOXO3, ETV1, IFI16, IRAK1 | SPI1, NFE2, FOXO3, ETV1, IFI16, IRAK1 | SPI1, NFE2, FOXO3, ETV1, IFI16, IRAK1 |
| **CEBP** | LYL1, ELF1, CEBP | LYL1, ELF1, CEBP | LYL1, ELF1, CEBP |

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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: EPSE and CHEA3. TFs inferred from both datasets (discovery: IBD, Character, replication: Ostorowski 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.

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 TGFB signalling. Some of the genes with high anti-inflammatory module membership cause very early-onset IBD when deficient [ZAP70, LRBA].

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 undermethyalted in systemic sclerosis in comparison with controls. SPI1 [PU.1] governs Th9 immunity, which in IBD is still insufficiently understood, but appears predominantly pro-inflammatory. Animal models, interleukin-9 deficiency protected mice against trinitrobenzene sulfonic acid [TNBS]- and oxazolone-induced colitis. 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. 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.

CEBPB was found to lie 150 kb downstream from an IBD risk locus. Interestingly, profiling of transcription start sites in IBD biopsies also highlighted CEBBP as potentially involved in the disease. It is overexpressed in the mucosa of patients with UC and strategically positioned at the interface analysis. CD177 encodes human neutrophil alloantigen 2a, which is present on cells exhibiting bactericidal activity in IBD, and impairs neutrophil migration. Interestingly, CD177+ neutrophils producing IL-17 are increased in allergic asthma. Olfactomedin 4 is a marker of intestinal stem cells and is known to be increased in the IBD mucosal proteome. The copy number of defensins AI–3 correlates with the diagnosis of CD and its colonic location. 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 defense deficiency in ileal CD.

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. GPR15- T cells secrete less IFN-γ, but more IL-17 after stimulation, suggesting a Th17-like phenotype. It has been shown that regulatory T cell GPR15 expression in UC patients is increased and, most recently, the dependence of GPR15 transcription on aryl hydrocarbon receptor signalling has been demonstrated.
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.

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 
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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.\(^\text{16}\) 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.

![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.](image)

![Table 4. Transcription factors implied in inflammatory bowel disease by this study point towards involvement of the Th9 immune response, monocytes/macrophages, and IFN-γ.](table)

| Biological significance                                      |
|-------------------------------------------------------------|
| NFE2  | Implied in megakaryocyte maturation and development of erythroid colonies.\(^\text{32,33}\) May bind IL-8 promoter.\(^\text{44}\) In late-stage megakaryocytes, NFE2 is upregulated by IRF2 [see below], but suppressed by IFNα.\(^\text{35,36}\) |
| SPI1 [PU.1] | Key regulator of Th9 immunity, marker of Th9 cells. Inhibits uncontrolled neutrophil activation.\(^\text{37}\) Together with CEBPB [see below] enables IL1B transcription.\(^\text{38}\) |
| CEBPB | Involved in monocyte survival,\(^\text{39}\) expression of IL-17-regulated genes,\(^\text{40}\) and development of Th2 cells. May form homo- or heterodimers with other CEBP proteins. |
| IRF2  | Induced by IFNY, competes with IRF1 to deactivate the expression of IFNα and IFNβ. Activates IL-7 and belongs to non-canonical inflammasome detecting cytosolic lipopolysaccharide.\(^\text{41,42}\) |

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

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