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Whole Blood Profiling of T-cell-Derived microRNA Allows the Development of Prognostic models in Inflammatory Bowel Disease

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Abstract

Background: MicroRNAs [miRNAs] are cell-specific small non-coding RNAs that can regulate gene expression and have been implicated in inflammatory bowel disease [IBD] pathogenesis. Here we define the cell-specific miRNA profiles and investigate its biomarker potential in IBD.

Methods: In a two-stage prospective multi-centre case control study, next generation sequencing was performed on a discovery cohort of immunomagnetically separated leukocytes from 32 patients (nine Crohn's disease [CD], 14 ulcerative colitis [UC], eight healthy controls) and differentially expressed signals were validated in whole blood in 294 patients [97 UC, 98 CD, 98 non-IBD, 1 IBDU] using quantitative PCR. Correlations were analysed with phenotype, including need for early treatment escalation as a marker of progressive disease using Cox proportional hazards.

Results: In stage 1, each leukocyte subset [$CD4^+$ and $CD8^+$T-cells and $CD14^+$ monocytes] was analysed in IBD and controls. Three specific miRNAs differentiated IBD from controls in $CD4^+$T-cells, including miR-1307-3p [$p = 0.01$], miR-3615 [$p = 0.02$] and miR-4792 [$p = 0.01$]. In the extension cohort, in stage 2, miR-1307-3p was able to predict disease progression in IBD (hazard ratio [HR] 1.98, interquartile range [IQR]: 1.20–3.27; logrank $p = 1.80 \times 10^{-3}$), in particular CD [HR 2.81; IQR: 1.11–3.53, $p = 6.50 \times 10^{-4}$]. Using blood-based multimarker miRNA models, the estimated chance of escalation in CD was 83% if two or more criteria were met and 90% for UC if three or more criteria are met.

Interpretation: We have identified and validated unique $CD4^+$ T-cell miRNAs that are differentially regulated in IBD. These miRNAs may be able to predict treatment escalation and have the potential for clinical translation; further prospective evaluation is now indicated.

Key Words: MicroRNA; T-cell; inflammatory bowel disease; crohn's disease; ulcerative colitis; biomarkers; proteins; prognosis; whole blood; mRNA; epigenetics

1. Introduction

Inflammatory bowel disease [IBD] is a global health burden with increasing incidence and prevalence in newly industrialized nations, with healthcare costs in the UK and Europe.[1,2] Despite tremendous progress in our understanding of the genetics in IBD, there remains a large proportion of disease variance that is unexplained. Studies are beginning to explore the epigenome as the next tier of information in complex immune-mediated diseases.[3–5] First implicated in 1993 in epigenetic regulation, microRNAs [miRNAs] have now been discovered in most species and within most body fluids in humans. MicroRNAs are non-coding RNAs that have the ability to regulate and fine-tune gene expression. There is strong evidence that IBD pathways are regulated by miRNAs, notably regulation of the Th-17 pathway by the NOD2-driven miR-29.[6] miR-196 regulates IRGM, which is a known IBD GWAS susceptibility gene; the known single nucleotide polymorphism [SNP] alters the binding site for miR-196, dysregulating xenophagy in Crohn's disease [CD].[7] Most recently, in a detailed genome-wide analysis of the disease-associated methylome, our group has shown differential hypo-methylation at the transcriptional start site for miR-21 and increased expression of pri-miR-21 in leukocytes and in inflamed intestinal tissue.[4] This miRNA has now been shown to have protective effects in miR-21-knockout [KO] mice exposed to dextran sodium sulphate [DSS] and deleterious effects when exposed to 2,4,6-trinitrobenzenesulfonic acid [TNBS].[8]

There are a number of methodological considerations that potentially confound miRNA analysis, both biological and technical; a key issue is cellular heterogeneity. Every cell type possesses its own unique epigenetic signature. Therefore, interpreting the relevance of miRNAs detected in heterogeneous samples [e.g. whole blood, intestinal biopsies] is challenging.[5] Applying next-generation sequencing to immune cell subsets provides hypothesis-free and cell-specific profiling of miRNAs. In this study, we have applied sequencing to generate unique circulating cell-specific signatures in IBD at diagnosis. In a multi-centre independent replication cohort, we further assess this signal as a biomarker in whole blood and characterize its accuracy in defining disease course. These miRNA models are then incorporated into prognostic models with conventional blood markers with the ability to accurately predict treatment escalation over time.

2. Materials and Methods

2.1. Study design

We conducted a prospective two-stage discovery and validation multi-centre case-control study as summarized in Figure 1. Patients with a new diagnosis of IBD were included in the study. All IBD cases met the standard diagnostic criteria for ulcerative colitis [UC], CD or inflammatory bowel disease unclassified [IBDU] following thorough clinical, microbiological, endoscopic, histological and radiological evaluation. The Lennard-Jones, Montreal and Paris criteria were used for diagnosis and classification of clinical phenotypes.[9–11]

2.2. Stage 1: Discovery cohort

For the discovery cohort, immune-magnetic cell separation was performed using a previously described protocol.[12] In brief, peripheral blood mononuclear cells were isolated from 18–36 mL of EDTA whole blood using Ficoll [Ficoll-Paque, GE Healthcare]. Cells labelled with antibody-coated microbeads [human $CD14^+$, $CD8^+$ and $CD4^+$ microbeads, 20 µL per 10^7 cells] were immunomagnetically separated using the autoMACs Pro cell separator [Miltenyi] and cell purity was estimated using fluorescent antibody staining and flow cytometry [FACS Aria II, BD]. Flow cytometric assessment demonstrated high purity of isolated cell populations following immunomagnetic cell separation ($CD14^+$ median: 92.4% [interquartile range {IQR} 87–94.9], $CD4^+$: 97.3% [93.8–98.9], $CD8^+$: 88.7 [80.5–93]).

A total of 90 leukocyte subset [$CD4^+$, $CD8^+$ and $CD14^+$ cells] samples were obtained from 32 patients [nine CD, 14 UC, one IBDU, eight healthy controls] with newly diagnosed IBD, who were naïve to therapy, and age- and sex-matched healthy individuals. Demographic and clinical data including drug therapies were collected [Table 1A]. Cell sample RNA was extracted using the Qiagen Allprep DNA/RNA miRNA universal kit as per the manufacturer's instructions. The Agilent Bioanalyzer platform and NanoChip kit were used for sizing, quantification and quality control [QC] of extracted miRNA from separated cells. All separated cells reached high-quality RNA integrity number [RIN] [mean RIN 9.2].

Libraries were prepared for 90 separated cell samples using the Trilink Clean Tag method. Library preparation involved ligating adenylated single strand DNA to the 3′ and 5′ ends of the RNA. RNA was then reverse transcribed into cDNA clones and PCR was used to amplify sequences, with the addition of barcodes to allow pooling of samples. The PCR products were size selected using gel electrophoresis to obtain small RNA libraries. Sequencing was performed using the Illumina NovaSeq platform.

2.3. Stage 2: Validation cohort

Whole blood miRNA for the replication cohort was collected using a standardized protocol across UK [Edinburgh] and European centres [Sweden, Norway, Spain, Netherlands] in Paxgene tubes and stored at −80°C. Total RNA was extracted from whole blood using a MagMax extraction kit according to the manufacturer's instructions. The validation cohort comprised 294 patients with suspected or confirmed IBD and a control group consisting of patients with gastrointestinal symptoms [symptomatic controls] who had no discernible clinical or pathological evidence of IBD at any time during follow-up, and healthy controls. Patients were recruited at presentation to gastrointestinal clinics across six clinical centres in the UK and Europe as part of the EU Character study [EU Character reference no. 305676]. Demographic and clinical data including drug therapies were collected [Table 1B]. Paired high-sensitivity C-reactive protein [hsCRP] and albumin were available in a subcohort of patients assayed as part of the IBD Character Consortium. Other routine markers including haemoglobin, white cell count and platelets were tested as part of clinical care. Clinical outcome data were collected at follow up for patients with IBD. A total of 73% of patients with IBD were naïve to medical therapies in the validation cohort.

A total of seven endogenous controls were identified from a literature review as potential controls in whole blood PCR experiments: miR-130b-3p, miR-130b-5p, miR-342-3p, U6, SNORD44, SNORD48 and SNORD49A. Controls were tested for their performance and stability across all samples and a GeNorm score was

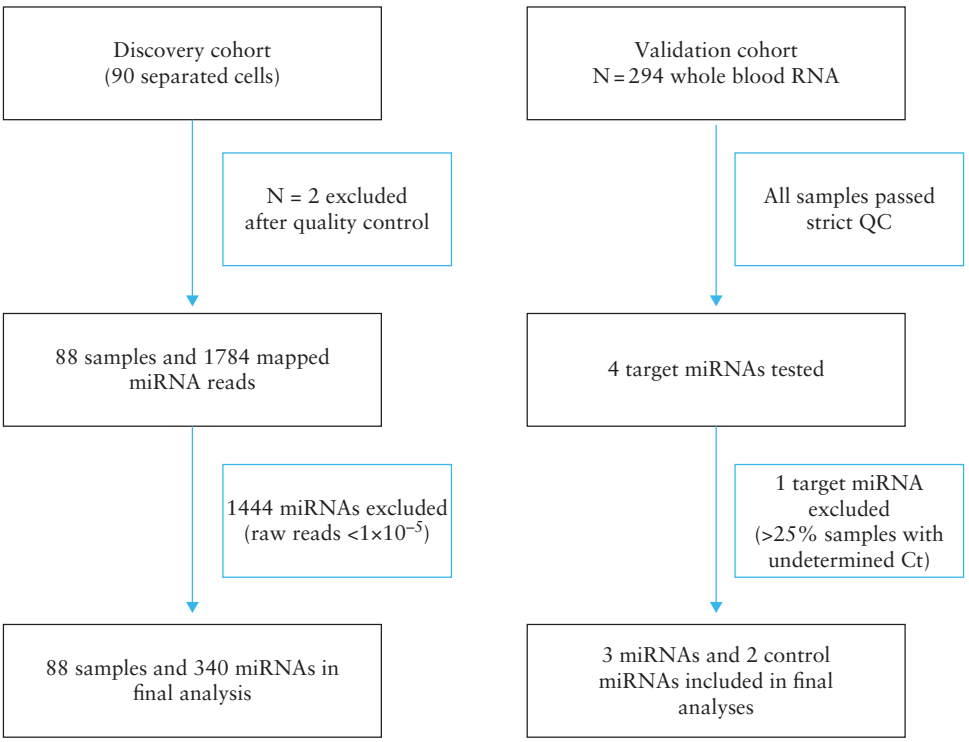

Figure 1. Flow diagram showing the two-stage discovery and validation design of the study.

Table 1A. Separated cell discovery cohort

miRNA sequencing discovery cohort		
Variable	IBD [*n* = 24]	HC [*n* = 8]
Diagnosis [CD:UC:IBDU]	9:14:1	8
Cell subsets [CD4:CD8:CD14]	23:20:24	7:8:8
Median age, years [range]	34 [18–68]	43 [20–59]
Sex [M:F]	16:8	4:4

CD4: $CD4^+$ T-cells; CD8: $CD8^+$ T-cells; CD14: $CD14^+$ monocytes; CD: Crohn's disease; UC: ulcerative colitis; IBDU: inflammatory bowel disease unclassified; HC: healthy controls; M: male; F: female. All numbers shown represent the number of patients.

given to each marker. GeNorm calculates stability based on pairwise variation and generates a stability score, a lower score representing higher stability.[13] miR-130b-5p [GeNorm 0.82] and miR-342-3p [GeNorm 0.8] had the lowest GeNorm score and were selected for further analyses.

miRCURY LNA miRNA PCR assays were designed and synthesized by Qiagen for the following mature miRNAs: miR-130b-3p, miR-130b-5p, miR-200b-3p, miR-342-3p, U6, miR-1307-3p, SNORD44, SNORD48 and SNORD49A. TaqMan Advanced miRNA assays were designed and synthesized by ThermoFisher for the following mature miRNAs: miR-3615, miR-4792, miR-130b-3p, miR-342-3p and miR-130b-5p.

Reverse transcription [RT] of miRNA templates was performed according to the manufacturer's protocol. In brief, 10 ng of RNA was polyadenylated and reverse transcribed into cDNA containing UniSp6 [Qiagen] spike-in using the miRCURY LNA RT Kit [Qiagen] and cel-miR-39 spike-in for the Taqman cDNA kit on a T100 thermal cycler [Bio-Rad]. Real-time qPCRs were performed with miRCURY LNA miRNA PCR assays and a miRCURY LNA SYBR Green PCR Kit [Qiagen] in a 384-well plate [HardShell 384-well PCR plates, Bio-Rad] on a QuantStudio 7 Flex Real-Time PCR System [ThermoFisher].

2.4. Clinical outcome data

In the IBD cohort, clinical outcome data were collected over time across all centres [Table 2]. Treatment escalation was defined as the need for two or more immunomodulators and/or surgery over time after initial disease remission.[14,15] Treatment naivety within the IBD cohorts was defined as no exposure to any IBD-related medical therapies such as oral or topical steroids, 5-aminosalicylic acid [5-ASA] therapies, biologics and immunosuppressants.

2.5. Gene expression profiling

Whole blood RNA underwent targeted RNA sequencing which was performed using an Ion AmpliSeq Human Gene Expression Core Panel, containing 20 802 genes. QC was performed using the Ion Library Taqman Quantitation kit. Sequence reads were aligned using the Torrent Suite Software [TSS] and the number of matches per amplicon was quantified. After filtering, 14 182 transcripts were available for further analysis.

2.6. Ethics statement

All centres were granted local ethics approval for this study and all patients gave written and informed consent prior to participating in this study. The study was funded by Crohn's and Colitis UK [grant number M2016/2].

3. Data Analysis

3.1. Stage 1: RNA sequencing

Raw reads were aligned to the human genome using miRDeep 2, with output restricted to those that aligned full length and were

Table 1B. Demographics of the quantitative polymerase chain reaction [qPCR] validation cohort

qPCR validation cohort demographics			
Variable	UC [*n* = 97]	CD [*n* = 98]	Non-IBD [*n* = 98]
Mean age, years [range]	30 [24–60]	30 [23–35]	26 [23–28]
Gender [M:F]	68:29	47:51	59:39
Centre [UK:Sweden:Norway:Spain]	35:16:41:5	34:22:31:11	20:51:14:13
Smoking status [current:ex:never:unknown]	9:29:57:2	34:15:45:4	17:20:55:6
Non-IBD: Healthy controls			66:32
Montreal location			
E1	23		
E2	29		
E3	45		
L1		37	
L2		26	
L3		34	
L4		1	
Montreal behaviour			
B1 + B1p		81	
B2		10	
B3 + B3p		6	
Not available		1	

CD: Crohn's disease; UC: ulcerative colitis; IBDU: inflammatory bowel disease unclassified; HC: healthy controls; M: male; F: female. All numbers shown represent the number of patients.

Table 2. Patient demographics for predicting disease course in inflammatory bowel disease [IBD]

qPCR validation cohort demographics		
Variable	Escalators [*n* = 80]	Non-escalators [*n* = 115]
Diagnosis [UC:CD:IBDU]	33:47:0	64:50:1
Median age, years [range]	27 [21–36]	29 [24–34]
Sex [M:F]	46:34	68:47
Centre [UK:Sweden:Norway:Spain]	38:15:23:4	31:23:49:12
Smoking status [current:ex:never:unknown]	20:18:38:4	24:25:64:2
UC classification		
E1 [proctitis]	0	23
E2 [left-sided colitis]	9	20
E3 [pancolitis]	24	21
CD classification		
L1 [terminal ileum]	18	18
L2 [colonic]	11	15
L3 [ileocolon]	18	16
L4 [upper gastrointestinal]	0	1
CD behaviour		
B1, B1p [non-stricturing and non-penetrating, +perianal]	34	46
B2, B2p [stricturing, +perianal]	8	2
B3, B3p [penetrating, +perianal]	5	1
Not available	0	1

Escalation was defined as the need for two or more immunomodulators and/or surgery after initial disease remission. CD: Crohn's disease; UC: ulcerative colitis; IBDU: inflammatory bowel disease unclassified; HC: healthy controls; M: male; F: female. All numbers shown represent the number of patients.

a perfect match. miRNAs with raw reads < 1×10^{-5} and samples with > 50% of miRNA reads < 5 were excluded from further analyses. Any cell type mismatches identified from principal component analysis were filtered out [*n* = 2]. A total of 340 miRNAs and 88 cellular samples were quantile-normalized and further analysed for differential expression. R 3.4.4 [R Foundation for Statistical Computing] was used for statistical and bioinformatics analysis. *p*-values for differentially expressed proteins were adjusted for multiple testing (Benjamini–Hochberg procedure; false discovery rate [FDR]].

3.2. Stage 2: qPCR statistical analysis [relative quantification]

Three technical replicates were performed for each assay. Raw Ct values were exported for downstream analysis. The expression levels of target miRNAs were normalized to two reference genes, miR-130b-5p and miR-342-3p. [16]Relative quantification [i.e. fold change] of miRNAs was calculated by the $2^{-\Delta\Delta Ct}$ method.

Means and standard deviations [SD] were generated for each sample and for the miRNA target and endogenous controls. An SD threshold filter of < 1.0 was used for QC. Target miRNAs in

which > 25% of samples had undetermined Ct values were excluded from further analyses. There were no samples excluded after filtering for QC and three target miRNAs passed QC: miR-1307-3p, miR-3615 and miR-4792. Fold change was calculated using the formula $2^{-\Delta\Delta Ct}$ [equivalent to relative endogenous expression of target miRNA].

3.3. Biomarker statistical analyses

Conventional laboratory inflammatory parameters [CRP and albumin], age and sex were included in multivariable models for IBD diagnosis and prognosis. CRP and the top miRNAs were $\log_{10}$-transformed to approximate a normal distribution for further multivariable analysis. The optimal models were then selected by performing backward stepwise regression using the lowest Akaike information criterion [AIC] values. Leave-one-out [LOO] cross-validation was used to test the performance of a multi-marker diagnostic model.

For the prognostic model, a Cox proportional hazards model was derived to assess the contribution of each variable to disease outcomes. Thresholds were then identified using receiver operating characteristic [ROC] analyses to allow stratification of patients to either a benign or an aggressive disease course [requiring treatment escalation and/or surgery], and to allow creation of survival curves.

4. Results

4.1. Stage 1: miRNA sequencing: Discovery cohort

A total of 90 separated cell samples were selected for sequencing, of which 88 samples and 340 miRNAs passed strict QC as described previously. There were 30 CD4$^+$ T-cell samples, 28 CD8$^+$ T-cell samples and 30 CD14$^+$ monocyte samples. Principal component analyses demonstrated distinct clustering based on cell-type [Supplementary Figure 1].

Each cell type was analysed for differential expression in IBD compared to controls adjusting for age, sex and batch effects. These data are summarized in Table 1A. Three miRNAs differentiated IBD from controls in CD4 T-cells: miR-1307-3p [FDR $p = 0.01$], miR-3615 [$p = 0.02$] and miR-4792 [$p = 0.01$]. In CD8 T-cells miR-200b-3p was the only miRNA that was differentially regulated in IBD compared to controls [Table 3]. This miRNA was down-regulated in UC [Supplementary Table 1]. There were no CD14-specific miRNAs that differentiated UC from controls in this cohort.

Only miR-10b-5p differentiated CD from controls [Supplementary Table 2] but no miRNAs differentiated UC from CD across all cell subsets.

4.2. Stage 2: Validating miRNA markers using qPCR

After QC, a total of 294 whole blood RNA samples and three target miRNAs were included for further analyses. Table 1B summarizes the demographics of the cohort. There were 97 UC, 98 CD, one IBDU and 98 non-IBD controls. In CD, 78% [$n = 76$] had a B1 [non-stricturing] phenotype at recruitment. In UC, 24% [$n = 23$] had limited proctitis while 46% [$n = 45$] had pancolitis at recruitment.

A total of 287 samples passed QC for miR-1307-3p. This miRNA was differentially up-regulated in IBD compared to controls (1.55-fold change [FC], IQR: 1.00–1.87; $p = 2.77 \times 10^{-5}$), consistent with the direction of change seen in the sequencing dataset. There was no significant difference seen between non-IBD symptomatic controls and healthy controls for miR-1307-3p [$p = 0.82$]. This miRNA was differentially up-regulated in UC [1.69 FC, IQR: 1.01–2.00; $p = 1.56 \times 10^{-6}$] and CD [1.42 FC, IQR: 0.84–1.70; $p = 0.01$] compared to controls and was more highly expressed in UC compared to CD [1.19 FC, $p = 0.02$; Figure 2]. Furthermore, miR-1307-3p was more highly expressed with progressive UC extent as defined by the Montreal Classification [Kruskal–Wallis $p = 0.03$] but was not associated with CD location or behaviour [$p = 0.13$].

The other miRNAs, miR-3615 and miR-4792, were differentially up-regulated in UC compared to controls [miR-3615: 1.21 FC, IQR: 0.91–1.48; $p = 8.26 \times 10^{-4}$; miR-4792: 1.91 FC, IQR: 0.81–2.56; $p = 9.21 \times 10^{-3}$]. The same miRNAs were overexpressed in pooled IBD vs control analyses, but the result failed to reach statistical significance.

4.3. miRNA expression and its association with inflammatory activity

Correlation analyses were performed using the top differentially expressed miRNAs and conventional blood-based inflammatory

Table 3. Differential expression of miRNAs in inflammatory bowel disease [IBD] vs healthy controls within separated CD4$^+$, CD8$^+$ and CD14$^+$ cells

miRNA	Log FC	Average relative expression	*p* value	FDR *p* value
CD4 T-cell analyses: IBD vs controls				
hsa-miR-4792	6.23	8.54	4.20E-05	0.01
hsa-miR-1307-3p	3.79	11.85	8.24E-05	0.01
hsa-miR-3615	2.69	11.43	2.00E-04	0.02
hsa-miR-320b	2.30	14.05	6.72E-04	0.05
hsa-miR-921	4.98	6.09	7.27E-04	0.05
CD8 T-cell analyses: IBD vs controls				
hsa-miR-200b-3p	–5.59	3.42	2.79E-05	0.01
hsa-miR-4792	5.26	8.80	2.69E-04	0.05
hsa-miR-30c-5p	–1.65	13.05	2.33E-03	0.26
hsa-miR-1246	2.35	10.67	3.73E-03	0.27
hsa-miR-3202	–4.33	4.12	3.95E-03	0.27
CD14 cell analyses: IBD vs controls				
hsa-miR-1261	–4.56	4.75	2.96E-03	0.37
hsa-miR-30c-5p	–1.63	14.13	0.02	0.37
hsa-miR-576-5p	–3.31	6.70	0.01	0.37
hsa-miR-126-5p	–3.31	8.01	0.01	0.37
hsa-miR-152-3p	–3.34	8.01	0.01	0.37

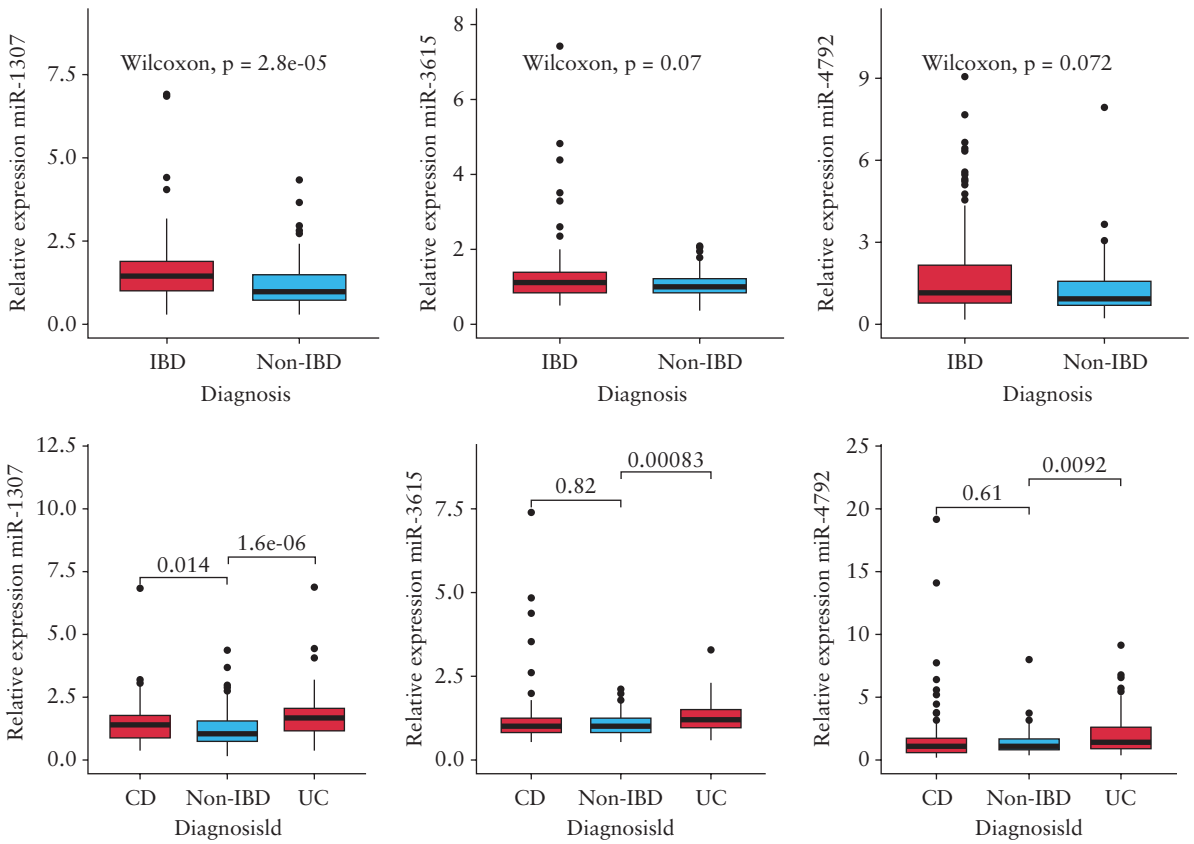

Figure 2. Relative expression of miR-1307-3p, miR-3615 and miR-4792 [*y*-axis] comparing inflammatory bowel disease [IBD] from non-IBD and differentiating IBD subtypes (Crohn's disease [CD] and ulcerative colitis [UC]) from non-IBD. Relative expression is depicted using the $2^{-\Delta\Delta Cq}$ method using miR-130b-5p and miR-342-3p as reference genes.

Table 4. Correlation analyses of conventional biomarkers with novel miRNA-based markers

miRNA	Hb	WCC	Platelet count	hsCRP	Albumin
miR-1307-3p	0.16 [0.01]	0.00 [0.96]	0.05 [0.40]	0.12 [0.06]	−0.04 [0.51]
miR-3615	0.25 [3.08×10^{-5}]	0.04 [0.45]	0.15 [0.02]	−0.02 [0.75]	0.07 [0.26]
miR-4792	0.23 [4.15×10^{-4}]	0.08 [0.24]	0.15 [0.02]	0.07 [0.31]	−0.09 [0.19]

Spearman analyses were performed and the data are depicted as rho[*p*-value]. FC: faecal calprotectin; WCC: white cell count; Hb: haemoglobin; hsCRP: high-sensitivity C-reactive protein.

markers such as hsCRP and albumin [data complete $n = 263$]. None of the miRNAs correlated with conventional blood-based tests such as hsCRP or albumin [Table 4].

4.4. Diagnostic biomarkers in IBD

The top differentially expressed miRNA had modest performance as a blood-based diagnostic marker. miR-1307-3p differentiated IBD from controls with area under the ROC curve [AUC] of 0.66 (95% confidence interval [CI]: 0.59–0.73) and performed on a par with hsCRP [AUC 0.67, CI 0.60–0.73; vs miR-1307-3p, $p = 0.88$] and albumin [AUC 0.65, CI 0.59–0.72; vs miR-1307-3p, $p = 0.89$]. In those who were naïve to medical therapy, the diagnostic performance of miR-1307-3p was similar [0.63, CI: 0.55–0.70, $p = 0.55$]. In CD, miR-1307 had a modest performance in CD [AUC 0.60, CI: 0.51–0.68]. In UC, miR-1307-3p performed on a par with miR-3615[p for comparison = 0.10] and miR-4792 [$p = 0.09$]. A combined three-miRNA marker provided no added benefit to the diagnostic UC model [AUC 0.66, CI: 0.57–0.74]. Figure 3 summarizes the diagnostic performance of the miRNAs in IBD and UC compared to controls.

Multivariable logistic regression analysis of predictors of IBD was performed on 263 cases [168 IBD, 95 non-IBD] where the data for predictors were complete and included miR-1307-3p, albumin and hsCRP. Age (odds ratio [OR]: 1.13, 95% CI: 1.08–1.20, $p = 8.73 \times 10^{-6}$), log[hsCRP] [OR: 2.11, CI: 1.26–3.63, $p = 5.60 \times 10^{-3}$], log[miR-1307] [OR: 6.40, CI: 2.08–20.98, $p = 1.56 \times 10^{-3}$] and albumin [OR: 0.92, CI: 0.86–0.99, $p = 0.04$] were significant predictors of IBD. These markers remained significant even after adjusting for treatment exposure. An LOO cross-validated diagnostic model incorporating these four predictors had an accuracy of 0.72 [95% CI: 0.65–0.78] and a positive and negative predictive value of 0.73 and 0.67 respectively.

In patients with a negative CRP [hsCRP < 5 mg/L], miR-1307-3p had an LOO cross-validated diagnostic accuracy of 0.65 [CI: 0.59–0.70] and performed on a par with albumin [accuracy 0.62, CI: 0.56–0.68].

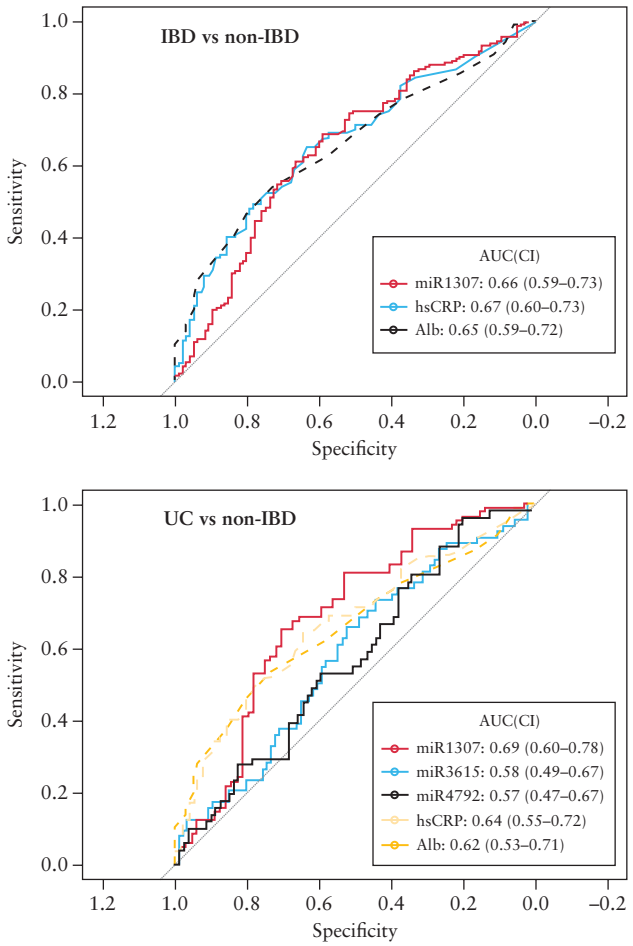

Figure 3. Receiver operator curve [ROC] analyses of miRNAs, high-sensitivity C-reactive protein [hsCRP] and albumin [Alb] in differentiating inflammatory bowel disease [IBD] from non-IBD and ulcerative colitis from non-IBD.

4.5. miRNAs as predictors of treatment escalation in IBD

Prognostic data were available for 195 IBD patients and the demographics are summarized in Table 2. A total 80 patients required treatment escalation over a median time of 371 days [IQR: 140–711]. There were no differences in age [$p = 0.37$] or sex [$p = 0.88$] between the escalator and non-escalator group. A total of 47 CD and 33 UC patients escalated treatment over time defined by the need for two or more immunomodulators and/or surgery over time after initial disease remission. miR-1307-3p was tested for its prognostic performance. Kaplan–Meier analyses were performed for 189 patients where data for the predictor had passed QC. miR-1307-3p was associated with disease course in IBD (hazard ratio [HR] 1.98, IQR: 1.20–3.27; logrank $p = 1.80 \times 10^{-3}$]. Analyses within disease sub-types showed that this marker was significantly associated with disease course in CD [HR 2.81; IQR: 1.11–3.53, $p = 6.50 \times 10^{-4}$] but not UC [$p = 0.061$].

Similarly, miR-3615 [UC: HR 2.55, IQR: 1.24–5.25, $p = 3.40 \times 10^{-3}$; CD: HR 2.01, IQR: 1.07–3.77, $p = 0.04$] and miR-4792 [UC: HR 2.29, IQR: 0.93–5.64, $p = 0.04$; CD: HR 2.42, IQR: 1.18–4.97, $p = 0.02$] predicted disease course in UC and CD [Table 5A and Supplementary Figure 2].

Table 5A. miRNAs as predictors of treatment outcomes in Crohn's disease and ulcerative colitis

Categorical variable	HR [IQR]	*p*-value for threshold
Crohn's disease		
miR-1307-3p > 1.29	2.81 [1.11–3.53]	6.50×10^{-4}
miR-3615 > 0.89	2.01 [1.07–3.77]	0.04
miR-4792 > 1.11	2.42 [1.18–4.97]	0.02
Ulcerative colitis		
miR-1307-3p > 1.43	2.11 [0.98–3.98]	0.06
miR-3615 < 0.95	2.55 [1.24–5.25]	3.40×10^{-3}
miR-4792 > 2.22	2.29 [0.93–5.64]	0.04

Categorical thresholds reported for miRNAs are relative expression as calculated by the $2^{-\Delta\Delta Ct}$ method. HR: hazard ratio; IQR: interquartile range.

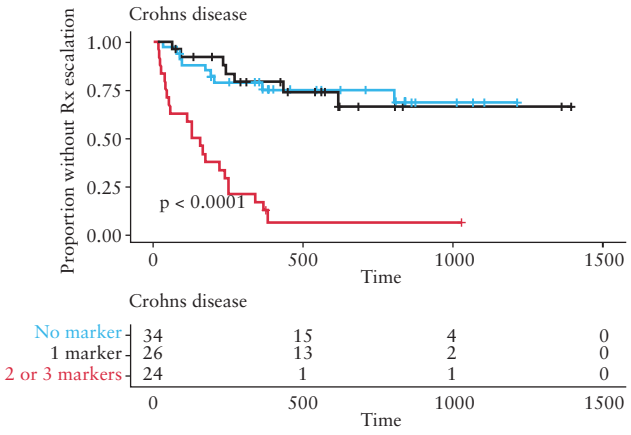

Figure 4. Kaplan–Meier curves of disease course based on blood markers in newly diagnosed Crohn's disease. '1 marker' represents either relative miR-1307-3p > 1.31 or albumin < 31 g/dL or age < 24 years. '2 or 3 markers' represents a combination of any of the mentioned variables.

4.6. Multi-marker prognostic models in CD

The prognostic performance of miRNAs was then compared to conventional predictors including hsCRP, albumin, age and sex. Kaplan–Meier analyses were performed in 167 patients where data for the predictors were complete. In CD [$n = 84$], age < 24 years, albumin < 31 g/dL and relative expression of miR-1307-3p > 1.31 were implicated by modelling [$p = 3.00 \times 10^{-8}$]. At 1 year, the estimated chance of escalation was 21% [CI: 6–34] for patients meeting none of the criteria, 21% [CI: 3–36] for patients meeting one criterion and 83% [CI: 58–93] for patients meeting two or more criteria [Figure 4 and Table 5B]. Similar prognostic analyses were performed in 141 patients [65 CD, 76 UC] in whom miRNA expression data for miR-3615 and miR-4792 were available. Including all target miRNAs in the multivariate model, miR-1307-3p, albumin and age still remained significant predictors in CD and the addition of miR-4792 and miR-3615 provided no additional benefit to the prognostic model. These markers remained significant even after adjusting for any treatment exposure at recruitment and smoking status.

4.7. Multi-marker prognostic models in UC

In UC, similar analyses were performed and included all differentially expressed miRNAs [miR-1307-3p, miR-3615, miR-4792] and conventional clinical and biomarker predictors including age, sex, hsCRP, albumin and pancolitis subtype, where data for predictors

Table 5B. Multiple categorical logistic regression of predictors of treatment outcomes in crohn's disease and ulcerative colitis. Categorical thresholds reported for miRNAs are relative expression as calculated by the 2^[-ddCt] method. Extensive colitis is defined as E3 as per Montreal disease extent classification

Categorical variable	HR [IQR]	AIC	*p*-value for threshold
Crohn's disease			
Age < 24 years	2.19 [1.14–4.24]	290.17	0.01
miR1307 > 1.31	2.12 [1.02–4.39]	288.65	0.04
Albumin < 31 g/dL	4.49 [2.08–9.75]	297.70	7.37×10^{-5}
Ulcerative colitis			
Extensive colitis	3.26 [1.31–8.12]	163.25	0.01
miR-3615 < 0.95	3.63 [1.52–8.66]	164.17	3.60×10^{-3}
Albumin < 39g/dL	7.10 [2.69–18.74]	165.50	7.53×10^{-5}
miR-4792 > 2.26	4.43 [1.77–11.11]	173.51	1.53×10^{-3}

Categorical thresholds reported for miRNAs are relative expression as calculated by the $2^{-\Delta\Delta Ct}$ method. Extensive colitis is defined as E3 as per the Montreal disease extent classification. AIC: Akaike information criterion; HR: hazard ratio; IQR: interquartile range.

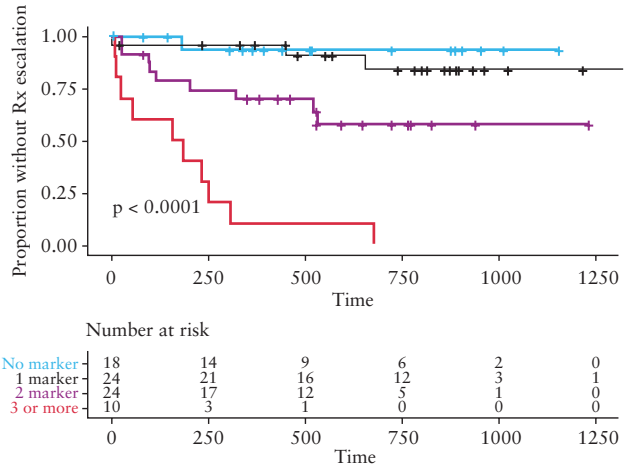

Figure 5. Kaplan–Meier curves of disease course based on blood markers in newly diagnosed ulcerative colitis. '1 marker' represents either relative miR-4792 > 2.26 or albumin < 39 g/dL or miR-3615 < 0.95 or extensive colitis [Montreal E3]. '2 marker' represents two combinations of any of the mentioned variables. '3 or more' represents three or more of the above mentioned variables.

were complete [$n = 76$]. Four markers predicted disease course [Figure 5 and Table 5B]: relative miR-3615 expression < 0.95, miR-4792 > 2.26, albumin < 39g/dL and extensive colitis [logrank $p = 6.93 \times 10^{-7}$]. At 1 year, the estimated chance of escalation was 6% [CI: 0–17] for patients meeting none of the criteria, 4% [CI:0–12] for patients meeting one criterion, 31% [CI: 9–48] for patients meeting two or more criteria and 90% [CI: 36–98] for patients meeting three criterion or more criteria. miR-1307-3p did not predict outcomes in UC and provided no additional benefit to the UC model. These markers remained significant even after adjusting for any treatment exposure at recruitment and smoking status.

4.8. miR-1307 and mRNA integrative network analyses

We performed miR-1307-3p target interactions using the miRNet online platform.[17] A total of 240 genes were targets of these T-cell-specific miRNAs [Supplementary Table 3] as predicted by miRNet. Paired whole blood mRNA expression profiles were available for all patients. Of the 240 predicted targets for miR-1307-3p, 63 remained significant after FDR correction with 40% [$n = 25$] of the targets negatively correlated with this miRNA. The top-most significant negative correlations included ZNF431 [rho –0.27, $p = 7.86 \times 10^{-5}$], ZNF841 [–0.33, $p = 1.67 \times 10^{-6}$], LRIG2 [–0.28, $p = 4.23 \times 10^{-5}$] and ZNF85 [–0.30, $p = 9.51 \times 10^{-5}$]. Positive correlations included MAPK1 [r 0.32, $p = 3.21 \times 10^{-6}$], IL6R [r 0.20, $p = 4.55 \times 10^{-3}$] and IL10RB [r 0.20, $p = 4.36 \times 10^{-3}$].

Of the predicted targets for both miR-3615 [$n = 39$] and miR-4792 [$n = 101$], none of the genes remained significant after adjusting for multiple testing.

We then performed GO term and KEGG analyses on the gene targets using an online platform [Gene Set Enrichment Analysis: GSEA].[18] Ten GO terms were enriched for miR-1307-3p [Table S5]. One of the top miR-1307-3p-specific GO terms in this analysis included the regulation of T-cell activation and included three gene targets: HMGB1 [rho 0.15, $p = 4.52 \times 10^{-2}$], IL6R [0.20, $p = 4.55 \times 10^{-3}$] and TMIGD2 [–0.19, $p = 9.37 \times 10^{-3}$]. Only one KEGG pathway was enriched and included the regulation of adherens junction [KEGG: M638]. This pathway included four gene targets from our data, MAPK1, ACTB, ACTG1 and WASF2; these were all positively correlated with miR-1307-3p.

5. Discussion

More recently, there have been rapid advances in our understanding of the clinical heterogeneity in IBD. Studies have identified unique molecular profiles that represent disease course, behaviour and response to therapy.[14,15,19–21] With this in mind, there has been immense interest in personalized medicine, to allow enhanced disease stratification at diagnosis in order to prevent long-term sequelae and improve clinical outcomes. In this study we have identified and validated a novel CD4 T-cell-specific miRNA profile that predicts IBD and its disease course over time, at disease inception.

The potential for clinical translation of miRNAs in our study lies in their ability to predict treatment escalation in CD and UC. Our top differentially expressed and validated miR-1307-3p is able to predict treatment escalation in IBD, in particular CD [HR 2.81; IQR: 1.11–3.53, $p = 6.50 \times 10^{-4}$]. Combined miRNA-based models with blood tests such as albumin further strengthens the performance of a prognostic model. These miRNAs do not correlate with conventional inflammatory markers and may not be driven by the inflammatory burden. Our markers have translational relevance as they have been validated using RT-qPCR in whole blood, without the need to extract immune cells or utilize a new platform that is yet to be established in clinical practice. Several studies have investigated

disease outcomes in IBD. These have identified unique genetic, gene expression, methylation, protein and glycomic profiles that associate with an aggressive disease course over time.[19–23] Studies have used varied criteria to define treatment escalation, including the escalation to two or more immunomodulators over time, mucosal healing, response to biological agents, and development of fistulizing or stricturing complications over time as end points. These are all relevant and explore unmet but distinct clinical scenarios. In our study, we recruited patients at disease inception who were uniquely positioned to investigate treatment escalations over time as defined by transcriptome and protein studies.[14,15,20,23] Our study provides another level of molecular depth in these patients by defining cell-specific miRNA markers that associate with disease course. Future studies integrating these multi-omic markers may provide mechanistic insights into aggressive clinical course and provide future drug targets. In CD, activated T-cells represent a key cell type within a unique cellular component [GIMATS] that when present in disease, has been shown to associate with anti-tumour necrosis factor drug resistance.[24] Our findings may be of relevance in the context of drug response, but this remains to be explored. Future studies incorporating these signals in treatment response/non-response would be of interest. Recently, there have been studies exploring the role of miRNA-based biomarkers as tools for disease monitoring and treatment response in IBD and other immune-mediated diseases. Circulating miR-146b-5p AUC [0.869, CI: 0.764–0.940] has been shown to better reflect mucosal inflammation in IBD compared to CRP [0.680, CI: 0.554–0.790, *p* for comparison = 0.0043]. Mucosal miRNA profiles generated in acute severe colitis patients identified a panel of nine miRNAs and five clinical factors that can differentiate responders vs non-responders with steroids [AUC 0.91], infliximab [AUC 0.82] and cyclosporine [AUC 0.79].[25] In rheumatoid arthritis, circulating levels of three miRNAs, miR-155-5p, miR-146a-5p and miR-132-3p, predict response to methotrexate, all with similar AUC [0.72–0.76].[26]

Published miRNA studies in peripheral blood of IBD patients are limited by cellular heterogeneity within the biological samples analysed.[5] In our study, we have profiled miRNAs in cell-specific peripheral blood cells and validated these signals in whole blood. All three validated miRNAs in whole blood show signals in CD4 T-cells and are up-regulated in IBD. Our methodology of utilizing RNA sequencing on separated cells has identified novel, yet undiscovered IBD-specific miRNAs. From GO term analysis, miR-1307-3p appears to target genes that control several cellular pathways, in particular regulation of T-cell activation. Correlation analysis also reveals positive correlations with pro-inflammatory gene targets such as IL6R and MAPK1, particularly relevant in chronic inflammatory disorders such as IBD. KEGG analysis reveals that this miRNA and its targets may also be involved in regulating intestinal barrier function, possibly through MAPK signalling. The exact role of miR-1307-3p in disease pathogenesis and T-cell function remain to be explored. Much of the literature on miR-1307-3p biology is in the field of cancer. In colon adenocarcinoma, miR-1307-3p targets isthmin1 [ISM1], inhibiting Wnt3a/β-catenin signalling and cell proliferation and promoting cell apoptosis.[27] Germline variation in pre-miR-1307-3p [rs7911488] was significantly associated with efficacy to capecitabine-based chemotherapy in colon cancer; rs7911488 C-allelic pre-miR-1307 was associated with poor drug response through the attenuation of mature miR-1307 levels and up-regulation of its target TYMS.[28] Conversely, miR-1307-3p appears to be detrimental in hepatocellular carcinoma and breast cancer, predicting poor clinical outcomes if over-expressed.[29,30] Within our prognostication data, there is divergent expression seen for miR-3615 in IBD subtypes amongst escalators and non-escalators. This warrants further exploration. Given that miRNAs regulate gene expression, their own expression may vary based on disease subtype and severity. An example includes the divergent influence of miR-21 in murine models of acute DSS-induced colitis vs chronic TNBS-induced colitis.[1] Studies are needed to investigate their mechanistic role in disease course and severity. Furthermore, studies exploring their dynamic differential regulation of the miRNAs over time are also needed.

Our findings provide an enriched resource for future studies to evaluate the function of this resource in IBD.

There are certain methodological considerations in our study that are worthy of discussion. Our study design, identifying and validating differentially expressed miRNAs in IBD compared to controls, may not capture all prognostic miRNAs relevant in IBD. However, this would require a much larger multi-centre pool of purified immune cell subsets in a treatment-naïve IBD cohort with follow-up data, beyond the scope of this study. Patients with IBD may have different cellular proportions compared to controls and this may influence cell-specific results. However, there was no correlation seen between the miRNAs and white blood cell count. Treatment may be escalated in response to blood markers, thereby confounding our findings of routine clinical markers predicting escalation. This is likely to explain the over-representation of albumin in most prognostic models. As decisions on treatment escalations are based on clinical tests such as CRP and albumin, it is noteworthy that the miRNA markers still remain significant predictors in IBD. Decisions regarding treatment escalation may vary across centres but it is important to highlight that in our study, all centres utilized a step-up approach when tailoring therapy. The major strengths of the study include a two-stage prospective study design including target validation, cell-specific profiling and a multi-centre recruitment of patients at disease inception. Erythropoietically derived miR486-5p and miR-451 reads can often be over-represented in small RNA sequencing studies that profile whole blood, resulting in inaccurate quantification and detectability of low-abundance signals that may in fact be relevant in disease pathogenesis.[31] We therefore used RT-qPCR to validate our findings in whole blood. Studies are now developing novel hybridization methods to deplete these miRNAs to allow detection of low-abundance miRNAs in whole blood.[31]

Our work adds to the valuable literature defining the epigenome in IBD, in particular cell-specific miRNAs. These data will allow future studies to explore the epigenetic alterations that associate with disease onset and outcomes and pave the way potentially for miRNA-based therapeutics.

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Conflict of Interest

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Author Contributions

Study design R.K., J.S. and A.B. Patient recruitment and sample processing N.T.V., R.K., D.B., S.V., A.T.A. Experimental work C.C., R.W., R.K., N.T.V., A.T.A., B.L.J. Data analysis R.K., N.A.K., A.I., A.T.A. R.K. wrote the manuscript. All authors were involved in critical review, editing, revision and approval of the final manuscript.

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Supplementary Data

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

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