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Whole blood profiling of T-cell derived miRNA 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. In our study, we define the cell-specific miRNA profiles and investigate its biomarker potential in IBD.

Methods: In a 2-stage prospective multi-centre case control study, Next Generation sequencing was performed on a discovery cohort of immunomagnetically separated leucocytes from 32 patients (9 CD, 14 UC, 8 healthy controls) and differentially expressed signals were validated in whole blood in 294 patients (97 UC, 98 CD, 98 non-IBD) 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 leucocyte 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 (HR 1.98, IQR:1.20-3.27;logrank $p=1.80\times10^{-3}$), in particular CD (HR 2.81; IQR: 1.11-3.53, $p=6.50\times10^{-4}$). Using blood-based multimarker miRNA models, the estimated chance of escalation in CD was 83% if 2 or more criteria were met and 90% for UC if 3 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.

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Introduction

Inflammatory bowel disease (IBD) is a global health burden with increasing incidence and prevalence in newly industrialised nations and healthcare costs in UK and Europe[1,2]. Despite tremendous progress in our understanding of the genetics in IBD, there still 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 RNA that have the ability to regulate and fine-tune gene expression. There is strong evidence that IBD disease pathways are regulated by miRNAs, notably the regulation of Th-17 pathway by the NOD2 driven miR-29[6]. miR-196 regulates IRGM, which is a known IBD GWAS susceptibility gene; the known 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 leucocytes and in inflamed intestinal tissue[4]. This miRNA has now been shown to have protective effects in miR-21 KO mice exposed to DSS and deleterious effects when exposed to TNBS[8].

There are a number of methodological considerations that potentially confound miRNA analysis, both biological and technical; a key issue being 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 characterise 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.

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Materials and Methods

Study Design

We conducted a prospective 2-stage discovery and validation multi-centre case-control study as summarised 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), Crohn's disease 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].

Stage 1: Discovery cohort

For the discovery cohort, immune-magnetic cell separation were performed using a protocol as described previously[12]. In brief, peripheral blood mononuclear cells were isolated from 18–36 mls of EDTA whole blood using Ficoll (Ficoll-Paque, GE healthcare, Bucks, UK). Cells labelled with antibody-coated microbeads (human $CD14^+$, $CD8^+$ and $CD4^+$ microbeads, 20 µl per $1x10^7$ cells) were immunomagnetic separated using the autoMACs Pro cell separator (Miltenyi, Germany) and cell purity was estimated using florescent antibody staining and flow cytometry (FACS Aria II, BD, Germany). Flow cytometric assessment demonstrated high purity of isolated cell populations following immunomagnetic cell separation (CD14+ median: 92.4% (IQR 87-94.9), CD4: 97.3% (93.8 98.9), CD8+: 88.7 (80.5-93)).

A total of 90 leucocyte subsets ($CD4^+$, $CD8^+$ and $CD14^+$ cells) samples obtained from 32 patients (9 CD, 14 UC, 1 IBDU, 8 healthy controls) patients with newly diagnosed IBD, naïve to therapy and age and sex matched healthy individuals. Demographic and clinical data including (**Table 1A**) drug therapies were collected. Cell sample RNA were extracted using QIAGEN® Allprep DNA/RNA miRNA universal kit as per manufacturer's instructions. The Agilent Bioanalyzer platform and NanoChip kit have been used for sizing, quantification and quality control (QC) of extracted miRNA from separated cells. All separated cells reached high quality RIN (mean RIN 9.2).

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

Stage 2: Validation cohort

Whole blood miRNA for the replication cohort were collected using a standardised protocol across UK (Edinburgh) and European centres (Sweden, Norway, Spain, Netherlands) in Paxgene tubes and stored in -80C. Total RNA was extracted from whole blood using MagMax™ extraction kit according to the manufacturer's instructions. The validation cohort comprised of 294 patients with suspected or confirmed IBD and a control group comprised 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 6 clinical centres in 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, 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 literature review as potential controls in whole blood PCR experiments. These included miR-130b-3p, miR-130b-5p, miR-342-3p, U6, SNORD44, SNORD48, SNORD49A. Controls were tested for their performance and stability across all samples and GeNorm score was given to each marker. GeNorm calculates stability based on pairwise variation and generates a stability score, 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, 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 qPCR reactions were performed with miRCURY LNA miRNA PCR Assays and 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).

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 2 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-ASA therapies, biologics and immunosuppressants.

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Gene expression profiling

Whole blood RNA underwent targeted RNA sequencing which was performed using Ion AmpliSeq Human Gene Expression Core Panel, containing 20,802 genes. Quality control 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 were quantified. After filtering, 14,182 transcripts were available for further analysis.

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. This study was funded by Crohn's and Colitis UK (grant number #M2016/2).

Data Analysis

Stage 1: RNA sequencing

Raw reads were aligned to the human genome using miRDeep 2, output restricted to those that aligned full length and were a perfect match. miRNAs with raw reads $<1\times10^{-5}$ 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 normalised and further analysed for differential expression. R 3.4.4 (R Foundation for Statistical Computing, Vienna, Austria) was used for statistical and bioinformatics analysis. P-values for differentially expressed proteins were adjusted for multiple testing (Benjamini Hochberg procedure; FDR).

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 normalised to 2 reference genes, miR-130b-5p and miR-342-3p[16]. Relative quantification (i.e. fold change) of miRNAs was calculated by the equation $2^{-\Delta\Delta Ct}$ method.

Averages and standard deviations (SD) were generated for each sample and for the miRNA target and endogenous controls. A SD threshold filter of <1.0 was used for quality control. Target miRNAs where >25% of samples had undetermined Ct values were excluded from further analyses. There were no samples excluded after filtering for quality control and 3 target miRNAs passed QC including miR-1307-3p, miR-3615 and miR-4792. Fold change was calculated using the formula below: $2^{-\Delta\Delta Ct}$ (equivalent to relative endogenous expression of target miRNA).

Biomarker statistical analyses

Conventional laboratory inflammatory parameters (CRP and albumin), age and gender were included in multivariable models for IBD diagnosis and prognosis. CRP and the top miRNAs were $\log_{10}$ transformed in order to approximate a normal distribution for further multivariable analysis. The optimal models were then selected by performing backward stepwise regression using the smallest Akaike information criterion (AIC) values. Leave-out one 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 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.

Results

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, gender and batch effects. These data are summarized in **Table 1A**. A total of 3 miRNAs differentiated IBD from controls in CD4 T cells and these included 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 S1**). There were no CD14 specific miRNAs that differentiated UC from controls in this cohort.

Only miR-10b-5p differentiated CD from controls (**Supplementary Table S2**) but no miRNAs that differentiated UC from CD across all cell subsets.

Stage 2: Validating miRNA markers using qPCR

After quality control, a total of 294 whole blood RNA samples and 3 target miRNAs were included for further analyses. **Table 1B** summarises the demographics of the cohort. There were 97 UC, 98 CD and 98 non-IBD controls and 1 IBDU. 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; $p=2.77\times10^{-5}$) and 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; $p=1.56\times10^{-6}$) 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: 1.21FC, IQR:0.91-1.48; $p=8.26\times10^{-4}$ and miR-4792 :1.91 FC, IQR: 0.81-2.56; $p=9.21\times10^{-3}$).The same miRNAs were overexpressed in pooled IBD versus control analyses, but the result failed to reach statistical significance.

miRNA expression and its association with inflammatory activity

Correlation analyses were performed in using the top differentially expressed miRNAs and conventional blood-based inflammatory markers such as hsCRP and albumin (data complete n=263). None of the miRNAs correlated with conventional blood-based tests such as hsCRP and albumin (**Table 4**).

Diagnostic biomarkers in IBD

The top differentially expressed miRNA has a modest performance as blood-based diagnostic marker. miR-1307-3p differentiated IBD from controls with area under the receiver operator characteristics curve (AUC) of 0.66 (95% CI: 0.59-0.73) and performed at 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 has a modest performance in CD (AUC 0.60, CI: 0.51-0.68). In UC, miR-1307-3p performs at par with miR-3615(p for comparison =0.10) and miR-4792 (p=0.09). A combined 3-miRNA marker provides no added benefit to the diagnostic UC model (AUC

0.66, CI: 0.57-0.74). Figure 3 summarises 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, $p=8.73\times10^{-6}$), log (hsCRP) (OR: 2.11, CI: 1.26-3.63, $p=5.60\times10^{-3}$), log (miR-1307) (OR: 6.40, CI:2.08-20.98, $p=1.56\times10^{-3}$) 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. A leave-one-out (LOO) cross-validated diagnostic model incorporating these 4 predictors has 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<5mg/L), miR-1307-3p had a LOO cross-validated diagnostic accuracy of 0.65 (CI: 0.59-0.70) and performed at par with albumin (accuracy 0.62, CI: 0.56-0.68).

miRNAs as predictors of treatment escalation in IBD

Prognostic data were available on 195 IBD patients and the demographics are summarised 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 gender (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 2 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 in 189 patients where data for the predictor had passed QC. miR-1307-3p was associated with disease course in IBD (HR 1.98, IQR:1.20-3.27;logrank $p=1.80\times10^{-3}$). 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, $p=6.50\times10^{-4}$) but not UC (p=0.061).

Similarly, miR-3615(UC: HR 2.55, IQR:1.24-5.25, $p=3.40\times10^{-3}$ and 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 and CD: HR 2.42, IQR: 1.18-4.97, p=0.02) predict disease course in UC and CD (**Table 5A and Supplementary Figure 2).**

Multi-marker prognostic models in Crohn's Disease

The prognostic performance of miRNAs was then compared to conventional predictors including hsCRP, albumin, age and gender. Kaplan-Meier analyses were performed in 167 patients where data for the predictors were complete. In CD (n=84), age<24 years, albumin<31g/dL and relative expression of miR-1307-3p>1.31 were implicated by modelling ($p=3.00\times10^{-8}$). 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 1 criterion and 83% (CI:58-93) if 2 or more criteria are met (**Figure 4 and Table 5B**). Similar prognostic analyses were performed in 141 patients (65 CD, 76 UC) in whom miRNA expression for miR-3615 and miR-4792 were available. Including all target miRNAs in the multivariate model, miR-1307-3p, Alb and age still remain significant predictors in CD and the addition of miR-4792 and miR-3615 provides no additional benefit to the prognostic model. These markers remain significant even after adjusting for any treatment exposure at recruitment and smoking status.

Multi-marker prognostic models in Ulcerative colitis

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, gender, hsCRP, albumin and pancolitis subtype, where data for predictors were complete (n=76). A total of 4 markers predicted disease course (**Figure 5 and Table 5B**) and include relative miR-3615 expression <0.95, miR-4792>2.26, Alb<39g/dL and extensive colitis (logrank $p=6.93x10^{-7}$). 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 1 criterion, 31% (CI:9-48) if 2 or more criteria are met and 90% (CI:36-98) if 3 or more criteria are met. miR-1307-3p does not predict outcomes in UC and provides no additional benefit to the UC model. These markers remain significant even after adjusting for any treatment exposure at recruitment and smoking status.

miR-1307 and mRNA integrative network analyses

We performed miR-1307-3p target interactions using the miRNet online platform[18]. A total of 240 genes are targets of these T-cell specific miRNAs (**Supplementary Table 3**) as predicted by miRNet. Paired whole blood mRNA expression profiles were available in 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 include ZNF431 (rho -0.27, $p=7.86\times10^{-5}$), ZNF841(-0.33, $p=1.67\times10^{-6}$), LRIG2 (-0.28, $p=4.23\times10^{-5}$), ZNF85(-0.30, $p=9.51\times10^{-5}$). Positive correlations included MAPK1(r 0.32, $p=3.21\times10^{-6}$), IL6R (r 0.20, $p=4.55\times10^{-3}$) and IL10RB (r 0.20, $p=4.36\times10^{-3}$).

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)[20]. Ten GO terms were enriched for miR-1307-3p **(Table S5)**. One of

the top miR-1307-3p specific GO term in this analysis included the regulation of T-cell activation and included 3 gene targets: HMGB1 (rho 0.15, $p=4.52\times10^{-2}$), IL6R (0.20, $p=4.55\times10^{-3}$) and TMIGD2 (-0.19, $p=9.37\times10^{-3}$). Only 1 KEGG pathway was enriched and included the regulation of adherens junction (KEGG: M638). This pathway included 4 gene targets from our data including MAPK1, ACTB, ACTG1 and WASF2; all positively correlated with miR-1307-3p.

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 have been immense interest in personalised 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 lie in its 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, $p=6.50\times10^{-4}$). 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 utilise 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 utilised varied criteria to define treatment escalation including the escalation to 2 or more immunomodulators over time, mucosal healing, response to biological agents, and development of fistulising 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 and 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-TNF drug resistance[24]. Our findings may be of relevance in the context of drug response, however this is yet 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 (ASUC) patients identified a panel of 9 miRNAs and 5 clinical factors that can differentiate responders versus non-responders with steroids (AUC 0.91), infliximab (AUC 0.82) and cyclosporine (AUC 0.79)[25]. In rheumatoid arthritis, circulating levels of 3 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 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 utilising RNA sequencing on separated cells have identified novel, yet undiscovered IBD-specific miRNAs. From GO term analysis, miR-1307-3p appears to targets 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 are yet 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 within pre-miR-1307-3p (rs7911488) significantly associated with efficacy to capecitabine-based chemotherapy in colon cancer; rs7911488 C-allelic pre-miR-1307 being 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 (HCC) 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 dextran sodium sulfate (DSS) induced colitis versus chronic 2,4,6-trinitrobenzenesulfonic acid (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 its function 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. There was however 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 centre but it is important to highlight that in our study, all centres utilised a step-up approach when tailoring therapy. The major strengths of the study include a 2-stage prospective study design including target validation, cell-specific profiling and a multi-centre recruitment of patients at disease inception. Erythropoietic 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 abundant 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 abundant 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 way potentially for miRNA-based therapeutics.

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Author Contributions: Study design RK, JS and AB. Patient recruitment and sample processing NTV, RK, DB, SV, ATA. Experimental work CC,RW,RK, NTV, ATA, BLJ. Data analysis RK, NAK, AI, ATA. RK wrote the manuscript. All authors were involved in critical review, editing, revision and approval of the final manuscript.

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Figure Legends

Figure 1: Flow diagram showing the 2-stage discovery and validation design of the study

Figure 2: The 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. The relative expression is depicted as $2^{-\Delta\Delta Cq}$ method using miR-130b-5p and miR-342-3p as reference genes.

Figure 3: Receiver operator curve (ROC) analyses of miRNAs, high sensitivity c-reactive protein (hsCRP) and albumin (Alb) in differentiating Inflammatory Bowel Disease from non-IBD and Ulcerative colitis from non-IBD.

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 marker' represents a combination of any of the mentioned variables.

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 2 combinations of any of the mentioned variables. '3 or more' marker represents 3 or more of the above mentioned variables.

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Tables

Table 1A: Separated cell discovery cohort. 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 number of patients

miRNA sequencing discovery cohort		
Variables	**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 (Range)	34 (18-68)	43 (20-59)
Gender (M:F)	16:8	4:4

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Table 1B: Quantitative polymerase chain reaction (qPCR) validation cohort demographics.

CD: crohn's disease; UC: ulcerative colitis; IBDU: Inflammatory bowel disease unclassified; HC: healthy controls; M: Male; F: Female. All numbers shown represent number of patients.

qPCR validation cohort demographics			
Variables	UC (n=97)	CD (n=98)	Non-IBD (n=98)
Mean age (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	

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Montreal Behaviour			
B1+B1p		81	
B2		10	
B3+B3p		6	
NA		1	

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Table 2: Patient demographics for predicting disease course in Inflammatory bowel disease (IBD). Escalation defined as the need for 2 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 number of patients

qPCR validation cohort demographics		
Variables	Escalators (n=80)	Non-Escalators (n=115)
Diagnosis (UC:CD:IBDU)	33:47:0	64:50:1
Median age (Range)	27 (21-36)	29(24-34)
Gender (M:F)	46:34	68:47
Centre **UK:Sweden:Norway:Spain**	38:15:23:4	31:23:49:12
Smoking status	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 GI)	0	1
CD Behaviour		

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B1, B1p (non-stricturing & non-penetrating, +perianal)	34	46
B2, B2p (stricturing, +perianal)	8	2
B3, B3p (penetrating, +perianal)	5	1
Not available	0	1

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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

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Table 4 : Correlation analyses of conventional biomarkers with novel miRNA-based markers. Spearman analyses have been performed and data depicted as rho(p-value).

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)

Footnote: FC: faecal calprotectin; WCC: white cell count; Hb: Haemoglobin; hsCRP: high sensitivity CRP

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Table 5A: miRNAs as 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 .

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

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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

Crohn's Disease			
Categorical Variable	HR(IQR)	AIC	p-value for threshold
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<31g/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}

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Figure 1

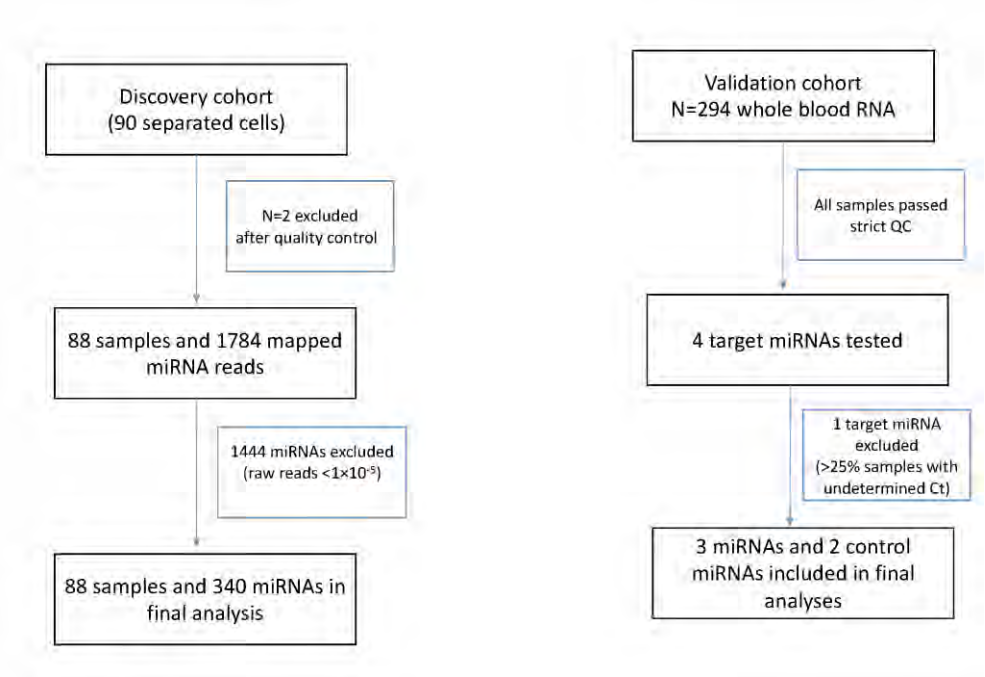

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Figure 2

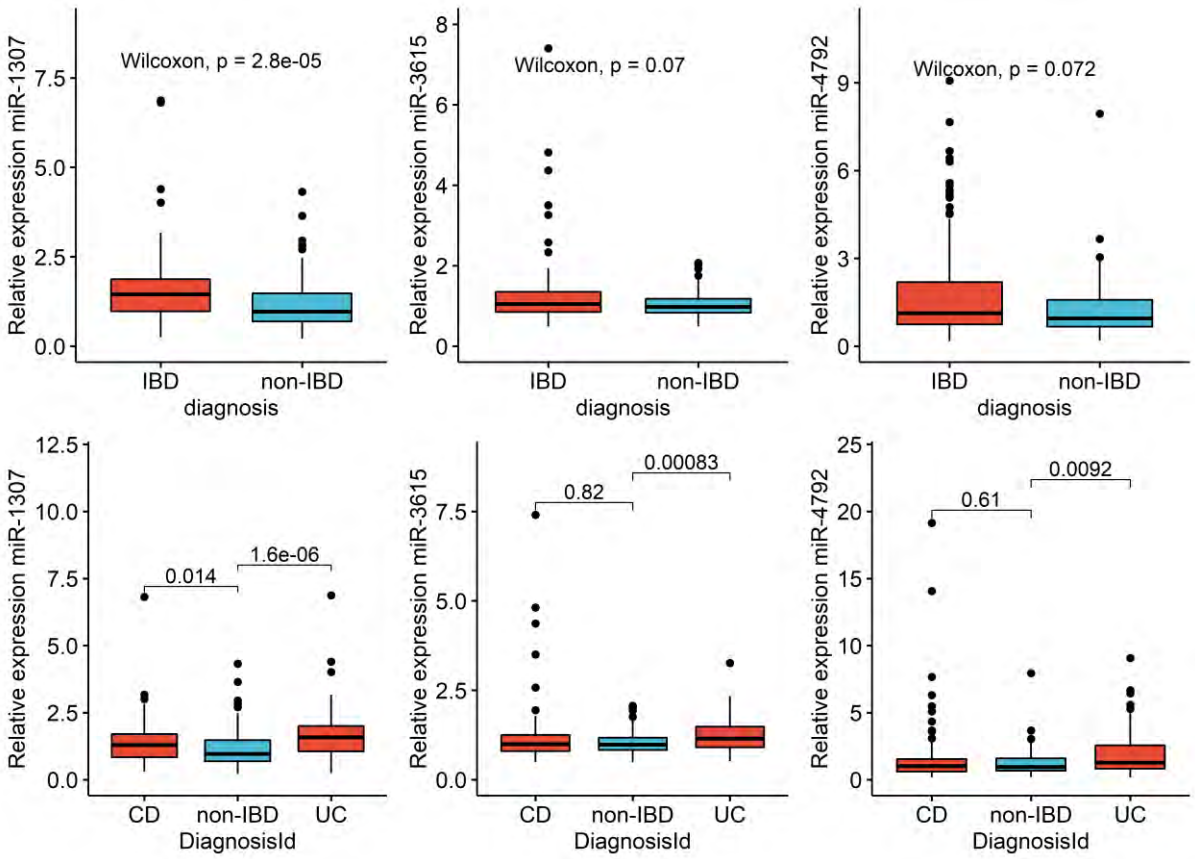

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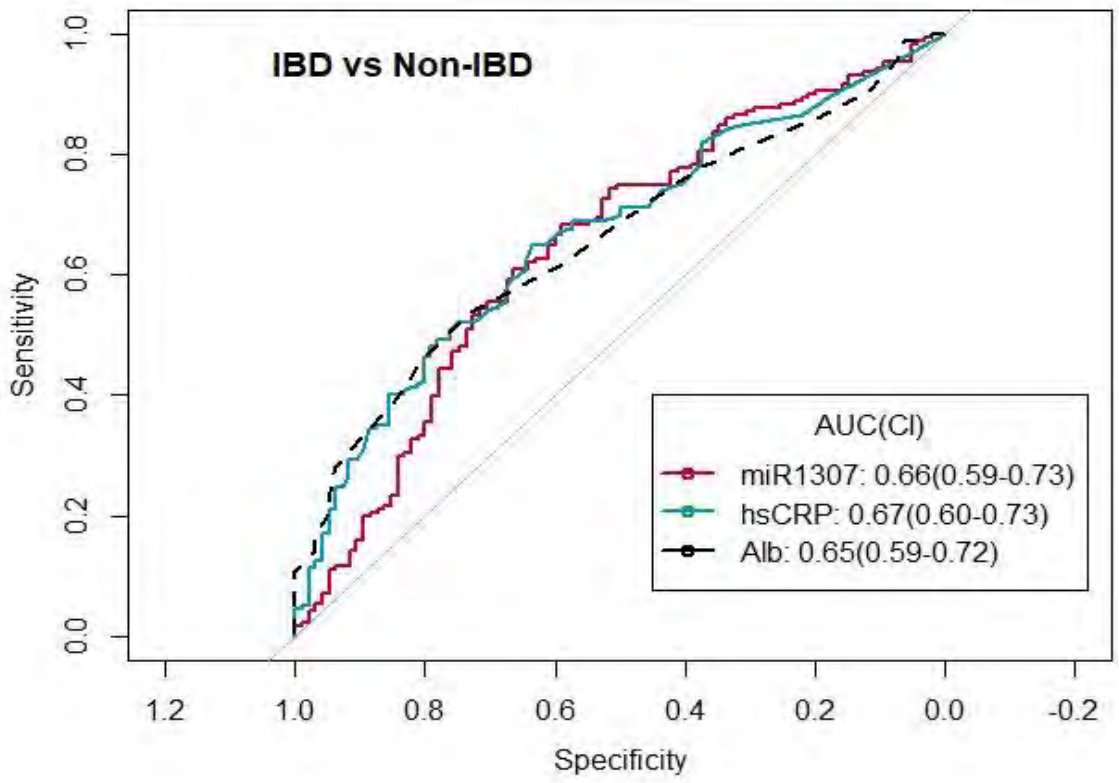
Figure 3



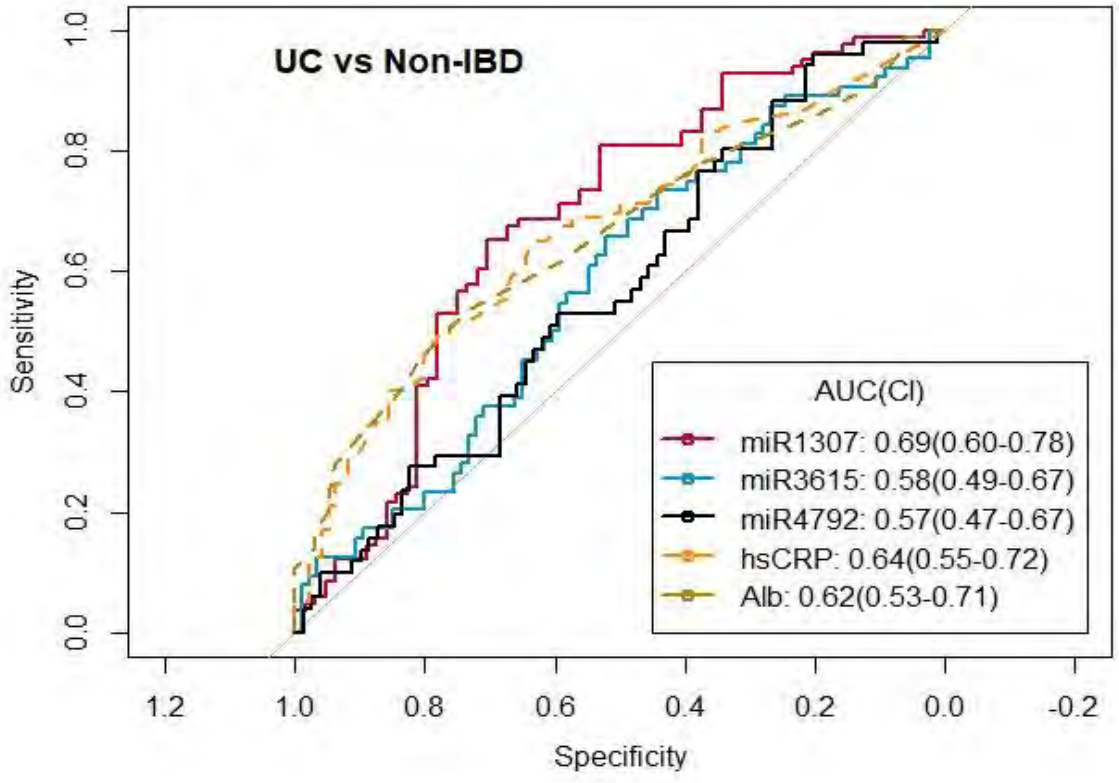


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Figure 4

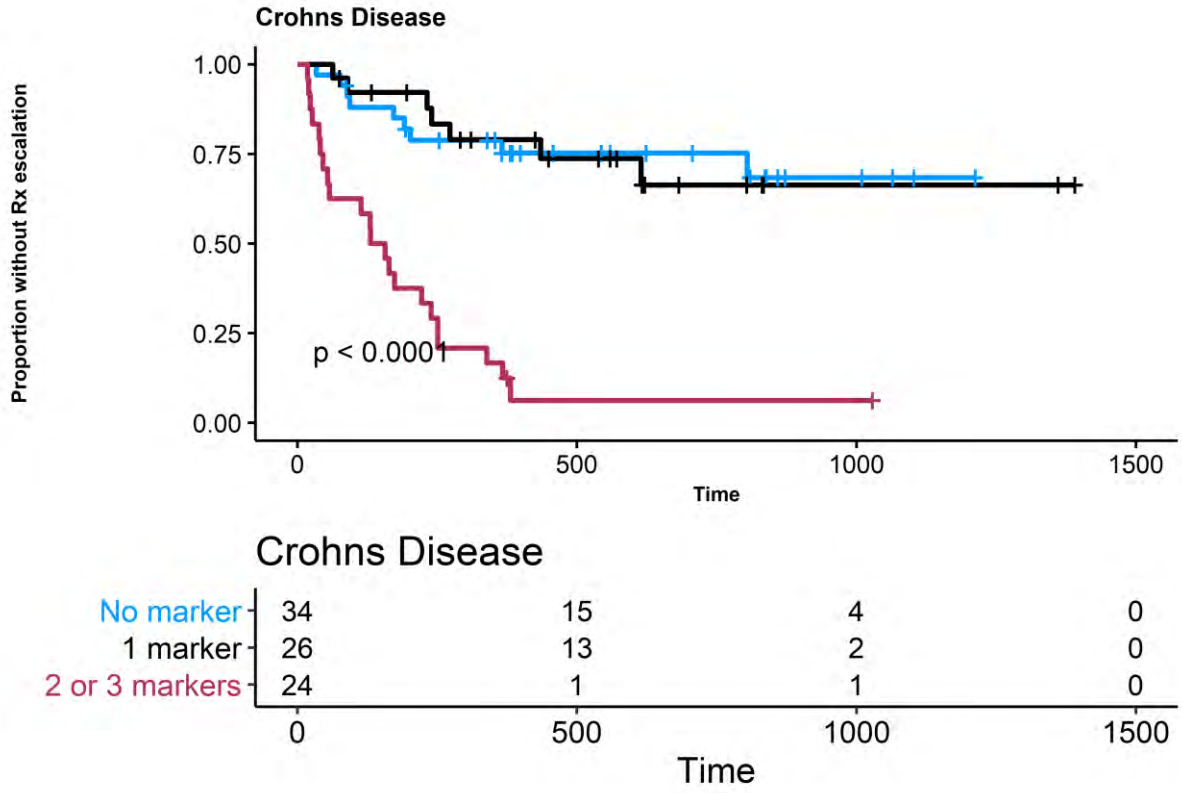

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Figure 5