Tissue MicroRNA Expression Signatures as Diagnostic Biomarkers and Predictors of Residual Disease Activity and Relapse in Treatment-Naïve Pediatric Inflammatory Bowel Disease

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Background: Identifying novel diagnostic and prognostic biomarkers for pediatric inflammatory bowel diseases (PIBD), including Crohn's disease (pCD) and ulcerative colitis (pUC), is essential for enhancing treatment outcomes. MicroRNAs (miRNAs) have been recognized for their broader relevance in PIBD pathogenesis. This study investigates their diagnostic potential and clinical utility in PIBD.

Methods: This prospective, monocentric study, with retrospective validation, involved 119 PIBD patients (58 pCD, 61 pUC) and 39 non-IBD controls. Small RNA next-generation sequencing was performed on fresh-frozen gut biopsies, targeting histopathologically confirmed inflamed areas. Twenty-five dysregulated miRNA candidates were validated via RT-qPCR in formalin-fixed, paraffin-embedded gut biopsies. Logistic regression was used to establish diagnostic and prognostic miRNA expression signatures.

Results: A diagnostic signature of 5 miRNAs (miR-223-3p, miR-34a-5p, miR-194-5p, miR-215-5p, miR-338-3p) distinguished pCD from non-IBD with 96.49% accuracy. Two miRNAs (miR-223-3p, miR-194-5p) differentiated pUC from non-IBD with 100% accuracy, and miR-215-5p distinguished pCD from pUC specimens with 83.54% accuracy. For treatment-naïve pCD patients, 7 miRNAs predicted residual disease activity at 3 months with 100% accuracy. Additionally, a distinct signature predicted the risk of relapse within 12 months with an accuracy of 84.21%.

Conclusions: In this study, we have established tissue miRNA expression signatures with significant diagnostic and prognostic potential for use in PIBD. These findings aid in stratifying disease severity and risk, paving the way for more precise and personalized management of pediatric IBD.

Lay Summary

MicroRNA signatures can accurately diagnose pediatric Crohn's disease and ulcerative colitis while predicting prognosis in treatment-naïve patients. They provide a foundation for precision medicine by enabling risk-based patient stratification and optimizing personalized therapeutic strategies in pediatric inflammatory bowel disease.

Introduction

Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are multifactorial disorders of the gastrointestinal tract characterized by chronic, relapsing inflammation.[1] The incidence of IBD is rising globally, particularly in pediatric populations, with CD being more prevalent than UC.[2] Pediatric IBD (PIBD) differ from adult IBD by their higher rates of extraintestinal manifestations, including growth failure, anemia, joint symptoms, and delayed puberty, thus necessitating more tailored management and therapeutic approaches.[3]

Current methods for diagnosing and monitoring PIBD include endoscopic and histological assessments, imaging techniques, clinical activity indexes (eg, weighted Pediatric Crohn's Disease Activity Index [wPCDAI] and Pediatric Ulcerative Colitis Activity Index [PUCAI]), as well as serum and fecal inflammatory markers such as C-reactive protein (CRP) and fecal calprotectin (FC).[4–7] Because of the heterogeneity in clinical presentations of PIBD, however, these

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Key Messages

What is already known?

- The clinical presentations of pediatric inflammatory bowel diseases (PIBD) are heterogeneous, and current prognostic factors have limitations in accurately predicting disease progression.

What is new here?

- This study establishes that specific tissue microRNA (miRNA) signatures can effectively diagnose PIBD and prognosticate residual activity and relapse in treatment-naïve patients.

How can this study help patient care?

- The incorporation of miRNA signature testing may lead to the advancement of precision medicine strategies in PIBD, enhancing clinical outcomes and optimizing therapeutic interventions.

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approaches have limitations in predicting disease progression and treatment response. Therefore, identifying novel biomarkers and prognostic factors is crucial for improving clinical outcomes and optimizing therapy.[8,9]

MicroRNAs (miRNAs) are endogenous, short (~22 nucleotides), noncoding RNA molecules that regulate gene expression post-transcriptionally, primarily by interacting with the 3′ untranslated region of target mRNAs, thereby resulting in mRNA degradation or translational repression.[10] As clinical biomarkers, miRNAs are particularly promising due to their stability, conserved structure, and ease of detection via quantitative methods, such as real-time polymerase chain reaction (PCR).[11] In IBD, miRNAs play key roles in regulating intestinal homeostasis, inflammatory responses, and autophagy of the intestinal epithelium.[12] Notably, miRNA expression profiles are altered across tissues, blood, and stool in IBD patients compared to healthy controls, making them potential diagnostic markers.[13] MiRNAs hold potential also as prognostic markers in adult IBD and PIBD, where they could help guide initial treatment decisions and identify patients most likely to benefit from timely and effective interventions.[14-16] Nevertheless, miRNA expression varies with age, and so pediatric-specific studies are needed.[17,18] The evidence supporting miRNAs as biomarkers for PIBD remains limited, with most studies conducted to date involving only small sample sizes.[15,19]

Our study investigates miRNA expression in a large cohort of newly diagnosed, treatment-naïve PIBD patients with the aim to develop diagnostic and prognostic miRNA signatures to differentiate PIBD from non-IBD, distinguish between pCD and pUC, and predict disease outcomes. Ultimately, these findings could guide more precise diagnoses and treatment strategies in PIBD management.

Materials and Methods

Study Cohort and Sample Collection

This study was designed as a monocentric, prospective study with retrospective validation. It was approved by the University Hospital Brno Ethics Committee (Approval Code: 27-100620/EK). The study samples were collected at the Department of Pediatrics, University Hospital Brno, Czech Republic. In compliance with the Helsinki Declaration, written informed consent was obtained from the parents of all enrolled children before any procedures.

Cases were children diagnosed with PIBD by experienced pediatric gastroenterologists according to ESPGHAN revised Porto criteria. The PIBD cohort consisted of children with proven pCD or pUC. IBD unclassified or other nonspecific cases were excluded. Patients with acute severe colitis (PUCAI >65) were excluded from the study due to a lack of representative material for examination. PIBD patients were treated following clinical guidelines relevant at the time of diagnosis and were regularly monitored.[4-7] The non-IBD control group included children undergoing endoscopy mostly because of chronic abdominal pain, but these patients did not show laboratory, radiological, endoscopic, or histological signs of IBD. None of the non-IBD patients were diagnosed with IBD during the entire study period.

For the prospective part of the study, we selected 40 treatment-naïve children with active PIBD and 20 non-IBD controls who were examined at the Department of Pediatrics, University Hospital Brno, between 2017 and 2022. From each individual, 2 fresh-frozen tissue biopsies were obtained from 3 gut regions: Terminal ileum (TEI), cecum-ascendens (right hemicolon, RHC), and rectosigmoidum (left hemicolon, LHC). One biopsy from each location was sent for pathological examination by an experienced pathologist at the Department of Pathology, University Hospital Brno, Masaryk University, to confirm or exclude microscopic inflammation. The second biopsy was stored in RNAlater Stabilization Solution (Invitrogen, Thermo Fisher Scientific) and preserved at –80 °C for further analysis. We also collected clinical data, including activity indexes (wPCDAI, PUCAI), hemoglobin levels, and CRP.

The retrospective part of the study included 79 selected children with PIBD (38 with pCD and 41 with pUC) and 19 non-IBD controls, all examined at the Department of Pediatrics, University Hospital Brno, between 2010 and 2017. From these treatment-naïve individuals, formalin-fixed, paraffin-embedded (FFPE) tissue samples were collected at the time of diagnostic colonoscopy, specifically from gut regions affected by microscopic inflammation (for PIBD cases) or non-inflamed tissues (for non-IBD controls). The clinical characteristics of the retrospective cohort were based on the data available at the time of these patients' diagnosis. Clinical activity indexes were calculated retrospectively (especially wPCDAI, which has been available since 2012) to better compare the full study cohort.[20]

The clinical disease activity was evaluated 3 months after diagnosis based on wPCDAI or PUCAI, where wPCDAI score >12.5 reflected residual pCD activity and PUCAI >10 indicated pUC residual activity.[21] In addition, relapse within 12 months after diagnosis of pCD or pUC was monitored as another prognostic clinical endpoint and/or response to treatment.[22] Relapse was defined as a flare of symptoms in PIBD patients in clinical and laboratory remission requiring treatment modification.[23] Every patient was completely investigated during relapse to rule out an infectious etiology. The study flow is shown in Figure 1.

RNA Extraction

Total RNA was extracted using the mirVana miRNA isolation kit from tissue samples with histopathologically confirmed microscopic inflammation (for PIBD cases) and non-inflamed
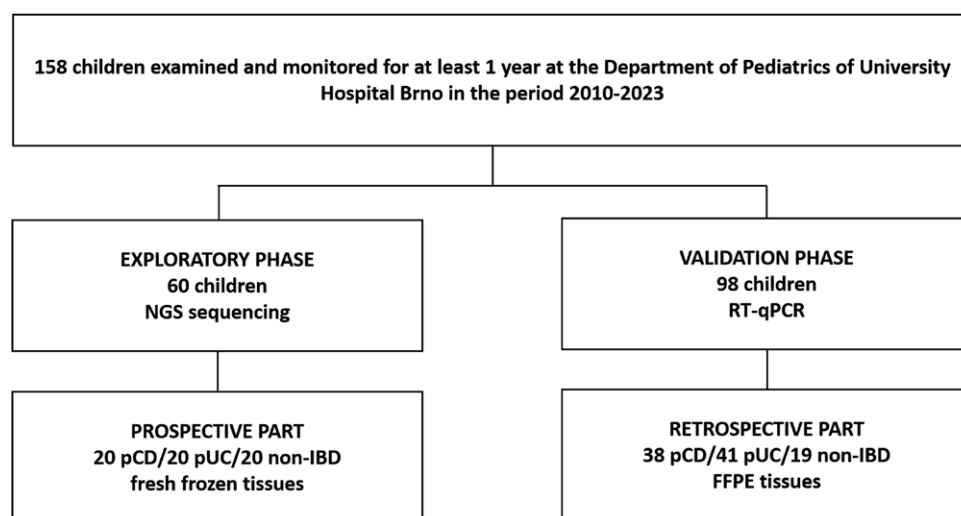
Figure 1. Study flowchart. Deeper explanation is provided in the text.

tissues (for non-IBD controls). The detailed procedure is given in Supplementary Text 1, Methods. The RNA quality was assessed using a NanoDrop 2000/2000c Spectrophotometer (Thermo Fisher Scientific), and the RNA quantity was measured with the QuantiFluor RNA System (Promega). Isolated RNA samples were stored at –80 °C until further analysis.

Exploratory Phase: Small RNA Sequencing

For the exploratory phase, 20 pCD, 20 pUC, and 20 non-IBD children were included. Small RNA libraries were prepared from 100 ng of tissue RNA per sample using the QIAseq miRNA Library Kit and QIAseq miRNA 96 Index Kit UDI A-D (Qiagen), following the manufacturer's instructions. The final library pool was sequenced using the NovaSeq 6000 Sequencing System and NovaSeq 6000 S2 Reagent Kit v1.5 (100 cycles; all Illumina). See Supplementary Text 1, Methods for more details.

Validation Phase: Reverse Transcription and Quantitative PCR

The retrospective validation cohort consisted of 38 pCD patients, 41 pUC patients, and 19 non-IBD controls, from whom FFPE tissue samples were collected at the time of diagnostic colonoscopy. Complementary DNA synthesis was performed using 10 ng of RNA isolated from these tissues, with the miRCURY LNA RT Kit (Qiagen). Quantitative real-time reverse transcription PCR (RT-qPCR) was conducted using miRCURY LNA miRNA Probe PCR Assays to evaluate 25 candidate miRNAs selected based on miRNA next-generation sequencing (NGS) profiling, and 3 miRNA endogenous controls (see Supplementary Text 1, Methods). The RT-qPCR analysis was performed in duplicate for each sample using the miRCURY LNA SYBR Green PCR Kit (Qiagen) on the QuantStudio 6 Real-Time PCR System (Applied Biosystems).

Bioinformatics and Biostatistical Analysis

In the exploratory phase, raw sequencing data were obtained as fastq files. Sequences were quality checked with fastQC and subsequently adapter-trimmed with cutadapt (version 3.3), mapped to miRBase (version 22), and counted using miraligner tool seqcluster (version 1.2.7) and seqbuster (version 3.2). Differential expression analysis was conducted using R/Bioconductor packages edgeR and DESeq2. The DESeq analyses were conducted without any covariates to ensure the validity of our findings across all pediatric patients, irrespective of gender and age. MiRNAs were identified as differentially expressed based on the following criteria: $P_{adj} < .05$ or $P < .01$, baseMean > 500 reads, and log2 fold change >0.58 or < –0.58. These miRNAs were then validated in an independent retrospective cohort. Endogenous control reference genes were selected based on sequencing data (uniform expression across samples with $P > .8$, baseMean > 500 reads), literature search,[24] and analysis with GeneNorm and NormFinder programs in GenEx software 6.0 (MultiD Analyses AB). The expression levels of selected miRNAs (Ct values) were normalized using the geometric mean of three selected reference genes by the $2^{-\Delta Ct}$ method. For statistical analysis, $2^{-\Delta Ct}$ values were log2-transformed to improve normality. Differences in miRNA expression between groups were analyzed using the Mann–Whitney U-test and receiver-operating characteristic (ROC) analysis in GraphPad Prism 9 software (GraphPad Software). P-values <.05 were considered statistically significant.

MiRNA signatures were identified using multivariate logistic regression. The optimal model for each outcome was determined through bidirectional stepwise selection, which iteratively added or removed miRNAs to minimize the Akaike information criterion. Predictive performance was evaluated through the sensitivity and specificity of the models, summarized in ROC curves (https://cran.r-project.org/web/packages/boot/index.html). A risk score formula for predicting individual outcomes was developed as a linear combination of miRNA expression levels, weighted by regression coefficients derived from the logistic regression model. Patients were stratified into high-risk and low-risk groups based on a threshold designed as the value maximizing the sum of sensitivity and specificity. Confidence intervals (CIs) for sensitivity, specificity, accuracy, positive predictive value, and negative predictive value for the threshold were calculated using Wilson's formula with 2000 bootstrap replicates of the ROC curve.[25,26] The CI for the area under the ROC curve (AUC) was computed using the DeLong method based on 2000 bootstrap replicates. To evaluate the model's predictive ability, leave-one-out cross-validation (LOOCV) was applied.

Results

Exploratory Phase: miRNA NGS Profiling

In the first stage of the study, miRNA NGS profiling was conducted on a well-characterized prospective cohort comprising newly diagnosed, treatment-naïve PIBD patients with active pCD (N = 20; 20 tissue samples) or pUC (N = 20; 20 tissue samples), and non-IBD children (N = 20; 40 tissue samples). For analysis, only 1 intestinal tissue sample from each patient with histopathologically confirmed microscopic inflammation from PIBD patients and 2 non-inflammatory tissue samples from different origins from non-IBD children were included. To account for the potential impact of differential miRNA expression across different intestinal sites, tissue origins were percentage-matched between PIBD and non-IBD groups. Specifically, the non-IBD group matched to pCD consisted of 75% terminal ileum (TEI) tissues, while the non-IBD group matched to pUC consisted of 95% left hemicolon (LHC) tissues (Table 1).

In the PIBD cohort, clinical and laboratory parameters (wPCDAI, PUCAI, CRP, Hb) were monitored at diagnosis and over 12 months. Based on these characteristics, patients were classified into 2 prognostic groups. The good prognosis was defined as wPCDAI < 12.5 or PUCAI < 10 within 3 months of diagnosis and with no relapse within 12 months. The poor prognosis was defined as wPCDAI > 12.5 or PUCAI > 10 within 3 months of diagnosis or a relapse occurring within 12 months (Table 1).

A total of 73 significantly dysregulated miRNAs were identified in comparisons between pCD and non-IBD tissues, 280 miRNAs between pUC and non-IBD tissues, and 167 miRNAs between pCD and pUC (P_{adj} < .05; Figure 2). The apparent clustering of samples according to pCD, pUC, and non-IBD conditions is shown in the Principal Component Analysis Plot (Figure S1). Diagnostic miRNAs were selected based on criteria of baseMean > 500 reads and fold change > 1.5×. The miRNAs selected for subsequent validation included miR-10a-5p, miR-21-5p, miR-34a-5p, miR-223-3p (pCD vs non-IBD to pCD; Figure S2), miR-21-5p, miR-31-5p, miR-126-3p, miR-146a-5p, miR-146b-5p, miR-223-3p, miR-378a-3p, miR-424-5p, miR-625-5p, miR-708-5p (pUC vs non-IBD to pUC; Figure S3), miR-194-5p, miR-215-5p, miR-338-3p, and miR-382-5p (pCD vs pUC). In terms of prognostic analysis, miRNAs were also compared based on disease outcomes, including residual disease activity and relapse, in pCD and pUC patients. Among pCD patients, 117 significantly dysregulated miRNAs were identified, 72 of which were associated with residual disease activity and 45 with relapse (P < .05). Similarly, 108 miRNAs were found to be dysregulated in pUC patients, with 83 related to residual activity and 25 to relapse (P < .05). The miRNAs selected for subsequent validation as prognostic markers included miR-30a-3p, miR-143-3p, miR-144-3p, miR-451a, miR-486-5p (pCD poor vs good prognosis), miR-30c-5p, miR-374a-5p, miR-374b-5p, and miR-1275 (pUC poor vs good prognosis). All significantly differentially expressed miRNAs with labeled candidate miRNAs for subsequent validation are listed in Supplementary Text 2, Selected miRNAs.

Validation Phase: RT-qPCR Validation of Selected miRNA Candidates

The retrospective validation cohort included 38 pCD patients, 41 pUC patients, and 19 non-IBD controls (Table 2). FFPE tissues from intestinal regions with microscopic inflammation (PIBD patients) and non-inflamed regions (non-IBD controls) were analyzed. In the pCD group, most samples were from the terminal ileum (TEI; 63.2%), while in the pUC group, most were from the left hemicolon (LHC; 92.7%). Non-inflammatory tissues in the non-IBD group were matched to the corresponding intestinal regions, with 73.7% TEI and 26.3% RHC for pCD comparisons, and 89.5% LHC and 10.5% RHC for pUC comparisons. As in the prospective cohort, patients in the retrospective cohort were stratified based on prognosis (Table 2).

Of the 16 diagnostic miRNAs selected from the exploratory phase, 13 were successfully validated in the retrospective cohort (Table 3). Only miR-10a-5p, which was significantly downregulated in the exploratory phase of the study, was not validated in the independent retrospective cohort. MiR-31-5p, which showed nonspecific amplification, and miR-382-5p, which repeatedly showed high standard deviation (more than cutoff of 0.5 Ct) between replicates, were excluded from further analyses. For prognostic miRNAs, miR-30a-3p, miR-143-3p, miR-144-3p, miR-451a, and miR-486-5p were upregulated in exploratory pCD patients with poor prognosis (residual activity). Similarly, miR-30c-5p, miR-374a-5p, miR-1275 (associated with residual activity), and miR-374b-5p (associated with relapse) were dysregulated in exploratory pUC patients with poor prognosis. However, none of these nine miRNAs were validated as prognostic markers in the independent retrospective cohort (P > .05; Table S1).

To improve diagnostic and prognostic power, a bidirectional stepwise logistic regression approach was applied. For the diagnostic models, we tested 14 diagnostic miRNAs (miR-10a-5p, miR-21-5p, miR-34a-5p, miR-223-3p, miR-194-5p, miR-215-5p, and miR-338-3p for diagnostic model pCD vs non-IBD; miR-21-5p, miR-126-3p, miR-146a-5p, miR-146b-5p, miR-223-3p, miR-378a-3p, miR-424-5p, miR-625-5p, miR-708-5p, miR-194-5p, miR-215-5p, and miR-338-3p for diagnostic model pUC vs non-IBD; and miR-194-5p, miR-215-5p, and miR-338-3p for diagnostic model pCD vs pUC). In the case of finding the best prognostic model, we tested all 14 diagnostics, and 9 prognostic miRNAs (miR-10a-5p, miR-21-5p, miR-34a-5p, miR-223-3p, miR-194-5p, miR-215-5p, miR-338-3p, miR-30a-3p, miR-143-3p, miR-144-3p, miR-451a, and miR-486-5p for pCD residual activity and relapse model; and miR-21-5p, miR-126-3p, miR-146a-5p, miR-146b-5p, miR-223-3p, miR-378a-3p, miR-424-5p, miR-625-5p, miR-708-5p, miR-194-5p, miR-215-5p, miR-338-3p, miR-30c-5p, miR-374a-5p, miR-374b-5p, and miR-1275 for pUC residual activity and relapse model). Multiple diagnostic and prognostic miRNA panels were developed and validated (Figure 3).

The signature of 5 miRNAs can diagnose pCD with high accuracy of 96.49% (95% CI, 0.8808-0.9903). Similarly, the signature for pUC, which is based on the examination of 2 miRNAs can diagnose pUC with 100% accuracy (95% CI, 0.9398-1.000), and the miR-215-5p can distinguish between pCD and pUC with 83.54% accuracy (95% CI, 0.7385-0.9012). Moreover, the combination of miR-10a-5p, miR-21-5p, miR-30a-3p, miR-34a-5p, miR-143-3p, miR-144-3p, and miR-451a can determine the pCD patients with high risk of residual activity (accuracy 100.00%, 95% CI, 0.9082-1.000), and the combination of miR-223-3p, miR-486-5p, and miR-194-5p can determine the pCD patients with a high risk of relapse (accuracy 84.21%, 95% CI, 0.6958-0.9256). No reliable model was found for the prediction of pUC residual activity. However, the 2 miRNAs can determine the pUC patients with a high risk of

Table 1. Clinical parameters of the exploratory cohort.

Exploratory cohort	pCD	pUC	non-IBD to pCD	non-IBD to pUC
Patients, *N*	20	20	20	
Age, mean ± SD	13.9 ± 2.4	13.6 ± 2.4	15.4 ± 2.8	
Sex, boys, %	60.0	55.0	40.0	
TEI, %	75.0	0.0	75.0	0.0
RHC, %	25.0	5.0	25.0	5.0
LHC, %	0.0	95.0	0.0	95.0

Prognostic factor	pCD RA		pCD R		pUC RA		pUC R	
	yes	no	yes	no	yes	no	yes	no
Patients, *N*	9	11	9	11	5	15	16	4
wPCDAI/PUCAI, mean								
Initial	34	16	33	17	38	28	32	25
3 months	20	6	18	7	17	1	7	0
12 months	8	4	8	4	3	5	6	0
CRP [mg/dL], mean								
Initial	29	17	24	21	2	5	4	3
12 months	8	2	8	2	1	6	5	2
Hb [g/L], mean								
Initial	120	118	117	121	109	121	115	130
12 months	120	125	120	125	128	133	130	140
Initial therapy, %								
EEN	56	64	67	55	0	0	0	0
CD-ED	33	27	33	27	0	0	0	0
AZA	78	82	78	82	0	40	31	25
5-ASA	0	0	0	0	100	87	88	100
GCS	11	0	0	9	20	60	50	50
MTX	22	0	11	9	0	7	6	0
ADA	0	9	0	9	0	0	0	0
IFX	0	9	0	9	0	0	0	0
Resection	0	0	0	0	0	0	0	0
Escalation up to 12 months, %	89	18	100	9	100	73	100	0

Abbreviations: 5-ASA, 5-aminosalicylates; ADA, adalimumab; AZA, azathioprine; CRP, C-reactive protein; CD-ED, Crohn's Disease Exclusion Diet; EEN, exclusive enteral nutrition; GSC, glucocorticoids; Hb, hemoglobin; IFX, infliximab; LHC, left hemicolon; MTX, methotrexate; RA, residual activity; R, relapse; RHC, right hemicolon; SD, standard deviation; TEI, terminal ileum.

relapse (accuracy 68.29%, 95% CI, 0.5302-0.8044). Formulas for the Score of each miRNA signature are shown in Table S2. All of these models were successfully validated by LOOCV, where the mean squared error (MSE) of each model is very similar to the MSE of LOOCV (Table 4).

Discussion

This study investigates tissue miRNAs as diagnostic and prognostic markers in PIBD. It benefits from a large, well-characterized PIBD cohort ($N = 119$), the consideration of tissue locations, and the use of small RNA sequencing to identify candidate miRNAs. Combining NGS profiling of fresh gut tissues with RT-qPCR validation in FFPE tissues highlights statistically significant miRNA associations with PIBD and demonstrates their predictive performance.

While evaluation of tissue miRNAs requires endoscopic examination and biopsy, thus limiting their diagnostic utility, their variations may serve as valuable prognostic markers and assist in differentiating between types of IBD. Prior evidence supports noninvasive IBD markers based on clinical variables supplemented by traditional laboratory monitoring, such as that for albumin, CRP, and FC. Although their clinical utility is obvious, they have some limitations, specifically as predictors of disease progression.[8] For example, FC with a 50 μg/g cutoff can distinguish IBD in children from non-IBD with 83% sensitivity and 85% specificity (AUC = 0.96).[27] However, its predictive accuracy for disease progression and relapse in PIBD is inconsistent, with sensitivities ranging from 28% to 100% and specificities from 43% to 98%, depending on IBD subtypes (CD/UC), patient age, and cutoff thresholds.[28,29] Similarly, CRP, erythrocyte sedimentation rate, Hb, albumin, and platelet count lack sufficient diagnostic/prognostic power.[27,30] For instance, the CRP/albumin ratio at diagnosis does not reliably predict disease course in pCD or pUC.[30] These limitations underscore the need for more robust biomarkers.

Larger, predictive biomarker studies focused on PIBD, such as the RISK or PROTECT cohort studies, have incorporated
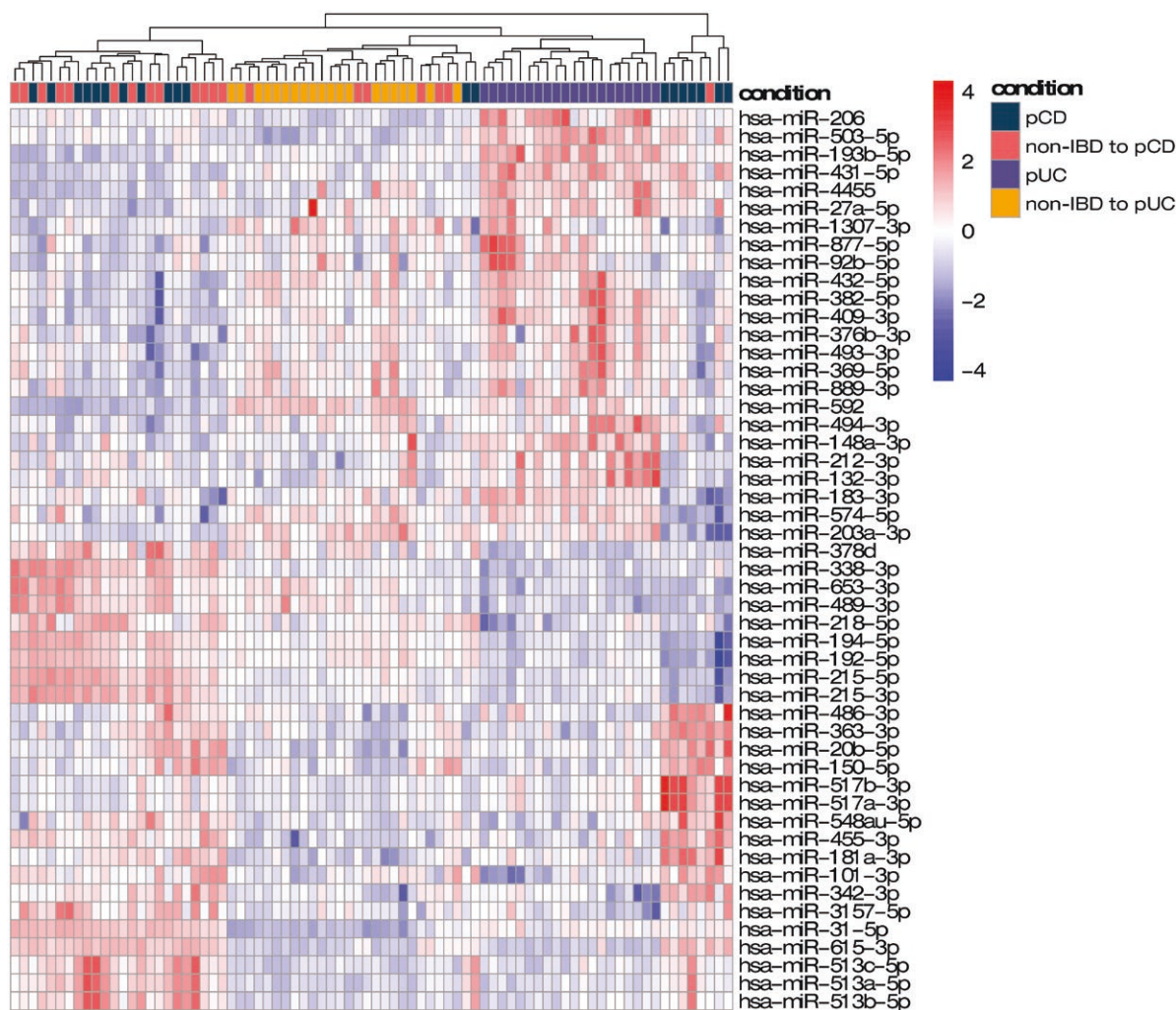
Figure 2. Hierarchical cluster-gram discriminating pCD and pUC patients and non-IBD children according to differentially expressed miRNAs. The heatmap shows the 50 most significantly dysregulated miRNAs in pCD and pUC comparison.

clinical, microbial, and genetic signatures to improve predictions. For instance, competing-risk models can predict complicated or stricturing diseases with AUC ≈ 0.7.[31] Other models consisting of clinical and biological predictors can predict remission without additional therapy beyond mesalamine (AUC = 0.75) or escalation therapy to anti-TNFα (AUC = 0.88).[32] MiRNAs, whether tissue-based or circulating, have also been explored as biomarkers, with sensitivity and specificity ranging from 62.5% to 100% and 66.7% to 100%, respectively.[19,33,34] Our diagnostic panel comprising miR-34a-5p, miR-194-5p, miR-215-5p, miR-223-3p, and miR-338-3p can differentiate pCD from non-IBD with an accuracy of 96.49% (AUC = 0.9850, SE = 94.74%, SP = 100%). In addition, the combination of miR-194-5p and miR-223-3p can distinguish pUC from non-IBD with 100% accuracy, and miR-215-5p can differentiate pCD from pUC with an accuracy of 83.54% (AUC = 0.8800, SE = 78.95%, SP = 78.71%). We observed increased expression of all these miRNAs in pCD, pUC, or both tissue types. MiR-34a suppresses the development and proliferation of Th17 cells and their migration into the colonic epithelium.[35] In adult IBD patients, miR-34a expression correlates variably with disease severity.[36,37] MiR-215-5p is significantly involved in cytokine-related signaling pathways linked to chronic inflammation

in colorectal cancer.[38] MiR-194-5p and miR-338-3p regulate the mitogen-activated protein kinase signaling pathway, and, along with miR-223-3p, contribute to the regulation of the intestinal barrier.[11,39] Additionally, miR-223-3p influences granulocyte activation and negatively regulates the level and activity of poly(ADP-ribose) polymerase-1 (PARP-1) in pCD intestinal tissues, leading to reduced DNA repair mechanisms.[11,40] In our previous study, miR-194-5p expression was associated with early disease relapse and primary sclerosing cholangitis.[15] MiR-194-5p, miR-215-5p, and miR-223-3p expression was also increased in inactive CD, and it is influenced by biopsy location (ileal vs colonic tissues).[41] Therefore, the tissue location needs to be integrated into personalized, location-specific diagnostic strategies.

Our study contributes novel findings to this body of work, particularly with the discovery of miRNA signatures predictive of residual disease activity and relapse in treatment-naïve PIBD patients. We introduce the signature of miR-10a-5p, miR-21-5p, miR-30a-3p, miR-34a-5p, miR-143-3p, miR-144-3p, and miR-451a that can predict residual disease activity (wPCDAI > 12.5) within 3 months of diagnosis of treatment-naïve pCD patients with 100% sensitivity and specificity. MiR-10a inhibits the conversion of Treg cells to

Table 2. Clinical parameters of the validation cohort.

Validation cohort	pCD	pUC	non-IBD to pCD	non-IBD to pUC
Patients, N	38	41	19	
Age, mean ± SD	13.5 ± 2.9	13.7 ± 2.6	14.5 ± 3.4	
Sex, boys, %	52.6	46.3	36.8	
TEI, %	63.2	0.0	73.7	0.0
RHC, %	36.8	7.3	26.3	10.5
LHC, %	0.0	92.7	0.0	89.5

Prognostic factors	pCD RA		pCD R		pUC RA		pUC R	
	Yes	No	Yes	No	Yes	No	Yes	No
Patients, N	6	32	11	27	9	32	9	32
wPCDAI/PUCAI, mean								
Initial	27	34	30	34	28	28	29	28
3 months	15	7	10	7	21	2	14	4
12 months	15	7	11	7	11	6	19	4
CRP [mg/dL], mean								
Initial	31	33	28	34	8	10	12	9
12 months	6	6	7	6	8	2	8	2
Hb [g/L], mean								
Initial	126	114	117	115	116	127	128	123
12 months	131	129	125	131	127	135	121	137
Initial therapy, %								
EEN	33	53	64	44	0	0	0	0
CD-ED	0	0	0	0	0	0	0	0
AZA	100	94	91	96	0	22	11	19
5-ASA	83	84	82	85	100	100	100	100
GCS	67	47	36	56	78	50	67	53
MTX	0	0	0	0	0	0	0	0
ADA	0	0	0	0	0	0	0	0
IFX	17	6	9	7	0	0	0	0
Resection	0	6	0	7	0	0	0	0
Escalation up to 12 months, %	17	19	27	15	11	19	44	9

Abbreviations: 5-ASA, 5-aminosalicylates; ADA, adalimumab; AZA, azathioprine; CRP, C-reactive protein; CD-ED, Crohn's Disease Exclusion Diet; EEN, exclusive enteral nutrition; GSC, glucocorticoids; Hb, hemoglobin; IFX, infliximab; LHC, left hemicolon; MTX, methotrexate; RA, residual activity; R, relapse; RHC, right hemicolon; SD, standard deviation; TEI, terminal ileum.

Th cells and the development of Th17 cells, highlighting its anti-inflammatory role.[35] In our study, miR-10a expression was found to be decreased in pCD patients. MiR-21-5p is critical in regulating cytokines, adaptive immune responses, and colon epithelial cell homeostasis, increases intestinal permeability during epithelial injury, and shows pro-apoptotic properties in colitis models; it also influences TLR4 stimulation and monocyte differentiation.[35] MiR-451a was part of a panel predicting nonspecific cases (IBDU) in patients who develop CD from those who develop UC with an AUC of 78.6%.[42] However, the roles of additional miRNAs in the panel regarding the pathogenesis of IBD are still not well understood. The panel of miR-223-3p, miR-486-5p, and miR-194-5p can predict disease relapse within 1 year of pCD diagnosis (AUC = 0.7950, SE = 54.55%, SP = 96.30%). MiR-486-5p levels were also correlated with CD activity and location in adult patients.[43] However, the involvement of miRNA in IBD pathogenesis may be different in children and adults.

For instance, pediatric patients show significantly lower expression of miR-21 than do adult IBD patients.[18] Therefore, miRNA biomarkers in the adult population need to be validated also in the pediatric population.

The study is limited by the variability in initial treatments within the cohort, with some cases requiring escalation over time. This may have influenced the assignment of patients to groups based on prognostic factors (ie, residual activity within 3 months of diagnosis and relapse within 1 year of diagnosis). Therapy always reflected current medical guidelines, however, and these changed during the study. In some cases within the retrospective cohort, the wPCDAI had to be retrospectively calculated, as it was not an established standard of care at the time of diagnosis. While cross-validation strengthens the study's robustness, further validation in a larger, independent, prospective cohort of various age groups is necessary to ensure the proposed miRNA signatures' analytical validity and broader applicability.

Table 3. Diagnostic miRNAs.

	Exploratory phase			Validation phase				
	BaseMean	log$_2$FC	P_{adj}	AUC	95%CI	SE%	SP%	P-value
pUC vs non-IBD								
miR-10a-5p	71 303	−0.69	3.55E−05	0.5914	(0.4404-0.7424)	68.42	57.89	.2702
miR-21-5p	74 247	0.61	7.71E−03	0.8393	(0.7363-0.9423)	76.32	73.68	<.0001
miR-34a-5p	7675	0.72	1.66E−02	0.6981	(0.5387-0.8575)	78.95	63.16	.0149
miR-223-3p	5524	1.67	6.73E−06	0.7742	(0.6271-0.9214)	76.32	73.68	.0003
pUC vs non-IBD								
miR-21-5p	74 247	1.15	2.05E−09	0.9499	(0.8986-1.000)	87.80	100.0	<.0001
miR-31-5p	32 033	4.12	4.79E−19	NA	NA	NA	NA	NA
miR-126-3p	154786	0.77	2.25E−10	0.8896	(0.8073-0.9719)	80.49	84.21	<.0001
miR-146a-5p	2335	1.46	4.89E−08	0.9653	(0.9254-1.000)	90.24	94.74	<.0001
miR-146b-5p	6584	1.34	1.07E−12	0.9718	(0.9320-1.000)	95.12	94.74	<.0001
miR-223-3p	5524	1.85	1.27E−07	0.9615	(0.9183-1.000)	80.49	94.74	<.0001
miR-378a-3p	17 065	−1.53	1.49E−08	0.9217	(0.8537-0.9897)	90.24	84.21	<.0001
miR-424-5p	968	1.24	7.79E−10	0.7356	(0.6075-0.8636)	60.98	63.16	.0031
miR-625-5p	2738	0.77	3.20E−07	0.7702	(0.6260-0.9144)	80.49	73.68	.0006
miR-708-5p	833	1.77	1.87E−07	0.9576	(0.9130-1.000)	90.24	89.47	<.0001
pCD vs pUC								
miR-194-5p	21 686	1.66	2.16E−06	0.8697	(0.7829-0.9565)	90.24	81.58	<.0001
miR-215-5p	2361	2.50	2.50E−08	0.8800	(0.7984-0.9616)	82.93	81.58	<.0001
miR-338-3p	1162	2.12	2.08E−11	0.7401	(0.6280-0.8521)	73.17	65.79	.0002
miR-382-5p	657	−1.04	3.85E−10	NA	NA	NA	NA	NA

Abbreviations: AUC, area under the ROC curve; CI, confidence interval; FC, fold change, NA, not applicable; SE, sensitivity; SP, specificity.
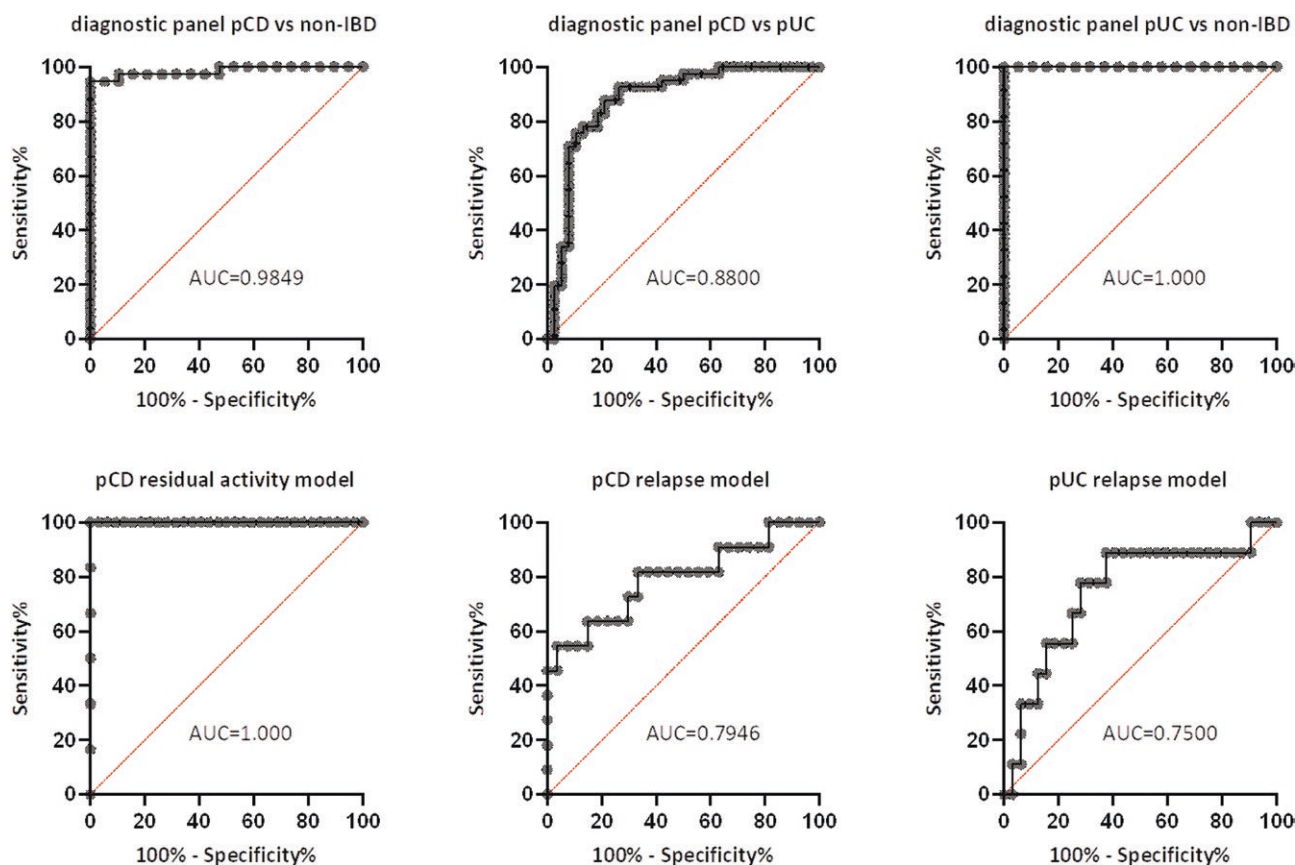
Figure 3. Models of disease diagnosis and prediction.

Table 4. Models of disease diagnosis and prediction.

Model	miRNAs in panel	AUC (95% CI)	SE (95% CI)	SP (95% CI)	MSE (model/LOOCV)
pCD vs non-IBD	miR-223-3p miR-34a-5p miR-194-5p miR-215-5p miR-338-3p	0.9850 (0.9586–1.000)	0.9474 (0.8684–1.000)	1 (1.000–1.000)	0.7108/0.9735
pUC vs non-IBD	miR-223-3p miR-194-5p	1.000 (1.000–1.000)	1.000 (1.000–1.000)	1.000 (1.000–1.000)	6.01E-10/5.92E-10
pCD vs pUC	miR-215-5p	0.88 (0.7979–0.962)	0.7895 (0.6579–0.8947)	0.8781 (0.7805–0.9512)	0.1436/0.1435
pCD residual activity	miR-21-5p miR-10a-5p miR-34a-5p miR-30a-3p miR-143-3p miR-144-3p miR-451a	1.000 (0.9082–1.000)	1.000 (1.000–1.000)	1.000 (1.000–1.000)	0.1826/0.1778
pCD relapse	miR-223-3p miR-486-5p miR-194-5p	0.795 (0.6148–0.9745)	0.5455 (0.2727–0.8182)	0.9630 (0.8889–1.000	0.1925/0.1919
pUC residual activity	None found				
pUC relapse	miR-194-5p miR-215-5p	0.7500 (0.5515–0.9485)	0.8889 (0.6667–1.000)	0.6250 (0.4688–0.7812)	0.1736/0.1732

Abbreviations: AUC, area under the curve; CI, confidence interval; LOOCV, leave-one-out cross-validation; MSE, mean squared error; SE, sensitivity; SP, specificity.

In conclusion, we have demonstrated the potential of miRNA signatures for diagnosing and predicting prognosis at the time of diagnosis in treatment-naïve PIBD patients. These signatures lay the groundwork for advancing precision medicine in pediatric IBD by enabling patient stratification based on risk and optimizing personalized therapeutic strategies. Further studies will be crucial to verify their utility in clinical practice and assess their broader applicability to independent pediatric or adult IBD cohorts.

Supplementary Data

Supplementary data is available at *Inflammatory Bowel Diseases* online.

Acknowledgments

Author Contributions

T.D. wrote the manuscript. D.T., L.R., and T.D. performed sequence and statistical data analysis. J.B., L.K., O.S., and P.J. were responsible for study design and funding acquisition. L.K., M.A., M.H., P.J., and T.P. were responsible for patient recruitment, clinical examination, and sample and clinical data collection. J.S., O.S., P.J., and T.M. performed supervision. All coauthors participated in reviewing and editing. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no competing interests.

Data Availability

Raw sequencing data generated during the current study are available in the SRA under BioProject IDs: PRJNA1213321. Data from RT-qPCR are available from the corresponding author upon reasonable request.

Ethical Considerations

The study was performed with the approval of the University Hospital Brno Ethics Committee (Approval Code: 27-100620/EK). Written informed consent was obtained from all participants before inclusion into the study, in line with the Helsinki Declaration.

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