Assessment of circulating microRNAs for the diagnosis and disease activity evaluation in ulcerative colitis patients by using the Nanostring technology

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Abstract

Background: Clinical decision and patient care management in inflammatory bowel diseases is largely based on the assessment of clinical symptoms, while the biomarkers currently in use poorly reflect the actual disease activity. Therefore, the identification of novel biomarkers will serve an unmet clinical need for IBD screening and patient management. We examined the utility of circulating microRNAs for diagnosis and disease activity monitoring in ulcerative colitis (UC) patients.

Methods: Blood serum microRNAs were isolated from UC patients with active and inactive disease and healthy donors. High-throughput microRNA profiling was performed using the Nanostring technology platform. Clinical disease activity was captured by calculating the partial Mayo score. C-reactive protein (CRP) was measured in UC patients as part of their clinical monitoring. The profiles of circulating microRNAs and CRP were correlated with clinical disease indices.

Results: We have identified a signature of 12 circulating microRNAs that differentiate UC patients from control subjects. Moreover, six of these microRNAs significantly correlated with UC disease activity. Importantly, a set of four microRNAs (hsa-miR-4454, hsa-miR-223-3p, hsa-miR-23a-3p, and hsa-miR-320e) which correlated with UC disease activity, were found to have higher sensitivity and specificity values than CRP.

Conclusions: Circulating microRNAs provide a novel diagnostic and prognostic marker for UC patients. The use of an FDA approved platform could accelerate the application of microRNA screening in a GI clinical setting. When used in combination with current diagnostic and disease activity assessment modalities, microRNAs could improve both IBD screening and care management.

Keywords: blood biomarkers, UC, disease activity, microRNAs

Introduction

Inflammatory bowel diseases (IBD), consisting primarily of ulcerative colitis (UC) and Crohn's disease (CD), are chronic idiopathic pathological conditions characterized by frequent inflammatory episodes in the gastrointestinal track.[1, 2] The prevalence of IBD around the world varies substantially with the higher prevalence recorded in Europe and the United States.[3] Moreover there is an increasing manifestation of IBD in the developing countries.[3] In the United States the prevalence of pediatric CD and UC is approximately 43 and 28 per 100,000 individuals, respectively.[4] In adults, the corresponding numbers are 201 and 238 per 100,000 individuals.[4] Overall in the United States approximately more than one million people suffer today from IBD.[5]

Current clinical decisions in IBD are based on clinical examinations and assessment of symptoms resulting in disease activity indices, in combination with histopathology, endoscopy, and imaging techniques.[6] Evaluating IBD disease activity from clinical indices is often inaccurate due to the subjective recording of symptoms.[7] Additionally, most of the imaging techniques are invasive, painful, time-consuming and expensive causing significant burden on the patient. To overcome the above challenges a number of biomarkers is routinely applied in combination to the above methods to facilitate diagnosis and assess disease activity.[8] Most commonly applied IBD biomarkers include fecal proteins calprotectin and lactoferin[9] and serological proteins such as C-reactive protein (CRP). CRP is a liver-derived protein that is produced in acute response to various inflammatory stimuli.[10] Although CRP correlates with IBD disease activity it shows low specificity and high expression heterogeneity in CD and UC patients.[11-13] Fecal biomarkers correlate with IBD disease activity and relapse but harvesting feces samples is a hurdle for IBD patients.[8, 9, 14] Thus even nowadays, diagnosis and management of IBD remain challenging to the most experienced physicians. Therefore, the identification of novel, accurate non-invasive IBD biomarkers to monitor disease activity would serve an unmet medical need.

MicroRNAs are small (18-25 nucleotides) non-coding RNA molecules that act as negative regulators of gene expression at the post-translational level.[15] Several studies have demonstrated differential microRNA expression patterns between healthy and diseased individuals particularly for cancer,[16] cardiovascular[17] and inflammatory diseases[18, 19] including UC and CD.[20, 21] Emerging evidence suggests that microRNAs can be found in serum and plasma as well as urine, at stable, reproducible and consistent expression levels.[22, 23] Additionally, recent studies have correlated the expression levels of circulating microRNAs with disease diagnosis and clinical outcomes.[24, 25]

The purpose of our study was to evaluate the potential of serum microRNAs as diagnostic and disease activity assessment tools in adult patients of UC. We have employed the Nanostring platform technology to perform microRNA expression profiling in serum samples of active and inactive UC patients and healthy controls. We have identified a microRNA gene signature that discriminates between healthy and UC and correlates with the respective disease activity index. This is the first report on a set of circulating microRNAs that correlate and can be reliably employed to assess disease activity in UC.

Materials and methods

Blood samples: Whole blood from UC patients and healthy donors (IRB#12-000420) was subjected to serum and peripheral blood mononuclear cells (PBMCs) isolation by centrifugation (1600g, 15 minutes, 4^{o}C) and Ficoll (Roche) gradient centrifugation (1600g, 10 minutes, 4^{o}C), respectively.

Partial Mayo index

Partial mayo index was calculated by the treating physician at every clinical visit based on assessment of stool frequency, rectal bleeding, and physician's global assessment. Every parameter was estimated on a scale from 0 to 4 as previously described.[26] Based on the partial Mayo index UC patients were categorized in remission (≤2), moderately active (3-4), severely active (≥5).

CRP measurements

C-reactive protein (CRP) detection in blood serum samples was performed in the main clinical laboratory of the Ronald Reagan Medical school of University of California, Los Angeles (UCLA). Venus blood was collected in yellow top BD Microtainer Laboratory Plastic Capillary Blood Collectors supplemented with Clot additive. Approximately, 1 mL of venous blood (0.5 mL serum) was used for the detection of CRP via nephelometry. The reference range of the detection method is <0.8 mg/dL.

RNA isolation

RNA was isolated from serum samples using the miRNeasy Serum/Plasma Kit (Qiagen) and from PBMCs with the miRCURY Cell and Plant RNA Isolation Kit (Exiqon) according to the manufacturer's instructions. Eluted RNA from serum samples and PBMCs was further purified and concentrated by using Amicon Ultra YM-3 columns (3000 kDa MWCO, Millipore).

Nanostring analysis

RNAs following hybridization reactions were processed using the nCounter Prep Station and subsequently the nCounter Digital Analyzer and analyzed by nSolver software, v1.1 (Nanostring Technologies). Normalization was performed using all the microRNAs with coefficient of variation less than 70%.

Statistical analysis

Statistical analyses were performed with the use of Origin software, version 8.6. Student's t-test was used to examine the statistical difference in microRNA levels between control samples and specimens derived from active and inactive UC patients. The correlation significance was determined by means of Spearman correlation analyses. A *P* value of 0.05 or less was considered to indicate statistical significance. The ROC curves were generated using ROCR package (http://rocr.bioinf.mpi-sb.mpg.de/ROCR.pdf) in R/Bioconductor. Area under the curve and its significance was calculated using the 'verification' package (http://cran.r-project.org/web/packages/verification/verification.pdf). Linear regression analysis using both CRP and microRNAs as independent variables and the partial Mayo score as the dependent variable to determine whether both measures contributed redundantly or synergistically to the prediction of disease severity. Regression analyses were conducted using PASW Statistics 21, Release Version 21.0.0 (SPSS, Inc.).

Results

Identification of a Serum MicroRNA Signature for UC patients

To identify microRNAs with potential diagnostic value, we performed microRNA profiling analysis using the Nanostring Technology platform and compared the levels of microRNAs expressed in the serum of healthy controls (n=21) relative to UC patients (n=46). The clinical characteristics of patient groups and healthy controls are presented in **Table 1**. This approach revealed 12 microRNAs to be differentially expressed between control and UC patients (**Figure 1A**). Specifically, 9 microRNAs were up-regulated (miR-223a-3p, miR-23a-3p, miR-302-3p, miR-191-5p, miR-22-3p, miR-17-5p, miR-30e-5p, miR-148b-3p, miR-320e) and 3 were down-regulated (miR-1827, miR-612, miR-188-5p) in UC patient vs control serum samples (**Figure 1B**).

Identification of a Serum MicroRNA Signature correlated with UC Disease Activity

To identify microRNAs that correlate with UC disease activity we performed microRNA profiling analysis in serum samples derived from UC active (n=24) and inactive (in remission) (n=22) patients. This approach revealed six microRNAs (miR-4454, miR-223-3p, miR-23a-3p, miR-148b-3p, miR-320e and miR-4516) significantly down-regulated in UC patients in remission relative to patients with active disease (**Figure 2A**). Analysis of the CRP, by using a nephelometry assay, revealed lower levels of CRP in inactive UC patients compared to active (**Figure 2B**) and calprotectin (**Supplementary Figure 1**) however neither reached statistical significance.

Subsequently, we investigated the correlation between the microRNA levels and the partial Mayo score, a well-established disease activity index for UC. UC patients were clustered according to their partial Mayo scores in three groups: a) patients in remission (n=22); b) patients with moderately active disease (n=10) and c) patients with severely active disease (n=14). The levels of miR-223-3p, miR-4454, miR-23a-3p, miR-148b-3p, miR-320e and miR-4516 positively correlated with disease severity (**Figure 3A)**. Five microRNAs (with the exception of miR-148b-3p, **Supplementary Figure 2**) were significantly higher in severely active UC patients in comparison to inactive patients. However, the difference between patients in remission and moderately active patients did not reach statistical significance. Most importantly, the CRP levels did not exhibit statistically significant differences between any of the same groups of UC patients (**Figure 3B**), suggesting that microRNA analysis consists a more reliable means in monitoring disease activity than CRP.

To compare the diagnostic value of the identified microRNAs we plotted their levels against the disease activity index or CRP. As shown in **Figure 4A** four microRNAs correlated significantly with the partial Mayo score of active and inactive UC patients (**Supplementary Figure 3**). However, CRP levels failed to reach significant correlation with the partial Mayo score (**Figure 4B**). Importantly, all microRNAs demonstrated a higher Spearman r value in comparison to CRP indicative of the higher correlation to the partial Mayo score than CRP. To the best of our knowledge this is the first demonstration of significant correlation between levels of circulating microRNAs and disease activity in UC. In accord, the above data propose that serum derived microRNAs might hold superior prognostic value than CRP.

Sensitivity and Specificity Analysis of the Serum UC MicroRNA Signature

In order to further explore the performance of the identified microRNA signature as disease activity biomarkers we calculated the specificity and sensitivity of each microRNA as well as of their combination. As benchmark we used CRP, for which we calculated the sensitivity and specificity in the UC patients. Sensitivity describes the ability of the test to identify correctly true positives, in this case patients with active disease as indicated by the disease activity index. On the other hand specificity, describes the predictive ability of the test to identify true negatives, which are patients in remission as indicated by the partial Mayo score. The threshold value for CRP to distinguish active from inactive IBD patients is 0.8 mg/mL as indicated by the guidelines of the UCLA Clinical Laboratory and Pathology Services (https://online.lexi.com/lco/action/doc/retrieve/docid/ucla/86972). By applying the above threshold we calculated that the sensitivity and specificity of CRP method for UC is 37% and 95% respectively (**Figure 5A**). In order to find the best threshold value of each microRNA we generated a receiver operating characteristics (ROC) curve by applying a R statistical package called “verification”. The sensitivity and specificity of miR-4454, miR-223-3p, miR-23a-3p, and miR-320e are 70%-68%, 79%-72%, 79%-68%, and 67%-67% (**Supplementary Figure 4**), respectively. Among all combinations of microRNAs the one with the highest sensitivity (78%) and specificity (81%) was the one of combining all four microRNAs (**Figure 5B**). This is the first comparison between serum microRNAs and CRP as disease activity biomarkers in IBD. The above data suggest that the analysis of the four circulating microRNAs provides superior sensitivity and comparable specificity to CRP for the detection of active UC.

Predictive values of Serum microRNAs and CRP in UC patients

To compare the utility of microRNAs and CRP independently or in combination to reflect disease severity we conducted a linear regression analysis. We used both CRP and microRNAs as independent variables and the partial Mayo score as the dependent variable. Simple regression analysis revealed that disease severity, as measured by partial Mayo score, is predicted by both microRNAs ($r=0.56$, $p<0.01$) and CRP ($r=0.09$, $p<0.59$). Importantly, using multiple linear regression, when both microRNAs and CRP were entered into a single prediction model, the CRP no longer reflected disease severity. In fact, when removing the common variance shared by microRNAs and CRP, microRNAs became the only significant predictor of disease severity, accounting for a total of 38% of its variance. These analyses suggest that the predictive/prognostic use of CRP is covered by the microRNAs. Furthermore, these results show that using both variables to predict the severity of disease provides no additional information than the measurement of microRNAs alone.

Discussion

The detection of blood circulating microRNAs has opened new avenues in the development of diagnostic and prognostic biomarkers for different human diseases, including inflammatory diseases.[22] Previous studies have performed microRNA analysis in different blood fractions (serum, plasma, PBMCs) or in whole blood and have identified different microRNA signatures involved with different clinicopathological parameters in human diseases.[27, 28] Interestingly, the microRNA profiles between serum and plasma have been very similar, suggesting that both fractions can be used for evaluation of circulating microRNAs.[22] Our aim in this study was to evaluate if circulating microRNAs could have diagnostic and/or prognostic value for UC patients relative to well-known biomarkers such as CRP and the Mayo score.

A major issue to potentially develop a diagnostic and/or prognostic microRNA blood test is the identification of the appropriate method of normalization for the microRNA expression levels. Different microRNA array technologies use different microRNAs for normalization, thus the data depend on each technology and cannot be easily compared. Importantly, there are no microRNAs or other small RNAs in serum samples have been shown to be preserved at abiding levels among different samples, suggesting the lack of standard 'housekeeping' genes. In fact, the use of housekeeping genes (such as actin or U6) in analyses of serum samples would rather reflect the presence or sample contamination with lysed blood cells. Thus, the traditional reference genes can be used not as standards but could serve as a quality control indicator to exclude samples with hemolysis. In addition, the inclusion of a spike-in RNA can rather serve as an internal control for monitoring the efficiency/consistency of microRNA isolation from serum samples than a reference gene. Therefore, here normalization was performed to the global levels of microRNAs present in the serum samples. A second important parameter is that evaluating the microRNA expression levels by PCR analysis or microarray analysis involves an amplification step that actually affects the outcome (microRNA levels). To evaluate the levels of microRNAs and limit the effects of the amplification step, we have used novel technology, called Nanostring technology, which recently received an FDA clearance (http://www.nanostring.com/company/corp_press_release?id=93). In this study, our findings fulfill these important criteria, pointing to the potential use of these UC microRNA signatures as diagnostic and/or prognostic tools in the clinic.

In the current study we report the identification of a panel of serum microRNAs as novel biomarkers of UC disease activity. Given the recent IBD prevalence data, there is an unmet medical need for improved assessment of disease activity and detection of early response to applied therapies. These goals should be reached in combination with decrease in the costs associated to the high levels of care utilization. Furthermore, with analyses focused more on specific subgroups of patients, the set of serum microRNAs identified here could be tested for its utility in patient risk stratification, monitoring disease activity, the timely detection of disease flares/relapse and the assessment of drug responses. These applications represent significant steps towards a prevention-oriented care medicine, and control of smoldering disease activity.

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Table I. Demographic and clinical characteristics of healthy controls and UC patients.

	Control (n=21)			UC (n=46)		
	Total	**F**	**M**	**Total**	**F**	**M**
Gender		**62%**	**38%**		**39%**	**61%**
Age (years)	**33±8**	**33±8**	**35±8**	**38±12**	**42±14**	**36±11**
Disease Duration	**0**	**0**	**0**	**8±3**	**8±6**	**9±8**
Smoking	**21%**	**16%**	**28%**	**11%**	**11%**	**11%**
Fistulas	**0%**	**0%**	**0%**	**4%**	**0%**	**7%**
Strictures	**0%**	**0%**	**0%**	**0%**	**0%**	**0%**
Surgery	**0%**	**0%**	**0%**	**15%**	**11%**	**18%**

Table II. Demographic and clinical characteristics of active and inactive UC patients.

	Active (N=24)		Inactive (N=22)	
	F	M	F	M
Gender	42%	58%	33%	67%
Age (yrs)	41±13	34±9	42±15	38±12
Disease Duration	9±5	6±6	6±6	11±9
Smoking	0%	14%	25%	7%
Fistulas	0%	7%	0%	7%
Strictures	0%	0%	0%	0%
Surgery	20%	21%	0%	7%

Figures

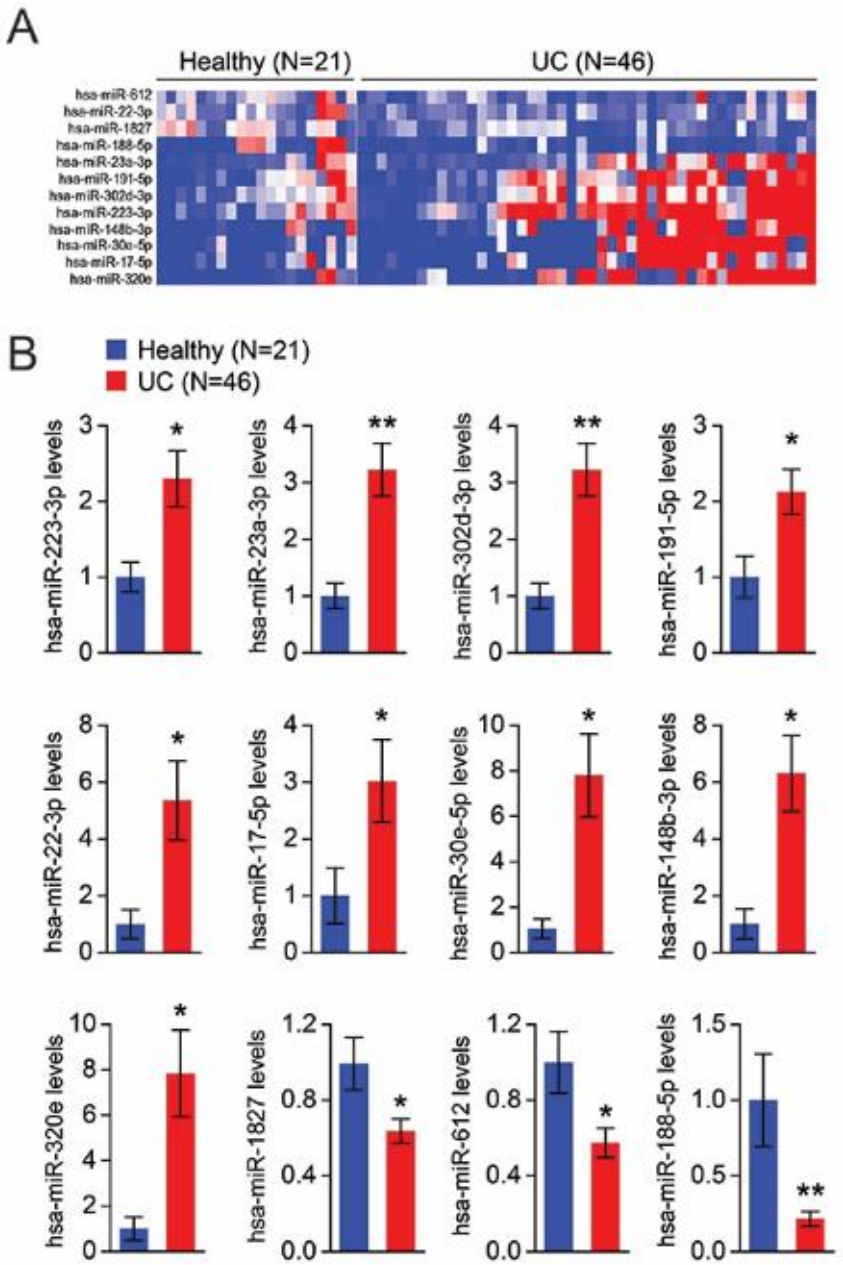

Figure 1. The levels of circulating microRNAs significantly differ in UC patients compared to healthy subjects. A. Heatmap of circulating microRNAs in UC patients (n=46) and healthy donors (n=21). The Heatmap represents the relative levels of microRNAs as assessed using the Nanostring platform. **B.** Circulating microRNAs with diagnostic value. Serum microRNAs with statistically different levels between healthy donors and UC patients. Data are represented as mean values ± s.e.m. * P <0.05, ** P <0.01, Student's t test.

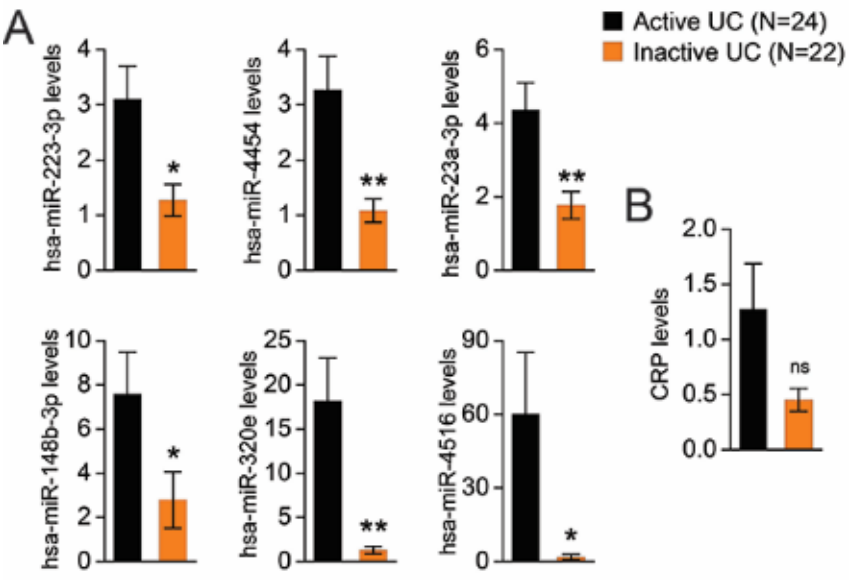

Figure 2. Circulating microRNAs as a prognostic tool. A. Circulating microRNAs with prognostic value. Serum microRNAs with statistically different levels between active (N=24) and inactive (N=22) UC patients. **B.** CRP levels in the same patients. Data are represented as mean values ± s.e.m. * *P* <0.05, ** *P* <0.01, Student's *t* test. ns, non significant.

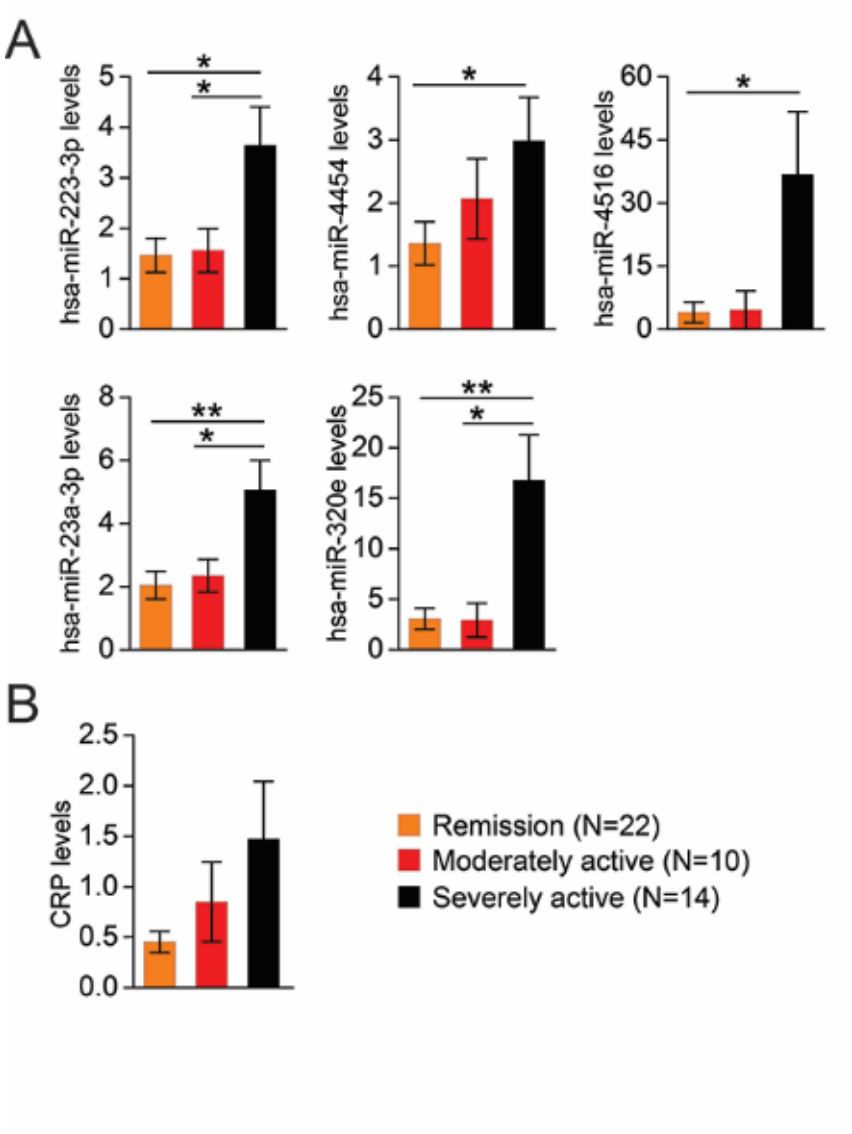

Figure 3. Correlation between the levels of circulating microRNAs and UC disease activity. **A.** Circulating microRNAs with prognostic value in UC patients grouped based on the partial Mayo score. **B.** CRP levels in the same patient groups. Data are represented as mean values ± s.e.m. Only statistically significant differences are denoted. * *P* <0.05, ** *P* <0.01, Student's *t* test.

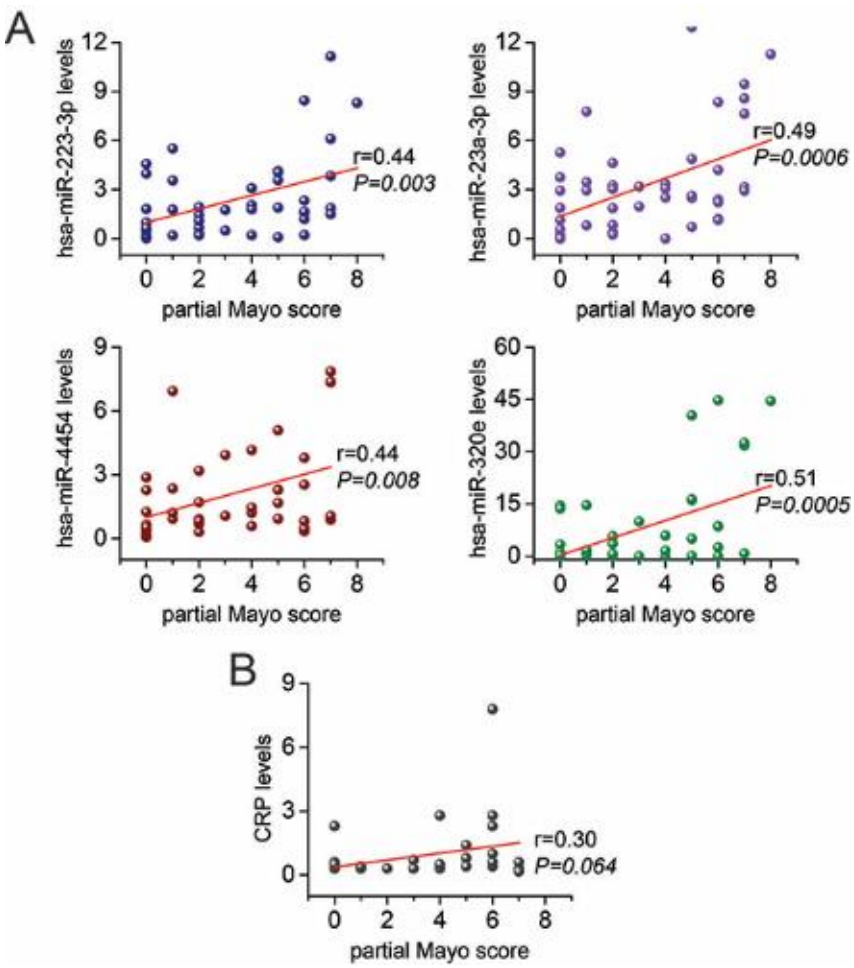

Figure 4. Correlation of circulating microRNAs and CRP with partial Mayo score in active and inactive UC patients. **A.** Spearman correlation analysis of the four microRNAs with prognostic value in active and inactive UC patients. **B.** Spearman correlation analysis of CRP in the same patients UC patients. ANOVA (*P*).

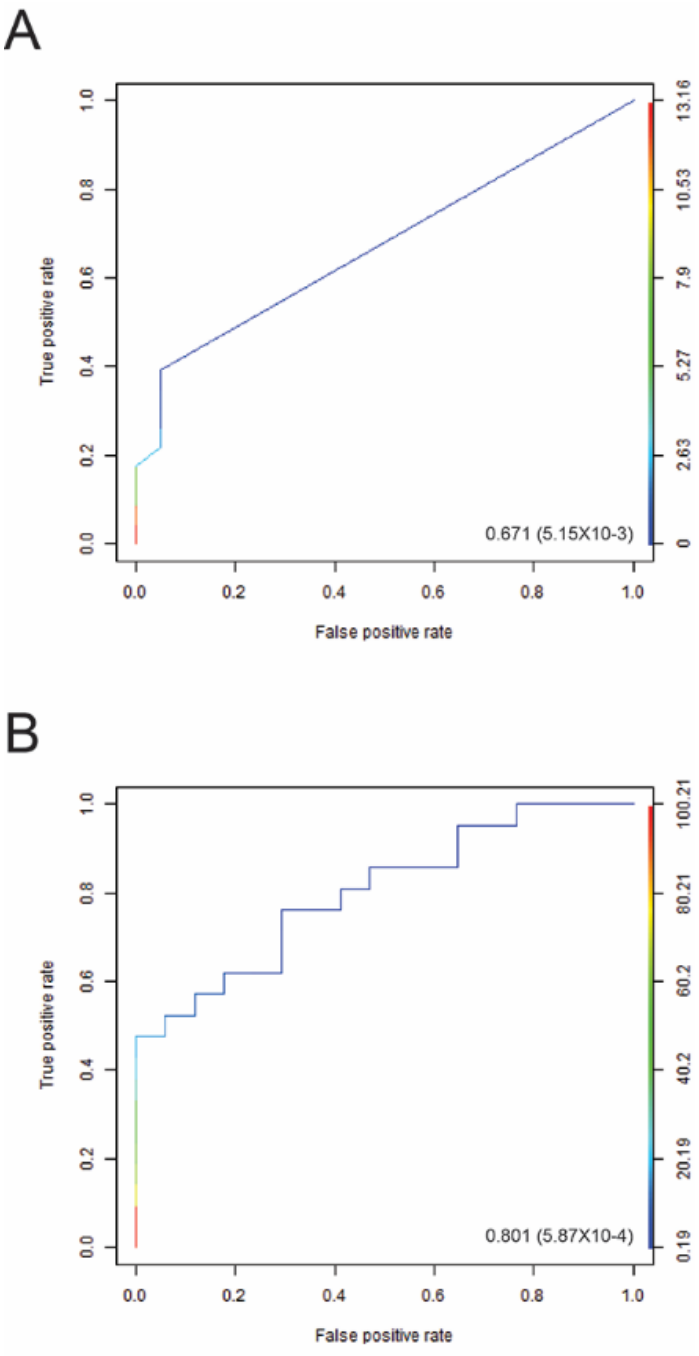

Figure 5. Sensitivity and specificity of circulating microRNAs and CRP tests in UC patients. **A.** ROC curve of four prognostic microRNAs (hsa-miR-4454, hsa-miR-223-3p, hsa-miR-23a-3p, hsa-miR-320e) combined in UC patients (N=46). **B.** ROC curve of CRP in the same patients. ROC curves were generated by applying the "verification" R statistical package. Values indicate the area under the curve and the statistical significance (in parentheses).

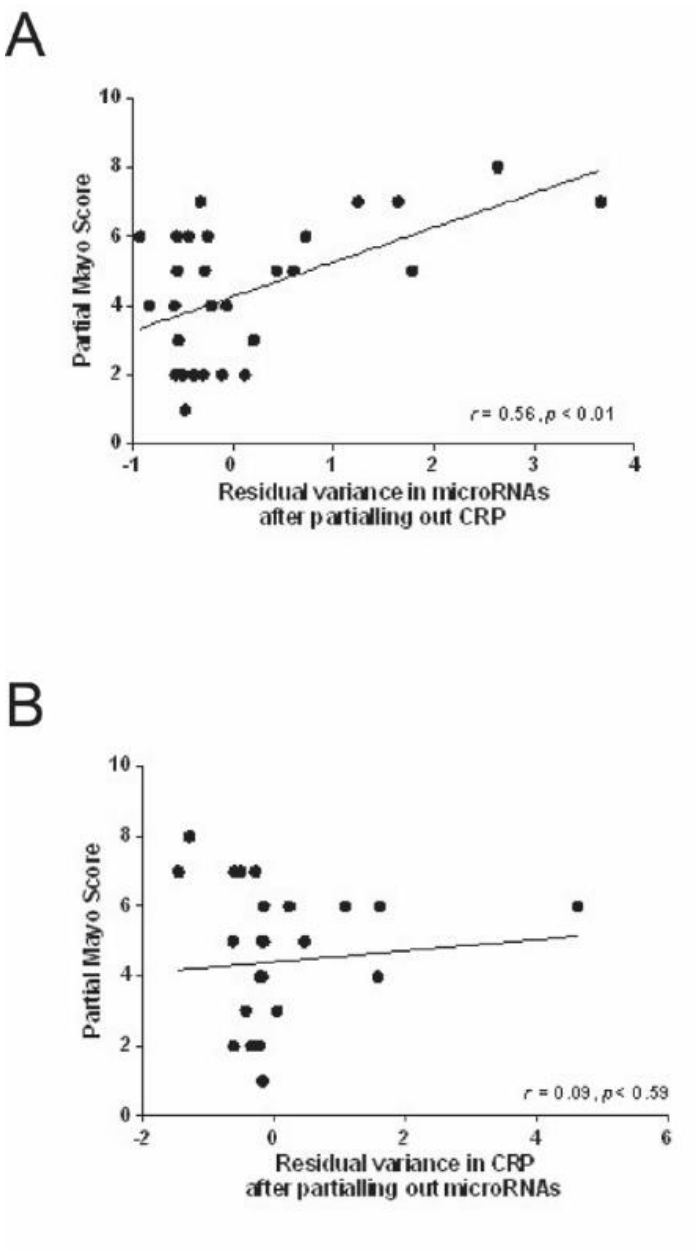

Figure 6. Predictive values of microRNAs and CRP in UC patients. A. Scatter plot shows that partial Mayo score (y-axis) is predicted by the microRNAs (index of all four microRNAs combined) (x-axis), over and above CRP. The x-axis displays the residual variance in the microRNAs after partialling out its shared variance with CRP. **B**. Scatter plot shows that partial Mayo score (y-axis) is no longer predicted by the CRP, when the shared variance between the CRP and the microRNAs was removed (x-axis). The x-axis displays the residual variance in the CRP, after partialling out its shared variance with microRNAs.