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Circulating miRNAs correlate with clinical evaluation of activity in ANCA-associated glomerulonephritis

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Abstract

Introduction Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis is an autoimmune necrotizing small vessel vasculitis, frequently resulting in severe renal manifestations such as rapidly progressive glomerulonephritis (AAV-GN). Monitoring disease activity and determining ongoing renal involvement remain significant clinical challenges due to the limitations associated with traditional biomarkers. This study focused on the potential of circulating microRNA (miRNA) as supplementary noninvasive biomarkers for disease activity in AAV-GN.

Methods This prospective follow-up study involved serum samples from 60 patients with biopsy-proven AAV-GN, collected at renal biopsy and at 3-, 6-, 12-, and 24-month intervals post-biopsy. Nine miRNAs (*miR-21-3p, miR-30b/d/e-5p, miR-142-5p, miR-150-5p, miR-181a-5p, miR-181b-5p,* and *let-7a-5p*) were selected based on the differential expressions in renal tissue and corresponding serum samples identified in the previous research phases. Expression analysis was performed using quantitative real-time polymerase chain reaction and correlated with disease activity based on the Birmingham Vasculitis Activity Score and other clinical parameters.

Results A significant correlation was identified between disease activity and the expression levels of the *miR-30* family members and *let-7a*. Specifically, these miRNAs demonstrated consistent correlation patterns across follow-up samples independent of the time elapsed post-biopsy, with down-regulation correlating with the presence of active disease. Notably, the miRNA expression profile in partial remission appeared analogous to that of complete remission, suggesting that many patients categorized as having partial remission may, in fact, be considered in true clinical remission.

Conclusion This study supports serum miRNA profiling as an adjunct noninvasive biomarker for assessing disease activity in AAV-GN. Such an approach could provide complementary information alongside traditional biomarkers and refine the future management of AAV-GN. However, it is important to acknowledge that our actual study cohort was small due to challenging technical aspects of miRNA expression analysis in the serum. Therefore, further research with larger cohorts is required to validate these results and assess their clinical applicability.

Keywords ANCA, Vasculitis, Glomerulonephritis, MicroRNA, Biomarker, BVAS, Epigenetics, Follow-up

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Background

ANCA-associated vasculitis (AAV) comprises a group of systemic autoimmune disorders characterized by necrotizing small vessel vasculitis. Renal involvement in the form of AAV-GN is a prevalent and severe manifestation of AAV that significantly impacts disease prognosis and management strategies as a major contributor to both morbidity and mortality. Precise noninvasive detection and monitoring of active AAV-GN remain a clinical challenge. Currently, circulating ANCAs serve as a principal biomarker for diagnosis and disease monitoring. However, the ANCA-based approach has several limitations, including the presence of ANCAs in diseases other than AAV-GN and the discordance between ANCA titers and clinical manifestations. Specifically, patients may exhibit persistent ANCA positivity despite clinical remission or, conversely, active disease in the absence of titer elevation [1–3]. The lack of sensitivity and specificity of ANCAs highlights the need for supplementary noninvasive biomarkers to establish the presence of active disease more accurately. Presently, a renal biopsy remains the diagnostic and prognostic standard in cases clinically suspected of AAV-GN.

MicroRNAs (miRNAs), a class of small noncoding RNAs that regulate gene expression post-transcriptionally, have emerged as promising biomarkers across a range of autoimmune diseases, including systemic lupus erythematosus, psoriasis, and rheumatoid arthritis [4–8]. Circulating miRNAs in body fluids such as serum exhibit inherent stability and are resistant to endogenous RNase, making them attractive candidates as minimally invasive biomarkers. The current knowledge of circulating miRNAs specific to active AAV-GN is limited and derived from studies that based on either restricted screening panels or a set of pre-selected miRNAs. Therefore, this study focused on the association between clinical disease activity in AAV-GN at follow-up and serum expression of selected miRNAs specific to AAV-GN we identified in our previous research through comprehensive screening [9] and subsequent validation [10].

Methods

Study design

This was a prospective follow-up study on serum samples of 60 consecutive cases of AAV after biopsy-proven renal involvement in the form of AAV-GN. The serum samples were collected at renal biopsy (initial diagnosis samples) and at 3-, 6-, 12-, and 24-month intervals post renal biopsy during clinical check-ups (follow-up samples). All serum samples were snap-frozen and stored at −80 °C within 72 h of collection.

In every case, the follow-up period terminated with one of the following predefined clinical events: last clinical follow-up at study closure, relapse, death, or need for kidney replacement therapy. Patient enrollment began in January 2018, and follow-up continued until December 2024.

Variables recorded for each case (and time point) included age, sex, estimated glomerular filtration rate (eGFR in ml/min/1.73 m2, calculated using the CKD-EPI 2021 equation), measured daily proteinuria (24 h urine total protein excretion in grams), ANCA titers, type of immunosuppressive therapy, and Birmingham Vasculitis Activity Score version 3 (BVAS) [11]. Renal biopsies were classified according to two established scoring systems in AAV-GN i.e., histologic class per 'Histopathologic classification of ANCA-associated glomerulonephritis [12]' (Berden class) and 'ANCA Renal Risk Score [13]' (ARRS) were assigned.

Biochemical serum and urinary analysis

ANCA specificity and titers were determined using enzyme-linked immunosorbent assay (ELISA, Wieslab AB, Malmö, Sweden) at the Institute of Pathology, Medical Faculty of Ljubljana. The positive cutoff levels were 8 IU/ml for MPO-ANCA and 6 IU/ml for PR3-ANCA. Additional routine laboratory parameters, such as C-reactive protein (CRP), were analyzed at the Department of Clinical Chemistry, University Medical Centre Ljubljana.

Assessment of disease activity and remission criteria

Disease activity was assessed by the treating nephrologist based on clinical judgment at the time of treatment and subsequently supplemented by a retrospective review of medical records by the study investigator using the BVAS.

Complete remission was defined as a BVAS3 score of 0. Standard remission criteria included the absence of clinical symptoms and signs of active vasculitis in any organ system, as confirmed by both the treating physician and the study investigator, no requirement for escalation of immunosuppressive therapy, and normalization of inflammatory markers (CRP).

For the purposes of this study, patients who were deemed to be in clinical remission by both the treating physician and the study investigator—based on a comprehensive review of clinical and laboratory data—but who nevertheless exhibited a BVAS3 score between >0 and ≤ 2 solely due to persistent but progressively improving proteinuria, were classified as being in partial remission. These patients demonstrated negative inflammatory markers, complete resolution of extra renal manifestations, and continuous improvement in renal function.

All other patients who did not meet the criteria for complete or partial remission were considered to have active disease.

Relapse was defined as the recurrence of disease after achieving complete remission, in patients who had completed induction immunosuppressive therapy. Relapse required the presence of new or worsening clinical symptoms and signs of vasculitis, a BVAS3 score > 2, and the need to intensify immunosuppressive therapy.

We aimed to include initial disease presentations only but subsequently enrolled five relapse cases after a sustained remission period without immunosuppressive therapy to increase sample size.

Selection of miRNAs for serum sample analysis

Nine miRNAs (*miR-21-3p, miR-30b-5p, miR-30d-5p, miR-30e-5p, miR-142-5p, miR-150-5p, miR-181a-5p, miR-181b-5p,* and *let-7a-5p*) with significant expression differences between AAV-GN and non-AAV GN, between MPO and PR3 subgroups of AAV-GN, and/or AAV-GN and bystander ANCA samples were selected by preceding screening [9] and subsequent validation on independent renal tissue and corresponding serum samples [10]. Bystander ANCA samples refer to cases with positive MPO- or PR3-ANCA without clinical or histologic evidence of AAV-GN.

Total RNA isolation from serum samples

For total RNA isolation, 200 µl of stored serum was processed with miRNeasy Serum/Plasma Advanced Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Elution was performed in 20 µL of nuclease-free water and the eluate was stored at −80 °C. Spike-in RNAs, namely UniSp2, UniSp4 and UniSp5 (Qiagen, Hilden, Germany), were used to verify the technical success of the isolation procedure.

Reverse transcription of serum RNA into cDNA

Reverse transcription (RT) was performed according to the manufacturer's instructions. For RT, miRCURY LNA RT Kit (Qiagen, Hilden, Germany) was used with 2 µl of isolated RNA in 10 µl reaction master mix. For RT quality control, UniSp6 was spiked into the RT. The reaction was run at 42 °C for 60 min and 85 °C for 5 min.

Quantitative real-time polymerase chain reaction (qPCR)

The obtained RT was diluted 30-fold and 3 µl was used in 10 µl qPCR reaction master mix containing 5 µl of 2X miRCURY SYBR Green Master Mix and 1 µl of appropriate 10X miRCURY LNA miRNA PCR Assay. All qPCR reactions were performed in duplicate on the QuantStudio 7 Pro platform (Thermo Fisher Scientific, Foster City, CA, USA) according to the manufacturer's instructions. The signal was collected at the endpoint of every cycle. To ascertain the specificity of the qPCR products, melting curve analysis was conducted using a ramping rate of 0.075 °C/1 s in the 60–95 °C range.

Before analysis, successful RT was tested using expression analysis of UniSp6 spike-in during RT reaction. Further, successful RNA isolation from serum was evaluated by expression analysis of spike-in RNAs during isolation, namely UniSp2, UniSp4 and UniSp5. Integrity of serum samples was analyzed by the test of hemolysis by quantifying the expression difference between *miR-23a* and *miR-451a*. As reference genes, *miR-103a-3p, miR-191-5p*, and *miR-423* were used according to the manufacturer's instruction. Only samples that successfully passed through all the control steps (expression of UniSp2/4/5/6, hemolysis test and expression of reference genes) were included.

Statistical methodology

The geometric mean of expression of reference genes for each sample was subtracted from the expression of analyzed miRNAs (ΔCq) [14]. For dependent samples, e.g., serum at the time of biopsy/diagnosis versus serum after 3, 6, 12, and 24 months, calculated ΔCq and paired Wilcoxon's test was used. ΔCq of samples after 3, 6, 12, and 24 months were normalized to initial serum samples (ΔΔCq). Differences in normalized miRNA expression values (ΔCq, ΔΔCq) were then tested for statistical significance using the average values of sample replicates. For independent groups of samples, e.g., complete remission versus active disease, the Mann–Whitney test was used. For correlation analyses, e.g., expression of miRNAs (ΔCq, ΔΔCq) versus ANCA titer, Spearman's rank correlation coefficient was calculated. We considered $p < 0.05$ as statistically significant.

Results

Characteristics of the study cohort

We included all cases with at least one technically adequate follow-up sample for correlation analyses in unpaired independent samples. The initial sample served as expression normalization reference and therefore was a prerequisite for paired analyses, but not for unpaired independent analyses.

Thirty-eight out of 60 cases initially considered eligible were ultimately selected after sample quality control. Twenty-two cases (19 MPO- and 3 PR3-positive) with technically adequate initial and at least one follow-up sample were included in both unpaired (global) and paired analyses. Sixteen additional cases (11 MPO- and 5 PR3-positive) without a technically adequate initial sample but with at least one follow-up sample were included in unpaired (global) analyses only.

The patients were followed up for a median of 38 months (range 3–73 months) up to the occurrence of a predefined clinical event as described in Materials & Methods. Selected clinicopathologic characteristics of the cohort are presented in Table 1. Detailed clinical characteristics of individual included cases are available in Supplementary Table 1.

Correlations between clinical parameters

Disease activity was negatively correlated to eGFR (Rho = −0.435, $p < 0.001$, Fig. 1) and positively to ANCA titer (Rho = 0.304, $p = 0.004$). eGFR was negatively and weakly correlated to ANCA titer (Rho = −0.223, $p = 0.040$). Since ANCA type (MPO versus PR3) was negatively correlated to disease activity (Rho = −0.225, $p = 0.023$) and ANCA titer (Rho = −0.353, $p = 0.001$), we subdivided the cases into two subgroups: PR3 and MPO. We repeated the analysis of disease activity correlations with eGFR and ANCA titers within each subgroup. The PR3 subgroup demonstrated the same correlation patterns observed in the overall cohort. In contrast, within the MPO subgroup, a significant negative correlation was found solely between disease activity and eGFR (Rho = −0.689, $p < 0.001$), with no notable association observed with ANCA titers.

Correlations of miRNA expressions with markers of disease activity

Expression of miRNAs in independent follow-up samples was correlated to disease activity, eGFR, ANCA titer and BVAS (both compounded and scored as persistent or new/worse). To disease activity, we observed a weak negative correlation with the expression of *let-7a-5p* (Rho = −0.212, $p = 0.032$). Specifically, we observed significant differential expression of *miR-30b-5p*, *miR-30e-5p* and *let-7a-5p* between complete remission and active disease ($p = 0.050$, $p = 0.024$, $p = 0.031$, respectively). We further observed differential expression for *miR-30e-5p* between active disease versus any kind of remission ($p = 0.046$), as well as differential expression for *let-7a-5p* between complete remission and any kind of persist disease ($p = 0.038$).

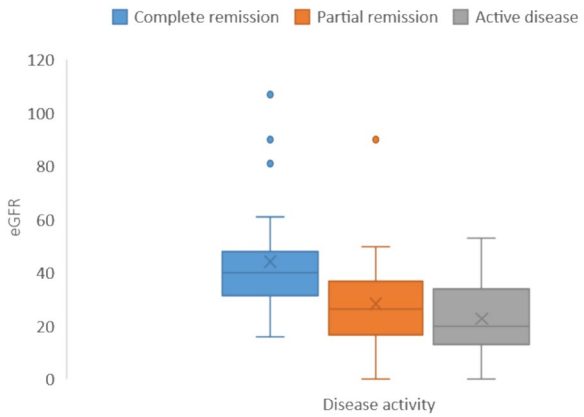

Fig. 1 Correlation between eGFR and disease activity. *eGFR* estimated glomerular filtration rate by CKD-EPI 2021 in ml/min/1.73 m.^{2}

To eGFR, we observed a weak positive correlation with the expression of *miR-30e-5p* to (Rho = 0.248, $p = 0.011$). To ANCA titer we observed a weak negative correlation of *miR-30d-5p* expression (Rho = −0.221, $p = 0.037$). To BVAS, we observed a weak negative correlation of *let-7a-5p* expression (Rho = −0.238, $p = 0.012$). The results are summarized in Table 2. Samples were further subdivided according to ANCA serotype. Whereas we did not observe any correlation between the expression of tested miRNAs and markers of disease activity in the MPO subgroup, we identified a weak positive correlation of expression of *miR-30e-5p* to eGFR (Rho = 0.374, $p = 0.001$) in the PR3 subgroup.

Expression of miRNAs in follow-up samples in comparison to initial serum samples

We compared the expression of miRNAs in follow-up samples (3-, 6-, 12-, and 24-month intervals months post initial samples) relative to the initial serum sample. We observed statistically significant differential expression of *miR-30b-5p* and *miR-30d-5p* in all time points after initial serum ($p < 0.05$ after 3 months, $p < 0.01$ for other time points), and *miR-181a-5p* and *miR-142-5p* in three of four time points after initial serum ($p < 0.05$).

Table 1 Core clinicopathologic characteristics of the study cohort

	Sex ratio	Age	BVAS	eGFR	Berden	ARRS	RRS class
AAV-GN	19:19	70 (12)	17 (12)	18.5 (16)	7:4:19:8	4 (7)	12:19:7
MPO	13:17	70 (13)	16 (11)	18.5 (17)	4:3:16:7	3.5 (7)	9:15:6
PR3	6:2	68.5 (16)	19 (12)	20 (15)	3:1:3:1	5 (7)	3:4:1

AAV-GN all patients with ANCA-associated glomerulonephritis, *MPO* MPO-positive AAV-GN, *PR3* PR3- positive AAV-GN, *BVAS* Birmingham Vasculitis Activity Score version 3, *eGFR* estimated glomerular filtration rate by CKD-EPI 2021 in ml/min/1.73 m^2, *Berden class* histologic class per 'Histopathologic classification of ANCA-associated GN', presented as focal:crescentic:mixed:sclerotic class ratio, *ARRS* ANCA Renal Risk Score, *RRS class* Renal Risk Score Class, deduced from ARRS and presented as low:moderate:high RRS class ratio. Sex ratio refers to male:female ratio and numerical variables are presented as median values and IQR (in brackets). All variables were recorded at kidney biopsy

Table 2 Correlations of miRNA expressions with markers of disease activity and miRNA expression differences between disease activity groups

Correlations	*miR-30b-5p*		*miR-30d-5p*		*miR-30e-5p*		*let-7a-5p*		*miR-21-3p*		*miR-142*	*miR-150*
	ΔCq	ΔΔCq	ΔCq	ΔΔCq	ΔCq	ΔΔCq	ΔCq	ΔΔCq	ΔCq	ΔΔCq	ΔΔCq	ΔΔCq
Disease activity	/	Rho = −0.381 $p = 0.007$	/	/	/	/	Rho = −0.212 $p = 0.032$	Rho = −0.312 $p = 0.023$	/	Rho = −0.514 $p = 0.041$	/	/
Complete remission	/	Rho = 0.444 $p = 0.001$	/	/	/	Rho = 0.291 $p = 0.043$	Rho = −0.215 $p = 0.029$	/	/	/	/	/
Active disease	/	Rho = −0.344 $p = 0.016$	/	/	Rho = −0.197 $p = 0.045$	/	/	Rho = −0.381 $p = 0.005$	/	/	/	/
BVAS	/	Rho = −0.432 $p = 0.002$	/	/	/	/	Rho = −0.238 $p = 0.012$	Rho = −0.341 $p = 0.012$	/	Rho = −0.576 $p = 0.020$	/	/
pBVAS	Rho = −0.228 $p = 0.021$	Rho = −0.480 $p < 0.001$	/	Rho = −0–375 $p = 0.008$	/	/	/	/	/	Rho = −0.667 $p = 0.003$	/	/
n/wBVAS	/	/	/	/	/	/	Rho = −0.241 $p = 0.14$	Rho = −0.384 $p = 0.005$	/	/	/	/
ANCA titer	/	/	Rho = −0.221 $p = 0.037$	/	/	/	/	/	/	/	/	/
eGFR	/	Rho = 0.533 $p < 0.001$	/	/	Rho = 0.248 $p = 0.011$	Rho = 0.446 $p = 0.001$	/	Rho = 0.364 $p = 0.009$	/	/	Rho = 0.526 $p < 0.001$	Rho = 0.315 $p = 0.024$
Mann–Whitney test												
Active disease versus Complete remission	$p = 0.050$	$p = 0.002$	/	/	$p = 0.024$	/	$p = 0.031$	$p = 0.050$	/	$p = 0.047$	/	/
Complete remission[a]	/	$p = 0.002$	/	/	/	$p = 0.044$	$p = 0.038$	/	/	/	/	/
Active disease[b]	/	$p = 0.017$	/	/	$p = 0.046$	/	/	$p = 0.006$	/	/	/	/

ΔCq delta Cq ($n = 38$ cases), *ΔΔCq* deltadelta Cq ($n = 22$ cases); quantitation cycle, *delta Cq* Cq of expressed miRNA in follow-up serum samples subdivided from geometric mean of reference genes, *deltadeltaCq* ΔCq of expressed miRNA in follow-up serum samples normalized to ΔCq of initial serum, *Rho* Spearman correlation coefficient, *BVAS* Compounded Birmingham Vasculitis Activity Score version 3, *pBVAS* BVAS for persistent symptoms, *n/wBVAS* BVAS for new or worse symptoms, *eGFR* estimated glomerular filtration rate by CKD-EPI 2021 in ml/min/1.73 m^2. Disease activity is scored as complete remission, partial remission or active disease

[a] compared to combined partial remission and active disease

[b] compared to combined partial and complete remissions

Interestingly, we detected the expression of *miR-181b-5p* exclusively in follow-up but not initial serum samples. We also checked whether miRNA expression levels were correlated with the time elapsed since the initial serum sampling (at 3-, 6-, 12-, and 24-months post-biopsy, converted to days). No temporal correlation was detected.

Correlations of normalized miRNA expressions with markers of disease activity

Since we observed differential expressions between paired serum samples (follow-up in comparison to initial serum sample), we normalized the expression of investigated miRNAs in follow-up serum samples to the initial one (ΔΔCq) and the normalized expression was again analyzed for correlation to disease activity.

With disease activity, we observed a weak negative correlation to expressions of *let-7a-5p* and *miR-30b-5p* (Rho = −0.312, $p = 0.032$ and Rho = −0.381, $p = 0.007$, respectively), and moderate negative correlation to the expression of *miR-21-3p* (Rho = −0.514, $p = 0.041$) across samples.

Specifically, a significant differential expression of *miR-30b-5p*, *let-7a-5p* and *miR-21-3p* between complete remission and active disease ($p = 0.002$, $p = 0.050$, p = 0.047, respectively), of *miR-30b-5p* and *let-7a-5p* between remission (complete and partial together) and active disease ($p = 0.017$ and $p = 0.006$, respectively), as well as of *miR-30b-5p* and *miR-30e-5p* between complete remission and any persistent disease (partial remission and active disease; $p = 0.002$, $p = 0.044$, respectively) was observed. Correlations with disease activity that were maintained after normalization to initial samples are depicted in Fig. 2. Interestingly, no miRNA showed statistically differential expression between complete and partial remissions. To BVAS, a negative correlation with the expression of *let-7a-5p* (Rho = −0.341, $p = 0.012$) was maintained after normalization (Fig. 3).

Additionally, negative correlations with the expression of *miR-30b-5p* and *miR-21-3p* (Rho = −0.432, $p = 0.002$, Rho = −0.576, $p = 0.020$, respectively) to BVAS were observed after normalization. To eGFR, a positive correlation with the expression of *miR-30e-5p* to (Rho = 0.446, $p = 0.001$) was maintained after normalization (Fig. 4). In addition, the expressions of *miR-30b-5p* (Rho = 0.533, $p < 0.001$), *let-7a-5p* (Rho = 0.364, $p = 0.009$), *miR-142-5p* (Rho = 0.526, $p < 0.001$), and *miR-150-5p* (Rho = 0.315, $p = 0.024$) were correlated to eGFR after normalization. The results are summarized in Table 2.

Discussion

In this prospective study, we sought to evaluate the utility of a circulating miRNA signature as a noninvasive biomarker for monitoring disease activity in AAV-GN. We identified a distinct circulating miRNA signature associated with active AAV-GN by preceding comprehensive screening [9] and validation of miRNA expression in renal tissue samples, followed by translation to matched serum samples [10]. In this phase, we analyzed the expression of these miRNAs in follow-up serum samples post renal biopsy and correlated the expression data with clinically established markers of disease activity.

The most important findings include correlations between the disease activity, BVAS, eGFR and ANCA titers, and the differential expressions of *miR-30* family members and *let-7a* in follow-up serum samples. Accurate determination of active disease remains a significant clinical challenge in AAV-GN. This is evident not only from clinical perspective, but also at the molecular level,

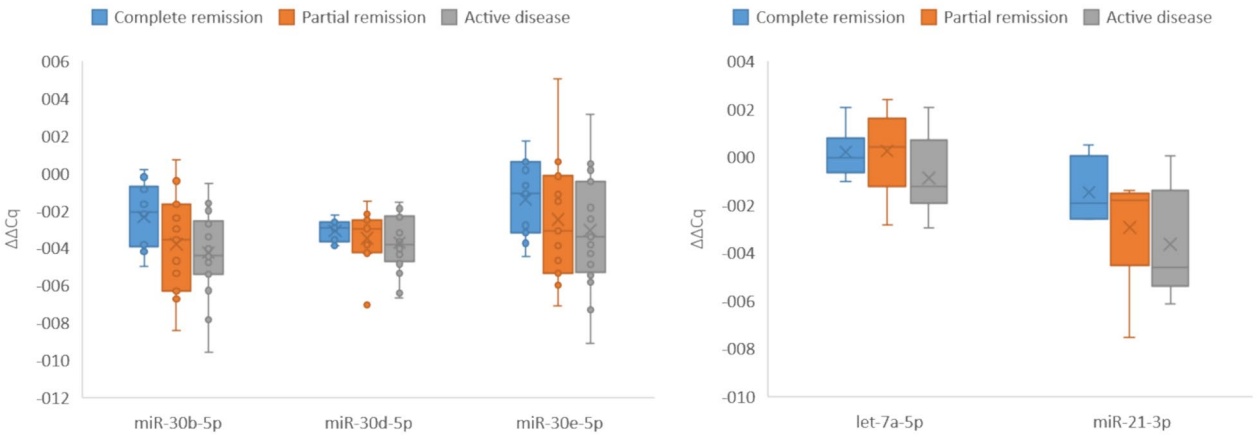

Fig. 2 Correlations of normalized miRNA expressions with disease activity. *ΔΔCq* deltadelta Cq, *Cq* quantitation cycle, *deltadelta Cq* delta Cq of expressed miRNA in follow-up samples subdivided from geometric mean of reference genes (delta Cq) and normalized to the delta Cq in the initial sample

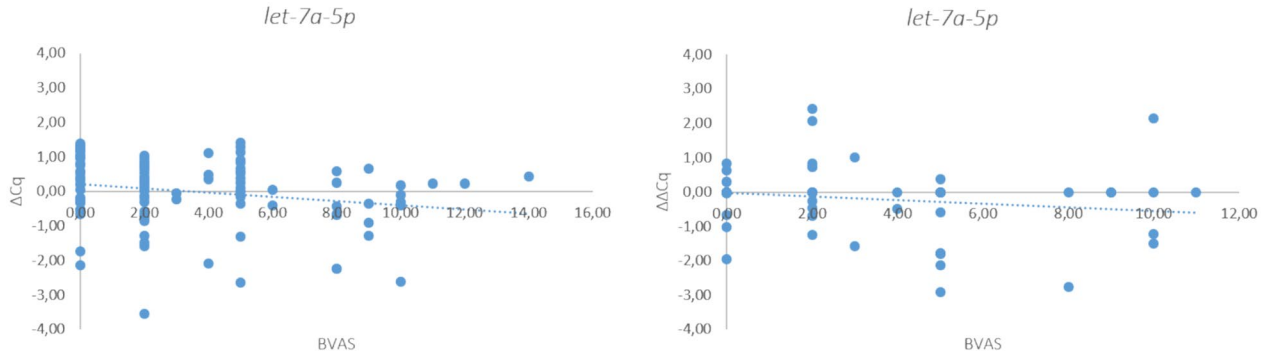

Fig. 3 Correlation of *let-7a-5p* to BVAS. *BVAS* Birmingham Vasculitis Activity Score version 3, *ΔCq* delta Cq, *ΔΔCq* deltadelta Cq, *Cq* quantitation cycle, *delta Cq* Cq of expressed miRNA in all follow-up serum samples subdivided from geometric mean of reference genes (left panel), *deltadeltaCq* ΔCq of expressed miRNA in follow-up samples normalized to ΔCq of initial sample (right panel)

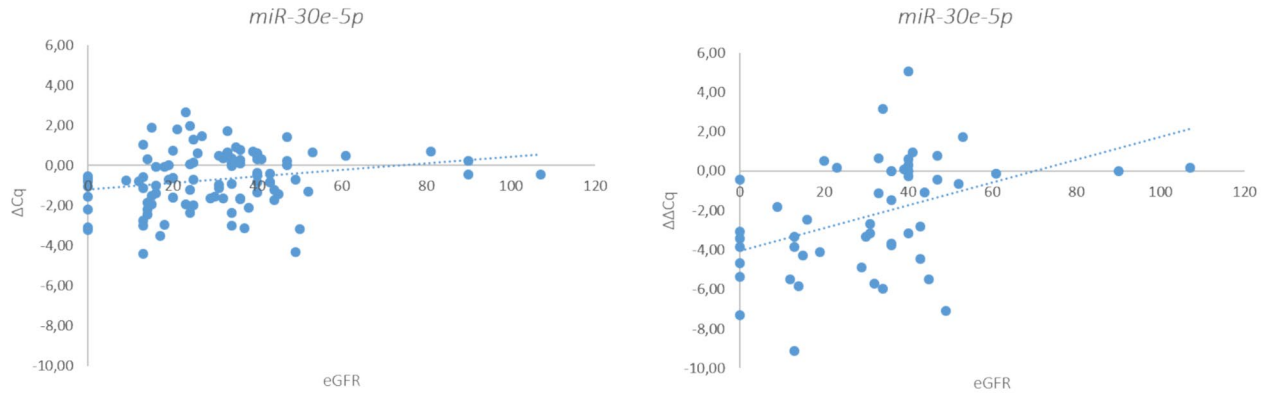

Fig. 4 Correlation of *miR-30e-5p* to eGFR. *eGFR* estimated glomerular filtration rate by CKD-EPI 2021 in ml/min/1.73 m.^{2}, *ΔCq* delta Cq, *ΔΔCq* deltadelta Cq, *Cq* quantitation cycle, *delta Cq* Cq of expressed miRNA in all follow-up serum samples subdivided from geometric mean of reference genes (left panel), *deltadeltaCq* ΔCq of expressed miRNA in follow-up samples normalized to ΔCq of initial sample (right panel)

as reflected in this study by the varied miRNA expression levels within the category of active disease. These observations underscore the imperative to refine existing criteria for defining true active disease, with a goal of delineating a more homogeneous patient subset in whom therapeutic intervention is warranted. In this context, miRNA expression profiling may offer some insights. Notably, we identified differential expression patterns of *miR-30* family and *let-7a-5p* that correlate with the presence of active disease, despite the inherent patient heterogeneity within the active disease category. The normalized expression levels of these miRNAs strengthened correlations with disease activity and BVAS across follow-up samples, irrespective of the time elapsed since renal biopsy, highlighting their potential as biomarkers for disease activity monitoring in follow-up samples. Interestingly, no miRNA showed statistically differential expression between complete and partial remissions. This finding could relate to imperfect clinical classification. Many patients categorized as having partial remission based on BVAS may represent true clinical remission, but are not classified as such due to persistent hematuria or proteinuria—features that commonly lag immunologic quiescence. These residual findings, although scored as activity in BVAS, may not represent ongoing active vasculitis. Thus, the miRNA expression profile in partial remission appears analogous to that of complete remission, reinforcing the potential of circulating miRNAs as more precise indicators of true disease quiescence.

Our study also confirmed the inverse relationship between disease activity and eGFR, underscoring the detrimental impact of active vasculitis on renal function. These findings align with previous reports that have demonstrated a significant association between higher disease activity scores and decreased eGFR [15]. In our overall cohort, a weak positive correlation was observed between disease activity and ANCA titers, and a similarly weak negative correlation was found between eGFR and

ANCA titers. This low correlation strength indicates that factors other than ANCA titers may play a more significant role in renal function decline. Interestingly, only the PR3 subgroup exhibited correlation patterns consistent with those observed in the overall cohort. In contrast, the MPO subgroup demonstrated a significant correlation only between disease activity and eGFR, indicating that in the MPO subgroup especially, the ANCA titers may not reliably reflect renal function or disease activity. Additionally, we did not observe any correlation between the expression of tested miRNAs and markers of disease activity in the MPO subgroup. While low sample size could be the reason, the findings still support the rationale that PR3-and MPO-positive AAV should be regarded as entities with distinct pathogeneses, clinical features, outcomes, and optimal patient management strategies [16, 17]. Additionally, weak correlations of disease activity and expressions of miRNAs to ANCA titers reinforce the notion that ANCA titers alone are inadequate markers of disease activity, underscoring the need for complementary biomarkers.

Current knowledge of circulating miRNAs in AAV-GN is minimal and inconsistent. Of note, the existent studies did not stem from comprehensive tissue-based screening with subsequent validation and employed a limited screening panel or analyzed pre-selected miRNAs in serum or urine samples [18–20]. Furthermore, these studies lacked control samples from patients with GN other than AAV-GN, potentially limiting their specificity. Conversely, our screening and validation data [9, 10] included patients with GN other than AAV-GN among control samples to enhance selection specificity of biomarker miRNAs. In addition, we were able to correlate tissue and biofluid expression of miRNAs in our previous research [10]. Of note, translating findings from renal tissue to serum is non-linear, as expression of individual miRNAs profiled in tissue is often not detectable in serum samples due to inherent limitations common to biofluid-based analysis of miRNAs [21]. This issue could be addressed by adjusting the sample volume or protocol (i.e., employing a more sensitive detection method, such as digital PCR). Regardless, we were still able to detect the majority of selected miRNAs in follow-up serum samples.

Among the most promising biomarker miRNAs, *let-7a-5p* correlated to disease activity, both as assessed clinically and as reflected by BVAS. Moreover, it is functionally implicated in the processes related to AAV pathogenesis, namely macrophage polarization [22, 23]. In our study, we observed a down-regulation in active disease compared to remission and therefore inverse correlation with disease activity. In a study by Zhu et al., *let-7a* downregulation correlated with macrophage polarization and activation, and the severity of rheumatoid arthritis [24].

We also included three out of five miRNAs belonging to *miR-30* family members, namely *miR-30b/d/e-5p*, that were expressed differentially in our renal tissue validation phase, followed by translation to matched serum samples [10]. Interestingly, the *miR-30* family miRNAs are considered podocyte-enriched. We observed down-regulation of *miR-30* family members with activity of the disease in the follow-up samples. Accordingly, marked down-regulations of *miR-30* family members were observed in active focal segmental glomerulosclerosis, predisposing podocytes to apoptosis and podocyte detachment. Interestingly, AAV-GN is also characterized by certain functional podocyte defects, such as detachment and reduced podocyte density, that associate with end-stage renal disease (ESRD) risk in AAV-GN [25].

We also observed a down-regulation of *miR-21-3p* expression in relation to disease activity. Whereas the main strand of *miR-21* i.e., *miR-21-5p* is recognized as an important contributor to renal fibrosis, there is very limited data regarding *miR-21-3p* strand. Notably, *miR-21-3p* has been associated with the process of macrophage polarization [26].

Two additional miRNAs (*miR-142-5p* and *miR-181a-5p*) should merit brief discussion, since their differential expression was also observed in the follow-up samples relative to the initial one, although they did not relate to disease activity. These two miRNAs were also related to monocytes/macrophages, which have been implicated in AAV pathogenesis as inductors and maintainers of vasculitic process [27]. In this context, the alternatively activated (i.e., M2-polarized) macrophages, linked to a Th2-dominant (anti-inflammatory and profibrogenic) response, have been considered the dominant macrophage phenotype in AAV and associated with a higher ESRD risk in AAV [28, 29]. Accordingly, *miR-142-5p* and *miR-181a-5p* have both been shown to regulate macrophage polarization [30, 31].

An interesting finding relates to *miR-181b-5p.* We detected the expression of *miR-181b-5p* exclusively in follow-up samples. Although we found no correlation with disease activity, this finding remains noteworthy, as *miR-181b-5p* was undetectable in the majority of active AAV-GN serum samples in our prior research [10] while consistently expressed in healthy controls. A plausible explanation is that *miR-181b-5p* expression reflects immunological quiescence or restoration of vascular homeostasis, potentially as a downstream effect of immunosuppressive therapy, highlighting the potential impact of treatment on miRNA expression dynamics. In this regard, it would be interesting to explore the modulation of miRNA profiles in response to specific

immunosuppressive regimens, as our sample size did not allow for subgroup analysis due to significant variability of maintenance therapy. Furthermore, the *miR-181* family is involved in regulating vascular inflammation through NF-κB signaling in endothelial cells. In this context, *miR-181b-5p,* which was undetectable in the majority of active AAV-GN in our research, suppressed an enriched set of proinflammatory NF-κB-responsive genes in endothelial cells, such as adhesion molecules VCAM-1 and E-selectin, and reduced leukocyte adhesion. Therefore, altered expression of *miR-181* family members (in our study, *miR-181a-5p* and *miR-181b-5p*) aligns with the observation of increased cardiovascular risk in AAV-GN, especially in patients with active disease [32, 33].

Although clinical application remains distant, functional annotations of the studied miRNAs to biological pathways and target genes relevant to AAV pathogenesis also support attractive therapeutic avenues by miRNA ago-/antagomirs and miRNA sponges.

As a limitation, it is important to acknowledge that our actual study cohort was small due to challenging technical aspects of biofluid-based miRNA expression analysis, resulting in constrained statistical power for subgroup comparisons and correlation analyses.

Conclusion

Collectively, our data suggest that serum miRNA expression profiling holds promise as a surrogate tool for non-invasive disease activity assessment in AAV-GN during follow-up, particularly when expression data are normalized to baseline samples. However, like ANCAs, the generally weak to moderate strength of identified associations limits their reliability as standalone markers, indicating that their greatest utility may lie in combination or as components of a broader, integrated model. To validate the monitoring utility of the identified miRNAs and to explore their integration into decision-making algorithms, future studies in larger cohorts should be performed.

Abbreviations

AAV	ANCA-associated vasculitis
AAV-GN	ANCA-associated glomerulonephritis
ANCA	Anti-neutrophil cytoplasmic antibody
BVAS	Birmingham Vasculitis Activity Score version 3
CRP	C-reactive protein
eGFR	Estimated glomerular filtration rate
miRNA	microRNA
MPO	Myeloperoxidase
PR3	Proteinase 3
RT	Reverse transcription

Supplementary Information

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Supplementary material 1.

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Author contributions

Conceptualization, E.B. and N.K.; methodology, E.B.; software, E.B.; formal analysis, E.B., Z.P.T.; investigation, M.B, E.B. and N.K.; data curation, M.B., N.K. and Ž.V.-H.; writing—original draft preparation, M.B., E.B. and Z.P.T.; writing—review and editing, E.B., N.K. and Ž.V.-H.; preparation of figures, E.B.; supervision, N.K. All authors reviewed the manuscript.

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Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The study observed the guidelines of the Declaration of Helsinki and was approved by the National Medical Ethics Committee (Republic of Slovenia, Ministry of Health, approval number 0120-102/2020/6). Written informed patient consent was obtained for serum samples, as required by the National Medical Ethics Committee of Republic of Slovenia.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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