A panel of plasma microRNAs improves the assessment of surrogate markers of cardiovascular disease in rheumatoid arthritis patients.

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ABSTRACT:

Objective: Patients with rheumatoid arthritis (RA) present increased risk of cardiovascular (CV) disease compared to the general population. Moreover, CV risk factors that have causal relationship with atherosclerosis do not seem to fully explain the accelerated process that they exhibit. We evaluated the association of a 10 microRNAs panel with surrogate markers of subclinical arteriosclerosis (carotid intima media thickness (cIMT), carotid plaque presence (cPP), pulse wave velocity (PWV) and distensibility) in a cohort of RA patients.

Methods: 199 patients with RA were included. Surrogate markers of arteriosclerosis were measured with My Lab 60 X-Vision sonographer. MicroRNAs were extracted from plasma and quantified with qPCR. Multivariate models and classification methods were performed.

Results: Multivariate models showed that microRNAs-24 (β = 15.48), 125a (β = 9.93), 132 (β = 11.52), 146 (β = 15.12), 191 (β = 13.25) and 223 (β = 13.30) were associated with cIMT globally. MicroRNA-24 (OR = 0.41), 146 (OR = 0.36) and Let7a (OR = 0.23) were associated with cPP in men. Including the microRNAs in a PLS-DA model properly classified men with and without cPP. MicroRNA-96 (β = -0.28) was associated with PWV in male patients. Finally, several miRNAs were also associated with cIMT, cPP and arterial stiffness in the high DAS28 group and in the earlier tertile groups of disease duration.

Conclusion: Plasmatic expression of microRNA-24, 96, 103, 125a, 132, 146, 191, 223 and Let7a were associated with surrogate markers of CV disease and could be predictors of CV risk in patients with RA.

Keywords: Rheumatoid arthritis, microRNAs, atherosclerosis, biomarker, epigenetics.

Key messages:

1) Several microRNAs are independent predictors of cIMT in rheumatoid arthritis patients.
2) MicroRNAs classify male RA patients with and without carotid plaque presence.
3) The studied microRNAs might be potential biomarkers of cardiovascular disease in rheumatoid arthritis patients.

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease that mainly affects synovial joints and causes chronic pain, bone erosion and progressive disability. It is the most common chronic inflammatory disease, presenting a prevalence between 0.5 and 1% in the general population (1,2). In addition to having joint involvement, patients with RA present an increased risk of cardiovascular (CV) disease that is estimated to be approximately 50% greater than that of the general population. This risk is in part due to the chronic inflammatory state of RA, which plays an important role in the development of atherosclerosis (2). However, the relationship between subclinical arteriosclerosis and RA is complex, and numerous CV risk factors that have causal relationships with atherosclerosis do not seem to fully explain the accelerated process that patients with RA exhibit. Therefore, the scientific community continues to seek new biomarkers that help prevent these CV events in patients with RA.

The assessment of subclinical atherosclerosis in RA patients can be performed by means of ultrasonographic determination of the carotid intima-media thickness (cIMT) and the presence of carotid plaque (cPP), which have been accepted as good predictors of CV events (3). In this sense, several longitudal studies support the use of cIMT and cPP as useful tools to establish the CV risk of patients with RA. It was observed that RA patients with cIMT greater than 0.91 mm had higher risk of suffering CV events in the 5 following years (5). Moreover, it was also shown that RA patients with carotid plaque presence presented increased risk of CV event and death in a follow-up of 5 years (6). Increased arterial stiffness is also one of the earliest stages of atherosclerosis (6), and its measurement, in terms of pulse wave velocity (PWV) and distensibility, is accepted as a precise and non-invasive method to assess subclinical arteriosclerosis.

MicroRNAs (miRNAs) are defined as small molecules of approximately 21-25 nucleotides of small noncoding RNAs that modulate gene expression, functioning predominantly as negative regulators of the expression of target genes by repressing translation or through direct cleavage of mRNAs (7). For each miRNA, single or multiple mRNA targets have been described, which participate in distinct processes such as cellular differentiation and proliferation, metabolism, homeostasis, apoptosis, senescence and immune response, as they are able to modulate the differentiation of B and T cells (8). Moreover, miRNAs are recognized as critical regulators in atherosclerosis (9), and some specific miRNAs are useful for the early detection of acute myocardial infarction (10). On the other hand, differential expression of miRNAs has also been identified in patients with coronary artery disease (11). Regarding RA, several plasma circulating miRNAs have been reported to be abnormally expressed and

have been associated with higher risk and progression of the disease as well as with clinical parameters (12–14). Other miRNAs are altered by other diseases such as arterial hypertension and dyslipidaemia and their expression is modulated by hypertensive treatment, altering the vascular homeostasis (15,16). Furthermore, we showed that miRNA-451 and miRNA-425-5p were associated with cIMT and PWV in patients with RA (17). However, the association of miRNAs with CV disease in patients with RA remains unclear, and although more evidence is needed, it is reasonable to consider them as potential biomarkers of CV disease in this type of patients (18).

In the present study, 10 miRNAs previously associated with CV risk (miRNA-24, miRNA-96, miRNA-103, miRNA-125a, miRNA-125b, miRNA-132, miRNA-146, miRNA-191, miRNA-223, and miRNA-Let7a) (19–23) were selected, and we studied their role in arteriosclerosis and CV disease in a cohort of 199 patients with RA with the aim of improving the early diagnosis of CV disease in this type of patient. Moreover, we aimed to study these associations stratified by disease activity and duration and also by sex to improve our understanding of the phenotypic differences that show men and women with CV disease (24).

Materials and methods

Patients: The RA population of the present study has been previously described (17,25). The 1987 American College of Rheumatology criteria for RA diagnosis were used to select patients who visited the University Hospital Sant Joan de Reus via external consultation. 199 patients between 20 and 80 years of age were included in the study, and on the same day of their medical visit and without changing their clinical therapy routine, we performed blood collection and carotid ultrasound. Clinical evaluation of the patients has been previously described (17) (Supplementary Data S1). The Clinical Research Ethics Committee of our hospital approved the study (11-04-28/4proj5) and all the participants gave written informed consent. We executed the investigation in accordance with our institution's guidelines and the Helsinki Declaration. Patients were not involved in the development of the study.

Laboratory measurements. Blood samples were collected from 199 patients who had fasted for at least 12 hours. Plasma was obtained by whole blood centrifugation at 3000 rpm for 10 minutes, and plasma samples were stored at -80 °C for analysis. Analytical determinations were performed by enzymatic and conventional methods and have been previously described (17) (Supplementary Data S2).

Ultrasound evaluation of intima-media thickness and arterial stiffness. To measure carotid intima media thickness (cIMT), we used My Lab 60 X-Vision sonographer (Esaote SpA, Genova, Italy). In vivo measurements of cIMT were performed of both common carotid arteries using the QIMT© radiofrequency image processing software (Esaote SpA, Genova, Italy). We defined plaque as a focal structure encroaching into the arterial lumen by at least 0.5 mm or 50% of the surrounding IMT value or a thickness > 1.5 mm (26). Arterial stiffness expressed by the PWV and carotid distensibility was measured directly at both common carotid arteries using the ultrasound linear probe (4-15 MHz) as a tannometer and analysed in vivo by Quality Arterial Stiffness (QAS©) radiofrequency software (Esaote SpA, Genova, Italy). The examination was performed according to standardized measurements (27).

Plasma microRNA expression. The candidate miRNAs (miRNA-24, miRNA-96, miRNA-103, miRNA-125a, miRNA-125b, miRNA-132, miRNA-146, miRNA-191, miRNA-223, and miRNA-Let7a) were studied in independent plasma samples from 199 RA patients. Aliquotes of 200µl were used for

haemolysis evaluation before RNA extraction. Haemolysis was discarded after spectrophotometer analysis at λ=414 nm, corresponding to oxyhaemoglobin contamination.

200µl of frozen plasma were used to extract the RNA containing the fraction of small RNAs by means of the commercial miRCURY RNA Isolation Kit (Exiqon) following the manufacturer's instructions. 1µl of a mixture of synthetic RNAs (UniSp2, UniSp4, and UniSp5) was spiked into the plasma to control for the efficiency of the RNA extraction. Additionally, 1.25 µL of MS2 RNA carrier (Roche) was added to improve RNA extraction. MiRNA candidates were measured by qPCR using commercial miRCURY LNA Universal RT microRNA PCR, ExiLENT SYBR Green master mix Kit (Exiqon, Denmark) and commercial primers for each miRNA (hsa-miR LNA™ PCR primer set, UniRT). Melting curve analysis was performed to control the specificity of the qPCR.

The cycle threshold (Ct) for each sample and miRNA was obtained with SDS v2.3 software (Applied Biosystems). miRNA-16-5p was chosen as reference for normalization, as it showed optimal stability after evaluation with RefFinder (28). The relative expression of each miRNA in each sample was calculated using the variable ΔCt, obtained as Ct miRNA candidate—Ct miRNa-16-5p. An increase in the ΔCt variable of a particular miRNA represented a decrease in the expression of that miRNA. Additional information on miRNA quantification is provided in Supplementary Data S3.

Statistical analysis. Continuous normal variables are presented as the mean and standard deviation (SD), while continuous nonnormal variables are presented as the median and interquartile range (IQR). Categorical variables are presented as the percentage and number of individuals. T tests, Mann–Whitney U tests and chi-squared tests were used to evaluate differences between normal, nonnormal and categorical variables, respectively. Bivariate correlations were estimated using the Pearson and Spearman coefficient for normal and nonnormal variables, respectively. Analyses were performed in the overall cohort and stratified by sex, high and low DAS28 (ESR) and tertiles of disease duration. Patients with DAS28 > 3.2 were considered in the high DAS28 group and patients with DAS28 ≤ 3.2 in the low DAS28 group. To evaluate miRNA associations with continuous dependent variables (cIMT, PWV and distensibility), multivariate linear models were adjusted. Multivariate logistic models were used to estimate the association of cPP and pathological cIMT (PAT-cIMT) with the candidate miRNAs. PAT-cIMT is a binary variable derived from the continuous cIMT where individuals above the 75^{th} percentile were considered positive for PAT-cIMT. Receiver operating characteristic (ROC) curves and area under the ROC curve (AUC) values were calculated as a measure of the classification accuracy of each model. Partial least square discriminant analysis (PLS-DA) was used as a supervised

classification method to discriminate between patients with or without carotid plaque. Details about PLS-DA are provided in Supplementary Data S4. All the models were adjusted for traditional and previously known confounders (29), including age, sex, BMI, disease duration, DAS28, hypertension, DMT2, dyslipidimia and treatments. Elastic net regressions were used in models not previously fitted to select the most influential confounders. R^2, ΔR^2 and Akaike information Criteria (AIC) were provided for each model (Supplementary Data S4). Finally, we computed a global miRNA expression score variable taking into account the individual expression of several miRNAs (Supplementary Data S4). This global miRNA expression score was analysed as tertiles of expression, being the first tertile the lowest expression score and the third the highest expression one. Statistical analyses were performed in R Studio, version 4.0.1. P values < 0.05 were considered statistically significant.

RESULTS

Characteristics of patients with RA

Table 1 shows the general characteristics of the RA cohort included in the study (n = 199), globally and stratified by sex. The mean age of the overall cohort was 57.8 (12.4) years, the mean disease duration was 8 years (3–13), and 66% of patients were female. 25.12% of the patients were in remission, 18.59% presented low disease activity, 45.72% moderate, and 10.55% high. 75% of the patients received conventional synthetic disease-modifying antirheumatic drugs (csDMARDs), 21.6% biological treatment, 57.28% nonsteroidal anti-inflammatory drugs (NSAIDs), 51% corticosteroids, 24.62% renin-angiotensin-aldosterone system (RAAS) inhibitors, 26.13% other hypotensive treatments, 16.08% statins and 0.15% other lipid lowering drugs. The mentioned treatments did not alter any of the carotid ultrasonographic measures (Supplementary Table S1). Men presented increased waist circumference, systolic blood pressure (SBP), diastolic blood pressure (DBP) and cIMT and decreased HDLc. Furthermore, incidence of hypertension, cPP and previous CV events was higher in men. Finally, DAS28 values were higher in women.

Associations of the selected miRNAs with cIMT and PAT-cIMT.

Multivariate linear regression analyses adjusted for known confounders, DAS28, disease duration and treatments showed that miRNA-24 ($\beta = 15.48$, $p = 0.02$, $\Delta R^2 = 2.33\%$), miRNA-125a ($\beta = 9.93$, $p = 0.03$, $\Delta R^2 = 1.79\%$), miRNA-132 ($\beta = 11.52$, $p = 0.04$, $\Delta R^2 = 0.46\%$), miRNA-146 ($\beta = 15.12$, $p = 0.01$, $\Delta R^2 = 2.54\%$), miRNA-191 ($\beta = 13.25$, $p = 0.01$, $\Delta R^2 = 2.54\%$) and miRNA-223 ($\beta = 13.30$, $p = 0.03$, $\Delta R^2 = 1.89\%$) were independent predictors of cIMT in the overall population (Figure 1). No associations were found when analyses were performed stratifying by sex. When we stratified our population by DAS28 (high and low) and by tertiles of disease duration, we showed that miRNAs 24, 125a, 146 and 191 were associated with cIMT in the high DAS28 group (Table 2, A) and miRNAs 24, 103, 125a, 125b, 132, 146, 191, 223 and Let7a were associated with cIMT in the first tertile group of disease duration (Table 2, C). No associations were found in the low DAS28 group nor in the second and third tertile groups of disease duration. All these associations showed that decreased expression of all the mentioned miRNAs was associated with increased cIMT. Furthermore, adding miRNAs to the models improved the explanation of the variability of the data as the AIC decreased and the amount of variability explained (R^2) increased (Figure 1 and Table 2).

We then adjusted multivariate logistic regression models to evaluate the associations between the selected miRNAs and PAT-cIMT in the overall cohort and stratified by sex, DAS28 and tertiles of disease duration. The odds ratio (OR) obtained showed that miRNA-146 (OR = 1.45, p = 0.03) and miRNA-191 (OR = 1.50, p = 0.01) expression levels were associated with PAT-cIMT in the overall population (Supplementary Table S2). No associations were found between miRNAs and PAT cIMT neither in men nor in women. Regarding stratification by DAS28 (low and high), the odds ratio showed that miRNA-24 (OR = 1.77, p = 0.03), miRNA-103 (OR = 2.13, p = 0.02), miRNA-125a (OR = 1.61, p = 0.009), miRNA-146 (OR = 2.34, p = 0.007), miRNA-191 (OR = 1.91, p = 0.01), miRNA-223 (OR = 1.70, p = 0.04) and miRNA-Let7a (OR = 2.31, p = 0.01) were associated with PAT-cIMT in patients with high DAS28 (Supplementary Table S2). No associations were found in the low DAS28 group nor in the tertiles groups of disease activity. A decrease in the expression of all the significant miRNAs was associated with a higher risk of having PAT-cIMT. R^2 as well as AUC, obtained from ROC curves (Supplementary Figure S1), increased and AIC decreased when the different miRNAs were added to the models, improving the predictive power and the explanation of the variability of the data (Supplementary Table S2).

Finally, when we computed the global miRNA expression score variable to evaluate the effect of the combined expression of several miRNAs on cIMT, adjusted models showed that patients with low global miRNA expression levels exhibited a significant increase in cIMT, which was on average 41.47 μm higher (p = 0.03) than patients with high global miRNA expression levels (Supplementary Table S3). When analyses were performed stratifying by sex, it was observed that in men, low global miRNA expression patients showed increased cIMT compared to patients with high global expression levels (β = 66.52, p = 0.047) (Supplementary Table S3). No significant associations were found in women. Finally, stratified analyses of DAS28 and tertiles of disease duration showed in the high DAS28 and in the first tertile of disease duration groups that patients with low global miRNA expression showed higher cIMT values than those with high global miRNA expression (β = 57.59, p = 0.02 and β = 106.02, p = 0.009, respectively) (Table 2).

Associations of the selected miRNAs with carotid plaque.

Multivariate logistic regression models adjusted for known confounders, DAS28, disease duration and treatments showed that OR from miRNA-24, miRNA-146 and miRNA-Let7a were statistically associated with cPP in men (p = 0.04, p = 0.02 and p = 0.04, respectively) (Table 3). No miRNAs were associated

with cPP in the overall cohort or in women. Stratification by DAS28 and tertiles of disease duration showed that OR from miRNA-125a was associated with cPP in the high DAS28 group (p = 0.04) (Table 2, B) and that OR of miRNAs 24, 125a, 125b, 146 and 223 were associated with cPP in the second tertile group of disease duration (p = 0.04, p = 0.01, p = 0.04, p = 0.02 and p = 0.02, respectively) (Table 2, E). No associations were found in patients with low DAS28 and in the other tertiles of disease duration. Decreased expression of these miRNAs decreased the odds of a patient presenting plaque, and adding them to the different models increased the amount of variability explained as well as the discriminative power of the models (Figure 2).

In addition, a PLS-DA model was used with the candidate miRNAs and the most influential confounders ir order to assess the classification power of the miRNAs in males with or without the presence of plaque. The optimal number of latent variables (LV) chosen was 2, which explained 50% of the variability of the data (LV1 11% and LV2 39%). A two-dimensional representation of the data is shown in Figure 3, which shows an appropriate classification of men with and without carotid plaque. The contribution of the variables in each LV is shown in Supplementary Figure S2, which shows that miRNAs 24, 146 or 223, which are mainly represented in LV2, play a pivotal role in classifying men with and without plaque presence.

Associations of the selected miRNAs with arterial stiffness.

Regarding PWV, multivariate linear models, adjusted for known confounders, DAS28 and disease duration, showed that decreased expression of miRNA-96 (β = -0.28, p = 0.04) was significantly associated with decreased PWV in men, contributing 4.89% to the total amount of variability explained by the model (R^2 = 42.19%). Adding miRNA-96 to the model also decreased the AIC, indicating a better fit (from 274.49 to 268.26). No multivariate associations were found in the overall cohort or in women. On the other hand, decreased expression of miRNA-132 was associated with decreased PWV in the second tertile group of disease duration (Table 2, F).

Regarding distensibility, no associations were observed in the overall population, nor when we stratified by sex or by high and low DAS28. However, when we stratified by tertiles of disease duration, we observed that decreased expression of miRNA-125b was associated with increased distensibility in the first tertile group of disease duration (β = 35.71, p = 0.046) (Table 2, D) and decreased expression of miRNA-132 with decreased distensibility in the second group of disease duration (β = -20.42, p =

0.01) (Table 2, G). Adding these miRNAs to the models increased the amount of variability explained and decreased the AIC, improving the fitness of the models.

Correlation of the selected miRNAs with clinical variables.

Correlations between the selected miRNAs and disease-related variables are shown in Supplementary Table S4. Regarding men, miRNA-24 (r = -0.28), miRNA-103 (r = -0.25), miRNA-125a (r = -0.27), miRNA-125b (r = -0.29), miRNA-132 (r = -0.27), miRNA-146 (r = -0.27) and miRNA-223 (r = -0.30) expression levels were positively correlated with DAS28. Moreover, miRNA-125a (r = -0.25), miRNA-146 (r = -0.29) and miRNA-Let7a (r = -0.29) were correlated with ESR, and miRNA-Let7a was correlated with CRP (r = -0.289). For women, miRNA-103 (r = 0.22), miRNA-146 (r = 0.19), miRNA-191 (r = 0.18) and miRNA-223 (r = 0.17) were correlated with years from disease onset. Moreover, miRNA-24 (r= -0.18), miRNA-146 (r= -0.23) and miRNA-Let7a (r = -0.22) were correlated with ESR, and miRNA-24 (r = -0.20), miRNA-191 (r = -0.23), miRNA-223 (r = -0.20), miRNA-96 (r = -0.28) and miRNA-Let7a (r = -0.25) were correlated with CRP. Finally, miRNA-191 (r = -0.18) and miRNA-96 (r = -0.18) were correlated with fibrinogen.

DISCUSSION

In the present study, we evaluated the role of a panel of miRNAs that were previously associated with CV disease as biomarkers of subclinical atherosclerosis in patients with RA, with the aim of identifying patients with high CV risk.

We showed that decreased expression of miRNA-24, miRNA-125a, miRNA-132, miRNA-146, miRNA-191 and miRNA-223 were associated with increased cIMT, being miRNA-24, miRNA-146 and miRNA-223 those more strongly associated and having the greatest effect on the explanation of cIMT variability. We also showed that expression levels of miRNA-146, and miRNA-191 increased the accuracy of the classification of RA patients with PAT-IMT. Furthermore, we showed that RA patients with lower global miRNA expression levels presented significantly increased cIMT. These miRNAs might be predictors of CV risk in RA since many studies have shown that cIMT is an important surrogate marker of CV disease and since patients with RA show increased cIMT compared to controls (30–32). Additionally, cIMT values above 0.9 mm are considered a high predictor of development of CV events over a 5-year follow-up period (33). In this direction, the absolute yearly risk of myocardial infarction associated with increased cIMT above 1 mm was 1.3% and 1.4% in men and women, respectively, and 0.5% for stroke in both sexes (34). Stratified by sex analyses did not show specificities in the effects of miRNAs on cIMT. It has been reported that disease activity, which seems to be higher in women, may affect the burden of atherosclerosis (35). We did not find sex-specific associations probably because all the analyses were adjusted by DAS28 and the heterogenety with respect to this variables was controlled.

Regarding cPP, we showed that increased expression of miRNA-24, miRNA-146 and miRNA-Let7a was associated with increased risk of cPP in men. cPP is another good predictor of CV disease, and patients with RA show increased prevalence of carotid plaque compared to healthy population (5). Additionally, we showed in the PLS-DA that the expression levels of the studied miRNAs played a pivotal role in differentiating patients with and without cPP. All these findings could be powerful tools to predict CV events in this kind of patient. Finally, increased expression of miRNA-96 was associated with increased PWV in men. The risk of a major CV event increases by nearly 14% for every 1 m/s increase in PWV, and these findings could help predict this increase and prevent future CV events (36).

Interestingly, we showed that miRNAs 24, 125a, 146 and 191 were associated with cIMT and that miRNA-125a was associated with cPP in the high disease activity group. We also observed that

several miRNAs were associated with cIMT, cPP and arterial stiffness in the first and second tertiles of disease duration. This interesting results affirm that the first years of the disease are decisive regarding the prognosis and evolution of the disease. Evidence shows that high disease activity, which implies a more inflammatory status, predicts the future development of subclinical atherosclerosis (37). Furthermore, we observed more associations in the earlier stages of the disease, as disease duration is inversely correlated with disease activity (38). However, the precise role that disease duration and disease activity have on miRNA expression levels requieres further studies designed specifically to answer this question.

Recent evidence shows that different miRNAs play pivotal roles in CV disease. It has been shown that increased expression of miRNA-24 and miRNA-132 are associated with heart failure and acute myocardial infarction (38, 39). Decreased expression of miRNA-96 has been preliminary related to pulmonary hypertension in women (41), and miRNA-223 has been identified as a predictor of CV death in a large cohort of patients with coronary artery disease (42). Moreover, miRNA-125 family has been related to arteriosclerosis and vascular diseases (21). Nevertheless, the association between these miRNAs and CV disease is a novel investigation in patients with RA. In this regard, our results added new miRNAs to a previous publication, in which we showed associations between two other miRNAs, miRNA-425-5p and miRNA-451, and subclinical arteriosclerosis in men and women, respectively, in the same cohort of RA patients (17). Our new results are in agreement with those of Ormesth et al., who found that miRNAs such as 125a and 425-5p improved the prediction of high coronary artery calcium in RA patients (43). Others also showed that miRNAs 146 and 223 may be involved in the cardiovascular pathogenesis of patients with RA (44). Nonetheless, the role of most of the miRNAs analysed in this study has not been previously evaluated in RA patients. However, it has been demonstrated that several of the selected miRNAs play a role in the pathophysiology and disease activity of RA (45–48). These new associations of the studied miRNAs with subclinical arteriosclerosis in patients with RA open the door to future research, such as the evaluation of the role of these miRNAs in prospective studies or the validation in different cohorts.

The study of the mechanisms involved in the miRNA's effects are beyond the scope of the present paper, but we showed that miRNAs 24, 103, 125a, 125b, 132, 146, 223 and Let7a expression levels were negatively correlated with disease activity parameters such as DAS28, ESR or CRP in male patients. This might indicate that the effects of these miRNAs might not be mediated through classical parameters but through other mediators modulated by genetic factors. Furthermore, miRNAs 103,

146, 191 and 223 were correlated with disease onset, and miRNAs 24, 96, 146, 191, 223 and Let7a were correlated with inflammatory parameters such as CRP, ESR and fibrinogen in women. These correlations could imply a role of these miRNAs in the modulation of the gene expression associated with the onset and severity of the disease.

It has been described that hypertension and dyslipidaemia induce changes in miRNAs expression (16,49). Furthermore, several studies have shown that treatments for RA, hypertension and dyslipidaemia may have an effect on cIMT and on miRNA expressions (15,37). In our study, the diferent treatments did not affect any of the ultrasonographic measurements. However, to avoid any heterogeneity that treaments may induce, we adjusted all the regression models for treatments and comorbidities. Thus, we ensure that our results are independent of these variables.

Our study has several limitations. Firstly, we cannot conclude causality of the miRNA effects on the studied ultrasonographic variables due to the corss-sectional desing of our study. Secondly, budget constrains have not allowed us to study other interesting miRNAs (15) associated with CV risc. For this reason, our results might be modulated by other miRNAs. Furthermore, the selection of our RA patients are regional focus, so the results might be too population-specific and difficult to generalize across other populations and larger series with measures of atherosclerosis and with follow-up would be needed to confirm the clinical relevance of the candidate miRNAs. However, the robustness of our statistical analyses support that the selected miRNAs have a potential role predicting CV risk that patients with RA show.

In conclusion, the results of our study demonstrate a relationship between miRNA-24, miRNA-96, miRNA-125a, miRNA-132, miRNA-146, miRNA-191, miRNA-223 and miRNA-Let7a with different surrogate markers of CV disease. Although more investigation is needed, this study provides evidence of a possible role of the mentioned miRNAs as useful epigenetic biomarkers of CV risk in RA patients, and the modulation of their expression levels could be a future therapeutic target to prevent CV events in this population.

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Competing interests

The authors declare no competing interests.

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Data availability statement

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

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