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Comprehensive analysis of glycoprotein profiles and their association with cardiovascular disease-related microRNAs in rheumatoid arthritis, metabolic disorders, and controls

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Rheumatoid arthritis (RA) is a chronic autoimmune disease that causes joint pain and disability. The connection between RA and cardiovascular (CV) disease is still being studied. This research aims to explore the relationship between CV-related microRNAs, inflammation, and glycosylated proteins to understand RA's inflammatory pathophysiology concerning CV disease. The study included 219 RA patients, 82 with metabolic disorders, and 64 controls. Clinical evaluations and blood samples were collected. Quantification of microRNAs (Let7a, 24, 96, 103, 125a, 125b, 132, 146, 191, 223, 425, 451) and measurement of glycoproteins (GlycA, GlycB, GlycF) using proton nuclear magnetic resonance (1 H-NMR) were performed. Multivariate linear models were applied. RA patients showed higher glycoprotein levels than those with metabolic disorders and controls. Significant associations between miRNAs 24, 451, Let7a and glycoprotein levels were found in RA patients, particularly in women. Glycoprotein levels were positively correlated with inflammatory markers, highlighting their role in indicating RA severity. This study highlights elevated glycoprotein levels in RA patients, indicating a severe inflammatory pattern. Moreover, glycoproteins were highly associated with CV-disease-related miRNAs, indicating that glycoproteins are involved in both inflammation and CV disease. Finally, the inflammatory profile of glycoproteins was validated as they were highly associated with inflammatory markers of RA.

Keywords Rheumatoid arthritis, Nuclear magnetic resonance, microRNAs, Inflammation, Cardiovascular disease

Rheumatoid arthritis (RA) is a chronic, autoimmune, inflammatory disease mainly affecting the joints that, if untreated, leads to chronic pain, cartilage and bone erosion, and progressive disability. With an approximate prevalence of 1%, it is the most common chronic inflammatory disease, with a higher incidence in women[1]. The inflammation resulting from RA affects other parts of the body beyond the joints, including rheumatoid nodules, pulmonary involvement or vasculitis, metabolic disorders, and increased cardiovascular (CV) disease, stemming from an accelerated atherosclerotic process mostly driven by inflammation since dyslipidemia is moderate[2,3]. However, the relationship between RA inflammation and its impact on extra-articular manifestations, including

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atherosclerosis, remains a subject of ongoing study. Therefore, new biomarkers are needed to better comprehend the underlying pathways of the inflammatory process of RA and its association with CV disease.

In this regard, several microRNAs (miRNAs) have previously been associated specifically with inflammatory pathophysiology of RA and with atherosclerosis and CV disease in the context of the same disease[4,5]. MiRNAs are small non-coding RNA molecules consisting of 21–25 nucleotides that regulate gene expression through translation repression or direct cleavage of RNA. Due to their promiscuous nature, with each miRNA capable of targeting multiple RNAs, they play crucial roles in various biological processes, including atherosclerosis and inflammation[6]. For this reason, numerous miRNAs have been linked to CV disease in the general population and also in RA patients[7]. In this regard, we have reported close associations between miRNAs 24, 146, and Let7a, and atherosclerosis and inflammatory markers in patients with RA[8,9]. Additionally, several miRNAs, including miRNA 125a, 132, 96, 425, or 451, were associated with subclinical atherosclerosis in RA[9,10]. Collectively, these results imply that miRNAs may play a crucial role in linking inflammation and CV disease in RA. However, the complete understanding of the involvement of miRNAs in the inflammatory process of RA largely depends on the inflammatory biomarker selected, which may reflect different aspects of the inflammatory response. In this direction, it has been observed that miRNAs are involved in the protein glycosylation process, which is linked with inflammation in multiple pathologies[11]. In this regard, proton nuclear magnetic resonance (H-NMR) has revealed novel inflammatory biomarkers such as glycoprotein A, B, and F (GlycA, GlycB, GlycF). These three glycoproteic parameters reflect a wide group of proteins with one or more chains of carbohydrates (glycans) bound. GlycA reflects the concentration of acetyl groups of protein-bound N-acetylglucosamine and N-acetylgalactosamine, GlycB reflects the concentration of N-acetylneuraminic acid, and GlycF reflects the concentration of acetyl groups not bound to any protein (free fractions)[12]. Interestingly, they reflect the concentration of a wide group of glycosylated proteins synthesized in the liver in response to systemic inflammation. This property makes them considered more sensitive indicators of low-grade inflammation than other conventional markers, such as C-reactive protein (CRP), which is a specific molecule[13,14]. In this direction, the inflammatory state induced by RA leads to an augmented production of these glycoproteins, especially those from the GlycA family, which have been identified as biomarkers of subclinical atherosclerosis, CV risk, and treatment response in RA[15,16]. However, the relationship between these glycosylated proteins and the inflammation and CV risk-associated miRNAs has not been previously studied.

In this work, we have extensively described the glycoprotein profile of patients with RA, measured with H-NMR, and we have compared it with healthy controls and patients with other high-CV risk metabolic disorders such as type 2 diabetes mellitus (T2DM) or hypertension. Moreover, for the first time, we have explored the associations of these molecules with informative miRNAs previously reported (Let7a, 24, 96, 103, 125a, 125b, 132, 146, 191, 223, 425, 451) to explore how these miRNAs might regulate glycoprotein concentrations. Finally, we also studied the relationship between the H-NMR glycoprotein profiles and the inflammatory and clinical markers of RA (erythrocyte sedimentation rate (ESR), DAS28-ESR, fibrinogen, CRP, DAS28-CRP, clinical disease activity index (CDAI)). All these analyses were performed in the overall cohorts and stratifying for sex in each one. Understanding the relationship between the expression of the studied miRNAs and the glycosylated proteins, and studying this relationship in subjects with other conditions might open a door for future therapeutic targets to reduce inflammation and CV risk exhibited by patients with RA.

Results

General characteristics of the cohorts

Table 1 presents the general characteristics of the three groups studied, including 219 patients with RA, 82 subjects with metabolic disorders, and 64 controls. Briefly, patients with RA and those with metabolic disorders were older and exhibited increased BMI, waist circumference, SBP, DBP, and TG levels compared to controls. The prevalence of hypertension, T2DM, and dyslipidemia was also higher in RA patients and in those with metabolic disorders. Regarding inflammation markers (ESR, CRP, and fibrinogen), RA patients showed elevated levels compared to patients with metabolic disorders and controls. *Supplementary Table 1* displays the general characteristics of the 219 patients with RA, both overall and stratified by sex. The cohort had a mean age of 57.59 ± 12.11, with 65.29% of the patients being female. The mean disease duration and DAS28-ESR score were 7 (2.50–13) and 3.48 (2.67–4.37), respectively. Male patients exhibited increased waist circumference, DBP, and a higher prevalence of hypertension than women. Conversely, women presented higher levels of HDL cholesterol, disease activity scores, CDAI, and health assessment questionnaire (HAQ) punctuation. *Supplementary Table 2* shows the general characteristics of the 82 patients with metabolic disorders and 64 controls, both overall and stratified by sex. In this regard, we observe that women patients with metabolic disorders are older and show increased ESR and fibrinogen values, while men patients show increased DBP. Furthermore, control women show increased waist circumference, HDL cholesterol, ESR and PCR.

H-NMR glycoprotein profile

We conducted an analysis of the H-NMR glycoprotein profile in patients with RA, patients with metabolic disorders, and controls (Table 2). Our findings revealed significant differences among these groups when post-hoc analyses were performed. Specifically, patients with RA exhibited elevated areas of GlycA ($p < 0.001$) and GlycB ($p < 0.001$) compared to the other groups. Patients with metabolic disorders also showed increased areas of GlycA and GlycB compared to controls ($p = 0.002$ and $p = 0.003$, respectively). Patients with RA showed increased levels of GlycF compared to controls ($p = 0.04$), but with no differences compared to patients with metabolic disorders. Additionally, we observed increased HW-GlycA ($p < 0.001$) and HW-GlycB ($p < 0.001$) in RA patients compared to the other groups. Patients with metabolic disorders also showed increased HW-GlycA and HW-GlycB compared to controls ($p = 0.002$ and $p = 0.005$). We also investigated whether glycoprotein parameters differed between men and women within the RA group, the patients with metabolic disorders, and

	RA ($n=219$)	Metabolic diseases ($n=82$)	Controls ($n=64$)	p
Sex – female (%, n)	143, 65.29%	58, 70.73%	50, 78.13%	0.15
Age (years, SD)	57.59 (12.11) *, +	61.50 (11.89) #	51.23 (9.12)	< 0.001
Body mass index (kg/m3, IQR)	26.89 (23.54–30.84) *	26.56 (23.34–29.69) #	24.58 (21.65–27.32)	< 0.001
Waist circumference (cm, SD)	92.18 (12.78) *	95.54 (13.92) #	86.28 (10.31)	< 0.001
SBP (mmHg, IQR)	135 (120–150) *	135 (123–149.8) #	120 (110–130)	< 0.001
DBP (mmHg, SD)	81.28 (12.34) *	80 (10.32) #	72.34 (9.70)	< 0.001
LDL cholesterol (mg/dL, SD)	114 (99–134.5)	123 (107–139)	115 (101.5–130.2)	0.11
HDL cholesterol (mg/dL, IQR)	66 (53–76)	62 (49.5–75)	69 (59.75–77.25)	< 0.001
Tryglycerides (mg/dL, IQR)	93 (69–128) *	93.5 (75–141.5) #	76 (57–93.25)	< 0.001
Glucose (mg/dL, IQR)	89 (82–98.5)	94.50 (90–102)	91.50 (85.75–97.28)	0.12
Current smoker (%, n)	59, 26.94% +	8, 9.75%	10, 15.63%	0.002
Hypertension (%, n)	127, 57.99% *	50, 60.97% #	7, 10.94%	< 0.001
Diabetes mellitus (%, n)	25, 11.42% *	5, 6.09% #	0, 0%	0.009
Dyslipidaemia (%, n)	89.40, 64% *	52, 65% #	0, 0%	< 0.001
ESR (mm/h, IQR)	39 (19–50) *, +	16 (9–26.5)	9.50 (5–14.25)	< 0.001
CRP (mg/dL, IQR)	0.5 (0.2–0.95) *, +	0.12 (0.07–0.25)	0.12 (0.06–0.2)	< 0.001
Fibrinogen (mg/dL, SD)	444.8 (95.12) *, +	345 (71.88)	313.3 (291–332.8)	< 0.001

Table 1. Description of the general characteristics of the RA cohort, the control subjects and the patients with metabolic disorders. n = number of individuals, SBP = systolic blood pressure, DBP = diastolic blood pressure, ESR = erythrocyte sedimentation rate, CRP = C-reactive protein, p = p value. *, differences between RA patients and controls. +, differences between RA patients and metabolic patients. #, differences between metabolic patients and controls.

	RA ($n=219$)	Met. Dis. ($n=82$)	Controls ($n=64$)	p-value
Area GlycA	657.8 (582.9–743.9) *, +	607.8 (543.2–695.3) #	546.5 (495.4–607)	< 0.001
Area GlycB	329.1 (301.3–366.3) *, +	303.6 (284.3–324.1) #	279.7 (255.2–295)	< 0.001
Area GlycF	185.3 (168.6–210.5) *	181.9 (164.7–207.8)	172.4 (160.5–186.5)	0.04
HW-GlycA	15.93 (14.19–17.90) *, +	14.59 (13.36–15.89) #	13.34 (12.34–14.03)	< 0.001
HW-GlycB	4.16 (3.79–4.64) *, +	3.82 (3.58–4.08) #	3.53 (3.22–3.73)	< 0.001

Table 2. Comparison of the different glycoproteic parameters between patients with RA, controls, and patients with metabolic disorders. *, differences between RA patients and controls. +, differences between RA patients and metabolic patients. #, differences between metabolic patients and controls.

controls. In this regard, no significant differences were observed in any parameter between men and women in any group.

Association of glycoprotein profile with microRNAs

Figure 1 shows the summaries obtained from the multivariate linear models of the miRNA statistically associated with the different parameters of the glycoprotein profile of the patients with RA, patients with metabolic disorders and controls. These models were adjusted for multiple confounders, including age, sex, BMI, and RA treatments (in the RA cohort). First, in the RA overall cohort, we observed that decreased expression of miRNAs 24, 125a and Let7a were associated with decreased GlycA area ($\beta = -0.14$ and $p = 0.04$, $\beta = -0.16$ and $p = 0.02$ and $\beta = -0.14$ and $p = 0.04$, respectively), and decreased expression of miRNAs 24 and Let7a with decreased H/W-GlycA ($\beta =$

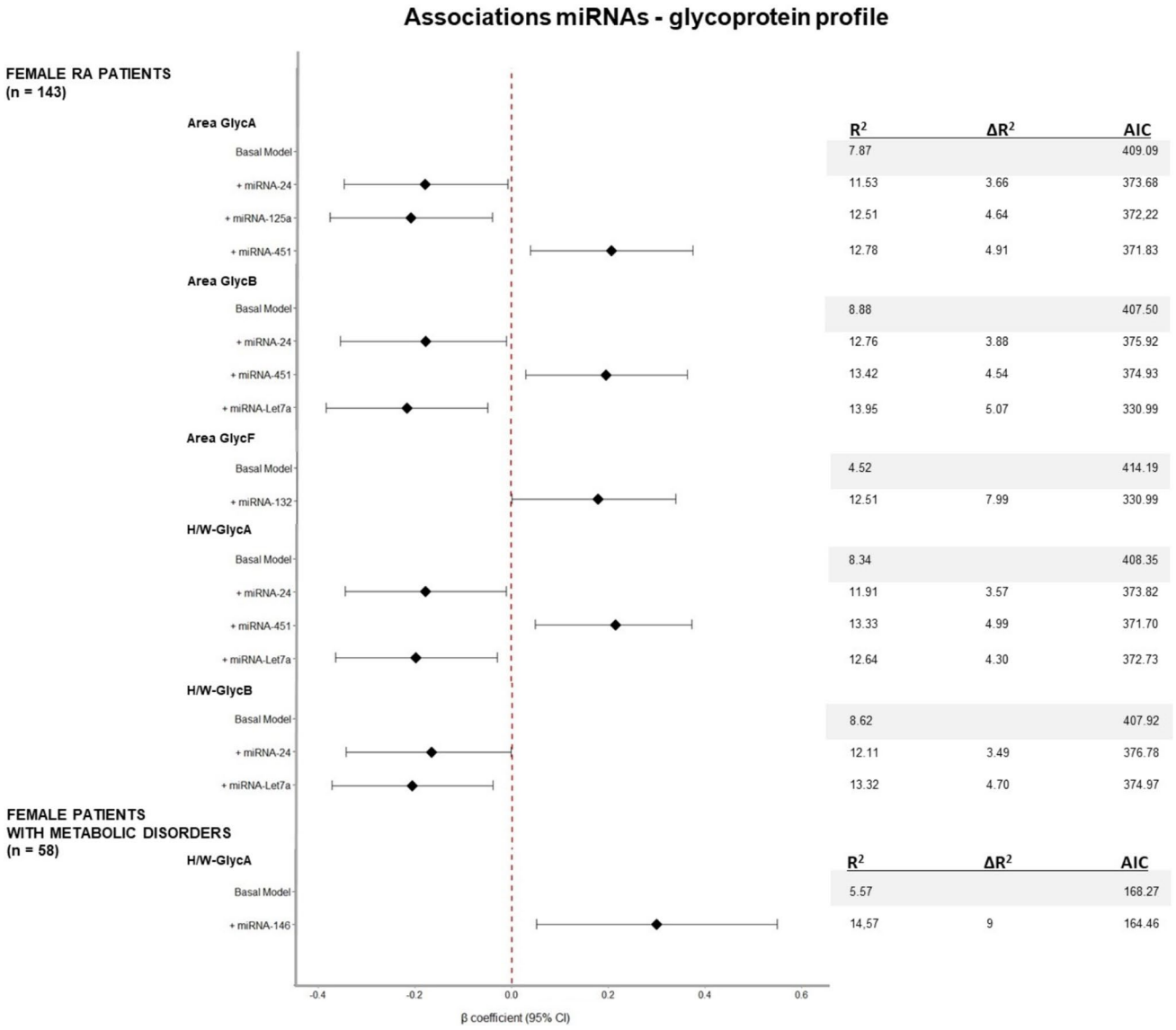

Fig. 1. Study of the associations between the different miRNAs and the glycoprotein parameters analysed by NMR. Multivariate lineal models, adjusted for age, sex and BMI were used. Models with RA patients were also adjusted for RA treatment. β = beta coefficient; p = p-value, AIC = Akaike information criteria.

-0.17, $p = 0.02$ and β = -0.19, $p = 0.008$, respectively). On the other hand, decreased expression of miRNA-451 was associated with increased GlycA area (β = 0.15, $p = 0.03$) and H/W-GlycA (β = 0.18, $p = 0.01$). Regarding GlycB, we observed that decreased levels of miRNAs 24 and Let7a were associated with decreased GlycB area (β = -0.16 and $p = 0.02$ and β = -0.20 and $p = 0.005$, respectively) and H/W-GlycB (β = -0.16 and $p = 0.03$ and β = -0.20 and $p = 0.006$, respectively). On the other hand, decreased levels of miRNA-451 were also associated with increased GlycB area and H/W-GlycB (β = 0.17, $p = 0.02$ and β = 0.17, $p = 0.02$, respectively). Finally, decreased expression of miRNA-125b was associated with increased GlycF area (β = 0.15, $p = 0.03$). When sex-stratified analyses were performed (*Supplementary Fig. 1*), we observed relevant differences in the associations according to the sex of the patients. In this regard, we observed that most of the associations that we observed in the overall cohort, were maintained in women patients. In addition, decreased expression of miRNA-132 was also associated with increased GlycF area (β = 0.18, $p = 0.03$). However, when we studied the associations in men, none of them remained significant. Regarding patients with metabolic disorders, we observed that decreased expression of miRNA-146 was associated with increased H/W-GlycA (β = 0.23, $p = 0.03$). When sex-stratified analyses were performed, this association remained significant in women (β = 0.30, $p = 0.02$) (*Supplementary Fig. 1*). No associations were found in men. Finally, no associations were found between the miRNAs and the glycoprotein parameters, neither in the overall cohort nor in men or women in the control group. Incorporating the different miRNAs into the models improved the overall model qualities, leading to an increase in the explained variability (ΔR^2) and a decrease in the Akaike information criteria (AIC) in every model.

Association of glycoprotein profile with the inflammatory markers of the disease

Finally, Table 3 shows the multivariate lineal models, adjusted for age, sex, BMI and RA treatments (in the RA cohort), to study the relationship between the different glycoproteins and several inflammatory markers in RA patients (DAS28-ESR, DAS28-CRP, ESR, CRP, fibrinogen and CDAI), patients with metabolic disorders (ESR, CRP and fibrinogen) and controls (ESR, CRP and fibrinogen). In this regard, areas of GlycA and GlycB and H/W-GlycA and H/W-GlycB were positively associated with DAS28-ESR, DAS28-CRP, CRP, fibrinogen and CDAI in the overall RA cohort. Area of GlycB as well as H/W-GlycA and H/W-GlycB were also positively associated with ESR. Sex-stratified analyses (*Supplementary Table 3*) showed that area of GlycB, H/W-GlycA and H/W-GlycB were also positively associated with DAS28-ESR, ESR CRP and fibrinogen in men patients. Finally, area of GlycA was also associated with fibrinogen. Regarding women with RA, we observed that area of GlycB, H/W of GlycA and H/W of GlycB were positively associated with DAS28-ESR, DAS28-CRP, ESR, CRP, fibrinogen and CDAI. Regarding patients with metabolic disorders, we observed that GlycB areas and H/W were positively associated with ESR. No associations were found when these patients were analysed sex stratified. Finally, regarding controls, we observed that GlycB area, H/W-GlycA and H/W-GlycB were positively associated with fibrinogen both in the overall cohort and in women (*Supplementary Table 3*). No associations were found in men controls. Adding the different glycoproteins to the models increased the variability explained and decreased the AIC of all models, increasing the overall model qualities. Complete summary of the models can be found in Table 3 and *Supplementary Table 3*.

Discussion

In the present study, we compared the advanced glycoprotein profile, measured with H-NMR, among a cohort of patients with RA, patients with metabolic disorders (dyslipidemia, obesity, and hypertension), and control subjects. Furthermore, we explored, for the first time, the associations between the glycoprotein parameters and miRNAs previously linked to CV disease and inflammation in three different cohorts. Lastly, we reviewed the associations between the glycoprotein parameters and inflammatory markers, including DAS28-ESR, DAS28-CRP, ESR, CRP, fibrinogen and CDAI. All these analyses were performed in the overall cohorts and stratifying by sex.

Regarding to comparing the glycoprotein profile of patients with RA to those with metabolic disorders and controls, we observed that patients with RA exhibited increased levels in all glycoprotein parameters compared to the other groups. Glycoprotein parameters have previously been found to be elevated in patients with RA when compared to control subjects and have been targeted as markers of the inflammatory pattern of RA, CV disease and treatment response in RA[12,15,16]. However, in our present study, we also observed that patients with RA showed higher glycoprotein levels compared to patients with metabolic diseases[17–19]. GlycA and GlycB reflect the overall number of N-glycan side chains attached to acute phase reactant proteins, which are produced regardless of the underlying trigger (e.g., infection, metabolic stress). Our results suggest a more intense glycosylation response in RA compared to other conditions, including metabolic disorders that also involve subclinical inflammation. This intense inflammatory pattern observed in RA, partly attributed to the increased glycosylation response, may be a key factor linking the disease to extra-articular manifestations, such as interstitial lung disease and accelerated atherosclerosis[20]. In our study, we did not find differences between men and women in any group, suggesting that the subclinical inflammation exhibited by glycoproteins is sex independent in the three situations that we studied (RA, metabolic disorders, and healthiness).

Moreover, we investigated the associations between the advanced glycoprotein profile and previously identified CV disease and inflammation-associated miRNAs, yielding several noteworthy findings. Specifically, we identified significant associations between miRNAs 24, 451, and Let7a and the parameters of GlycA and GlycB, both in the overall RA cohort and among female participants. In this regard, decreased expression levels of miRNAs 24 and Let7a were associated with decreased levels of GlycA and GlycB parameters, and decreased levels of miRNA-451 levels were associated with increased levels of GlycA and GlycB. Notably, our research group had previously established associations between miRNAs 24 and Let7a and specific inflammatory markers in RA that align with those identified in this study[8]. Interestingly, decreased expression of these miRNAs was also associated with decreased odds of presenting carotid plaque presence[9]. Additionally, decreased expression levels of miRNA-451 had previously been linked to subclinical atherosclerosis in RA[10]. However, this study represents the first exploration of these specific associations in this context. Moreover, glycosylation is one of the major targets of miRNAs and are critical regulators of glycosylation in human cells[21,22]. In this context, it has been demonstrated that miRNA-24 directly targets O-GlcNAc transferase, down-regulating O-GlcNAcylation[23]. This down-regulation aligns with our results, as we have observed that a decrease in the expression of miRNA-24 is associated with more glycated proteins. A similar pattern was observed with miRNA-451, which acts as a regulator of the gene *PMM2*. Among its functions, *PMM2* regulates glycan synthesis. MiRNA-451 is indirectly involved in this process by suppressing the expression of *PMM2*[24]. The Let-7 family has also shown broad indirect involvement in the glycosylation process, targeting the *GALNT2* gene and the sialyltransferase family of enzymes, both of which are regulators of the mechanisms of glycosylation[25,26]. Given their multiple connections to inflammation and glycosylation and the associations we have uncovered between these miRNAs and GlycA, GlycB and GlycF, it is plausible to speculate that these miRNAs could be modulators of the inflammatory processes through the regulation of glycosylation in RA. Interestingly, we found that miRNA-146 was exclusively associated with H/W-GlycA in patients with metabolic disorders. This association could imply that miRNA-146 is involved in an inflammatory pathway not exclusively related to RA. In fact, the downregulation of circulatory miRNA-146 or miRNA-146 deficiency is associated with inflammatory disorders manifested in various organs such as the lungs, heart, brain, or skin, and underlies vascular or autoimmune diseases[27]. The observed associations were primarily identified in the RA group, suggesting that these miRNAs may play a role in stress

	β	*p*	R^2	ΔR^2	AIC
OVERALL RA COHORT (n = 219)					
DAS28-ESR					
Basal Model			12.66		720.59
Area GlycA	0.21	0.02	15.01	2.35	716.625
Area GlycB	0.32	< 0.001	18.34	5.68	707.88
H/W GlycA	0.33	< 0.001	18.69	6.03	706.94
H/W GlycB	0.33	< 0.001	18.58	5.92	707.24
DAS28-CRP					
Basal Model			14.74		603.57
Area GlycA	0.14	0.04	16.44	1.7	601.15
Area GlycB	0.16	0.01	17.13	2.39	599.35
H/W GlycA	0.20	0.002	18.45	3.71	595.81
H/W GlycB	0.17	0.01	17.33	2.59	598.82
ESR					
Basal Model			10.33		2026.52
Area GlycB	7.90	< 0.001	19.52	9.19	2004.85
H/W GlycA	6.65	< 0.001	16.81	6.48	2012.11
H/W GlycB	7.93	< 0.001	19.58	9.25	2004.67
CRP					
Basal Model			6.29		561.64
Area GlycA	0.13	0.02	8.51	2.22	558.38
Area GlycB	0.25	< 0.001	13.80	7.51	545.33
H/W GlycA	0.25	< 0.001	14.22	7.93	544.27
H/W GlycB	0.25	< 0.001	13.84	7.55	545.23
Fibrinogen					
Basal Model			6.43		2619.07
Area GlycA	15.01	0.02	8.72	2.29	2615.64
Area GlycB	31.69	< 0.001	16.73	10.3	2595.53
H/W GlycA	29.34	< 0.001	15.23	8.8	2599.45
H/W GlycB	31.70	< 0.001	16.75	10.32	2595.48
CDAI					
Basal Model			11.90		610.75
Area GlycA	0.13	0.04	13.56	1.66	608.59
Area GlycB	0.16	0.01	14.43	2.53	606.36
H/W GlycA	0.20	0.003	15.47	3.57	603.69
H/W GlycB	0.17	0.01	14.53	2.63	606.10
OVERALL METABOLIC DISORDER COHORT (n = 82)					
ESR					
Basal Model			27.72		220.10
Area GlycB	0.24	0.02	32.73	5.01	216.07
H/W GlycB	0.22	0.03	32.12	4.40	216.83
OVERALL CONTROL COHORT (n = 64)					
Fibrinogen					
Basal Model			7.98		168.76
Area GlycB	0.43	0.002	23.64	15.66	159.95
H/W GlycA	0.36	0.008	19.47	11.49	163.03
H/W GlycB	0.40	0.003	21.95	13.97	161.21

Table 3. Study of the associations between the different inflammatory parameters and the glycoprotein parameters analysed by NMR. Multivariate lineal models, adjusted for age, sex and BMI were used. Models with RA patients were also adjusted for RA treatment. DAS28 = disease activity score 28; ESR = erythrocyte sedimentation rate; CRP = C reactive protein; CDAI = clinical disease activity index. β = beta coefficient; p = p-value, AIC = Akaike information Criteria.

conditions, either metabolic or inflammatory, which are less prominent in normal physiological conditions. Our findings further support this notion, as there were minimal associations detected in control subjects.

Finally, we have also found several associations between the glycoprotein parameters and DAS28-ESR, DAS28-CRP, ESR, CRP and fibrinogen. These associations align with previously published evidence, which has already linked glycoproteins to inflammatory markers of RA and identified them as potential inflammatory pattern markers of RA[12,28]. Moreover, for the first time, we have also associated GlycA and GlycB parameters with CDAI, which is an indicator of severity of RA using only clinical data, indicating that glycoprotein levels are also related with clinical RA manifestations. In this sense, knowledge of more sensitive inflammation biomarkers could contribute to better management of RA. The current treatment approach aims to control disease activity, achieving remission or low activity to minimise disease-associated morbidity and mortality. Controlling clinical inflammation and inflammatory markers also helps reduce the risk of CV disease and other RA-related complications[20,29]. We also found some associations between glycoprotein parameters and certain inflammatory markers in subjects with metabolic disorders and in controls, indicating that glycoproteins capture different sources of subclinical inflammation.

Our study does have several limitations. Firstly, we cannot establish causality in any of the associations we found due to the cross-sectional design of our study. Therefore, longitudinal studies are needed to further investigate these relationships. Secondly, our cohort of patients with metabolic disorders, while exhibiting a similar inflammatory pattern, is relatively heterogeneous. Lastly, the associations we discovered do not fully elucidate the metabolic interactions between the studied miRNAs and the glycoprotein particles identified. Consequently, conducting experimental and in vitro studies is imperative to gain a deeper understanding of the relationships between these parameters.

In summary, our study presents a comprehensive comparison of advanced glycoprotein profiles across three distinct cohorts: patients with RA, controls, and individuals with metabolic disorders. Significantly, we identified marked differences among these groups. Moreover, we discovered intriguing associations between this advanced profile and several miRNAs in the group of patients with RA, suggesting that these miRNAs play a regulatory role in the glycosylation process, which is notably elevated in RA. Furthermore, our findings underscore the robust connection between glycoprotein variables and various inflammatory markers. Importantly, we established, for the first time, an association with CDAI, providing insights into a more clinically relevant state of inflammation in RA. These results enhance our understanding of metabolic alterations in RA patients and highlight potential therapeutic miRNAs.

Materials and methods

Patients and clinical variables

The RA cohort in this current research has been previously described[9,30] and is composed by individuals who visited University Hospital Sant Joan de Reus through external appointments and met the classification criteria for RA outlined by the American College of Rheumatology in 1987. Those who were over 80 years old or under 18 years old, individuals with acute concurrent illnesses, and those whose disease diagnosis had changed were excluded from the study. Recruitment of patients occurred between November 2011 and January 2015. A total of 219 patients aged between 18 and 80 years were enrolled in the investigation, and blood samples were collected on the same day as their medical appointments. Clinical evaluation of the patients has been previously described (Supplementary Data S1). As a measure of disease activity and inflammation, disease activity score (DAS28) was calculated according to the ESR and CRP (DAS28-ESR and DAS28-CRP) in RA patients. Clinical disease activity (CDAI) was also calculated in RA patients as a measure of the clinical manifestation of the disease.

In addition, we incorporated 82 patients who voluntarily took part in the study and were receiving care at the Vascular Medicine and Metabolism Unit of our hospital for issues related to lipid metabolism disruptions and related conditions, including obesity, type 2 diabetes mellitus (T2DM), and metabolic syndrome. Diagnosis of obesity, T2DM, and metabolic syndrome was based on established clinical criteria[31–33]. Furthermore, we enlisted 64 control subjects who were drawn from the hospital's personnel pool as individuals free from RA, T2DM, obesity, metabolic syndrome, or any other medical ailments.

The study was approved by The Clinical Research Ethics Committee of Hospital Sant Joan de Reus (patients with RA: 11-04-28/4proj5, controls and patients with metabolic disorders: CEIm: 222/2020) and all the participants gave written informed consent. We executed the investigation in accordance with our institution's guidelines and the Helsinki Declaration.

Laboratory measurements

We obtained blood samples from all participants, who had observed a fasting period of no less than 12 h. Plasma was separated from the whole blood through centrifugation at 3000 rpm for a duration of 10 min, and these plasma samples were subsequently preserved at -80 °C for further analysis. Analytical assessments were carried out using enzymatic and standard methods. These assessments encompassed the measurement of rheumatoid factor (RF), anti-citrullinated cyclic peptides (anti-CCP), and inflammatory markers (ESR, CRP, and fibrinogen) using conventional techniques.

Plasma miRNA expression

We examined a set of miRNAs, including Let7a, 24, 96, 103, 125a, 125b, 132, 146, 191, 223, 425, and 451, in separate plasma samples of the patients with RA, patients with metabolic disorders and controls. The selection of these miRNAs was based on their previous associations with various surrogate markers of atherosclerosis in both RA patients and the general population. Additionally, miRNAs 24, 146, and Let7a have also been linked to the inflammatory processes underlying RA. Prior to RNA extraction, we conducted a hemolysis assessment using 200 µl aliquots, with any hemolysis being identified and discarded through spectrophotometer analysis

at λ = 414 nm, which corresponds to oxyhemoglobin contamination. The comprehensive procedure for plasma miRNA extraction can be found in Supplemental Data S2. For normalization purposes, miRNA-16-5p was selected as the reference. The relative expression of each miRNA in each sample was calculated using the variable ΔCt, derived as Ct miRNA candidate—Ct miRNa-16-5p. An increase in the ΔCt value for a specific miRNA indicated a reduction in the expression of that miRNA.

2D-1 H-NMR glycoprotein profile

The glycoprotein profile was analysed in plasma samples using 1 H-NMR, following established protocols[34]. 200 µl of serum were mixed with 50 µl of deuterated water and 300 µl of a 50 mM phosphate buffer solution at pH 7.4. The 1 H-NMR spectra were recorded at 305.95 K using a Bruker Avance III 600 spectrometer operating at a proton frequency of 600.20 MHz (14.1 T). We focused on the spectral region where glycoproteins resonate (2.15–1.90 ppm) and employed various functions based on chemical shifts, including glycoprotein A, B and F (GlycA, GlycB and GlycF). For each function, we calculated the total area which represents the concentration based on the number of sugar–protein bonds. This allowed us to determine the concentrations of GlycA, GlycB and GlycF. Moreover, we also obtained another marker, which is the ratio between the height and weight of GlycA and GlycB (H/W-GlycA and H/W-GlycB). This function depends on the height of the glycoproteins, which is related to the concentration, and its width, which is related to the flexibility and the aggregation of the molecules generating the signal[35].

Statistical analysis

Mean and standard deviation (SD) is provided for normal variables, while median and interquartile range (IQR) is provided for nonnormal variables. Percentage and number of individuals is provided for categorical variables. T tests, Mann–Whitney U tests and chi-squared tests were used to evaluate differences between normal, nonnormal and categorical variables, respectively. We employed analysis of variance (ANOVA) to examine distinctions among patients with RA, patients with metabolic disorders and healthy subjects. Subsequently, when significant differences were identified, we conducted Tukey's post-hoc tests to determine which specific groups exhibited disparities. To evaluate miRNA associations with the different glycoprotein and lipoprotein subfractions, multivariate linear models were adjusted. All the models were adjusted for age, sex, body mass index (BMI), RA treatments and analysed in the overall cohort and stratified by sex. R^2, ΔR^2 and Akaike information Criteria (AIC) were provided for each model. The R-squared (R^2) statistic estimates the amount of variability explained by the model. ΔR^2 shows the increase in variability explained when the different miRNAs were included in the models. Finally, AIC estimates the quality of the model. A lower AIC value implies a better model quality. Statistical analyses were performed in R Studio, version 4.0.1. P-values < 0.05 were considered statistically significant.

Data availability

The datasets generated and/or analysed during the current study are not publicly available due to privacy policies but are available from the corresponding author on reasonable request.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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