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RESEARCH ARTICLE

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MicroRNA profiles in plasma samples from young metabolically healthy obese patients and miRNA-21 are associated with diastolic dysfunction via TGF-β1/Smad pathway

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Abstract

Background: Metabolically healthy obese patients accounts for a large part of obese population, but its clinical significance and cardiac dysfunction are often underestimated. The microRNA profiles of metabolically healthy obese patients were investigated in the study, and the selected microRNA (miRNA) based on our microarray assay will be further verified in a relatively large metabolically healthy obese population.

Methods: microRNA microarray was performed from six metabolically healthy obese and 6 health control blood samples. Based on the bioinformatics analysis, we further measured RT-PCR, fibrosis markers, echocardiograms, and TGF-β1/Smad signaling pathway in 600 metabolically healthy obese population.

Results: We found that miRNAs expression characteristics in metabolically healthy obese groups were markedly different from healthy control group. MiRNA-21 was significantly increased in the samples of metabolically healthy obese patients. Besides, miRNA-21 levels were associated with cardiac fibrosis marker. Meanwhile, higher miRNA-21 levels were related to elevated E/E′. Besides, patients with the highest miRNA-21 quartile showed the lowest ratio of E/A. These associations between miRNA-21 and diastolic function parameters were independent of obesity and other confounding variables. Of note, TGF-β1and Smad 3 were significantly upregulated while Smad 7 was downregulated according to the miRNA-21 quartiles in metabolically healthy obese group.

Conclusions: We demonstrated the profiles of circulating microRNAs in metabolically healthy obese patients. Increased plasma miRNA-21 levels were related to impaired diastolic function independent of other relevant confounding variables. MiRNA-21 could be one of the mechanistic links between obesity and diastolic dysfunction through regulating cardiac fibrosis via TGF-β1/Smad signaling pathway in obese hearts, which may serve as a novel target of disease intervention.

KEYWORDS

biomarker, diastolic dysfunction, metabolism, microRNAs, obesity

1 | INTRODUCTION

Obesity poses significant global public health concerns, being one of the leading risk factors for overall morbidity and mortality.[1] It is well known that obesity may lead to prominent cardiac structure and function alterations, even in the absence of obesity-related metabolic comorbidities such as diabetes mellitus, hypertension, and dyslipidemia.[2] Obese individuals without metabolic abnormalities have been regarded as "metabolically healthy obese," who account for a large part of obese population.[3] It is worth noting that there is increasing evidence that among obese individuals, increased mortality and cardiovascular risk independent of metabolic status challenge the view that metabolically healthy obese is a benign disease.[4-6] Findings from animal studies have revealed that different mechanisms, including increased adiponectin, decreased inflammatory pathway signaling, and mitochondrial transmembrane transport, were involved in the pathogenesis of metabolically healthy obese compared with metabolic unhealthy obese.[7,8] Therefore, in view of the magnitude and impact of the obesity epidemic, personalized prevention and treatment strategies should be noted. Although the interaction between obesity and LV diastolic dysfunction is well documented, little was known about diastolic dysfunction in metabolically healthy obese. As shown in the previous study, diastolic dysfunction was more frequent in high BMI metabolically healthy patients, suggesting that obesity per se, other than its related metabolic complications, has an effect on LV structure and function.[9]

In addition, previous studies have demonstrated a link between diastolic dysfunction and circulatory miRNAs in cardiomyopathy patients, which suggests the potential role of miRNAs as biomarkers of diastolic function.[10] MicroRNAs are small non-coding RNAs that regulate target genes expression. Dysregulation of miRNAs is involved in multiple pathological processes of various cardiovascular diseases. Therefore, we used miRNA microarray and bioinformatics analysis to explore the profile of miRNAs expression in metabolically healthy obese patients. Based on our result, we found a significant increase in miRNA-21 in metabolically healthy obese patients. The abnormal levels of miRNA-21 were closely related to cardiac hypertrophy, heart failure, and pulmonary artery remodeling.[11-13] The crucial regulatory role of miRNA-21 was confirmed in LV remodeling. Meanwhile, the circulating miRNA-21 levels are significantly increased in severe aortic valve stenosis patients, revealing remarkable myocardial fibrosis.[14] MiRNA-21 deficiency can facilitate inflammation and exacerbate cardiac systolic dysfunction post-MI through targeting KBTBD7.[15] Besides, it has been reported that miRNA-21 can regulate cardiac fibrosis through TGF-β1/Smad7/Smad3 signaling pathway.[16] Therefore, in this study, we investigated the correlation between circulating miRNA-21 levels with diastolic dysfunction in metabolically healthy obese patients.

2 | METHODS

2.1 | Subjects and study design

This single-center retrospective study was approved by our hospital ethics committee, and all study participants have signed informed consent. The miRNA profiles were detected by miRNA microarray on six healthy and six metabolically healthy obese patients. Bioinformatics analysis was further performed to discover the potential biomarker. In order to test and verify our results, 600 young metabolically healthy obese patients were enrolled. The study population consisted of 600 young (aged 18-40 years old) metabolically healthy obese who underwent echocardiography between 2015 and 2018. The patients were recruited from the consecutive series of patients undergoing regular medical examinations in the department of cardiology in our hospital. Individuals with diabetes mellitus, hypertension, hyperlipidemia, coronary artery disease, valvular or congenital heart disease, cardiomyopathy, atrial fibrillation, and heart failure were excluded. Furthermore, individuals with EF < 50%, extreme BMIs (>50) and a history of malignancy, liver, kidney, and autoimmune disease were excluded. Clinical information including age, gender, smoking, BMI, waist, Hip, and blood pressure and et.al was obtained by medical records at enrollment. Laboratory measurements including total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, creatinine, urea nitrogen, and high-sensitivity CRP were performed in our hospital. Venous blood samples were drawn into EDTA tubes after a 12-hour overnight fast using standardized methods. All assays were assessed in duplicate by investigators blinded to the characteristics and clinical outcome of patients.

2.2 | MiRNA microarray and bioinformatics analysis

microRNA profile was detected by Affymetrix GeneChip miRNA arrays according to the manufacturer's instructions. Affymetrix expression console software was used for the further data analysis. Significantly, changed miRNAs were defined as either downregulated or upregulated at least 1.5-fold and false discovery rate (FDR) <0.05. GO analysis based on the DAVID bioinformatics resources databases was performed to explore the molecular function, cellular component, and biological process.

2.3 | Echocardiography

Two-dimensional, M-mode and Doppler echocardiography were performed by two experienced cardiologists who were blinded to patients in a standard manner with the same equipment. LV dimensions including LV diameters and wall thickness of the interventricular septum and posterior wall were calculated from the parasternal long-axis views. LVEF was evaluated from the apical two- and four-chamber views. Peak early mitral velocity (E wave), peak late mitral velocity (A wave), E/A ratio, tissue Doppler peak early diastolic mitral annular velocity (E′), and E/E′ ratio were measured to evaluate LV diastolic function. All recordings were averaged at least three consecutive cardiac cycles.

2.4 | RT-PCR assay

Peripheral blood samples of the participants were collected and centrifuged for RNA extraction. Total RNA in the plasma was extracted

referring to the protocol provided by the manufacturer with TRIzol LS reagent (Thermo Fisher Scientific, Inc). RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc) was used to perform reverse transcription, and qPCR was done on an ABI Sequence Detection system (Thermo Fisher Scientific, Inc). The relative expression of miRNA was calculated by 2−ΔΔCt. The internal control in our study was U6.

2.5 | Elisa analysis

The plasma concentration of TGF-β1 (Abcam), Smad3, and Smad7 (Novus Biologicals) was detected by Elisa kit according to the manufacturer's instructions.

2.6 | Statistical analysis

Statistical analysis was performed using SPSS 19.0 (SPSS, Inc). The differences between groups were compared by one-way ANOVA. Circulating levels of miRNA-21 were divided into quartiles. Correlations between miRNA-21 and the variables were analyzed by partial correlation analysis or ANCOVA. *P* values < .05 were considered statistically significant.

3 | RESULTS

Clinical characteristics of six healthy volunteers and six metabolically healthy obese patients for miRNAs microarrays were shown in Table 1. Our results demonstrated that circulating microRNA profile of metabolically healthy obese was significantly differed from health volunteers. The top 10 miRNAs upregulated or downregulated were listed in Figure 1A. For further bioinformatics analysis, Gene ontology analysis was carried out, including biological process, molecular function, and cellular component (Figure 2A-C). Cell-cell adhesion and translational initiation were highest enriched in biological process, nucleoplasm and nucleus were highest enriched in cellular component, while protein binding and DNA binding were highest enriched in molecular function. To confirm the microarray results, the expression of the miRNAs was detected by RT-PCR. Of note, the expression of hsa-miR-21 increased most in metabolically healthy obese group than in the control group (Figure 1B). Previous study has evidenced that miR-21 was involved in cardiac remodeling through cardiac fibrosis. We further verified the relation between miRNA-21 and diastolic function in a larger population.

Baseline characteristics of the study population based on miRNA-21 levels are presented in Table 2. No difference was found in plasma total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, systolic and diastolic blood pressure, plasma creatinine, and urea nitrogen between miRNA-21 quartiles. The proportion of gender, BMI, waist and hip circumference, waist-hip ratio, high-sensitivity C-reactive protein (CRP), and smoking history were significantly different between miRNA-21 quartiles before adjustments. It is noteworthy that female subjects were more common in the highest miRNA-21 quartile.

TABLE 1 Clinical characteristics of patients for miRNA Microarray

	Control	Metabolically healthy obese	*P*
n	6	6	
Age (y)	33.8 (7.61)	34.2 (7.35)	N
Sex M/F (%)	50/50	50/50	N
BMI (kg/m2)	22.5 (1.89)	33.7 (1.01)	<0.05
Smoking (%)	33.3	33.3	N
Diabetes mellitus (%)	0	0	N
Hypertension (%)	0	0	N
Dyslipidemia (%)	0	0	N
BUN (mmol/L)	5.84 (4.37)	5.97 (3.32)	N
Plasma creatinine (μmol/L)	77.5 (11.80)	78.0 (17.89)	N

Note: Data are means (SD) or percentages.
Abbreviation: BMI, body mass index.

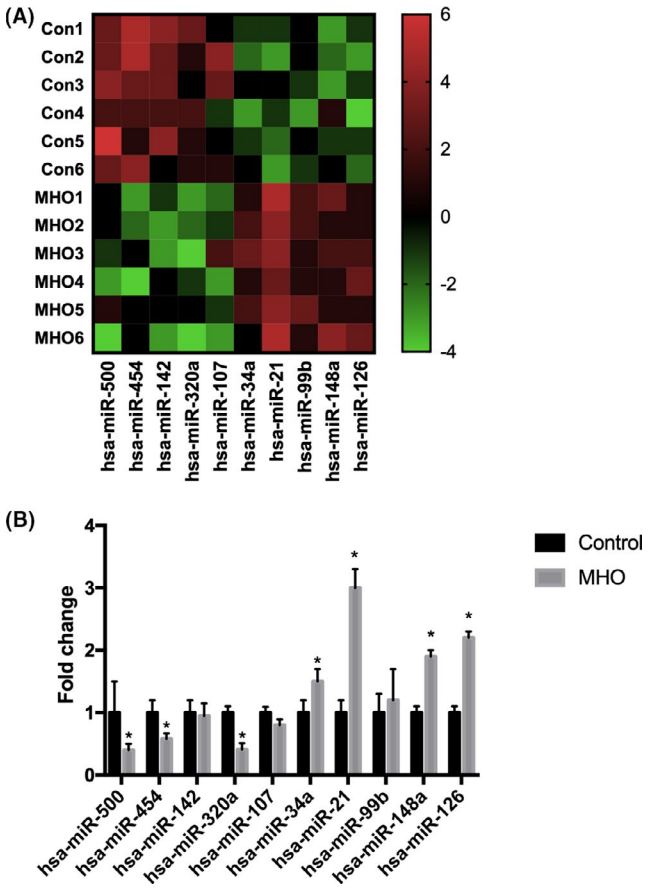

FIGURE 1 A, Heatmap of the differentially expressed circulating miRNAs in the plasma of metabolically healthy obese patients and healthy volunteers. B, The expression of selected miRNAs in the plasma of metabolically healthy obese patients and healthy volunteers. MHO, metabolically healthy obese. *P < .05

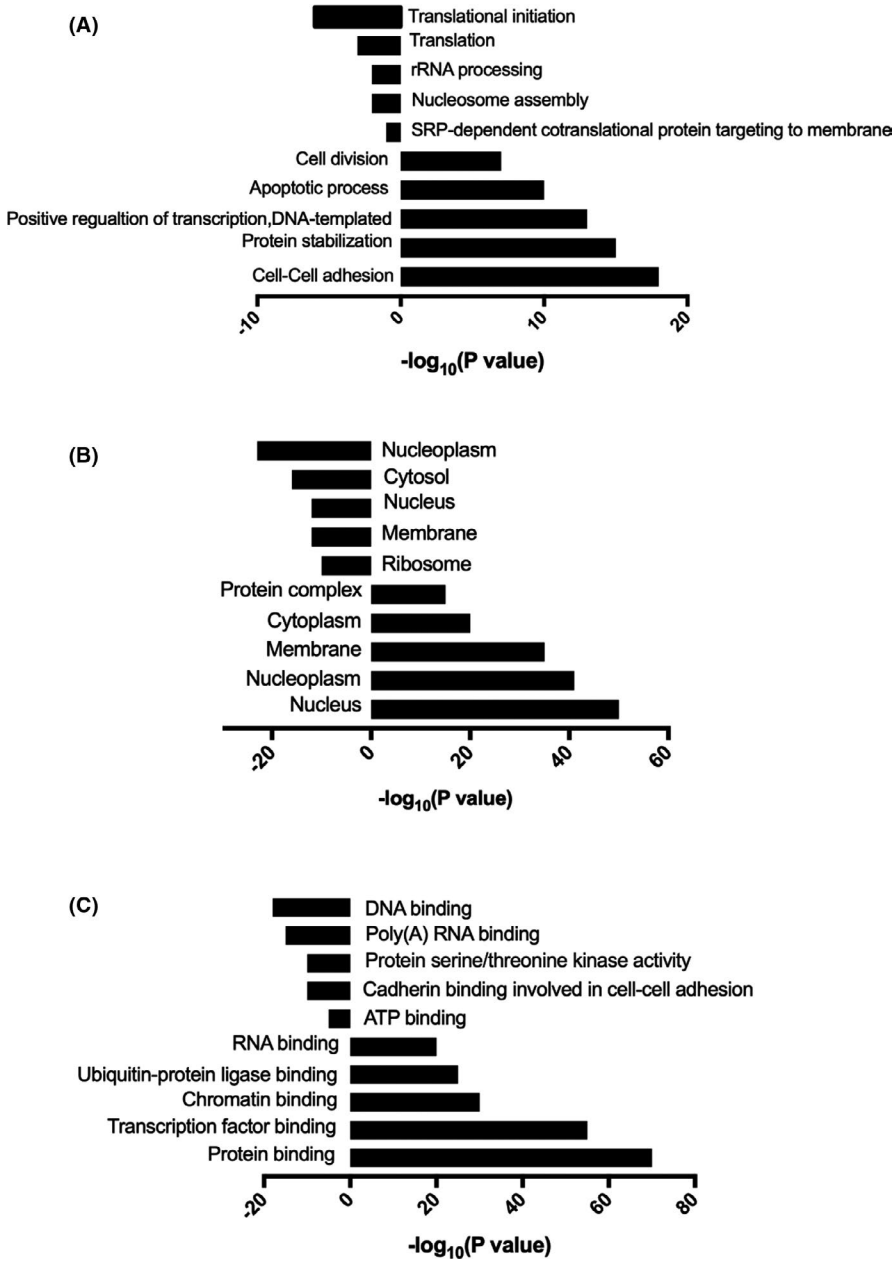

FIGURE 2 TOP changed functional annotations in metabolically healthy obese patients based on GO analysis: A: biological process, B: cellular component, and C: molecular function, ranked by enrichment score ($-\log_{10}$[*P* value])

Highest BMI, waist and hip circumference, waist-hip ratio, and high-sensitivity CRP were found in the individuals of highest miRNA-21 quartile compared with individuals with the lowest miRNA-21 quartile. After age and gender adjustment, the correlation between miRNA-21 and BMI, waist circumference, hip circumference, and waist-hip ratio was still persisted ($P < .05$ for all). However, no significant difference of high-sensitivity CRP or smoking history was observed between the miRNA-21 quartiles after adjustment for age, gender, and BMI.

Echocardiographic parameters of the subjects are demonstrated in Table 3. There was no significant difference in LV EF and LV dimensions evidenced by left ventricular end-diastolic diameter (LVEDD), end-systolic diameter (LVESD), and wall thickness between the miRNA-21 quartiles. However, diastolic dysfunction parameters illustrated by E′, E/E′, and E/A were significantly different between miRNA-21 quartiles. After adjustments for age, gender, BMI, waist circumference, hip circumference, waist-hip ratio, high-sensitivity CRP, and smoking history, significant differences among groups were still distinct for E′, E/E′, and E/A. The highest quartile of miRNA-21 displayed the highest ratio of E/E′ (Figure 3A). Accordingly, patients with the highest miRNA-21 quartile showed lowest ratio of E/A (Figure 3B). MiR-21 can activate fibrotic gene programme and accelerate cardiac fibrosis. Besides, it is well known that cardiac fibrosis is one of the leading cause of diastolic dysfunction. Therefore, fibrosis marker of a-SMA, CoL-I, and CoL-III was detected. Patients with the highest miRNA-21 quartile showed the highest increase in a-SMA, CoL-I, and CoL-III (Figure 4), which may reveal that the link between miRNA-21 and diastolic dysfunction may be partly mediated by cardiac fibrosis. It was confirmed in the previous study that miRNA-21 can

TABLE 2 Characteristics and general cardiovascular risk factors of the subjects according to the circulating level of miRNA-21

Level of miRNA-21quartile	1st	2nd	3rd	4th	*P**	*P***
miRNA-21 relative expression level	<4	4-8	8-12	>12		
n	150	157	161	132		
Age (y)	33.7 (7.61)	34.5 (7.72)	33.8 (7.35)	34.1 (7.16)	N	
Sex M/F (%)	70/30	68/32	64/36	42/58	<.05	
BMI (kg/m^2)	31.3 (1.89)	32.2 (2.01)	34.8 (1.57)	36.6 (1.35)	<.05	<.05
Waist (cm)	91 (7.81)	94 (7.89)	96 (6.97)	102 (7.32)	<.05	<.05
Hip (cm)	96 (7.33)	98 (7.46)	98 (7.78)	101 (8.32)	<.05	<.05
Waist-hip ratio	0.95 (0.06)	0.96 (0.07)	0.97 (0.1)	1.03 (0.06)	<.05	<.05
Smoking (%)	31.3	33.8	32.5	39	<.05	N
TC (mmol/L)	4.31 (0.85)	3.89 (0.82)	3.93 (0.78)	4.25 (0.90)	N	
TG (mmol/L)	1.47 (0.34)	1.48 (0.28)	1.50 (0.41)	1.46 (0.38)	N	
HDL-C (mmol/L)	1.36 (0.47)	1.32 (0.35)	1.37 (0.68)	1.34 (0.74)	N	
LDL-C (mmol/l)	2.95 (0.76)	3.01 (0.68)	2.91 (0.83)	2.97 (0.86)	N	
SBP (mm Hg)	135 (4.12)	137 (2.81)	134 (5.22)	136 (1.89)	N	
DBP (mmHg)	87 (2.89)	80 (5.36)	82 (4.89)	86 (3.11)	N	
BUN (mmol/L)	5.99 (4.37)	6.21 (3.68)	5.89 (3.32)	6.01 (2.79)	N	
Plasma creatinine (μmol/L)	78.5 (11.80)	78.2 (15.08)	77.4 (17.89)	77.9 (19.4)	N	
High-sensitivity CRP (mg/L)	0.87 (1.13)	1.25 (1.49)	2.56 (3.81)	4.89 (2.59)	<.05	N

Note: Data are means (SD) or percentages. *P* values obtained before (*) and after (**) adjustments.
Abbreviations: BMI, body mass index; BUN, blood urea nitrogen; CRP, C-reactive protein; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SBP, systolic blood pressure; TC, total cholesterol; TG, triglycerides.

regulate cardiac fibrosis through TGF-β1-Smad signaling pathway. We found that the plasma TGF-β1 and Smad 3 were significantly increased in metabolically healthy obese patients than in healthy controls, while Smad 7 was significantly decreased in metabolically healthy obese patients (Figure 5).

4 | DISCUSSION

In this study, we performed miRNAs microarray to demonstrate the significantly different profiles of circulating miRNAs between metabolically healthy obese patients and healthy individuals. Based on the informatics analysis, we found a close relationship between miRNA-21 levels and LV diastolic function in metabolically healthy obese patients with preserved ejection fraction. High circulating miRNA-21 levels were found correlated with impaired diastolic function as well as increased cardiac fibrosis markers via TGF-β1/Smad signaling pathway. To our knowledge, those results have not been reported in previous studies. The findings of this study supported the possibility that increased expression of circulating miRNA-21 could be a potential diagnostic marker for diastolic dysfunction in metabolically healthy obese population.

Obesity is widely recognized as a global health crisis, which is caused by the imbalance between energy intake and expenditure. It not only induces adipose tissue excessive accumulation, but also brings a variety of obesity-related metabolic dysfunction inducing diabetes, hypertension, and hyperlipidemia.[17] Obesity is associated with increased cardiovascular mortality, which has been conclusively confirmed by numerous large epidemiological studies, though the mechanism is far from clear.[2] Previous studies mainly focused on the cardiac dysfunction in obesity metabolically unhealthy. However, obesity metabolically healthy constitutes a large part of obesity, which is more frequent in the whole population. It is widely accepted that cardiac remodeling induced by obesity is far more than the consequence of obesity-related metabolic dysfunction, the process of systemic inflammation, cytokine secretion, adipose tissue modulation, etc.[18]

Some recent study showed that a positive correlation between BMI and increased risk of diastolic dysfunction.[9] In line with this study, we found that high BMI did increase the risk of diastolic dysfunction among metabolically healthy obese. Diastolic dysfunction refers to abnormity in ventricular relaxation, distensibility, or filling. It is implicated that anomalous mechanical properties, disturbance of energy metabolism, and stiffness changes of the left ventricle took part in its pathogenesis.[19-21] Indeed, in the light of accumulating evidence from previous studies, cardiac fibrosis has been hypothesized to be the most crucial component in the development of diastolic dysfunction.[22-24]

TABLE 3 Echocardiographic parameters of the subjects according to the circulating level of miRNA-21

Level of miRNA-21 quartile	1st	2nd	3rd	4th	*P**	*P***
miRNA-21 relative expression level	<4	4-8	8-12	>12		
n	150	157	161	132		
EF (%)	64.8 (7.5)	65.6 (8.5)	66 (8.5)	65.3 (9.3)	N	
LVEDD (mm)	47.1 (4.2)	47.8 (5.5)	47.5 (4.6)	48.1 (6.3)	N	
LVESD (mm)	28.5 (4.7)	28.8 (6.6)	29.5 (4.8)	29.7 (4.0)	N	
IVS (mm)	9.9 (1.7)	10.5 (1.5)	10.8 (1.8)	11.0 (2.1)	N	
LVPW (mm)	8.2 (1.5)	8.6 (2.3)	8.8 (2.7)	9.1 (1.8)	N	
E′ (m/s)	0.08 (0.01)	0.07 (0.02)	0.07 (0.01)	0.06 (0.01)	<.05	<.05
E/E′	8.69 (3.4)	9.75 (3.3)	9.94 (3.5)	11.54 (3.2)	<.05	<.05
E/A	1.31 (0.1)	1.15 (0.1)	1.05 (0.2)	1.01 (0.1)	<.05	<.05
IVRT (ms)	90 (11.8)	89 (8.1)	88 (10.9)	91 (9.2)	N	

Note: Data are means (SD). *P* values obtained before (*) and after (**) adjustments.

Abbreviations: E/A, ratio of peak early mitral velocity to peak late mitral velocity; E/E′, ratio of peak early diastolic mitral velocity to tissue Doppler-derived peak early diastolic mitral annular velocity; E′, tissue Doppler-derived peak early diastolic mitral annular velocity; EF, ejection fraction; IVRT, isovolumic relaxation time; IVS, Interventricular septal diameter; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LVPW, Left ventricular posterior wall diameter.

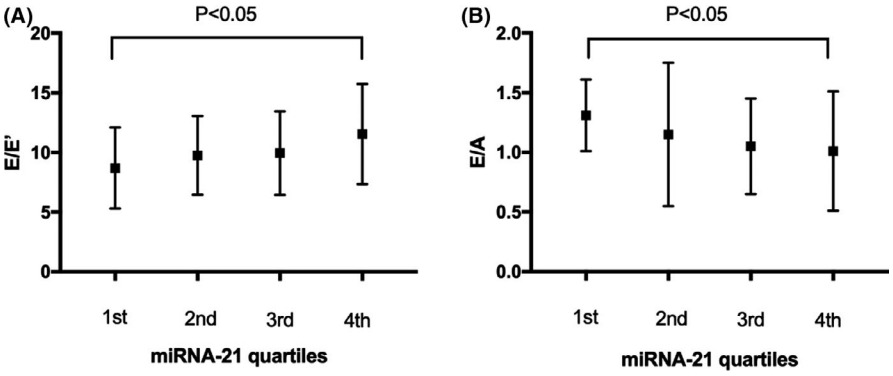

FIGURE 3 A, Ratio of peak early diastolic mitral velocity to tissue Doppler-derived peak early diastolic mitral annular velocity (E/E′) of the subjects (n = 600) according to the miRNA-21 quartiles. B, Ratio of peak early diastolic mitral velocity to late diastolic mitral velocity (E/A) of the subjects (n = 600) according to the miRNA-21 quartiles

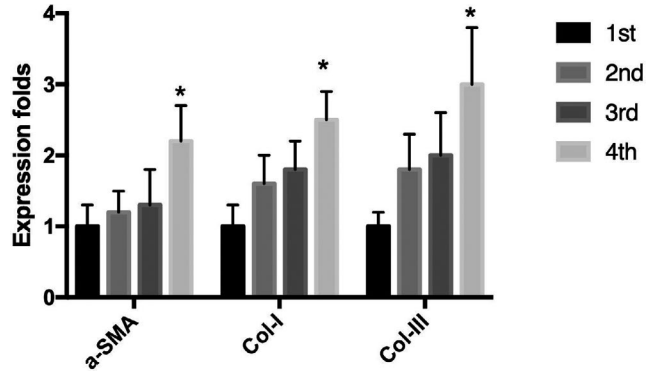

FIGURE 4 The mRNA expression of a-SMA, Col-I, and Col-III in the plasma of metabolically healthy obese patients according to the miRNA-21 quartiles. *$P < .05$

Numerous studies have shown that miRNAs were stable in circulation and could be reliably obtained from human plasma, suggesting that they may be served as a diagnostic tool for cardiovascular diseases.[25-27] A small cohort study revealed that the circulating miRNA levels of miR-1246 and miR-124-5p could be used to assess diastolic dysfunction in patients with dilated cardiomyopathy.[10] MiRNA-21 is located on chromosome 17q23.2 and highly and constantly expressed in vascular smooth muscle cells, cardiocytes, and cardiac fibroblasts. It was shown to play an important role in a variety of cardiovascular diseases.[28-30] The aberrant expression of miRNA-21 is involved in the development of myocardial infarction, heart failure, and pulmonary hypertension.[12,13,28] Besides, in patients with aortic valve stenosis, it was confirmed that miRNA-21 levels were related to myocardial fibrosis as well as regional and global LV strain impairment.[14] Previous findings highlighted the fibrogenic mechanism of miRNA-21 in the heart, including the activation of TGF β 1-smad7-smad3 pathway.[16] In this regard, inhibition of miR-21 may be a promising strategy to target cardiac fibrosis development. Of note, obesity could induce significant myocardial fibrosis, which would lead to diastolic dysfunction and heart failure.[31] In our study, miRNA-21 was closely associated with diastolic dysfunction and increased fibrosis markers. Therefore,

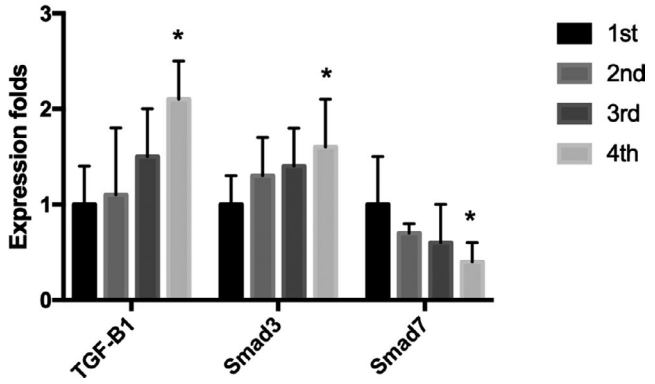

FIGURE 5 The concentration of TGF-β1, Smad3, and Smad7 in the plasma of metabolically healthy obese patients according to the miRNA-21 quartiles. *$P < .05$

it could be hypothesized that miRNA-21 is a mechanistic link between metabolically healthy obese and diastolic dysfunction mediated by cardiac fibrosis.

4.1 | Limitations of the Study

Small sample size and lack of follow-up are the main limitations of this study. Besides, the evaluation of LV fibrosis from MRI or endo-myocardial biopsy is also lacking. Although assaying circulating miRNA-21 is meaningful, it should be verified carefully in larger cohort studies to clarify its diagnostic and prognostic role in clinical settings.

5 | CONCLUSIONS

We found that the level of circulating miRNA-21 levels was associated with impaired LV diastolic function in metabolically healthy obese, but independently of common metabolic syndromes and other variables. MiRNA-21 connects obesity with diastolic dysfunction possibly through its profibrotic effects in the heart. The present findings can help deepen the understanding of obesity-related cardiac dysfuction. Furthermore, specific miRNA-21 antagonist may be of great clinical significance for reducing diastolic dysfunction in obese population.

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