Molecular Biology Reports (2021) 48:3503–3513
https://doi.org/10.1007/s11033-021-06352-7

ORIGINAL ARTICLE

Identification of miR-17, miR-21, miR-27a, miR-106b and miR-222 as endoplasmic reticulum stress-related potential biomarkers in circulation of patients with atherosclerosis

Pelin Telkoparan-Akillilar[1] · **Dilek Cevik**[1]

Received: 20 January 2021 / Accepted: 12 April 2021 / Published online: 15 April 2021

Abstract

Atherosclerosis and related cardiovascular diseases are among the most common causes of death worldwide. Unfolded protein response, also known as Endoplasmic reticulum stress, has a critical role in many diseases including atherosclerosis. Small non-coding microRNAs (miRNA), which generally suppress gene expression, regulate UPR signalling and they may also be involved in the progression of atherosclerosis. We aim to investigate the expression levels of miR-17, miR-21, miR-27a, miR-106b, miR-222 and CHOP gene in circulation of atherosclerosis patients compared to healthy controls to establish a link between ER stress and atherosclerosis. miRNA containing whole RNA was isolated from blood samples of 25 patients with atherosclerosis and 26 healthy controls. Expression levels of miRNAs and CHOP were measured via Real Time PCR method. miR-17 and miR-106b were significantly increased while miR-21, miR-27a, and miR-222 were significantly decreased in patients compared to controls. CHOP gene was also dramatically and significantly induced in patient samples. miR-17, miR-21, miR-27a, miR-106b, miR-222 and CHOP were significantly differentially expressed in patients with atherosclerosis. Each miRNA and CHOP might regulate atherosclerotic plaque progression and they can be used as a biomarker in the diagnosis and follow-up of atherosclerosis-related cardiovascular diseases.

Keywords ER-Stress · Atherosclerosis · CHOP · MicroRNA · MiR-17 · MiR-21

Abbreviations

ATF6	Activating transcription factor 6
CAD	Coronary artery disease
CHOP	CCAAT-enhancer-binding protein homologous protein
CVDs	Cardiovascular diseases
ER	Endoplasmic reticulum
EPCs	Endothelial progenitor cells
HMGCR	Hydroxy-3-methylglutaryl- coenzyme A reductase gene
IRE1	Inositol-requiring enzyme 1
IMA	Internal mammary arteries
LPL	Lipoprotein lipase
LDL-C	Low-density lipoprotein cholesterol
LDL-R	Low-density lipoprotein receptor
miRNAs	MicroRNAs
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
UPR	Unfolded protein response
UA	Unstable angina

✉ Pelin Telkoparan-Akillilar
pelintelkoparan@gmail.com

[1] Department of Medical Biology, Faculty of Medicine, Yuksek Ihtisas University, Ankara, Turkey

Background

Cardiovascular diseases (CVDs) are the most important causes of morbidity and mortality worldwide [1]. Atherosclerosis remains one of the most attractive areas of cardiovascular research. Atherosclerotic vascular disease is a chronic and progressive pathological condition that is characterized by lipid accumulation and inflammation in the arterial walls, leading to the development of cardiovascular diseases [2]. Atherosclerotic lesion initiation and progression occur with the involvement of vascular endothelium, inflammatory cells (especially macrophages), smooth muscle cells, some growth factors and cytokines. These cells and molecules contribute to the progression of the disease and ultimately to the formation of thrombosis [2, 3]. Atherosclerosis eventually results in life threating health problems

including heart attack and stroke. For this reason, developing treatment strategies by discovering novel biomarkers and molecular targets is an urgent need to reduce atherosclerosis-related mortality rates globally.

Endoplasmic reticulum (ER) is an organelle that functions in protein synthesis and folding, calcium homeostasis and lipid metabolism [4]. Several pathological and physiological factors including hypoxia, ischemia, inflammation and oxidative stress can interfere with the homeostatic function of ER and lead to the accumulation of unfolded proteins within the cell, which is called ER stress [5]. ER stress then activates the cell's adaptive mechanism known as unfolded protein response (UPR) [6]. Then, UPR tries to restore cellular homeostasis by activating three ER-localized stress sensory proteins; namely inositol-requiring enzyme 1 (IRE1), protein kinase RNA-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6) [6]. Recent studies suggest that ER stress plays an important role in atherosclerosis and atherosclerotic plaque rupture [7, 8]. ER stress response also contributes to the pathogenesis of other cardiovascular diseases [7, 9–11]. In addition, it was shown that the number of apoptotic macrophages is increased in advanced atherosclerotic lesions. In these macrophages, ER Stress-C/EBP homologous protein (CHOP) mediated apoptosis was implicated to have a role in the instability of atherosclerotic plaques [12, 13]. CHOP is also known as DDIT3 (DNA damage-inducible transcript 3) and it is encoded by the gene *DDIT3* [14].

MicroRNAs (miRNAs) are non-coding single-stranded RNAs of approximately 22 nucleotides long, derived from pre-miRNAs (precursors) of 75–80 nucleotides containing the hairpin structure [15]. miRNAs suppress transcription or translation by binding to their target genes' mRNAs at the 3 ′ UTR regions [16]. Recently, miRNAs that generally suppress gene expression emerged as key regulators of ER homeostasis and important players in UPR-dependent signalling [17, 18]. Furthermore, miRNAs that can be detected in circulating blood may be useful as biomarkers for the detection of certain diseases, including atherosclerosis [19]. The importance of miRNAs in the treatment, prognosis and diagnosis of cardiovascular diseases has been extensively reported [15, 20]. Studies have shown that some of these ER stress related miRNAs are important for myocardial function and their expression changes in many cardiovascular diseases [21, 22]. Under ER stress conditions, IRE1's RNase domain is activated, then it cleaves miR-17-5p and down-regulates its expression [23]. Besides, PERK/ATF4 arm of UPR reduces the expression level of miR-106b and induces intracellular apoptotic response via pro-apoptotic BCL-2 family members [24]. In another study, miR-27a was suggested to induce apoptosis by increasing CHOP's level [25]. Furthermore, under ER stress conditions, miR-222 is down regulated by a CHOP dependent mechanism preventing cells from ER stress related apoptosis in HCC cells [26]. The expression level of miR-222 was also investigated in atherosclerotic plaques, but no significant difference was found between patients and healthy controls [19].

In this study we investigated the expression levels of miR-17, miR-21, miR-27a, miR-106b and miR-222 in the peripheral blood of atherosclerosis patients and healthy controls together with an ER stress related gene, CHOP mRNA level to establish a link between ER Stress and atherosclerosis. By this way, we will be able to detect novel biomarker candidates as circulating miRNAs that can be used both in the diagnosis and treatment phases of atherosclerosis.

Methods

Sample collection and storage

The Yuksek Ihtisas Hospital Ethics Committee (File number: 2018-8663) approved the current study under the principles outlined in the Declaration of Helsinki and written informed consent was signed by all participants. Human peripheral blood samples were obtained from 25 patients with stable angina who underwent coronary angiography and had an operation decision by joint decision of Cardiology and Cardiovascular Surgery Clinics according to angiographic evaluation. The patient group was selected for their severe stenosis in at least one of the major coronary vessels that was evidenced by at least 70% of clogged according to their angiography results. In order to compare the expression levels of selected miRNAs in the circulation of patients and controls, peripheral blood samples were taken from 26 healthy individuals without active infection and history of cardiovascular disease belonging to similar age was used as controls. The flow chart describing patient and control samples is displayed in Fig. 1.

Whole blood samples were taken into 10-mL EDTA K2 tubes (BD Vacutainer, Cat No: 367525). To separate plasma from whole blood, samples were immediately centrifuged at 1900 g for 10 min, supernatant was translated to a microcentrifuge tube followed by a second centrifugation for another 10 min at 10.000 g at 4 °C. Isolated plasma samples were transferred into RNase free tubes and stored at − 80 °C for further experiments (Fig. 1).

miRNA isolation and real-time PCR

Once the number of samples required for the study was reached, frozen plasma samples were allowed to stand at room temperature until thawed. Approximately 200µL of plasma sample was used for miRNA isolation. miRNA-containing total RNA was isolated with miRNeasy Serum/Plasma kit (Qiagen, Cat No: 217184) following the

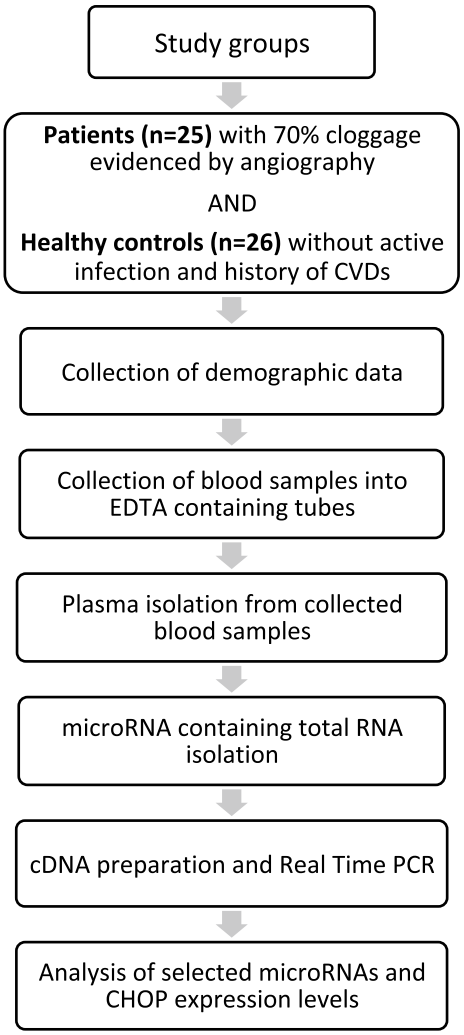

Fig. 1 Patient flow diagram starting from blood samples collection to data analysis

manufacturer's instructions. Then, cDNA synthesis was performed in accordance with the miScript RT kit's protocol (Qiagen Cat No: 218161) and cDNAs were stored at − 20 °C until using. Real-time PCR was performed with Rotor-Gene Q5plex HRM Instrument (Qiagen) by using the miScript SYBR Green PCR Kit (Qiagen Cat No: 218073) for microRNAs and Syber Green (Roche) for mRNAs.

After extensive literature search, we selected several miRNAs to be enrolled in this study. miR-21, miR-17-5p, miR-106b, miR-27a and miR-222 were analysed in atherosclerosis patients since they were already implicated to be associated to ER Stress by different researchers [23–26]. miScript Primer assays specific for miR-17, miR-21, miR-27a, miR-106b and miR-222 (MS00029274, MS00009079, MS00003241, MS00003402, MS00007609) and SNORD44 primer assay (MS00007518) were purchased from Qiagen. Human GAPDH and SNORD44 were used as internal controls. Real Time PCR reaction conditions are: 94 °C for 15 min, 45 cycles at 94 °C for 15 s, 55 °C for 30 s and 70 °C 30 s. mRNA primer sequences were taken from the literature and the primer sequences are as following;

GAPDH [7]**:** Forward 5′GACCACAGTCCATGCCATCACT-3′,

Reverse 5′TCCACCACCCTGTTGCTGTAG-3′;

CHOP [27]: Forward 5′-TTCTCTGGCTTGGCTGACTG-3′,

Reverse 5′-CTGCGTATGTGGGATTGAGG-3′.

Statistical analysis

The quantification of microRNAs was performed with the ΔΔCt (Ct: threshold cycle) method by using Qiagen miScript miRNA PCR Arrays & Assays Data Analysis Tool of the manufacturer's website (https://dataanalysis.qiagen.com/mirna/arrayanalysis.php). Internal spike-in control was used for normalization in miRNA experiments. miRNA expression analysis was first performed with patient group vs control group and then, repeated for hypercholesterolemia subgroup vs control group, and diabetes subgroup vs control group. mRNA expression analysis was performed with the ΔΔCt method as previously described [28]. GAPDH was used for normalization of mRNAs. In order to compare statistical differences among clinical sample groups according to their demographic characteristics, we used Vassar Statistics Tool (http://vassarstats.net) available online. Student's *t*-test was used for continuous variables for group-wise comparisons. For categorical variables, Fisher's exact test was used. All tests were 2-sided. A significance level of $P < 0.05$ was considered to be statistically significant.

To analyse mRNA expression levels by calculating the differences between groups, results were analysed with student's *t*-test. $P < 0.05$ was considered to be significant.

Bioinformatic analysis of miRNAs

Differentially expressed miRNAs were analysed using the mirPath V.3 online tool which is available at DIANA-Tools (www.microrna.gr). This tool performs an enrichment analysis of miRNAs and matches them to the signaling pathways in the KEGG database (Kyoto Encyclopedia of Genes and Genomes) (http://www.genome.jp/kegg/pathway.html) to determine biological pathways regulated by the expression of miRNA queries [29]. The analysis for the prediction of binding sites between miR-222 within the 3′UTR of CHOP (DDIT3) mRNA was performed using TargetScan database available online at (http://www.targetscan.org/) [30].

Results

Demographic and clinical parameters of blood samples

51 peripheral blood samples (25 cases with atherosclerosis and 26 healthy controls) were collected for this study. Clinical and demographic characteristics including age, gender, LDL cholesterol, Triglyceride, Hypercholesterolemia, diabetes mellitus, urea, AST, ALT, histories of smoking, family history of CAD were recorded (Table 1). The mean age of patients was 63,2 ± 8,8 and the mean age of controls was 54,69 ± 8,2. No significant difference was found among groups in any of the laboratory and demographic analyses except gender. Gender was a significantly different variable between cases and controls $p < 0.05$, and this is caused by the high number of available male patient samples compared to a very low number of female cases during the sample collection phase of the study.

Table 1 Demographic and clinical characterization of blood samples

	Cases (n = 25)	Control(n = 26)	*p* value
Age (mean ± SD)	63.2 ± 8.8	54.69 ± 8.2	0.08
LDL cholesterol (mean ± SD)	120.6 ± 49.5	116.46 ± 26.8	0,35
Triglyceride (mean ± SD)	157.4 ± 96.4	144.69 ± 44.8	0.89
Gender			
Female	2	9	**0.04**
Male	23	17	
Hypercholesterolemia			
Absent	18	16	0.55
Present	7	10	
Diabetes mellitus			
Absent	9	13	0.40
Present	16	13	
Urea (mg/dL)	39.88 ± 11.04	27.95 ± 13.0	0.47
AST (IU/L)	24.56 ± 12.46	23.88 ± 11.1	0.09
ALT (IU/L)	23.29 ± 12.45	27.07 ± 13.5	0.81
Smoker			
Absent	8	10	0.77
Present	17	16	
Family history of CAD			
Absent	11	7	0.24
Present	14	19	

microRNA quantification

In the present study, we compared expression levels of miR-21, miR-17-5p, miR-106b, miR-27a and miR-222 between patients with overt atherosclerosis and healthy controls in their peripheral blood samples and found that these miRNAs were differentially expressed in patients (Fig. 2). The expression level of miR-17 was significantly increased by 7.78-fold in the blood of patients with atherosclerosis compared to the control samples (Table 2).

When we group the patients according to the presence of Hypercholesterolemia or Diabetes, miR-17 was again significantly elevated more than eightfold in both hypercholesteremia and diabetes subgroups (Table 2).

The other miRNA whose expression is elevated in patient samples is miR-106b (Table 2). We detected miR-106b level to be up-regulated significantly, with a fold change of 3.29 in patients. Additionally, miR-106b expression level is significantly different in patients' subgroups with hypercholesterolemia or diabetics (Table 2).

Apart from up-regulated microRNAs, we spotted miR-21, miR-27 and miR-222 to be significantly down-regulated in patients compared to controls, $p < 0.05$ and fold changes were 0.276, 0.403 and 0.245 respectively (Table 3). When

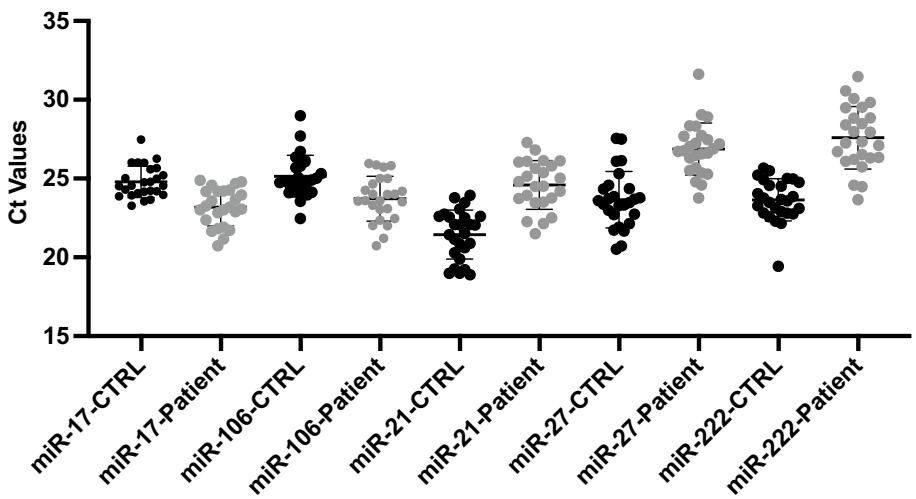

Fig. 2 Scatter plots of miRNAs in patients' blood samples vs healthy controls. This figure shows the Ct (threshold cycle) values of miR-17, miR-106b, miR-21, miR-27 and miR-222 that were analysed via qRT-PCR. Error bars indicate standard deviation. The mean Ct values between patients and controls are statistically significant for each miRNA ($P \leq 0.05$)

Table 2 Fold changes, OR, *p* values and Standard deviations of up-regulated miRNAs depending on demographic parameters

Up-regulated miRNAs	hsa-miR-17-5p				hsa-miR-106b-5p			
Patients with atherosclerosis vs healthy controls	Fold Change	OR (95% CI)	*p* value	Standard deviation	Fold Change	OR (95% CI)	*p* value	Standard deviation
Total (n = 25)	7.778	(4.38, 11.17)	**0.000001**	1.04	3.293	(2.15, 4.44)	**0.000429**	1.12
Hypercholesterolemia (n = 7)	8.088	(3.78, 12.40)	**0.000001**	0.80	2.771	(1.63, 3.91)	**0.000E + 00**	0.71
Diabetes (n = 16)	8.067	(4.16, 11.98)	**0.000002**	1.03	2.970	(1.77, 4.17)	**0.000007**	1.07

Hypercholesterolemia was defined as LDL cholesterol > 130 mg/dl or total cholesterol > 200 mg/dl. *p* values are significant for all groups.

subgrouped patients are considered, miR-21 was significantly down regulated in diabetes group but not the hypercholesterolemia group. miR-27 level was also decreased significantly in diabetes group but not the hypercholesterolemia group. On the other hand, miR-222 was significantly down regulated in both hypercholesterolemia and diabetes group (Table 3).

CHOP mRNA quantification and prediction of binding miR-222 to 3′ UTR of CHOP gene DDIT3

Together with ER stress related miRNAs, we also checked the expression of ER stress effector CHOP in our samples in order to establish a link between atherosclerosis and ER stress. CHOP is a transcription factor that is normally found at low levels, and its expression is triggered in response to ER stress. We observed an enormous increase in the CHOP expression, corresponding to 20-fold significant up-regulation in patient samples compared to the controls (Fig. 3). Once we detected a critical increase in CHOP mRNA level, we further investigated the connection between differentially expressed miRNAs and CHOP (DDIT3). TargetScan analysis revealed that there is a predicted binding site between miR-222-5p and 3′-UTR of DDIT3 mRNA (Fig. 4). Since miR-222 was downregulated in our patient group, it is expected to see an increase in CHOP levels in response.

Identification of pathways involved with differentially expressed miRNAs

Next, we analyzed differentially expressed miRNAs, namely miR-17, miR-21, miR-27a, miR-106b, miR-222 by using the mirPath V.3 tool of DIANA-Tools (www.microrna.gr) to determine the biological pathways enriched in KEGG pathways in relation with one or more miRNAs. As a result, 78 pathways came up with our query, each with a different p value and number of genes involved. For the ease of track, we represent these pathways in Fig. 5 as a heatmap, instead of giving it as a list (Fig. 5). Among enriched pathways, four pathways with very significant p values, Protein processing in endoplasmic reticulum, Cell cycle, p53 signaling pathway and Sphingolipid signaling pathway appeared to be very relevant to our study. Moreover, these pathways were associated by at least three miRNAs and the number of genes targeted in these pathways in relation with specific miRNAs were very high changing between 36 and 76 (Table 4).

Discussion

In this current study, we observed that expression levels of miR-17 and miR-106b and CHOP mRNA significantly increased; while miR-21, miR-27a, and miR-222 significantly diminished in circulation of patients with atherosclerosis compared to control group.

We observed more than seven fold significant upregulation of miR-17 expression in blood samples of patients versus controls. Additionally, miR-17 expression was also 8 times more in both hypercholesterolemia and diabetic patients' subgroups (Table 2). The relation of miR-17 with atherosclerosis was suggested in both animal and clinical studies [31, 32]. Hypercholesterolemia augments the risk of cardiovascular diseases by causing a significant increase in low-density lipoprotein cholesterol (LDL-C) levels. Circulating LDL particles are engulfed in the cells via the LDL receptor (LDL-R) [33]. This in turn results in LDL-C accumulation and triggers plaque progression. Furthermore, depletion of miRNA-17 expression was shown to alleviate atherosclerotic lesions in Apolipoprotein E Knockout ($Apoe^{-/-}$) Mice. In another study performed in Chinese population with overt atherosclerosis, miR-17 was found to have a role in initiation and development of coronary atherosclerosis [34]. Together with previous studies, our findings indicate that miR-17 may function as a useful biomarker with high sensitivity and specificity to follow development and progression of atherosclerosis.

We detected miR106-b to be elevated in patients with atherosclerosis with a rate of more than threefold up-regulation. miR-106b level was significantly higher in patients with hypercholesterolemia or diabetics (Table 2). In a previous study, it was shown that the expression of miR-106b in CAD patients' plasma is not expressed differently in patients versus healthy controls [35, 36]. Increased miR-106b gene

Table 3 Fold changes, OR, *p* values and Standard deviations of downregulated miRNAs depending on demographic parameters

Down-regulated miRNAs	hsa-miR-21-5p				hsa-miR-27a-3p				hsa-miR-222-3p			
Patients with atherosclerosis vs healthy controls	Fold Change	OR (95% CI)	*p* value	Standard deviation	Fold Change	OR (95%CI)	*p* value	Standard deviation	Fold Change	OR (95% CI)	*p*-value	Standard deviation
Total (n = 25)	0.276	(0.01, 0.54)	**0.0084**	1.71	0.403	(0.23, 0.57)	**0.00009**	1.01	0.245	(0.13, 0.36)	**0.00043**	1.214
Hypercholesterolemia (n = 7)	0.591	(0.22, 0.97)	0.1743	0.96	0.497	(0.19, 0.81)	0.0698	1.00	0.202	(0.07, 0.34)	**0.0179**	1.21
Diabetes (n = 16)	0.176	(0.00001, 0.41)	**0.0123**	1.71	0.428	(0.23, 0.62)	**0.0019**	0.90	0.264	(0.14, 0.38)	**0.0021**	1.04

Hypercholesterolemia was defined as LDL cholesterol > 130 mg/dl or total cholesterol > 200 mg/dl. Significant *p* values are shown in bold

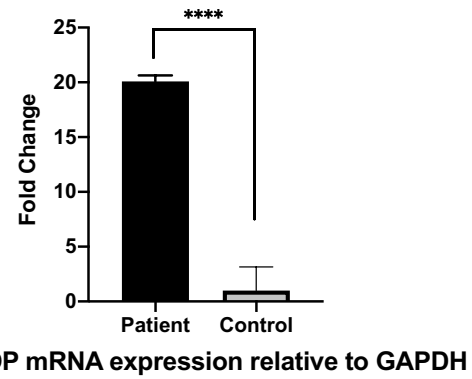

Fig. 3 Relative expression of ER stress indicator CHOP in patients blood samples vs healthy controls. This figure demonstrates the fold change between patients and control samples. qRT-PCR data was analysed by ΔΔCt method. CHOP mRNA level is significantly increased in patient samples ($P \leq 0.0001$)

expression was detected in microparticles that were isolated from patients with unstable angina (UA) [37]. When researchers performed microarray analysis from blood of three patients with UA and three healthy controls and increased levels of miR-106b and miR-17 were observed in patient samples. However, they could not validate the upregulation of these miRNAs in a larger study group performed with 67 patients with CAD and 67 healthy controls [38]. Our study group is composed of patients with stable angina and we compared them with healthy controls. In a more detailed future study, we could test the expression levels of these miRNAs by including three groups being the patients with acute coronary syndrome, the patients with stable angina and a healthy control group to see if we could discriminate these patients from each other by the levels of these molecular markers to support the personalized clinical practice in the upcoming years.

We found that miR-222, miR-21, and miR-27 were significantly down-regulated in patients with atherosclerosis as compared to healthy controls (Table 3). We detected expression of miR-222 to be reduced significantly, around 4-times in patients compared to controls. Deregulation of miR-222 has been implicated formerly in various cardiovascular diseases. In one study investigating expression of miR-222 in coronary artery atherosclerotic plaques (CAAP) versus internal mammary arteries (IMA) samples and blood samples of the patients, no significant difference was found between the groups [19]. During atherosclerotic progression, proliferation of epithelial cells are increased, miR-222 expression was decreased in lesion area, thus miR-222 was shown to have a role in regulation of this progression [39, 40]. In a following study, miR-222 expression was examined in Endothelial progenitor cells (EPCs) obtained from patients (CAD) under lipid lowering therapy and miR-222 was observed to be significantly decreased [41]. Together

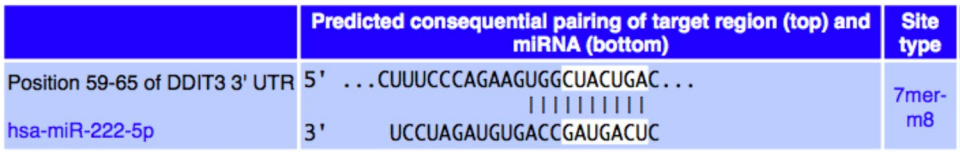

Fig. 4 In silico analysis of miR-222-5p binding to CHOP gene *DDIT3*. The predicted binding site between miR-222-5p and the 3′-UTR sequences of *DDIT3* mRNA was detected by TargetScan analysis

with the studies on the role of miR-222 endothelial dysfunction, our findings indicate that miR-222 may have potential to be used as a biomarker in the follow-up and treatment of atherosclerosis.

In the present study, miR-21 diminished 3.5 times in blood samples of patients compared to healthy controls. Furthermore, miR-21 was also found more than fivefold down-regulated in Diabetics patients.

There is a recent report supporting our findings by showing that depletion of miR-21 expression level in macrophages triggered atherosclerosis plaque progression [42]. miR-21 level was also found significantly down regulated in micro-dissected fibrous caps of ruptured plaques in animal models [43]. However, there are a number of studies reporting controversial. For example; the expression level of miR-21 was found 4-times up-regulated in atherosclerotic plaques samples taken from 50 patients in comparison to healthy controls [44]. In another investigation, plasma miR-21 level was found to be elevated in subclinical atherosclerosis in patients with hypertension when compared to the healthy controls [45]. Since miRNAs observed in plasma are not only derived from circulating blood cells but also from other tissues [46], the research results on different populations may be affected by various intrinsic and extrinsic factors, which may result in variations observed in the level of mir-21 by different scientists. Therefore, the potential of miR-21 to be used, as a biomarker for atherosclerosis should be further investigated in more detail with increased sample sizes.

We discovered that miR-27 was significantly reduced 2.5-fold in patients. miR-27 was also significantly down-regulated approximately twofold in diabetes subgroup, but not in Hypercholesterolemia group. There are critical findings regarding the role of miR-27a on atherosclerosis that support our results. To begin with, in high cholesterol diet-fed Apoe$^{-/-}$ mice, miR-27a was detected as a critical regulator of cholesterol homeostasis weakening lipid accumulation on atherosclerotic lesions [47]. Additionally, the investigation of the fate of atherosclerotic lesions in ApoE$^{-/-}$ mice by treating the mice with miR-27a/b agomir (mimic) or miR-27a/b antagomir (silencer) led to the observation that the number and size of plaques were decreased when miR-27a/b mimic was added while silencing of miR-27a/b antagomir treatment caused the advancement of atherosclerosis [48]. Furthermore, miR-27a plasma level was compared in heart failure patients with or without atherosclerosis and there was an inverse correlation between miRNA level and plaques progression evidenced by lower level of miR-27a with increasing risk of atherosclerosis progression [49]. To this end, miR-27a can be considered as a biomarker candidate for atherosclerosis.

To sum up, when we consider all of these results together with ours, it is clear that there are parallel data as well as contradictory ones. In order to discover more specific and reliable biomarkers, new detection methods need to be developed and the number of study groups should be increased if possible.

We observed a statistically significant 20-fold increase at CHOP expression level in patients (Fig. 3)**.** We also detected a predicted binding site of miR-222 in the 3′ UTR of CHOP mRNA (Fig. 4). There are many studies showing that ER stress response is associated with advanced human coronary atheroma [7, 9, 12, 50]. The role of ER stress marker CHOP was previously examined in atherosclerotic plaques and it was increased in atherosclerotic lesions in both humans and ApoE$^{-/-}$ mice compared to healthy controls [12, 51, 52]. This indicates that CHOP, a marker of UPR activation, can significantly contribute to the advancement atherosclerotic plaque progression. Very interestingly, siRNA knockdown of CHOP was previously shown to prevent thapsigargin treatment mediated miR-222 downregulation in HCC cell lines [26]. Considering the presence of miR-222 binding site in CHOP's UTR, it can be speculated that there may be other interaction partners controlling the regulation of expression of CHOP and miR-222 through mechanisms that are not yet discovered. Our results provide an evidence supporting the previous works related to CHOP's function in atherosclerosis and may establish a link between the miRNAs and ER stress for the further projects.

On top of the experimental data, DIANA tools KEGG pathway analysis revealed Protein processing in endoplasmic reticulum, Cell cycle, p53 signaling pathway and Sphingolipid signaling pathways to be enriched by the miRNAs of interest. This computation analysis offered us more clarification in order to connect the three critical aspect of our study: ER stress, miRNAs and atherosclerosis. First of all, protein processing in ER Stress was the one with the highest number of genes (76 genes). Secondly, ER stress is an important player in modulation of cell cycle [53]. Third, p53

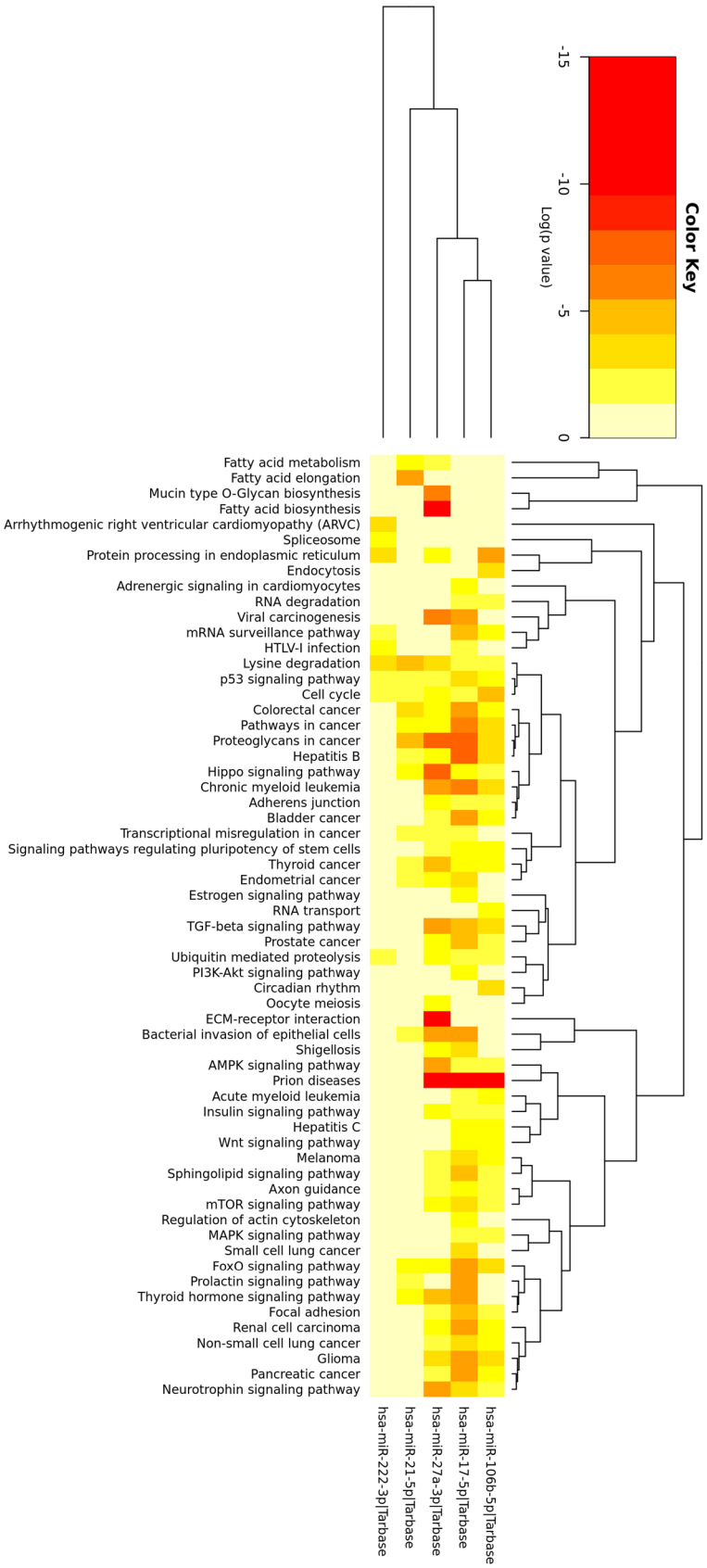

Fig. 5 Differentially expressed miRNAs vs KEGG Pathways heat map. Heat map was created by DIANA-miRPath v3.0. miRNA target enrichment analysis. Orange and red colour represent lower enrichment p -values while shades of yellow represent higher enrichment p-values

Table 4 Pathway names, *p* values, number of genes involved, and miRNAs related to the specific pathways

KEGG pathway	p value	#genes	miRNAs
Protein processing in endoplasmic reticulum	1.81E-08	76	miR-106b, miR-27a, miR-222
Cell cycle	3.33E-08	69	miR-106b, miR-17, miR-21, miR-27a, miR-222
p53 signaling pathway	9.68E-08	36	miR-106b, miR-17, miR-21, miR-27a, miR-222
Sphingolipid signaling pathway	5.11E-05	48	miR-106b, miR-17, miR-27a

signaling is controlled by ER Stress through different mechanisms [54]. Furthermore, one of the ER stress proteins that regulate p53 function directly is CHOP [55]. Forth, various spingolipids were involved in ER stress and the usage of the members of spingolipid pathways are suggested as future targetable candidates for ER stress modulation [56]. Last but not the least, specific spingolipids were implicated in the development of atherosclerosis and they were discussed as therapeutically targetable molecules in the fight against atherogenesis [57]. In brief, KEGG pathway analysis proves that miRNAs that are detected to be differentially expressed in this study are at the center of ER Stress and atherosclerosis connection.

Limitations

There are several limitations in this study. Firstly, the number of patients is relatively small due to the difficulty of collecting consent forms from the patients suitable to the study. Second, the samples were collected from only one centre. Third, the distribution of males and females are not even in both patients and control groups. Lastly, patients' samples could not be collected at a specific time span since the surgeries were scheduled independently from the research group. Increasing the sample size and inclusion of more centres could provide us more reliable data for the further studies.

Conclusion

In the present study, we demonstrate that miR-17 and miR-106b levels are significantly up-regulated in circulation of atherosclerosis patients whereas and miR-21, miR-27a and miR-222 levels are significantly down-regulated. ER stress activator CHOP's expression level is also increased in the same patients' samples compared to controls. In conclusion, CHOP and miRNAs that were detected to be differentially expressed in patient samples are powerful candidates that can be used as a biomarker in the progression of atherosclerosis. miRNAs that are detected in this study to be upregulated or downregulated can be used to design a custom high throughput diagnostic test panel. By the use of this panel in large cohorts with different cardiovascular diseases, the clinicians will be able to stratify the patients according to their molecular characteristics. Furthermore, miRNA mimics or miRNA antagomirs can be used to regulate the patient's miRNA levels in order to manage the disease conditions. Clinicians can integrate other available molecular diagnostics test together with miRNAs to have a more personalized therapy for each patient.

Author contributions Design and implementation of the research: PTA and DC. Performed the experiments: PTA and DC. Analysed the data: PTA and DC. Wrote the paper: PTA and DC. Both authors have read and approved the final version of the manuscript and they have met the criteria for authorship.

Funding This work was supported by the Scientific Research Projects Coordination Unit of Yuksek Ihtisas University under Grant Number 2018/01.001.

Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request without sharing patients' information.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethical approval The ethical approval was taken from The Yuksek Ihtisas Hospital Ethics Committee (File number: 2018-8663) meeting the criteria outlined in the Declaration of Helsinki and all participants signed written informed consent.

Informed consent Informed consent was obtained from all individual participants included in the study.

References

1. Mendis S (2014) Global status report on noncommunicable diseases 2014, 1st edn. WHO Press, Geneva, pp 1–11
2. Libby P, Ridker PM, Hansson GK (2011) Progress and challenges in translating the biology of atherosclerosis. Nature 473(7347):317–25. https://doi.org/10.1038/nature10146

3. Tabas I, García-Cardeña G, Owens GK (2015) Recent insights into the cellular biology of atherosclerosis. J Cell Biol 209(1):13–22. https://doi.org/10.1083/jcb.201412052
4. Berridge MJ (2002) The endoplasmic reticulum: a multifunctional signaling organelle. Cell Calcium 32(5–6):235–49. https://doi.org/10.1016/S0143416002001823
5. Chaudhari N, Talwar P, Parimisetty A, dHellencourt CL, Ravanan P. (2014) A molecular web: endoplasmic reticulum stress, inflammation, and oxidative stress. Front Cell Neurosci 8(JULY):1–15. https://doi.org/10.3389/fncel.2014.00213
6. Hetz C (2012) The unfolded protein response: controlling cell fate decisions under ER stress and beyond. Nat Rev Mol Cell Biol 13(2):89–102. https://doi.org/10.1038/nrm3270
7. Tufanli O, Akillilar PT, Acosta-Alvear D, Kocaturk B, Onat UI, Hamid SM et al (2017) Targeting IRE1 with small molecules counteracts progression of atherosclerosis. Proc Natl Acad Sci USA 114(8):E1395-404. https://doi.org/10.1073/pnas.1621188114
8. Hong J, Kim K, Kim JH, Park Y (2017) The role of endoplasmic reticulum stress in cardiovascular disease and exercise. Int J Vasc Med. https://doi.org/10.1155/2017/2049217
9. Çimen I, Kocatürk B, Koyuncu S, Tufanlı Ö, Onat UI, Yildirim AD et al (2016) Prevention of atherosclerosis by bioactive palmitoleate through suppression of organelle stress and inflammasome activation. Sci Transl Med 8(358):1. https://doi.org/10.1126/scitranslmed.aaf9087
10. Chen X, Guo X, Ge Q, Zhao Y, Mu H, Zhang J (2019) ER stress activates the NLRP3 inflammasome: a novel mechanism of atherosclerosis. Oxid Med Cell Longev. https://doi.org/10.1155/2019/3462530
11. Ren J, Bi Y, Sowers JR, Hetz C, Zhang Y (2021) Endoplasmic reticulum stress and unfolded protein response in cardiovascular diseases. Nat Rev Cardiol. https://doi.org/10.1038/s41569-021-00511-w
12. Tsukano H, Gotoh T, Endo M, Miyata K, Tazume H, Kadomatsu T et al (2010) The endoplasmic reticulum stress-C/EBP homologous protein pathway-mediated apoptosis in macrophages contributes to the instability of atherosclerotic plaques. Arterioscler Thromb Vasc Biol 30(10):1925–32. https://doi.org/10.1161/ATVBAHA.110.206094
13. Sun Y, Zhang D, Liu X, Li X, Liu F, Yu Y et al (2018) Endoplasmic reticulum stress affects lipid metabolism in atherosclerosis via CHOP activation and over-expression of miR-33. Cell Physiol Biochem. 48(5):1995–2010. https://doi.org/10.1159/000492522
14. Jauhiainen A, Thomsen C, Strömbom L, Grundevik P, Andersson C, Danielsson A et al (2012) Distinct cytoplasmic and nuclear functions of the stress induced protein DDIT3/CHOP/GADD153. PLoS One 7(4):e33208. https://doi.org/10.1371/journal.pone.0033208
15. Van Rooij E, Olson EN (2007) MicroRNAs: powerful new regulators of heart disease and provocative therapeutic targets. J Clin Invest 117(9):2369–76. https://doi.org/10.1172/JCI33099
16. Lee RC, Feinbaum RL, Ambros V, The C (1993) elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75:843–854
17. Byrd AE, Brewer JW (2013) Micro(RNA)managing endoplasmic reticulum stress. IUBMB Life 65(5):373–81. https://doi.org/10.1002/iub.1151
18. Maurel M, Chevet E, Faraldi M, Gomarasca M, Sansoni V, Perego S et al (2011) Normalization strategy is critical for the outcome of miRNA expression analyses in the rat heart. Sci Rep 8(1):1–9. https://doi.org/10.1152/ajpcell.00061.2013
19. Bildirici AE, Arslan S, Özbilüm Şahin N, Berkan Ö, Beton O, Yilmaz MB (2018) MicroRNA-221/222 expression in atherosclerotic coronary artery plaque versus internal mammarian artery and in peripheral blood samples. Biomarkers 23(7):670–675. https://doi.org/10.1080/1354750X.2018.1474260
20. Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116(2):281–97
21. Belmont P, Chen W, Thuerauf D, Glembotski C (2012) Regulation of microRNA expression in the heart by the ATF6 branch of the ER stress response. J Mol Cell Cardiol 52(5):1176–82. https://doi.org/10.1016/j.yjmcc.2012.01.017
22. Chen M, Ma G, Yue Y, Wei Y, Li Q, Tong Z et al (2014) Down-regulation of the miR-30 family microRNAs contributes to endoplasmic reticulum stress in cardiac muscle and vascular smooth muscle cells. Int J Cardiol 173(1):65–73. https://doi.org/10.1016/j.ijcard.2014.02.007
23. Upton JP, Wang L, Han D, Wang ES, Huskey NE, Lim L, Truitt M, McManus MT, Ruggero D, Goga A, Papa FR, Oakes SA (2012) IRE1α cleaves select microRNAs during ER stress to derepress translation of proapoptotic Caspase-2. Science 338:818–22. https://doi.org/10.1126/science.1226191
24. Gupta S, Read DE, Deepti A, Cawley K, Gupta A, Oommen D et al (2012) Perk-dependent repression of miR-106b-25 cluster is required for ER stress-induced apoptosis. Cell Death Dis 3(6):e333-10. https://doi.org/10.1038/cddis.2012.74
25. Chhabra R, Dubey R, Saini N (2011) Gene expression profiling indicate role of ER stress in miR-23a~27a~24–2 cluster induced apoptosis in HEK293T cells. RNA Biol. https://doi.org/10.4161/rna.8.4.15583
26. Dai R, Li J, Liu Y, Yan D, Chen S, Duan C et al (2010) MiR-221/222 suppression protects against endoplasmic reticulum stress-induced apoptosis via p27 Kip1- and MEK/ERK-mediated cell cycle regulation. Biol Chem 391(7):791–801. https://doi.org/10.1515/BC.2010.072
27. Jia LX, Zhang WM, Li TT, Liu Y, Piao CM, Ma YC et al (2017) ER stress dependent microparticles derived from smooth muscle cells promote endothelial dysfunction during thoracic aortic aneurysm and dissection. Clin Sci 131(12):1287–99. https://doi.org/10.1042/CS20170252
28. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 25(4):402–8
29. Vlachos IS, Hatzigeorgiou AG (2017) Functional analysis of miRNAs using the DIANA tools online suite. Methods Mol Biol 1517:25–50. https://doi.org/10.1007/978-1-4939-6563-2_2
30. Agarwal V, Bell GW, Nam JW, Bartel DP (2015) Predicting effective microRNA target sites in mammalian mRNAs. Elife. https://doi.org/10.7554/eLife.05005
31. An JH, Chen ZY, Ma QL, Wang HJ, Zhang JQ, Shi FW (2019) LncRNA SNHG16 promoted proliferation and inflammatory response of macrophages through miR-17–5p/NF-κB signaling pathway in patients with atherosclerosis. Eur Rev Med Pharmacol Sci 23(19):8665–8677. https://doi.org/10.26355/eurrev_201910_19184
32. Fichtlscherer S, De Rosa S, Fox H, Schwietz T, Fischer A, Liebetrau C et al (2010) Circulating microRNAs in patients with coronary artery disease. Circ Res 107(5):677–84. https://doi.org/10.1161/CIRCRESAHA.109.215566
33. Brown MS, Goldstein JL (1976) Familial hypercholesterolemia: a genetic defect in the low-density lipoprotein receptor. N Engl J Med 294(25):1386–90. https://doi.org/10.1056/NEJM197606172942509
34. Chen J, Xu L, Hu Q, Yang S, Zhang B, Jiang H (2015) MiR-17-5p as circulating biomarkers for the severity of coronary atherosclerosis in coronary artery disease. Int J Cardiol 197:123–124. https://doi.org/10.1016/j.ijcard.2015.06.037
35. Semo J, Chernin G, Jonas M, Shimoni S, George J (2019) Deletion of the Mir-106b~ 25 microRNA cluster attenuates atherosclerosis

in apolipoprotein e knockout mice. Lipids Health Dis 18(1):1–9. https://doi.org/10.1186/s12944-019-1155-8
36. Han H, Qu G, Han C, Wang Y, Sun T, Li F et al (2015) MiR-34a, miR-21 and miR-23a as potential biomarkers for coronary artery disease: a pilot microarray study and confirmation in a 32 patient cohort. Exp Mol Med 47(2):e138-7. https://doi.org/10.1038/emm.2014.81
37. Ren J, Zhang J, Xu N, Han G, Geng Q, Song J et al (2013) Signature of circulating MicroRNAs as potential biomarkers in vulnerable coronary artery disease. PLoS ONE. https://doi.org/10.1371/journal.pone.0080738
38. Zhou J, Shao G, Chen X, Yang X, Huang X, Peng P et al (2016) MiRNA 206 and miRNA 574–5p are highly expression in coronary artery disease. Biosci Rep. 36(1):1–7. https://doi.org/10.1042/BSR20150206
39. Dentelli P, Rosso A, Orso F, Olgasi C, Taverna D, Brizzi MF (2010) MicroRNA-222 controls neovascularization by regulating signal transducer and activator of transcription 5A expression. Arterioscler Thromb Vasc Biol 30(8):1562–8. https://doi.org/10.1161/ATVBAHA.110.206201
40. Chamorro-Jorganes A, Araldi E, Suárez Y (2013) MicroRNAs as pharmacological targets in endothelial cell function and dysfunction. Pharmacol Res 75:15–27. https://doi.org/10.1016/j.phrs.2013.04.002
41. Minami Y, Satoh M, Maesawa C, Takahashi Y, Tabuchi T, Itoh T et al (2009) Effect of atorvastatin on microRNA 221/222 expression in endothelial progenitor cells obtained from patients with coronary artery disease. Eur J Clin Invest 39(5):359–67. https://doi.org/10.1111/j.1365-2362.2009.02110
42. Canfrán-Duque A, Rotllan N, Zhang X, Fernández-Fuertes M, Ramírez-Hidalgo C, Araldi E et al (2017) Macrophage deficiency of miR-21 promotes apoptosis, plaque necrosis, and vascular inflammation during atherogenesis. EMBO Mol Med 9(9):1244–62. https://doi.org/10.15252/emmm.201607492
43. Jin H, Li DY, Chernogubova E, Sun C, Busch A, Eken SM et al (2018) Local delivery of miR-21 stabilizes fibrous caps in vulnerable atherosclerotic lesions. Mol Ther 26(4):1040–55. https://doi.org/10.1016/j.ymthe.2018.01.011
44. Raitoharju E, Lyytikäinen LP, Levula M, Oksala N, Mennander A, Tarkka M et al (2011) MiR-21, miR-210, miR-34a, and miR-146a/b are up-regulated in human atherosclerotic plaques in the tampere vascular study. Atherosclerosis 219(1):211–7. https://doi.org/10.1016/j.atherosclerosis.2011.07.020
45. Cengiz M, Yavuzer S, Klçkran Avc B, Yürüyen M, Yavuzer H, Dikici SA et al (2015) Circulating miR-21 and eNOS in subclinical atherosclerosis in patients with hypertension. Clin Exp Hypertens 37(8):643–9. https://doi.org/10.3109/10641963.2015.1036064
46. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F et al (2006) A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci USA 103(7):2257–61
47. Khan A, Agarwal H, Reddy SS, Arige V, Natarajan B, Gupta V et al (2020) MicroRNA-27a is a key modulator of cholesterol biosynthesis. Mol Cell Biol. https://doi.org/10.1128/mcb.00470-19
48. Xie W, Li L, Zhang M, Cheng HP, Gong D, Lv YC et al (2016) MicroRNA-27 prevents atherosclerosis by suppressing lipoprotein lipase-induced lipid accumulation and inflammatory response in apolipoprotein E knockout mice. PLoS ONE 11(6):1–20. https://doi.org/10.1371/journal.pone.0157085
49. Vegter EL, Ovchinnikova ES, van Veldhuisen DJ, Jaarsma T, Berezikov E, van der Meer P et al (2017) Low circulating microRNA levels in heart failure patients are associated with atherosclerotic disease and cardiovascular-related rehospitalizations. Clin Res Cardiol 106(8):598–609. https://doi.org/10.1007/s00392-017-1096-z
50. Minamino T, Kitakaze M (2010) ER stress in cardiovascular disease. J Mol Cell Cardiol 48(6):1105–10. https://doi.org/10.1016/j.yjmcc.2009.10.026
51. Thorp E, Li G, Seimon TA, Kuriakose G, Ron D, Tabas I (2009) Reduced apoptosis and plaque necrosis in advanced atherosclerotic lesions of Apoe−/− and Ldlr−/− mice lacking CHOP. Cell Metab 9(5):474–81. https://doi.org/10.1016/j.cmet.2009.03.003
52. Myoishi M, Hao H, Minamino T, Watanabe K, Nishihira K, Hatakeyama K et al (2007) Increased endoplasmic reticulum stress in atherosclerotic plaques associated with acute coronary syndrome. Circulation 116(11):1226–33
53. Solley S, Zappa F, Acosta-Alvear D (2020) Regulation of the mammalian cell cycle by the unfolded protein response. FASEB J 34:1–1. https://doi.org/10.1096/fasebj.2020.34.s1.08710
54. Qu L, Koromilas AE (2004) Control of tumor suppressor p53 function by endoplasmic reticulum stress. Cell Cycle 3(5):567–70
55. Engel T, Sanz-Rodgriguez A, Jimenez-Mateos EM, Concannon CG, Jimenez-Pacheco A, Moran C et al (2013) CHOP regulates the p53-MDM2 axis and is required for neuronal survival after seizures. Brain 136(2):577–92. https://doi.org/10.1093/brain/aws337
56. Park W-J, Park J-W (2020) The role of sphingolipids in endoplasmic reticulum stress. FEBS Lett 594(22):3632–51. https://doi.org/10.1002/1873-3468.13863
57. Yu Z, Peng Q, Huang Y (2019) Potential therapeutic targets for atherosclerosis in sphingolipid metabolism Sphingolipids metabolism overview of sphingolipid biosynthesis. Clin Sci 133:763–776. https://doi.org/10.1042/CS20180911

Terms and Conditions

Springer Nature journal content, brought to you courtesy of Springer Nature Customer Service Center GmbH (“Springer Nature”).
Springer Nature supports a reasonable amount of sharing of research papers by authors, subscribers and authorised users (“Users”), for small-scale personal, non-commercial use provided that all copyright, trade and service marks and other proprietary notices are maintained. By accessing, sharing, receiving or otherwise using the Springer Nature journal content you agree to these terms of use (“Terms”). For these purposes, Springer Nature considers academic use (by researchers and students) to be non-commercial.
These Terms are supplementary and will apply in addition to any applicable website terms and conditions, a relevant site licence or a personal subscription. These Terms will prevail over any conflict or ambiguity with regards to the relevant terms, a site licence or a personal subscription (to the extent of the conflict or ambiguity only). For Creative Commons-licensed articles, the terms of the Creative Commons license used will apply.
We collect and use personal data to provide access to the Springer Nature journal content. We may also use these personal data internally within ResearchGate and Springer Nature and as agreed share it, in an anonymised way, for purposes of tracking, analysis and reporting. We will not otherwise disclose your personal data outside the ResearchGate or the Springer Nature group of companies unless we have your permission as detailed in the Privacy Policy.
While Users may use the Springer Nature journal content for small scale, personal non-commercial use, it is important to note that Users may not:

1. use such content for the purpose of providing other users with access on a regular or large scale basis or as a means to circumvent access control;
2. use such content where to do so would be considered a criminal or statutory offence in any jurisdiction, or gives rise to civil liability, or is otherwise unlawful;
3. falsely or misleadingly imply or suggest endorsement, approval , sponsorship, or association unless explicitly agreed to by Springer Nature in writing;
4. use bots or other automated methods to access the content or redirect messages
5. override any security feature or exclusionary protocol; or
6. share the content in order to create substitute for Springer Nature products or services or a systematic database of Springer Nature journal content.

In line with the restriction against commercial use, Springer Nature does not permit the creation of a product or service that creates revenue, royalties, rent or income from our content or its inclusion as part of a paid for service or for other commercial gain. Springer Nature journal content cannot be used for inter-library loans and librarians may not upload Springer Nature journal content on a large scale into their, or any other, institutional repository.
These terms of use are reviewed regularly and may be amended at any time. Springer Nature is not obligated to publish any information or content on this website and may remove it or features or functionality at our sole discretion, at any time with or without notice. Springer Nature may revoke this licence to you at any time and remove access to any copies of the Springer Nature journal content which have been saved.
To the fullest extent permitted by law, Springer Nature makes no warranties, representations or guarantees to Users, either express or implied with respect to the Springer nature journal content and all parties disclaim and waive any implied warranties or warranties imposed by law, including merchantability or fitness for any particular purpose.
Please note that these rights do not automatically extend to content, data or other material published by Springer Nature that may be licensed from third parties.
If you would like to use or distribute our Springer Nature journal content to a wider audience or on a regular basis or in any other manner not expressly permitted by these Terms, please contact Springer Nature at

onlineservice@springernature.com