



Expression of miR21, miR122, miR146a and miR196 in Symptomatic Carotid Disease

Anna Sioziou¹, Hector Katifelis¹, Evangelia Legaki¹, Nikolaos Patelis², Dimitrios Athanasiadis², Theodoros Liakakos², Christos Klonaris², Maria Gazouli^{1,*}

¹Department of Basic Medical Sciences, Laboratory of Biology, Medical School, National and Kapodistrian University of Athens, Athens, Greece

²First Department of Surgery, Vascular Unit, National and Kapodistrian University of Athens, Laiko General Hospital, Athens, Greece

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ABSTRACT

Background: Carotid disease is one of the many forms of cardiovascular disease, which may lead to chronic disability and death. It is a multifactorial inflammatory disease, greatly affected by an individual's habits like smoking, lack of exercise, and a diet high in fats. MicroRNAs (miRs) are known to be involved in vascular inflammation.

Objectives: We aimed to analyse in a case-control study the expression profile of selected miRs from patients with symptomatic carotid disease and to examine their involvement in the disease pathogenesis.

Patients and Methods: Samples from 38 symptomatic patients who underwent carotid endarterectomy were collected and adjacent healthy regions from 15 patients were used as control samples. Fold change in the expression of miR21, miR122, miR146a and miR196a was measured using reverse transcription-real time PCR. Western blot was used to quantify the levels of MMP2 protein whose gene is a target of miR21.

Results: Compared to control samples, all patients showed upregulation of miR21, miR122, miR146a and miR196a. No statistical significance was found to exist from patients with high or low miRs expression and clinical/laboratory parameters. The levels of MMP2 were found to be decreased in patients when compared to control samples.

Conclusions: Our results revealed miRs which showed different expression in endarterectomy specimens from patients with symptomatic carotid disease, suggesting that these miRs correlated with vascular inflammation. Furthermore, miR21 seems an appealing pharmaceutical target since by targeting MMP2 can favour a stable plaque since low levels of the protein of its gene MMP2 target prevent the fibrous cap of the atheroma from getting thinner. Thus, miR21 seems to prevent rupture but further research is required.

1. Background

Carotid disease is a potentially life-threatening situation since carotid plaque is responsible for serious clinical manifestations including ischaemic stroke which in turn can cause permanent disability and even death (1). Formation of carotid plaque is a process triggered by fatty streak formation, vessel wall trauma, endothelial dysfunction, arterial remodelling, and chronic inflammation (2). Inflammation greatly affects the formation, progress, and vulnerability of the atheromatic plaque. Among the first

incidents related to inflammation during plaque formation are circulating monocytes and lymphocytes that are recruited to the carotid wall. This action is mainly mediated via the oxidized LDL cholesterol on endothelial cells which leads to the production of several pro-inflammatory molecules, including adhesion molecules. Oxidized cholesterol however is not the only factor that triggers an inflammatory response. Changes in the haemodynamic flow, postmenopausal hormone levels shift; even periodontal disease seem to be involved (3-5). ROS have also a role in this condition since they result in a highly oxidised LDL cholesterol which is collected by macrophages turning them into foam cells (4). The study of genetic variation involved in lipoprotein metabolism, polymorphisms in Toll-like

*Corresponding author: Maria Gazouli, Department of Basic Medical Sciences, Laboratory of Biology, Medical School, National and Kapodistrian University of Athens, Athens, Greece, Michalakopoulou 176, 11527 Athens, Greece, Tel: +30-2107462231, E-mail: mgazouli@med.uoa.gr.

Receptors (TLRs) and Scavenger Receptors (SRs) could allow a better understanding of the atheromatosis process (6) and subsequently of carotid artery disease. Further research on the role of genes involved in inflammation and other factors that affect gene expression will improve our current knowledge towards carotid disease, since evidence suggests that inflammation is basically involved in the pathogenesis of atherosclerosis (7). The inflammatory process leads to the development of plaques, which progressively can cause arterial stenoses and occlusions. Thus gene biomarkers related to the inflammation may predict the individuals risk for progression of atherosclerosis and carotid artery disease. For that reason, all miRs (miR21, miR122, miR146a, and miR196a) chosen in this study target genes related to inflammation and other contributing factors of the disease. These molecules are important gene expression regulators for many physiological and pathological processes in most organisms (8). MiRs are molecules 20 - 23 nucleotides long, single stranded that regulate gene expression by inhibiting translation or by leading their targets (mRNAs) to degradation (9). Polymerase II and sometimes Polymerase III transcribes miR genes to pri-miR which is a primary form of mature miR (10) Pri-miR is then processed by Drosha, an RNase III enzyme and precursor- miR (pre-miR) is formed. Via exportin 5, pre-miR is exported to the cytoplasm where a second enzyme known as Dicer processes pre-miR resulting to a mature two stranded miR. At that point, the RNA induced silencing complex (RISC) is assembled. The two strands of mature miR are called passenger strand and guide strand and are marked as miR* and miR respectively. The former is degraded allowing the latter to target mRNA and regulate gene expression (11).

2. Objectives

This study aims to investigate the expression of miRs that are linked to symptomatic carotid artery disease and inflammation and their effect on pathways that are involved. Elucidating the cellular patterns that govern plaque formation and progress will result in better, non-surgical treating options to be developed, reducing the morbidity and mortality of carotid artery disease.

3. Patients and Methods

3.1. Subjects and Specimens

Carotid plaque samples were collected from patients (n = 38) who underwent carotid endarterectomy as a surgical treatment for carotid stenosis. The surgical operations were performed at the Laiko General Hospital of Athens for a period of two years. The extracted carotid tissue was collected from these patients, transferred to the Athens School of Medicine Biology Laboratory where the samples were stored at -80°C. This study was performed under

the standards of the most recent update of the Helsinki Declaration. All patients gave their informed consent for their samples to be used in the study. Adjacent healthy regions of the samples were used as control (n = 15).

3.2. RNA Extraction and cDNA Synthesis

Total RNA was extracted using the TRIzol method. The TAKARA kit was used for cDNA synthesis from total RNA. The quantities used for each cDNA reaction were 6.5 µL of Master Mix (containing PrimeScript Buffer, PrimeScript RT Enzyme Mix I, specific reverse primer for each miR and RNase-free dH₂O) and 3.5 µL of whole RNA extracted from the tissues used. All reactions were held on Thermal Cycler (Kyratec SuperCycler). The reaction conditions were as follows: 37°C for 30 minutes and 85°C for 5 seconds in order to deactivate reverse transcriptase.

3.3. Real Time PCR and Gene Expression Analysis

In order to perform real time PCR, the KAPA SYBR FAST qPCR Kit was used. The purpose of this assay was to measure the expression of miR21, miR122, miR146a and miR196a in both plaque and control specimens. U6 was used as a gene of reference. The sequences of miR21, miR122, miR146a, miR196a and u6 primers are in table 1. The quantities used for each reaction were as follows: 10 µL of KAPA SYBR FAST Master mix, 0.25 µL of miR/u6 primers (forward and reverse), 7.5 µL of PCR grade water and 2 µL of cDNA. In order to quantify miR expression SYBR Green dye was used. All specimens were duplicated to ensure reproducibility. The reactions were held at Light Cycler 480 System, Roche. Differentiation between gene expression in carotid artery plaque and healthy tissue was measured as a fold change by making use of the $2^{-\Delta\Delta CT}$ method and the results are presented as log-transformed ratios.

3.4. Protein Quantification and Western Blot Analysis

Proteins were extracted from carotid tissue (from sites of atheromatosis and from healthy adjacent regions) using a lysis buffer that contained proteinase inhibitor and tissue homogenisation was performed using mechanical means on ice. Protein concentration was estimated using Bradford method. All samples were loaded into an 8 - 12% SDS-polyacrylamide gel having equal concentrations and they were electrotransferred. Non-specific sites were blocked by incubating the membrane in solution rich in protein (milk) at 4°C for 12 - 16 h. The membrane was incubated at the same conditions with the primary antibody. The immune complexes that were formed were detected by using a peroxidase-conjugated secondary antibody using chemiluminescence (Pierce ECL Western Blot Substrate). Protein expression was normalised by using GAPDH. The

Table 1. Primer Sequences

Gene	Forward Primer	Reverse Primer
miR21	5'-CCACTGTCTAGCAGACACTAA-3'	5'-CACTGTCTAGCAGACACTAATCAACATCA-3'
miR146a	5'-TAACCGAATCTTGCCATACGCA-3'	5'-CGGAGTCTGAGAAGTGAATTTCCA-3'
miR196a2c	5'-CCCCTTCCCTTCTCCTCCAGATA3'	5'-CGAAAACCGACTGATGTAACCTCCG-3'
miR122	5'-ACACTCCAGCTGGGTGGAGTGTGACAATCC-3'	5'-TGGTGTCTGGAGTTCG-3'
U6sn	5'-ATTGCAACGATACAGAGAAGATT-3'	5'-GGAACGCTTCACGAATTTG-3'

primary antibody was mouse anti-MMP2 (1:1000, Acris antibodies, AM00257AF-N) and mouse anti-GAPDH (1:1000, Merck) and the secondary anti-mouse-HRP (1:1000, R&D Systems, HAF007).

3.5. Statistical Analysis

All statistical analyses were performed using GraphPad version 3.00 (GraphPad Software, San Diego, Ca, USA). $P < 0.05$ was considered significant.

4. Results

Samples from 38 patients with carotid plaque were studied. Adjacent regions of 15 samples free of plaque were used as controls. Tables 2 and 3 show data from both clinical and laboratory parameters. As shown in table 2, most patients had hypertension, increased cholesterol (hypercholesterolemia) and triglyceride (TG) levels and almost half of them had coronary artery disease (CAD). On the contrary, most patients did not have diabetes, chronic obstructive pulmonary disease (COPD), peripheral artery disease (PAD), chronic kidney disease (CKD) whereas 1 out of 3 patients had a cerebrovascular event (CVE). Most patients were tobacco users and under pharmacological therapy using statins and antiplatelet drugs.

Fold change in the expression of miR21, miR122, miR146a and miR196a was measured in regions of atheromatic plaque as well as in control regions. Compared with the control regions, all atheromatic ones showed upregulation of all miRs used in this study (Figure 1). As shown on

table 4, miR21 had the highest upregulation with a mean value of 7.166 ± 0.445 in carotid plaque whereas control regions had a mean value of 1.243 ± 0.104 . MiR122 had a mean value of expression fold change in carotid plaque of 3.947 ± 0.349 and 1.596 ± 0.207 in control regions. MiR146a had a mean value 3.127 ± 0.218 for carotid plaque and 1.822 ± 0.115 for control regions. MiR196a had a mean value of 2.797 ± 0.213 for carotid plaque and 1.796 ± 0.151 for control regions. The difference in mean values between carotid plaque regions and control regions were statistically significant ($P < 0.05$). In order to investigate the existence of a connection between miR upregulation and clinical/laboratory parameters, the patients of this study were divided into two groups for each separate miR; the first group included patients whose carotid plaque showed low expression (patients of this group showed miR expression below mean value) and the second group included patients whose carotid plaque showed high expression (patients of this group showed miR expression above mean value) as shown in table 5. The expression of miR21, miR122, miR146a and miR196a does not appear to be associated with clinical parameters such as cholesterol, hypertension and smoking. Levels of MMP2 protein were also measured in atheromatic and control regions since it has been linked to carotid plaque development and carotid disease (12, 13). MMP2 is also targeted by miR21 which among all miRs studied had the highest upregulation. MMP2 protein levels in carotid plaque were found to be lower by 1.17 times compared to control regions (Figure 2).

Table 2. Demographics and Clinical Characteristics

Parameter	Value	P; OR [CI95%]	
Gender (F/M)	9/29		
Age (years \pm SD)	67.5 \pm 8.29		
Parameter	Yes (%)	No (%)	
Diabetes	21.05	78.95	< 0.0001; 0.07[0.04 - 0.14]
Hypertension	84.21	15.79	< 0.0001; 27.56[12.94 - 58.72]
Hypercholesterolemia	73.68	26.32	< 0.0001; 8.10[4.30 - 15.24]
Triglycerides	68.42	31.58	< 0.0001; 4.52[2.49 - 8.18]
CAD	44.74	55.26	0.203; 0.67[0.38 - 1.17]
COPD	18.42	81.58	< 0.0001; 0.05[0.23 - 0.09]
PAD	5.26	94.74	< 0.0001; 0.003[0.0008 - 0.01]
CVE	36.84	63.16	0.0001; 0.32[0.18 - 0.56]
Smoking	63.16	36.84	0.0001; 3.16[1.77 - 5.63]
CKD	0.21	99.79	< 0.0001; 0.0001[6.3 - 6 - 0.002]
Antiplatelet drugs	86.84	13.76	< 0.0001; 44.78[19.64 - 102.14]
Use of statins	73.68	26.32	< 0.0001; 8.10[4.30 - 15.24]

Table 3. Laboratory Parameters

Parameter	Value
HCT \pm SD (%)	40.1 \pm 3.63
PLTs \pm SD (PLTs/ μ L)	230 \pm 58.8
Total Chol \pm SD (mg/dL)	187.6 \pm 38.95
TG \pm SD (mg/dL)	143 \pm 46
LDL \pm SD (mg/dL)	110 \pm 36
HDL \pm SD (mg/dL)	47 \pm 12
Ur \pm SD (mg/dL)	40 \pm 12
Cre \pm SD (mg/dL)	0.95 \pm 0.23
Glu \pm SD (mg/dL)	111 \pm 34

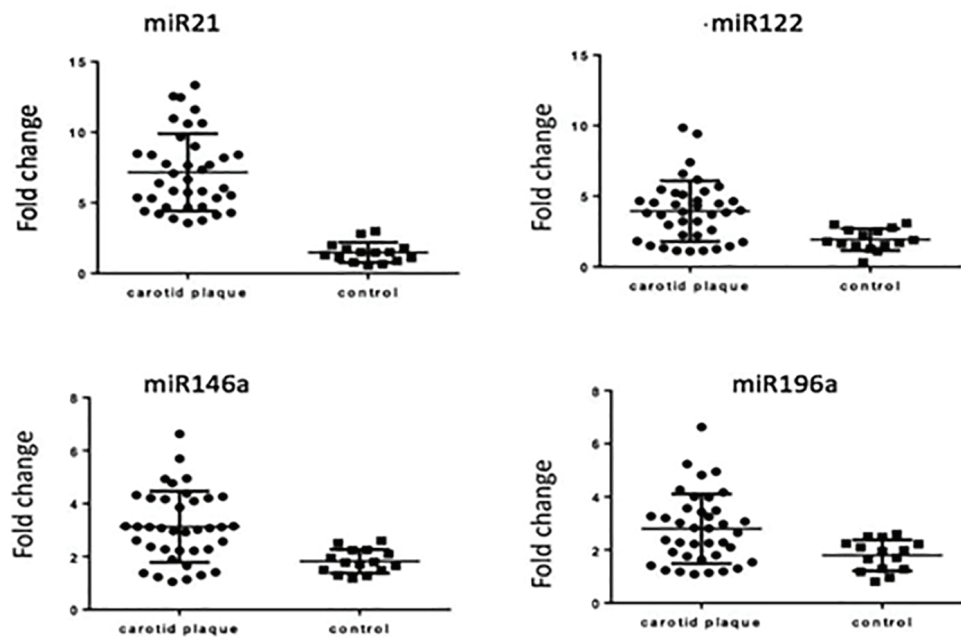


Figure 1. miR21, miR122, miR146a and miR196a Expression in Different Carotid Atheromatic Lesion Samples in Comparison with Adjacent Regions Used as Controls

Table 4. Mean Values of miR21, miR122, miR146a and miR196a Expression Fold Change

miR	Mean Value of Atheromatic Regions \pm SD (n = 38)	Mean Value of Control Regions \pm SD (n = 15)	P value
miR21	7.166 \pm 0.445	1.243 \pm 0.104	< 0.0001
miR122	3.947 \pm 0.349	1.596 \pm 0.207	0.001
miR146a	3.127 \pm 0.218	1.822 \pm 0.115	0.006
miR196a	2.797 \pm 0.213	1.796 \pm 0.151	0.065

Table 5. Percentage of Patients Falling in the Low or High Categories of miR21, miR122, miR146a and miR196a According to Chosen Parameters

Parameter	miR21		miR122		miR146a		miR196a	
	Low, < 7.17	High, > 7.17	Low, < 3.95	High, > 3.95	Low, < 3.13	High, > 3.13	Low, < 2.79	High, > 2.79
Diabetes (%)	37.50	62.50	37.5	62.5	71.43	28.57	62.5	37.5
Hypertension (%)	53.12	46.88	53.12	46.88	57.14	42.86	53.12	46.88
Hyper-cholesterolemia (%)	44.00	56.00	53.57	46.43	60.71	39.29	53.57	46.43
TG (%)	53.85	46.15	57.69	42.31	61.54	38.46	53.85	46.15
Smoking (%)	54.17	45.83	54.17	45.83	62.5	37.5	41.67	58.33
COPD (%)	66.67	33.33	42.86	57.14	100	0	57.14	42.86
CVE (%)	61.54	38.46	64.29	35.71	66.66	33.34	57.14	42.86
CAD (%)	50.00	50.00	52.95	47.05	33.33	66.64	47.06	52.94
Use of statins (%)	50.00	50.00	53.58	46.42	58.62	41.38	50.00	50.00
Antiplatelet drugs (%)	56.25	43.75	57.58	42.42	64.71	35.29	54.55	45.45
Total cholesterol \pm SD (mg/dL)	189.82 \pm 46.15	42 \pm 8.70	184.2 \pm 28.64	55 \pm 15	191.88 \pm 40.07	48.7 \pm 9.54	181.92 \pm 40.12	45.5 \pm 16.1
HDL cholesterol \pm SD (mg/dL)	188.5 \pm 40.36	43 \pm 6.8	186.42 \pm 34.09	55 \pm 17	187.59 \pm 41.54	48 \pm 11	187.64 \pm 38.58	46 \pm 17
LDL cholesterol \pm SD (mg/dL)	117 \pm 40	95 \pm 28	112 \pm 34.3	107 \pm 41.8	108 \pm 36	114 \pm 43	111 \pm 36	106 \pm 42
TG \pm SD (mg/dL)	146 \pm 46	139 \pm 43	140 \pm 48.1	148 \pm 46.3	153 \pm 49	131 \pm 41	136 \pm 49	155 \pm 42

* No statistical significance was found between high/low expression and the parameters listed on this table.

5. Discussion

Carotid artery disease is the result of a complex and yet not fully understood mechanism. The reason these miRs (miR21, miR122, miR146a and miR196a) were selected in this study is due to their involvement in inflammation and other important aspects of the diseases pathophysiology. Our results showed that all four miRs were upregulated in the patient carotid plaque specimens compared to adjacent healthy regions.

Of all the miRs studied, miR21 showed the greatest expression fold change. Like our findings, miR21 was also found to be upregulated in other studies about cardiovascular diseases (11, 14). An important mechanism that leads to changes in the expression of miR21 are DAMPs (Damage Associated Molecular Patterns) including the cholesterol crystals that can be found on the surface of the arterial wall (15, 16). Circulating levels of miR21 were also found to be elevated in another study in patients with hypertension

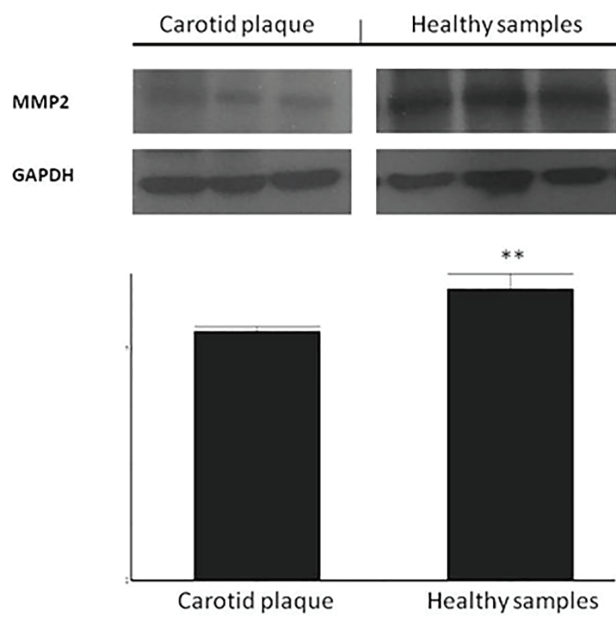


Figure 2. Fold Change in the Levels of MMP2 Protein between Patient and Control Samples. MMP2 Levels Are 1.17 Times Lower in Atheromatic Lesions Compared to Healthy Adjacent Regions ($P = 0.0375$)

and it is believed that miR21 could be important at the early stages of atheromatosis, by decreasing eNOS (endothelial Nitric Oxide Synthase) levels. Low levels of this synthase indicate endothelial dysfunction and inflammation (17). However according to another research (18), miR21 seems to be atheroprotective since it increases NO bioavailability and inhibits endothelial cell apoptosis. To better understand the role of miR21, which showed the greatest upregulation, we decided to look for its gene-targets in the online databases. Of all its targets, MMP2 was of the greatest importance since it has already been linked to the atheromatosis process.

However, the relationship between MMP2 expression and unstable plaques seems to be controversial since there are studies supported that MMP2 is not related to carotid instability, and that increased MMP2 activity is associated with the presence of smooth-muscle cells, suggesting a stable lesion phenotype (19), but also evidence shows that MMP2 protein reduces the atheromas stability, and thus making it prone to rupture (20). In our research, protein MMP2 was quantified and its levels were found to be lower than those of control samples. Our results are in agreement with findings by Kuge et al (21) and Heo et al (20) supported that MMP2 is responsible for plaque destabilisation, and atherogenesis, and that low levels of MMP2 can be beneficial in terms of plaque stability. Even though the question whether MMP2 prevents or promotes rupture remains to be answered, the role of miR21 seems to be important. By targeting MMP2, miR21 arises as an appealing pharmaceutical target. An appropriate interference could change the levels of miR21 to promote a stable plaque phenotype and thus preventing a life threatening rupture. Our finding suggests that overexpression of miR21 might have a protective role towards rupture. However, to ascertain whether miR21 upregulation is beneficial or not

for the disease, further research regarding its gene-targets is required.

MiR122 regulates genes involved in cholesterol and fatty acid metabolism (22, 23). In our study, miR122 was upregulated. MiR122 was also found to regulate plasma cholesterol and triglyceride levels but the molecular mechanism behind this remains unclear (24). Interestingly, a study about miR levels in hepatic cells showed that miR370 upregulates miR122 and thus regulates important genes for lipid metabolism (25). An investigation of whether this upregulation is caused by miR370 or not could show a similar connection between these two miRs in carotid plaque, and in turn reveal a part of the interaction network of different miRs in carotid disease. Better understanding of this complicated network could allow a pharmaceutical interference with a single miR, which would in turn affect other miRs regulated by it and their gene-targets, offering a novel treatment for carotid and other cardiovascular diseases.

Changes in the expression of miR146a are important in cardiovascular disease since they are also connected with inflammation (26, 27) and with LDL releasing mechanisms from the liver (11, 28). Regarding its inflammatory role, miR146a seems to activate inflammatory cytokine pathways and to provoke an imbalance between Th1 and Th2 cell populations. These events seem to favour plaque destabilisation, but further research in animal models is necessary (29).

The role of miR196a in the process of atheromatosis is poorly understood. To our knowledge, ours is the first study to investigate an expression fold change of miR196a in carotid plaque specimens. Two studies examined a possible association between miR196a polymorphisms and the incidence of an ischaemic stroke in an Iranian and a Chinese population but their results were contradictory (30, 31). One of its gene-targets (32), ANXA1 (Annexin A1) seems to be part of the pathophysiology mechanism of several chronic inflammation-related diseases (33). Quantification of ANXA1 protein levels in carotid plaque samples would be the next step that could reveal a dysregulation of these protein levels, suggesting an inflammatory mechanism mediated by miR196a.

The lack of statistical significance between the groups of high and low expression regarding the patients clinical and laboratory parameters could indicate that there is no linear connection between the expression fold change of a single miR and these parameters. It is more likely that miRs form a complicated network of interactions that affects these parameters.

Taken together, these miRs target genes (according to the current miR database, mirtarbase.mbc.nctu.edu.tw) that affect extracellular matrix structure (MMP2, TIMP3, VEGF-A, ADAM10, ADAM17, NRP1), genes related to inflammation (CXCL12, TLR2, S100A9) and genes coding proteins that are key players in cellular signalling and gene expression (GALNT10, ROCK1, BACH1). In this study, all patient samples showed overexpression of these miRs, a fact that reflects the great number of genes involved in this disease.

In conclusion, carotid plaque regions exhibit a very

different miR expression profile from adjacent healthy ones and this seems to be an important aspect of plaque formation and progression. However, despite progress in the study of miRs and their role in cardiovascular diseases, more research is needed and many questions remain to be answered such as why miR overexpression is caused, how different miRs together affect carotid disease and how they could be used in the diagnosis and treatment of the disease.

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Authors' Contribution

Anna Sioziou and Maria Gazouli initiated the research program. Nikolaos Patelis, Dimitrios Athanasiadis and Christos Klonaris collect the samples and patients data. Anna Sioziou, Hector Katifelis, Evangelia Legaki and Maria Gazouli carried out the experiments and the statistical analyses. Maria Gazouli, Christos Klonaris and Theodoros Liakakos supervised the project. Anna Sioziou, Hector Katifelis and Maria Gazouli wrote the paper.

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