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Does MicroRNA Profile Differ in Early Onset Coronary Artery Disease?

Erken Başlangıçlı Koroner Arter Hastalığında MiRNA Profili Farklı mı?

ABSTRACT

Objective: MicroRNAs have been explored as potential biomarkers for many pathological processes including coronary artery disease. In this study, we aimed to compare the circulating levels of selected atherosclerosis-associated miRNAs in patients with a history of early-onset coronary artery disease with that of age- and sex-matched healthy controls and older patients with late-onset coronary artery disease.

Methods: Study population consisted of 30 patients with early onset coronary artery disease, 31 age- and sex-matched healthy controls, and 30 patients with late-onset coronary artery disease. Plasma levels of 13 microRNAs (endothelial cell-related miR-126, -92a/b; vascular smooth muscle cell-related miR-145; inflammation-related miR-16, -21, -125b, -146a/b, -147b, -150, -155; lipometabolism-related miR-27b, -122, -370) were evaluated by using real-time polymerase chain reaction.

Results: In patients with early onset coronary artery disease, plasma expressions of the lipometabolism-related miR-27b, miR-122; inflammation-related miR-125b, miR-146a/b, miR-147b, miR-150, miR-155; and VSMC-related miR-145 were significantly downregulated and endothelial cell-related miR-126 was significantly upregulated compared to age- and sex-matched healthy controls. Circulating microRNA profile of patients with early onset coronary artery disease was also different from that of older patients with late-onset coronary artery disease. Plasma levels of miR-21, miR-27b, miR-122, miR-125b, miR-146b, miR-147b, and miR-155 were lower and plasma levels of miR-16 and miR-92a were higher in patients with early onset coronary artery disease compared to older patients with late-onset coronary artery disease.

Conclusion: MicroRNAs are promising biomarkers for early onset coronary artery disease.

Keywords: Biomarkers, coronary artery disease, early onset coronary artery disease, microRNA

ÖZET

Amaç: MikroRNA'lar (MiRNA), koroner arter hastalığını (KAH) da içeren pek çok patolojik süreçte potansiyel biyobelirteçler olarak araştırılmaktadır. Bu çalışmada, erken başlangıçlı KAH öyküsü olan hastalarda, aterosklerozla ilişkili seçilmiş MiRNA'ların plasma düzeylerini, bu hasta grubuna yaş ve cinsiyet olarak eşleştirilmiş sağlıklı kontrol grubu ve geç başlangıçlı KAH'ı olan daha yaşlı hastalar ile karşılaştırmayı amaçladık.

Yöntemler: Çalışmaya erken başlangıçlı KAH'ı olan 30 hasta, bu hasta grubuna yaş ve cinsiyet açısından eşleştirilmiş 31 sağlıklı kontrol ve geç başlangıçlı KAH'ı olan 30 hasta dahil edildi. On üç MiRNA'nın (endotel hücre ilişkili miR-126, -92a/b; vasküler düz kas hücre ilişkili miR-145; enflamasyonla ilişkili miR-16, -21, -125b, -146a/b, -147b, -150, -155; lipo-metabolizma ile ilişkili miR-27b, -122, -370) dolaşımdaki düzeyleri real-time PCR yöntemi ile değerlendirildi.

Bulgular: Erken başlangıçlı KAH'ı olan hastalarda, yaş ve cinsiyet açısından eşleştirilmiş sağlıklı kontrol grubuna göre lipo-metabolizma ile ilişkili miR-27b, -122, enflamasyonla ilişkili miR-125b, miR-146a/b, miR-147b, miR-150, miR-155 ve vasküler düz kas hücre ilişkili miR-145'in plasma düzeyleri anlamlı olarak daha düşük, endotel hücre ilişkili miR-126'nın plasma düzeyi ise anlamlı olarak daha yüksekti. Erken başlangıçlı KAH'ı olan hastalarda, dolaşımdaki MiRNA profili geç başlangıçlı KAH'ı olan daha yaşlı hastalara göre de daha farklıydı. Erken başlangıçlı KAH'ı olan hastalarda, geç başlangıçlı KAH'ı olan daha yaşlı hastalara göre miR-21, miR-27b, miR-122, miR-125b, miR-146b, miR-147b ve miR-155'in plasma düzeyleri daha düşük, miR-16 and miR-92a'nın plasma düzeyleri ise daha yüksekti.

Sonuç: MikroRNA'lar erken başlangıçlı KAH için ümit vaat eden biyobelirteçlerdir.

Anahtar Kelimeler: Biyobelirteçler, erken başlangıçlı koroner arter hastalığı, koroner arter hastalığı, mikroRNA

ORIGINAL ARTICLE
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MicroRNAs (miRNAs) are a class of short, non-coding, single-stranded RNA molecules, approximately 22 nucleotides in length, that mostly negatively regulate gene expression at the post-transcriptional level.[1] They bind sequences in the target messenger RNA 3'-untranslated regions through complementarity and form RNA–RNA complex, leading to mRNA degradation or translational inhibition. By influencing protein translation, miRNAs have emerged as powerful regulators of a wide range of biological processes.[2] Since, microRNAs are remarkably stable and readily detectable in blood, circulating miRNAs have been explored as potential biomarkers for many pathological processes including cardiovascular diseases.[3]

Coronary artery disease (CAD) is the leading cause of death in the world.[4] Multiple risk factors for CAD have been identified, including modifiable factors, such as hypertension, hypercholesterolemia, obesity, smoking, and diabetes (DM) and non-modifiable factors, such as family history, sex, and age.[5] Coronary artery disease is highly heritable (estimated 57% heritability), especially when of early onset.[6] There is scarce information about the role of miRNAs, which regulate gene expression at the post-transcriptional level, on the development of premature CAD. In this study, we aimed to investigate the plasma levels of selected miRNAs that are involved in atherosclerosis and vascular inflammation, in the serum of patients with a history of early onset CAD as compared to that of age- and sex-matched healthy control subjects and older patients with late-onset CAD.

Methods

In this cross-sectional single-center study, 30 consecutive patients with early onset CAD (onset of disease <40 years old), 31 age- and sex-matched healthy controls, and 30 patients with late-onset CAD (onset of disease >55 years old in males and >65 years old in females) were enrolled in the study. All patients included in this study had an angiographic documentation of CAD defined as ≥70% stenosis in ≥1 coronary artery. Patients with a history of recent (within last 4 weeks) myocardial infarction (MI), unstable CAD, heart failure, impaired left ventricular ejection fraction (<45%), DM, rheumatologic disease, malignancy, severe renal or hepatic insufficiency were excluded. The control group were selected from healthy volunteers with normal electrocardiogram with no evidence of cardiovascular disease that is without a history of angina pectoris, myocardial infarction, family history of early onset CAD and without symptoms.

The data about demographic characteristics, lifestyle habits (cigarette smoking, regular physical activity), anthropometric data, history of hypertension, hyperlipidemia, pharmacological therapies, and echocardiographic were collected. Regular physical activity was defined as physical activity ≥30 minutes/day at least 3 times a week. A fasting blood sample was obtained from all participants to determine plasma glucose, serum total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol, low-density lipoprotein cholesterol (LDL-C), urea, creatinine levels, and miRNA analysis.

ABBREVIATIONS

CAD	Coronary artery disease
Ct	Threshold cycle
DM	Diabetes
LDL-C	Lipoprotein cholesterol
MI	Myocardial infarction
miRNAs	MicroRNAs
PCR	Polymerase chain reaction
RT	Real-time
TC	Total cholesterol
TG	Triglyceride
VSMC	Vascular smooth muscle cell

RNA Preparation and Real-Time Polymerase Chain Reaction

Real-time (RT) polymerase chain reaction (PCR) was used to detect and quantify the miRNA expression in the samples. RNA was extracted from the 9 mL plasma samples using miRCURY RNA Isolation Kit enrichment of miRNAs in accordance with the manufacturer's instructions. TaqMan® microRNA assay quantification was performed using 2-step RT-PCR. In reverse transcription process, cDNA was obtained from total RNA samples by TaqMan® MicroRNA Reverse Transcription Kit using specific miRNA primers. In the second step, PCR products were amplified using TaqMan® MicroRNA Assay and TaqMan® Universal PCR Master Mix by LightCycler 480 (Roche, Mannheim, Germany). The expressions of 13 miRNAs (endothelial cell-related miR-126, -92a/b; vascular smooth muscle cell (VSMC)-related miR-145; inflammation-related miR-16, -21, -125b, -146a/b, -147b, -150, -155; lipometabolism-related miR-27b, -122, -370) were evaluated. The u6-snRNA was used as a control to normalize differences in total RNA levels in each sample. The relative amount of each miRNA to u6-snRNA was expressed using equation $2^{-\Delta\Delta Ct}$, where ΔΔCt = (Ct miRNA – Ct U6). The value of each control sample was set at 1 and was used to calculate the fold change in targets.

The study was approved by the İzmir Katip Çelebi University Faculty of Medicine (No: 65), and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

Statistical Analysis

For the statistical analysis, miRNA expressions were calculated by using RT2 Profiler PCR Array Software (SABiosciences http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php) and a *P* value <.05 was considered statistically significant. The data were normalized by geometric mean to the U6 snRNA expression and a threshold cycle (Ct) cut-off was set at 35 cycles. MicroRNA expression interpreted from Ct of each miRNA was normalized to Ct of U6 snRNA (ΔCt = Ct miRNA – Ct U6 snRNA). The actual power of the study was calculated using G* Power 3.1.9.2 software as 0.95 with an effect size of 0.473 (total sample size = 60) and alpha error of 0.05.

Demographic and clinical variables were compared between groups using chi-square test or Fisher's exact test for categorical variables. For continuous variables, distributions of the variables were determined by the Kolmogorov–Smirnov test and group differences were examined by *t*-test or by Mann–Whitney *U* test, as appropriate.

Results

Baseline demographic and clinical characteristics of the patients with early and late-onset CAD and the healthy control group are given in Table 1. The mean age of the patients with early onset

Table 1. Demographic and Clinical Characteristics of the Study Participants

	Age- and Sex-Matched Controls (n=31)	Patients with Early Onset CAD (n=30)	Patients with Late-Onset CAD (n=30)	*P*[1]	*P*[2]
Men (%)	87	87	90	1.00	1.00
Age (years)	35.0 ± 4.4	37.0 ± 4.1	64.9 ± 7.1	.06	<.01
Age at first coronary event	-	35.3 ± 4.2	61.9 ± 6.8	-	<.01
BMI (kg/m^2)	25.9 ± 3.6	26.8 ± 3.6	28.4 ± 3.5	.30	.06
Current smoker (%)	42	47	27	.71	.13
Regular physical activity (%)	47	40	34	.15	.66
Hypertension (%)	0	27	47	.002	.11
TK (mg/dL)	192 ± 46	188 ± 40	201 ± 55	.67	.30
LDL-C (mg/dL)	120 ± 38	116 ± 35	128 ± 46	.32	.31
HDL-C (mg/dL)	46 ± 11	38 ± 9	40 ± 9	.002	.34
TG (mg/dL)	135 ± 75	172 ± 62	161 ± 70	.02	.52
Familial hypercholesterolemia (%)	0	10	0		
Single vessel disease (%)		67	40		.04
Multivessel disease (%)		33	60		
Previous MI (%)	-	90	67	-	.02
STEMI		67	33		
NSTEMI		23	34		
Previous PCI (%)	-	97	73	-	.01
Previous CABG (%)	-	7	26	-	.06
EF (%)	-	57 ± 9	60 ± 8	-	.10
Medications (%)				-	
ASA	-	97	93		.53
Clopidogrel	-	63	37		.05
Statin therapy	-	63	60		.71
Beta blocker	-	87	67		.07
ACE inhibitor	-	47	47		.88
ARB	-	7	20		.11
CCB	-	13	20		.45
Nitrates	-	10	43		.006

P[1] value for the comparison of the patients with early onset CAD and control subjects; P[2] value for the comparison of the patients with early onset CAD and late-onset CAD.
Continuous variables are presented as mean ± standard deviation.
ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blocker; ASA, acetylsalicylic acid; BMI, body mass index; CABG, coronary artery bypass grafting; CAD, coronary artery disease; CCB, calcium channel blocker; EF, ejection fraction; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; MI, myocardial infarction; NSTEMI, non-ST-segment elevation myocardial infarction; PCI, percutaneous coronary intervention; STEMI, ST-segment elevation myocardial infarction; TG, triglyceride; TK, total cholesterol.

CAD, late-onset CAD, and the control group was 37.0 ± 4.1 years, 64.9 ± 7.1 years, and 35.0 ± 4.4 years, respectively. The mean age at the onset of disease in patients with early and late-onset CAD was 35.3 ± 4.2 years and 61.9 ± 6.8 years, respectively. Eighty-seven percent of the patients with early onset CAD and the healthy control group and 90% of the patients with late-onset CAD were male.

The relative expression of plasma miRNAs in patients with early onset CAD compared to age- and sex-matched heathy controls and compared to patients with late onset CAD is presented in Figures 1 and 2. In patients with early onset CAD, plasma expressions of the miR-27b, miR-122, miR-125b, miR-145, miR-146a, miR-146b, miR-147b, miR-150, and miR-155 were significantly downregulated and miR-126 was significantly upregulated compared to age- and sex-matched healthy controls. Some of the miRNA expressions were also significantly different in patiets with early and late-onset CAD. Plasma levels of miR-21, miR-27b, miR-122, miR-125b, miR-146b, miR-147b, and miR-155 were significantly downregulated and plasma

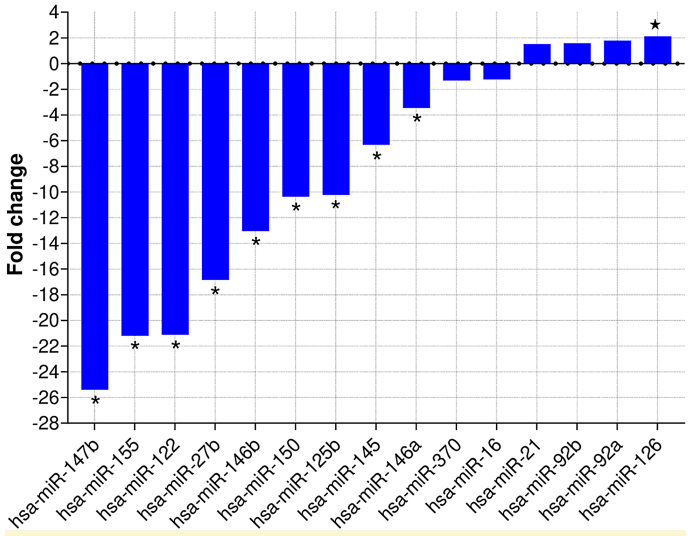

Figure 1. Relative expression of miRNAs in patients with early onset CAD compared to heathy controls. *$P < .05$.

levels of miR-16 and miR-92a were significantly upregulated in patients with early onset CAD compared to patients with late-onset CAD.

Discussion

The miRNAs have received increasing attention due to their central role in various biological processes. They play important role in the pathophysiology of atherosclerosis by regulation of endothelial function, communication between endothelial cells and vascular smooth muscle cells, differentiation of the vascular smooth muscle cells, and modulation of the inflammatory cells.[7] The present study demonstrates that circulating endothelial and inflammatory cell-derived and lipometabolism-related miRNAs are differently expressed in patients with early onset CAD compared to their age- and sex-matched healthy controls. This study also demonstrates that different plasma miRNA expression patterns exist in young patients with early onset CAD and older patients with late-onset CAD.

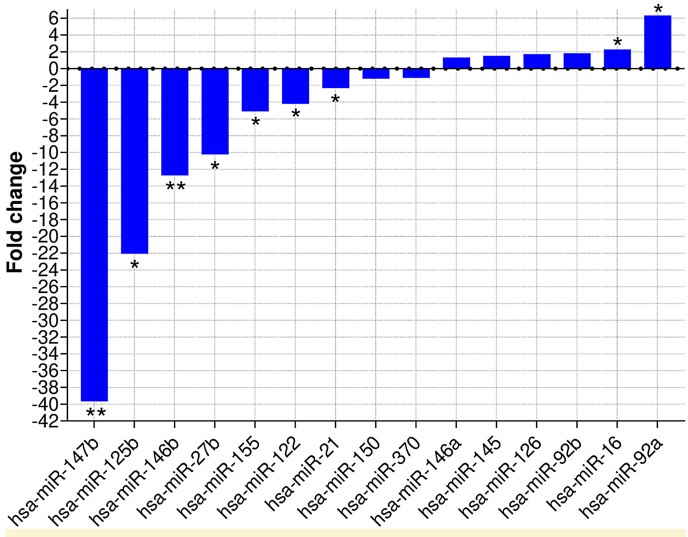

Figure 2. Relative expression of miRNAs in patients with early onset CAD compared to patients with late-onset CAD. *$P < .05$, **$P < .01$.

MiRNA-155 is a multifunctional miRNA that regulates multiple pathophysiological pathways including roles in hematopoietic cell differentiation, immunity, vascular remodeling, and inflammatory signaling pathways.[8] Studies have reported that miR-155 may have a preventive role in atherosclerosis development and progression.[9,10] Zhu et al[12] reported that miRNA-155 may be a part of a negative feedback loop in the atherosclerotic pathophysiological processes. Increased miRNA-155 expression in atherosclerotic lesions may reduce inflammation, while reduced expression causes atherosclerotic plaque instability and promotes atherogenesis.[9,10] Fichtlscherer et al[11] reported that circulating level of miR-155 was significantly downregulated in patients with CAD. Similarly, Zhu et al[12] reported that miRNA-155 expression was significantly lower in patients with CAD than those in controls. In line with these data, in the present study, patients with early onset CAD had miR-155 expression 21-fold lesser than age- and sex-matched healthy controls. Besides, plasma level of miR-155 in patients with early onset CAD was also significantly lower (−5 fold) than the patients with late-onset CAD. Therefore, miR-155 may be a good biomarker for premature CAD in young non-diabetic subjects.

MiR-126 is highly expressed in endothelial cells and hematopoietic cell lineages, and it has been shown to play an important role in angiogenesis and vascular integrity.[13,14] Under normal homeovascular conditions, miR-126 has atheroprotective properties suppressing the inflammatory cascade and mediating leukocyte adherence in atherosclerosis.[14,15] There are studies reporting that plasma miR-126 level is downregulated in patients with CAD.[11,16] However, there are also studies reporting contradictory results. In the study by Sun X et al[17] miR-126 was not significantly up or downregulated in patients with CAD, but miR-126 was inversely correlated with LDL cholesterol level. Gao J et al[18] reported that plasma miR-126 level increased and correlated with the presence and severity of cerebral atherosclerosis. Likewise, in our study, plasma miR-126 expression was increased (2-fold higher) in patients with early onset CAD compared with healthy controls, whereas miR-126 expression was similar in patients with early or late-onset CAD. The discrepancies between studies with respect to miR-126 regulation in CAD may be due to several factors. First, miR-126 regulation might be changing depending on the stage of atherosclerosis. In the second place, the differences in patients' characteristics, race, and ethnicity may have led to the discrepancies between studies with respect to miR-126 regulation in CAD. In our study, we excluded diabetic patients in order to exclude inflammatory disorders that may have an impact on miRNA profile. On the other hand, most of the mentioned studies above included diabetic patients. In a large population-based study, it has been shown that reduction in plasma level of miR-126 was consistently associated with DM and the miR-126 content in endothelial apoptotic bodies was reduced in a glucose-dependent fashion.[19] Since DM is a major risk factor for CAD, and its prevalence is high in patients with CAD, the presence of DM may be a confounding factor for plasma miR-126 expression in patients with CAD. Additionally, platelets are an important source of circulating miR-126 and it

has been reported that the usage of antiplatelet drugs may have an effect on the expression of miR-126.[20] Discrepancies between antiplatelet drug usage in the studies might also be a confounding factor for different miR-126 expression patterns observed in different studies.

MiR-122 and miR-370 have been found to play a crucial role in lipid metabolism.[21,22] It has been shown that miR-122 and miR-370 are over-expressed in the livers of hyperlipidemia animals. MiR-122 has been highlighted as a promising target for lowering plasma cholesterol. Inhibition of miR-122 expression by antisense oligonucleotides in mice resulted in increased oxidation of fatty acids in the liver and reduced cholesterol synthesis.[22] Gao et al[21] reported that plasma levels of lipometabolism-related miR-122 and miR-370 were significantly increased in patients with hyperlipidemia compared with controls. The levels of miR-122 and miR-370 were positively correlated with TC, TG, and LDL-C levels in both patients with hyperlipidemia and controls. On the other hand, statin-treated patients had lower circulating miR-122 and miR-370 levels than statin-free subjects. Wang et al[23] reported that patients with CAD had higher miR-122 expression than the control group, and serum level of miR-122 was associated with coronary atherosclerotic lesion severity. In this study by Wang et al.[23] patients with CAD had significantly higher TK, LDL-C, and TG levels than controls, whereas statin usage was similar between patients and controls. On the other hand, D'Alessandra et al[24] investigated the plasma miRNA levels in patients with acute myocardial infarction compared to healthy controls. MiR-122 plasma levels at 5 and 30 days after MI were persistently lower in healthy subjects. In the present study, miR-122 was significantly downregulated in patients with early onset CAD compared to healthy controls (21-fold) and also compared to patients with late-onset CAD (4-fold). On the other hand, miR-370 levels were similar between groups. Contradictory results with regard to association of miR-122, miR-370, and CAD might be related to differences in lipid profiles of the study participants, statin usage, clinical stage of atherosclerosis, race, and ethnicity in different studies. In our study, plasma TK and LDL-C levels of the 3 groups were similar. However, 63% of the patients with early onset CAD and 60% of the late-onset CAD patients were on statin therapy, while none of the control subjects were using statin. According to our results, miR-122 might be a biomarker for premature CAD in young adults without DM. However, statin usage might have influenced our results. So further studies are needed to investigate the role of miR-122 as a potential diagnostic marker for early onset CAD.

MiR-145 has a pivotal role in VSMC differentiation and phenotype switching.[25] It has been shown that miR-145 is upregulated in endothelial cells in response to shear stress and subsequently is exported in exosome-like vesicles that regulate VSMC phenotype.[26] Therefore, miR-145 may function as a signaling molecule that communicates between endothelial cells and VSMCs. The miR-145 cluster has been found to be downregulated in the proliferative VSMCs of atherosclerotic arteries in ApoE-knockout mice which suggests that downregulation of miR-145 may contribute to atherogenesis.[27] Fichtlscherer et al[11] reported that mir-145 was downregulated in patients with stable CAD. We also found that miR-145 was significantly downregulated in patients with early onset CAD compared to healthy controls; while patients with early and late-onset CAD had similar miR-145 levels.

MiR-146 family (miR-146a/b) regulates cytokine signaling through a negative-feedback regulation loop.[28] It has been shown that the enhancement of miR-146a in monocytes and macrophages by cellular apoE suppresses NF-κB (nuclear factor κB)-mediated inflammation and atherosclerosis.[29] Additionally, in endothelial cells, miR-146a and miR-146b are induced upon exposure to pro-inflammatory cytokines and repress the inflammatory signaling by suppressing the activation of pro-inflammatory transcriptional programs.[30] It is reported that the overexpression of miR-146a can delay oxidized low-density lipoprotein accumulation by inhibiting the activation of toll-like receptor 4-dependent signaling pathway.[31] However, there are very limited clinical data about circulating miR-146 level in patients with CAD. Pereira-da-Silva et al[32] found that miR-146 was downregulated in patients with atherosclerotic disease compared to controls and they found an association between the plasma miR-146 expression and severity of the atherosclerotic disease. Plasma miR-146 level was lower as the severity of coronary, lower extremity, and carotid atherosclerosis increased. On the other hand, in the study by Takahashi Y et al.[33] miR-146a/b and the inflammatory cytokines were markedly upregulated in CAD patients which seems to be contradictory to the data of the experimental studies. In the present study, mir-146a/b was significantly downregulated in patients with early onset CAD compared to healthy controls. Additionally, patients with early onset CAD had lower miR-146b levels compared to patients with late-onset CAD.

In experimental studies, miR-27b has been shown to have an antiatherogenic role by suppressing lipoprotein lipase-induced lipid accumulation and reducing inflammatory response in mice.[34] MiR-27 plays important role in lipid metabolism and has been reported to regulate the expression of key lipid-metabolism genes.[35] Data on circulating miR-27b expression in patients with atherosclerotic vascular disease are very limited. Pereira-da-Silva et al[32] reported that miR-27b expression was downregulated in the presence of atherosclerotic vascular disease. Lower expression levels of atheroprotective miR-27b were associated with more severe atherosclerotic disease in the coronary, lower extremity, and carotid arteries. Stather et al[36] investigated the circulating miRNAs in patients with peripheral arterial disease and miR-27b was found to be downregulated in the presence of lower extremity atherosclerosis. In our study, miR-27b was significantly downregulated (17-fold change) in patients with early onset CAD compared to controls. Patients with early onset CAD had also lower circulating miR-27 (10-fold change) compared to patients with late-onset CAD.

MiR-147 regulates pro-inflammatory response of macrophages to multiple toll-like receptor ligands in a negative feedback loop, where it represses pro-inflammatory cytokine (i.e., tumor necrosis factor-alpha and interleukin-6) secretion preventing excessive inflammatory response.[37] Hoekstra et al[38] investigated circulating peripheral blood mononuclear cell miRNA profile of patients with CAD. MiR-147 was decreased to 4-fold in CAD patients compared to healthy controls which suggests that peripheral blood mononuclear cells of patients with CAD exhibit

a microRNA-driven change in their inflammatory capacity, shifting to a more pro-inflammatory phenotype. In our study, circulating miR-147 was also significantly downregulated in patients with early onset CAD compared to both healthy controls and patients with late-onset CAD.

Experimental studies have shown that MiR-150 regulates inflammatory processes by modulating T- and B-cell development and macrophage differentiation.[39] Saadatian et al[40] investigated the STAT1 expression, which is an essential player in regulating cytokine signaling, and its regulator, mir-150 expression, in peripheral blood mononuclear cells of patients with CAD and healthy controls. They found decreased expression of miR-150 in patients with CAD compared to healthy controls and the decreased expression of miR-150 was associated with increased STAT1 expression. Weber et al[41] also reported that plasma miR-150 expression was significantly reduced in patients with CAD compared to healthy subjects. In our study, circulating miR-150 was significantly lower in patients with early onset CAD as compared to healthy controls, and plasma miR-150 expression was similar in patients with early or late-onset CAD.

MiR-125b has been reported to have antifibrotic and cardioprotective properties against ischemia-reperfusion injury potentially by decreasing myocyte apoptosis.[42] It has also been reported that miR-125b derived from mesenchymal stem cells effectively reduced inflammation and blood lipid and relieved the symptoms of atherosclerosis in atherosclerotic model mice.[43] In our study, miR-125b was significantly downregulated in patients with early onset CAD compared to both healthy controls (10-fold) and older patients with late-onset CAD (22-fold). To the best of our knowledge, there are no clinical data on the association between circulating miR-125b and atherosclerotic CAD. According to our results, miR-125b might be a good biomarker for early onset CAD. However, further studies are required to demonstrate the role of miR-125b as a potential diagnostic marker for CAD in young adults.

Most of the clinical data on the association of circulating miRNAs and CAD comes from older age patient populations, since the prevalence of CAD is higher in older adults. There are few studies in the literature investing the circulating miRNA profiles in patients with early onset CAD. Brittan et al[44] isolated endothelial cells from the blood vessel wall of patients with premature coronary artery disease and healthy controls and expanded them in vitro for phenotypic and functional characterization and for the analysis of microRNA expression levels. They assessed the expression levels of miRs -10 a, -27b, -let7b, -126, and -181b that are related to endothelial function in the vessel wall endothelial cells and in the endothelial outgrowth cells. They showed that the expression of these microRNAs was reduced in vessel wall endothelial cells which might be associated with the impairment in endothelial cell function observed in this study. Tong et al[45] investigated the circulating miRNAS in young (<45 years old) patients with acute coronary syndrome. They reported significantly lower expressions of miR-134-5p, miR-15a-5p, and let-7i-5p in patients with STEMI, and higher expression of miR-183-5p in NSTEMI patients, compared to healthy controls. However, smooth muscle-enriched miR145; inflammation-associated miR-155, -146a/b, -147b, -150, -155; and endothelial-enriched miR-126 were absent in their list of significantly dysregulated plasma miRNAs. MicroRNA expressions have been reported to differentiate among different clinical presentations of CAD.[3,46] In our study, patients with recent MI or unstable angina pectoris were excluded which may explain the discrepancy in miRNA expressions in our study and the study by Tong et al.[45]

Age has been found to be a strong confounder on circulating miRNA patterns.[47] It has been reported that there is an age-related increase in the plasma miR-21 level in the 65-95 years of age healthy subjects compared to younger subjects.[48] MiR-92a has been found to be associated with vascular aging. MiR-92a has been found to be downregulated in older adults and experimental reductions of miR-92a in young mice led to impaired endothelium-dependent dilation and increased aortic stiffness.[49] However, there are limited data regarding the age-related discrepancies in circulating miRNA levels among patients with CAD. In this study, we evaluated not only the differences in circulating miRNA expressions between patients with early onset CAD and healthy controls but also the differences in miRNA expressions between young patients with early onset CAD and older patients with late-onset CAD. To the best of our knowledge, our study is the first study in the literature investigating circulating levels of selected miRNAs that are associated with atherosclerosis and vascular inflamation in the plasma of young patients with early onset (<40 years old) CAD compared to those of healthy controls and also to those of older patients with late-onset CAD. MicroRNA expression in young patients also revealed differences as compared to older patients. Mir-16 and miR-92a were significantly lower and miR-21, miR-27b, miR-122, 125b, miR-146b, 147b, and mir-155 were significantly higher in older patients compared to patients with premature CAD. Discrepancies in miRNA expressions among young and old patients with CAD may indicate that different pathological processes occur in these patient populations. Hence, age should be taken into consideration when evaluating miRNAs as potential biomarkers for CAD.

There are some limitations of our study that must be considered. First, the number of study participants in each group was relatively small so further larger studies are needed to confirm our findings. Secondly, the usage of medications in the CAD patients might have influenced the results. Thirdly, diabetic patients were excluded in order to avoid a confounding factor for miRNA expression. Therefore, our observations may not be generalizable to young diabetic patients. Finally, we have missing data about the non-traditional risk factors for the development of CAD in the young population like drug abuse and thrombophilic disorders which might be related to dysregulation in miRNA expression. Future larger studies may be performed to investigate the relationship between MiRNA expression and non-traditional risk factors for the development of early onset CAD.

Conclusion

In conclusion, we found that miRNAs that are associated with the pathogenesis of CAD in experimental studies are dysregulated in patients with premature CAD compared to healthy subjects. Thus, miRNAs are promising biomarkers for early onset CAD. Additionally, miRNA expression patterns in young patients with

premature CAD also differ from that of older patients with late-onset CAD. Therefore, age should be considered a confounding factor in miRNA expression profile among patients with CAD.

Ethics Committee Approval: The study was approved by the medical ethics committee of İzmir Katip Çelebi University Faculty of Medicine (No: 65).

Informed Consent: Written informed consent was obtained from all participants who participated in this study.

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