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Differential MicroRNA Expression Profiles in Peripheral Arterial Disease

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Background—Peripheral arterial disease (PAD) is a clinical condition caused by an atherosclerotic process affecting the arteries of the limbs. Despite major improvements in surgical endovascular techniques, PAD is still associated with high mortality and morbidity. Recently, microRNAs (miRNAs), a class of short noncoding RNA controlling gene expression, have emerged as major regulators of multiple biological processes.

Methods and Results—A whole-miRNA transcriptome profiling was performed in peripheral blood from an initial sample set of patients and controls. A 12-miRNA PAD-specific signature, which includes let 7e, miR-15b, -16, -20b, -25, -26b, -27b, -28-5p, -126, -195, -335, and -363, was further investigated and validated in 2 additional sample sets. Each of these 12 miRNAs exhibited good diagnostic value as evidenced by receiver operating characteristic curve analyses. Pathway enrichment analysis using predicted and validated targets identified several signaling pathways relevant to vascular disorders. Several of these pathways, including cell adhesion molecules, were confirmed by quantifying the expression level of several candidate genes regulating the initial stages of the inflammatory atherosclerotic process. The expression level of 7 of these candidate genes exhibits striking inverse correlation with that of several, if not all, of the miRNAs of the PAD-specific miRNA signature.

Conclusions—These results demonstrate the potential of miRNAs for the diagnosis of PAD and provide further insight into the molecular mechanisms leading to the development of PAD, with the potential for future therapeutic targets. (*Circ Cardiovasc Genet.* 2013;6:490-497.)

Key Words: biological markers ■ microRNAs ■ peripheral artery disease ■ peripheral vascular diseases ■ vascular diseases

The prevalence of peripheral arterial disease (PAD) is reported to be 15% to 20% in people over the age of 70,^{1,2} with the prevalence of symptomatic intermittent claudication reaching 6% in people over the age of 60.³ Several risk factors have been associated with PAD, including ethnicity,⁴ male sex, age, smoking,⁵ diabetes mellitus,⁶ hypertension, hypercholesterolemia,⁷ and renal insufficiency.⁸ PAD is an atherosclerotic disease, which occurs through a complex process involving endothelial dysfunction, lipid disturbance, platelet activation, thrombosis, oxidative stress, vascular smooth muscle cell activation, altered matrix metabolism, remodeling, and inflammation.⁹ PAD is usually associated with atherosclerosis in the whole vasculature, including the coronary arteries, and is, therefore, regarded as a clinical manifestation of generalized atherosclerosis, a leading cause of mortality worldwide. Although there has been much research into the risk factors and mechanisms involved in atherosclerosis, the underlying pathophysiology remains unclear. Previous studies

have reported differentially regulated mRNAs in the peripheral mononuclear cells of patients with various atherosclerotic conditions.¹⁰⁻¹² However, the association of PAD with microRNAs (miRNA) is yet to be examined.

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miRNAs are short (19–26 nucleotide) noncoding RNAs involved in the regulation of human gene expression by binding to mRNA and inhibiting protein synthesis.¹³ miRNA sequences seem to be highly conserved among plants, microorganisms, and animals, suggesting that they represent an important regulatory pathway.¹⁴ There are currently >1500 human miRNAs in the miRBase database,¹⁵ which may target ≈60% of human genes.¹⁶ Assessing miRNA expression in the circulation of patients with PAD is likely to yield potential biomarkers for both diagnosis of PAD and prediction of future PAD risk, as well as potential treatment pathways for PAD in the future. Some studies have initiated

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the analysis of miRNAs in plasma from patients with vascular disease.^{17–19} However, the use of plasma miRNAs in gene expression studies faces technical limitations because of the extremely low concentrations of miRNAs freely circulating in plasma. Also, the absence of ribosomal RNAs in these samples makes the assessment of the RNA quality difficult. This is an important factor because accurate gene expression analyses are sensitive to the homogeneity of the samples to be compared. This is also a drawback to standardize and translate the use of plasma miRNAs in a clinical context where samples from various origins and qualities will have to be processed. To circumvent that problem, we hypothesized that miRNAs from peripheral blood could be used. Previous studies have reported specific mRNA expression signatures in peripheral blood from patients with coronary artery disease compared with healthy individuals.^{20,21} Furthermore, peripheral blood contains peripheral blood mononuclear cells, which comprise immune cells such as lymphocytes and monocytes. Immune cells are key components of the inflammatory response. They are in close contact with the atherosclerotic plaque and are involved in the first stages of the atherosclerotic process by expressing cell surface adhesion molecules.²² Recently, Hoekstra et al²³ have reported the dysregulation of several miRNAs in the peripheral blood mononuclear cells from patients with coronary artery disease.

In this study, we aimed to determine whether circulating miRNAs are differentially expressed in patients with PAD. This is the first human miRNome (miRNA transcriptome-wide) research in patients with PAD.

Materials and Methods

Sample Collection

The initial sample set consisted of 5 patients with PAD and 6 control individuals. Two validation sample sets consisting of 10 patients with PAD and 10 controls each were subsequently recruited to confirm results obtained in the initial sample set. Control samples were obtained from men attending a local vascular screening program. Control samples were obtained from men with no evidence of aortic atherosclerosis and no symptoms of intermittent claudication. Cases were obtained from vascular surgery clinics. Cases were selected based on both symptomatic and radiological evidence of PAD in the legs, including symptoms of intermittent claudication and with Trans-Atlantic Inter-Society Classification II³ type B or type C lesions seen on imaging. Patients with chest pain, ulcers, or necrotic tissue were excluded. All participants were white men. Patients with diabetes mellitus, systemic autoimmune or inflammatory conditions, infectious conditions, or malignancy were excluded. Ethical approval for this study was obtained from Leicestershire, Northamptonshire, and Rutland Research Ethics Committee (Ref: 6891). All participants provided written informed consent.

miRNA and mRNA Expression Profiling

Total RNA including the small RNA fraction was extracted using the Paxgene blood miRNA kit (PreAnalytiX Hombrechtikon, Switzerland; Method in the online-only Data Supplement). Whole-genome miRNA expression profiling was performed by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) using TaqMan Array Human MicroRNA A+B Cards Set v3.0 (Life Technologies Corporation, Foster City, CA), which allows quantification of 754 miRNAs. Reverse transcription of 150 ng total RNA using the Life technologies Megaplex RT Human Pool A and B (V2.1 and V2.0, respectively) followed by preamplification of samples using Megaplex PreAmp Primers Human Pool A and B were performed as described by the manufacturer's protocol. qRT-PCR was performed on an Applied Biosystems 7900HT

Fast Real-Time PCR System. Amplification data were analyzed using RQ Manager (Applied Biosystems, Foster City, CA). qRT-PCR curves for each miRNA were checked, with those miRNAs not amplified in all samples excluded (n=392). miRNAs with suboptimal amplification quantitative PCR curves (absence of clear S-shaped curve) were also excluded. Quantification of individual miRNAs was performed using TaqMan MicroRNA individual assays (Life Technologies Corporation, Foster City, CA) after reverse transcription and preamplification of all miRNAs with the Megaplex RT Primers and Megaplex PreAmp primers pools. MammU6 (mammalian spliceosomal RNA U6) and RNU48 (small nucleolar RNA, C/D box 48) were confirmed as stable reference genes using geNorm, and the geometric mean of their expression levels was used as normalization factor in all miRNA qRT-PCR data obtained in this study.²⁴ The list of miRNA individual assays is available as Table I in the online-only Data Supplement. For mRNA quantification, 150 ng total RNA was reverse transcribed using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, United Kingdom) according to the manufacturer's protocol. qRT-PCR was performed in a final volume of 5 μ L using 1 \times Maxima SYBR Green qPCR master mix (Thermo Scientific, United Kingdom) and 3.3 μ mol/L of forward and reverse primers for each mRNA tested. Quantitative PCR expression data were normalized using the geometric mean of *HPRT-1* (hypoxanthine phosphoribosyltransferase 1), *SDHA* (succinate dehydrogenase complex, subunit A), and *PGK1* (phosphoglycerate kinase 1) as normalization factors (Table II in the online-only Data Supplement).

Statistical Analysis

Background analysis was performed using Fisher exact test and Mann-Whitney *U* test. The difference in miRNA expression level between control and case groups was assessed by Mann-Whitney *U* test using IBM SPSS v.18 (PASW). Principal component analysis was performed using ArrayTrack.²⁵ Spearman rank correlation coefficients and receiver operating characteristic curves were generated using IBM SPSS v.18 (PASW). Identification of experimentally validated miRNA targets was performed using miRWalk²⁶ and miRTarBase²⁷ databases, 2 manually and regularly updated curated databases of miRNA targets. miRNA target prediction was performed with MiRWalk server that allows one to retrieve the genes predicted by ≤ 10 algorithms. Pathway enrichment analysis was performed with ArrayTrack using the Kyoto Encyclopedia of Genes and Genomes database. Pathways significantly enriched in the list of predicted targets were determined using a Fisher exact test at a $P < 0.001$. Empirical Bayes cross-experiment normalization²⁸ was performed using the web-based package ArrayMining.²⁹ Gene expression microarray data published by Masud et al¹² were retrieved from the Gene Expression Omnibus,³⁰ a public repository of gene expression data (<http://www.ncbi.nlm.nih.gov/geo/>). Differential expression of the genes of interest was determined using GEO2R, an interactive web-based tool using the GEOquery³¹ and limma R packages³² from the Bioconductor project, allowing analysis of high-throughput genomic data and available online (<http://www.ncbi.nlm.nih.gov/geo/geo2r/>).

Results

Background Analysis

To ensure that miRNA variations were not caused by individual patient comorbidities other than PAD or medication use, a background comparison was performed. This revealed no significant difference between controls and patients for most cardiovascular risk factors, including smoking history, history of myocardial infarction, hypertension, hypercholesterolemia, angina, cerebrovascular accident, chronic obstructive pulmonary disease, and all additional medications. However, we observed a significant increase in the use of aspirin in the PAD group in the initial analysis, which was not identified in either validation sample set. This was expected because aspirin is recommended for all patients with PAD. We also noted

a slight increase in age in the PAD group in the second validation sample set, which was not identified in the initial or first validation set (Table III in the online-only Data Supplement).

miRNA Expression Profiling in Patients With PAD

In a view to identify miRNAs differentially expressed in peripheral blood cells from patients with PAD, we applied a stepwise strategy. First, a whole-genome miRNA expression profiling was performed in an initial sample set. This allowed us to identify 362 miRNAs confidently detected in all samples. Using a cutoff $P < 0.1$, 53 miRNAs were found to be differentially expressed between the 2 groups (Table IV in the online-only Data Supplement), of which 34 were downregulated and 19 upregulated. Although the variability in expression within each group was large, principal component analysis demonstrated that these miRNAs can discriminate the 2 groups of samples with no ambiguity (Figure), thus suggesting that miRNAs are valid tools to classify patients with PAD from controls. Therefore, we ranked the 53 genes according to abundance to focus on the most expressed miRNAs (a cutoff Ct value < 25) and according to their significance score π^{33} (arbitrary π cutoff value > 0.5). The significance score combines the fold change and the P value from a hypothesis test under a unique score that allows better gene selection. In the obtained list, we added the miRNAs that were potentially relevant to atherosclerosis based on the literature. This selection allowed us to retrieve a subset of 14 miRNAs (Let 7e, miR-15b, -16, -20b, -25, -26b, -27b, -28-5p, -126, -195, -335, -363, -720, -1274), which we subjected to further analyses. To control for potential technical bias and to confirm the P values, we quantified the selected miRNAs in a second sample set, referred as first validation sample set, by

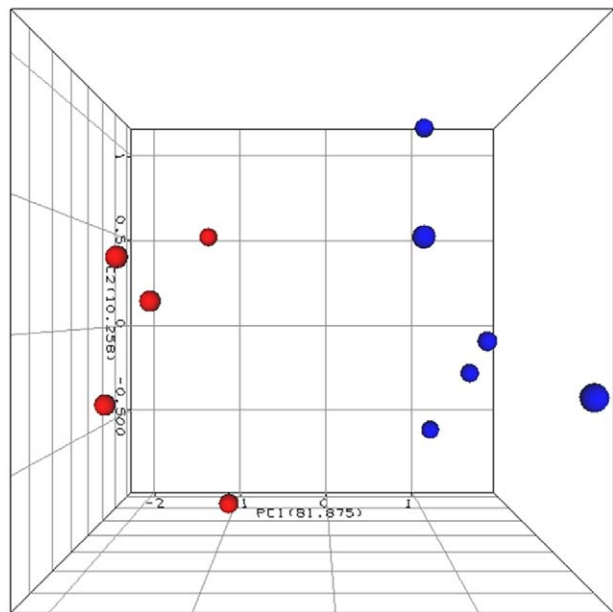


Figure. Principal component analysis of the 53 miRNAs differentially expressed in patients with peripheral arterial disease (PAD) compared with controls. Control samples are represented in blue. Patients with PAD are represented in red. The first principle component (PC1) axis accounts for 81.8% of the variance in the data set and segregates patients with PAD from controls. The second and third principle components (PC2 and PC2) account for 10.2% and 5.35% of the variance, respectively.

qRT-PCR using miRNA individual assays. Two miRNAs (miR-720 and miR-1274) did not yield any significant differential expression between patients with PAD and controls ($P > 0.05$) and were excluded from downstream analyses. The remaining significant miRNAs were again validated in a second validation sample set processed separately from the first one. Results showed that all 12 miRNAs (Let-7e, miR-15b, miR-16, miR-20b, miR-25, miR-26b, miR-27b, miR-28-5p, miR-126, miR-195, miR-335, and miR-363) remained differentially expressed between the 2 groups ($P < 0.05$). When combining the 3 sample sets, the Fisher-combined P value was < 0.001 , and the average fold change was > 1.5 for all miRNAs tested (Table 1).

Diagnostic Value of the miRNA Signature

In a view to ascertain whether the 12 miRNAs we identified here can be used as biomarkers for the diagnosis of PAD, we tested the performance of each individual miRNA in classifying PAD and healthy individuals. To do so, we combined the 2 validation sample sets and constructed receiver operating characteristic curves using the expression data. The analysis of each receiver operating characteristic curve revealed that miR-16, miR-363, and miR-15b had the best predictive value with an area under the curve > 0.92 ($P < 0.001$). Only 2 miRNAs (Let-7e and miR-20b) exhibited a poor discriminative value with an area under the curve < 0.75 (Table 2). Receiver operating characteristic curves for each individual miRNA are provided in Figure I in the online-only Data Supplement.

Pathway Enrichment Analysis

To gain further insight into the signaling pathways regulated by the PAD-specific miRNAs we identified, we explored miRWalk (release march 2011) and miRTarbase (v3.5) databases and retrieved the predicted and experimentally validated targets of these miRNAs. Altogether, we identified 892 experimentally validated and 7975 predicted targets. With regard to the predicted targets, only those genes predicted by ≥ 5 algorithms were selected. We considered this strategy as a good balance between the specificity and the sensitivity of our prediction. Further analyses showed that a prediction using > 5 algorithms generated a shorter gene list, but the percentage of validated targets correctly detected fell to $\approx 50\%$. Conversely, a prediction using < 5 algorithms slightly increased the detection of true targets (85%) but generated a considerably larger list that, therefore, increases the chance of predicting false-positives. We combined the validated and predicted target list and performed a pathway enrichment analysis using the Kyoto Encyclopedia of Genes and Genome database. Several pathways with a clear involvement in vascular physiology were found to be significantly enriched, such as the mitogen-activated protein kinase (MAPK) signaling pathway,³⁴ focal adhesion,³⁵ insulin signaling pathway,³⁶ adherens junctions,³⁷ transforming growth factor (TGF)- β signaling pathway,³⁸ and the Wnt signaling pathway³⁹ (Table V in the online-only Data Supplement). Not surprisingly, several pathways are involved in immune response and inflammation, such as the Toll-like receptor signaling pathway, NOD-like receptor signaling pathway, RIG-I-like receptor signaling pathway, and the chemokine signaling pathway (Kyoto Encyclopedia of Genes and Genome database).

Table 1. List of miRNAs Differentially Expressed in Patients With PAD Compared With Controls With the P Value and Fold Changes

	Initial Sample Set, PAD (n=5), Controls (n=6)		Validation Sample Set 1, PAD (n=10), Controls (n=10)		Validation Sample Set 2, PAD (n=10), Controls (n=10)		Combined (All 3 Sample Set)	
	P Value	Fold Change	P Value	Fold Change	P Value	Fold Change	P Value	Fold Change
hsa-let-7e	3.03×10 ⁻²	-1.84	4.90×10 ⁻²	-1.53	1.00×10 ⁻³	-2.46	1.45×10 ⁻⁴	-1.95
hsa-miR-15b	8.71×10 ⁻³	-2.27	4.00×10 ⁻³	-1.56	1.00×10 ⁻³	-3.51	7.10×10 ⁻⁶	-2.45
hsa-miR-16	4.31×10 ⁻³	-2.82	2.00×10 ⁻³	-2.88	1.00×10 ⁻³	-4.36	2.23×10 ⁻⁶	-3.35
hsa-miR-20b	3.03×10 ⁻²	-2.86	8.00×10 ⁻³	-2.07	2.80×10 ⁻²	-2.57	5.31×10 ⁻⁴	-2.50
hsa-miR-25	8.23×10 ⁻²	-1.52	5.90×10 ⁻²	-1.34	1.00×10 ⁻²	-1.64	2.50×10 ⁻³	-1.50
hsa-miR-26b	3.03×10 ⁻²	-2.57	1.30×10 ⁻²	-2.17	1.00×10 ⁻³	-6.44	4.58×10 ⁻⁵	-3.73
hsa-miR-27b	4.31×10 ⁻³	-2.87	8.00×10 ⁻³	-2.83	3.00×10 ⁻³	-4.21	2.03×10 ⁻⁵	-3.30
hsa-miR-28-5p	5.19×10 ⁻²	-1.44	1.90×10 ⁻²	-1.78	3.40×10 ⁻²	-1.91	1.92×10 ⁻³	-1.71
hsa-miR-126	8.71×10 ⁻³	-3.25	1.30×10 ⁻²	-3.61	2.00×10 ⁻³	-4.32	3.71×10 ⁻⁵	-3.73
hsa-miR-195	4.31×10 ⁻³	-3.15	7.00×10 ⁻³	-2.05	1.00×10 ⁻³	-3.71	6.81×10 ⁻⁶	-2.97
hsa-miR-335	1.73×10 ⁻²	-2.93	3.40×10 ⁻²	-2.08	1.00×10 ⁻³	-2.81	7.20×10 ⁻⁵	-2.61
hsa-miR-363	4.31×10 ⁻³	-3.31	1.30×10 ⁻²	-1.91	1.00×10 ⁻⁵	-5.40	1.89×10 ⁻⁷	-3.54
hsa-miR-720	4.31×10 ⁻³	+6.05	5.97×10 ⁻¹	-1.41	Not tested		N/A	
hsa-miR-1274A	8.71×10 ⁻³	+3.45	7.62×10 ⁻¹	+1.05	Not tested		N/A	

The miRNAs upregulated and downregulated in patients with PAD compared with controls are indicated by + and -, respectively (for full list of differentially expressed miRNAs in the initial sample set, see Table IV in the online-only Data Supplement). miRNA indicates microRNA; N/A, not applicable; and PAD, peripheral arterial disease.

Quantification of the mRNA Targets

We then aimed to validate the pathway enrichment analyses and gained further insight into the molecular mechanisms of this PAD-specific miRNA signature. In the combined list of validated and predicted targets, we explored the genes expressed by immune cells and potentially involved in the atherosclerotic process. The vascular endothelial growth factor A (*VEGFA*) and the TGF- β receptor II (*TGFBR2*) were of particular interest because they are relevant to vascular pathophysiology. Data from the miRWalk and miRTarBase databases demonstrate that *VEGFA* is regulated by 6 of the miRNAs we identified (miR-let7e, miR15b, miR-16,

miR-20b, miR-126, and miR-195) and *TGFBR2* by 1 miRNA (miR-126). *VEGFA* and *TGFBR2* are associated with several of the pathways enriched in the target list, including the MAPK and the TGF- β signaling pathways (Tables V and VI in the online-only Data Supplement). In addition to these genes, we also investigated the genes expressed in leukocytes and encoding the proteins controlling the rolling and adhesion of immune cells onto the endothelium of vessels or involved in their transmigration through this endothelium. This leukocyte adhesion cascade is well known as the key initial stages of the atherosclerotic process.^{22,40} Furthermore, the cell adhesion molecule signaling pathway is one of the pathways we found to be significantly enriched in the list of target genes (Tables V and VI in the online-only Data Supplement). Based on a bibliographic search, we identified 14 candidate genes. These genes are all predicted or validated targets of miRNAs of the PAD-specific miRNA signature we identified. We reasoned that the molecular consequences of the miRNA dysregulation observed in patients with PAD could be mediated, at least in part, by these candidate genes. Therefore, we quantified their expression levels in peripheral blood cells from 10 patients with PAD and 10 controls selected in our 2 validation sample sets using qRT-PCR. The full list of selected genes and the oligonucleotides used are listed in Table II in the online-only Data Supplement. Two genes, *JAM2* (junctional adhesion molecule 2) and *VCAM1* (vascular cell adhesion molecule 1), were considered but were excluded from downstream analyses because they could not be confidently detected in our samples. Interestingly, we found that *VEGFA*, *TGFBR2* plus 5 of the candidate genes selected, intercellular adhesion molecule 1 (*ICAM1*), *F11R* (Junctional adhesion molecule A), *ITGA6* (integrin, alpha 6), nuclear factor of κ B1, and *SELPLG* (selectin P ligand), were significantly upregulated in patients with PAD compared with controls ($P < 0.02$;

Table 2. ROC Curve Analysis of the miRNAs of the PAD-Specific miRNA Signature

miRNAs	Area Under the Curve	SE	P Value	95% Confidence Interval	
let-7e	0.71	0.08	0.02	0.55	0.88
miR-15b	0.92	0.05	<0.001	0.82	1.00
miR-16	0.93	0.04	<0.001	0.86	1.00
miR-20b	0.69	0.08	0.04	0.52	0.86
miR-25	0.75	0.08	0.01	0.59	0.91
miR-26b	0.77	0.08	<0.001	0.62	0.93
miR-27b	0.87	0.06	<0.001	0.75	0.99
miR-28-5p	0.86	0.06	<0.001	0.75	0.97
miR-126	0.88	0.05	<0.001	0.77	0.98
miR-195	0.89	0.06	<0.001	0.79	1.00
miR-335	0.76	0.08	<0.001	0.61	0.91
miR-363	0.93	0.04	<0.001	0.85	1.00

miR-15b, -16, and -363 have the best discriminative value with an area under the curve >0.93. miRNA indicates microRNA; PAD, peripheral arterial disease; and ROC, receiver operating characteristic.

Figure II in the online-only Data Supplement). Masud et al¹² performed a whole-genome mRNA expression profiling in peripheral blood mononuclear cells from patients with PAD using microarrays. To confirm our results, we retrieved their data from the Gene Expression Omnibus (GSE27034), and we compared the expression of these candidate genes between patients with PAD and controls using the GEO2R interactive web tool. Several probes corresponding to *SELPLG* (209880_s_at), *ICAM1* (202637_s_at, 215485_s_at), *VEGFA* (211527_x_at, 212171_x_at, 210513_s_at, 210512_s_at), and *F11R* (224097_s_at) mRNAs also exhibited significant upregulation in patients with PAD, which is consistent with our findings (Figure II in the online-only Data Supplement). These results shed light into the mechanisms of action of the miRNAs identified in patients with PAD.

Correlation of miRNA–mRNA Expression

The interactions between the candidate genes selected and the PAD-specific miRNAs were initially retrieved using a list of both predicted and validated targets (Table 3). To confirm all these interactions, we computed the Spearman correlations between the expression level of these genes and that of each miRNA of the PAD-specific miRNA signature. Theoretically, if an mRNA–miRNA interaction exists, the expression of the mRNA is expected to be upregulated when the miRNA is downregulated and vice versa. Strikingly, we found that the expression of the candidate genes upregulated in patients with PAD is inversely correlated with several, if not all the expression of the PAD-specific miRNAs. For instance, *TGFBR2*, nuclear factor of κ B1, *ITGA6*, and *SELPLG* mRNA expression levels are inversely correlated with the expression of 12, 10, 10, and 9 of these miRNAs, respectively (P values ranging from $5.10E-2$ to $5.26E-6$; Table 3). *VEGFA*, *F11R*, and *ICAM1* mRNA expression is inversely correlated with 9, 6, and 5 miRNAs, respectively ($P < 0.05$; Table 3). Conversely, no correlation was observed with the genes not differentially expressed. Altogether, these results show that the miRNAs dysregulated in peripheral blood cells from patients with PAD are implicated in PAD physiopathology by controlling the expression of the genes involved in the interactions of leukocytes with blood vessel walls. Furthermore, we found a significant inverse correlation between miR-335 and *ESAM* (endothelial cell adhesion molecule) (Spearman coefficient = -0.51 ; $P = 2.55E-2$), *ITGB2* (Spearman coefficient = -0.58 ; $P = 9.13E-3$), *JAM3* (Spearman coefficient = -0.47 ; $P = 4.5E-2$), and *SELP* (Spearman coefficient = -0.54 ; $P = 1.78E-2$) mRNA expressions, which also suggest that miR-335 likely regulates these genes. However, this will have to be confirmed experimentally. The interactions between miR-335 and *ITGB2* were computationally predicted but have not been confirmed before. Conversely, we did not find any significant correlation between the expression of *ESAM* mRNA and miR-15b, -16, and -195 and between the expression of *JAM3* mRNA and miR-15b, -16, -20b, -27b, and -28-5p. This suggests that no interaction occurs between these mRNAs and miRNAs in peripheral blood cells, although they were computationally predicted.

Discussion

The pathophysiological mechanisms underlying PAD is complex and currently not fully understood. Identification of the

molecular causes leading to the development of PAD is crucial to permit new therapeutic approaches. Furthermore, there is a need for minimally invasive biomarkers for the diagnosis of PAD, the early detection of patients at risk, and the management of disease progression. In this study, we performed a miRNA transcriptome profiling in peripheral blood cells from patients with PAD compared with controls. We identified a 12-miRNA signature specific to PAD, which we further validated in 2 additional sample sets processed independently. We then demonstrated the potential of each miRNA of this signature in discriminating patients with PAD from healthy individuals, and we showed that these miRNAs are involved in the regulation of key genes responsible for initiating the atherosclerotic process.

This is the first time that a miRNA signature is identified in patients with PAD. This finding is of importance because blood miRNAs can easily be monitored. Therefore, the miRNAs identified here (Table 1) potentially represent a convenient and minimally invasive tool for the diagnosis of PAD and patient stratification and may complement existing methods. The diagnosis of PAD is usually performed using ankle brachial index. Ankle brachial index is easy to implement in primary care practice. The sensitivity and specificity have been shown to be good in detecting stenosis $>50\%$. However, ankle brachial index is less accurate to detect mild PAD. Also, the test may not be accurate in case of arterial calcification, which is common in patients with long-standing diabetes mellitus or kidney disease, or some elderly patients. A significant proportion of patients also presents with atypical symptomatology. In all these cases, the circulating miRNAs identified here could prove to be useful individually or in combination to complement ankle brachial index and to refine the diagnosis of PAD. Also, predicting the level and progression of atherosclerosis and the risk of secondary cardiovascular events such as stroke, myocardial infarction, or claudication is also of importance in clinical practice to stratify patients with generalized atherosclerosis and improve disease management. Index scores or prediction models incorporating these miRNAs will now have to be tested. The confirmation of the dysregulation of some of the mRNAs targeted by this miRNA signature is also of importance as this sheds light into the mechanisms of action of these miRNAs and the underlying causes of the disease. Among the genes controlled by this miRNA signature, we chose to focus on the genes involved in the rolling and adhesion of immune cells as these genes have been widely described as key factors of the first stages of the atherosclerotic process and are appealing therapeutic targets. We found P-selectin glycoprotein ligand (*SELPG*), *ICAM1*, *ITGA6*, (*F11R*), and nuclear factor of κ B to be upregulated in patients with PAD, and we showed that these mRNAs are targeted by the PAD-specific miRNAs. *SELPG* binds to E-, L-, and P-selectins. This is responsible for leukocyte rolling. In addition, *ICAM1* binds to leukocyte integrins, such as *ITGA6*, and participates in stopping leukocyte rolling and promotes leukocyte transendothelial migration, along with *JAM* (*F11R*). Nuclear factor of κ B is involved in inflammatory diseases and is associated with the upregulation of these adhesion molecules.^{40,41} Our results suggest that these

Table 3. List of the Candidate Genes Quantified in Controls and Patients With PAD With Correlation Analysis

Candidate Genes	Regulation in PAD	Interaction Candidate Genes:		Correlation Between the Candidate Genes and the PAD-Specific miRNAs													
		Predicted MIRNAs	Validated MIRNAs	let-7e	miR-15b	miR-16	miR-20b	miR-25	miR-26b	miR-27b	miR-28-5p	miR1-26	miR-195	miR-335	miR-363		
F11R (F11 receptor, JAMA)	Up	miR-26b, miR-27b, miR-25, miR-335, miR-20b, miR-16, let-7e, miR-28-5p, miR-15b, miR-363, miR-195	None	-0.38	-0.60	-0.44	-0.36	-0.35	-0.47	-0.48	-0.45	-0.34	-0.50	-0.74	-0.42		
				P-value	1.08×10^{-1}	$7.24 \times 10^{-3*}$	5.92×10^{-2}	1.35×10^{-1}	1.41×10^{-1}	$4.49 \times 10^{-2*}$	$3.88 \times 10^{-2*}$	1.61×10^{-1}	$2.93 \times 10^{-2*}$	$3.36 \times 10^{-4*}$	7.66×10^{-2}		
ICAM1 (intercellular adhesion molecule 1)	Up	None	miR-31	-0.39	-0.55	-0.45	-0.35	-0.21	-0.47	-0.41	-0.40	-0.31	-0.46	-0.64	-0.42		
				P-value	9.44×10^{-2}	$1.41 \times 10^{-2*}$	$5.48 \times 10^{-2*}$	1.37×10^{-1}	3.83×10^{-1}	$4.05 \times 10^{-2*}$	7.80×10^{-2}	2.01×10^{-1}	$4.87 \times 10^{-2*}$	$3.14 \times 10^{-3*}$	7.53×10^{-2}		
ITGAE6 (integrin alpha 6)	Up	miR-26b, miR-30c	none	-0.46	-0.51	-0.54	-0.56	-0.51	-0.55	-0.63	-0.41	-0.54	-0.55	-0.65	-0.43		
				P-value	$4.58 \times 10^{-2*}$	$2.44 \times 10^{-2*}$	$1.74 \times 10^{-2*}$	$1.31 \times 10^{-2*}$	$2.49 \times 10^{-2*}$	$3.73 \times 10^{-3*}$	8.52×10^{-2}	$1.65 \times 10^{-2*}$	$1.49 \times 10^{-2*}$	$2.37 \times 10^{-3*}$	6.63×10^{-2}		
NFKB1 nuclear factor of kappa light polypeptide gene enhancer in B-cells 1)	Up	miR-195, miR-15b	miR-16, miR-195, miR-15b, let-7e, miR-26b	-0.44	-0.58	-0.56	-0.39	-0.49	-0.50	-0.48	-0.58	-0.35	-0.54	-0.85	-0.56		
				P-value	$5.68 \times 10^{-2*}$	$9.76 \times 10^{-3*}$	$1.23 \times 10^{-2*}$	1.02×10^{-1}	$3.44 \times 10^{-2*}$	$2.79 \times 10^{-2*}$	$3.96 \times 10^{-2*}$	1.47×10^{-1}	$1.73 \times 10^{-2*}$	$5.26 \times 10^{-6*}$	$1.28 \times 10^{-2*}$		
SELP1G (selectin P ligand)	Up	miR-26b, miR-16, miR-15b, miR-195, miR-20b	none	-0.48	-0.53	-0.46	-0.43	-0.33	-0.57	-0.47	-0.46	-0.35	-0.51	-0.76	-0.51		
				P-value	$3.97 \times 10^{-2*}$	$1.92 \times 10^{-2*}$	$4.68 \times 10^{-2*}$	6.63×10^{-2}	1.63×10^{-1}	$1.08 \times 10^{-2*}$	$4.31 \times 10^{-2*}$	1.43×10^{-1}	$2.49 \times 10^{-2*}$	$1.80 \times 10^{-4*}$	$2.49 \times 10^{-2*}$		
TGFBR2 (transforming growth factor, beta receptor II)	Up	none	miR-126	-0.56	-0.75	-0.62	-0.50	-0.54	-0.61	-0.65	-0.65	-0.51	-0.60	-0.86	-0.63		
				P-value	$1.97 \times 10^{-2*}$	$5.61 \times 10^{-4*}$	$7.61 \times 10^{-3*}$	$3.98 \times 10^{-2*}$	$2.55 \times 10^{-2*}$	$1.00 \times 10^{-2*}$	$4.78 \times 10^{-3*}$	$3.66 \times 10^{-2*}$	$1.12 \times 10^{-2*}$	$1.20 \times 10^{-5*}$	$7.01 \times 10^{-3*}$		
VEGFA (vascular endothelial growth factor A)	Up	none	Let-7e, miR-15b, miR-16, miR-20b, miR-195	-0.45	-0.69	-0.59	-0.45	-0.21	-0.58	-0.60	-0.35	-0.53	-0.64	-0.55	-0.62		
				P-value	$5.17 \times 10^{-2*}$	$1.14 \times 10^{-3*}$	$7.45 \times 10^{-3*}$	5.59×10^{-2}	3.91×10^{-1}	$9.40 \times 10^{-3*}$	$6.61 \times 10^{-3*}$	1.41×10^{-1}	$2.06 \times 10^{-2*}$	$2.93 \times 10^{-3*}$	$5.00 \times 10^{-3*}$		

The candidate genes are listed in the left part of the table. The miRNAs of the PAD-specific miRNA signature targeting these genes are also indicated. The interactions miRNA-mRNA are based on miRWalk or miRTarBase databases. The Spearman rank correlation coefficient (r) between the expression level of each candidate gene and each miRNA of the PAD-specific miRNA signature is indicated in the right part of the table. Significant correlations are indicated with an *. All the candidate genes selected are predicted or validated targets of ≥1 miRNAs of the PAD-specific miRNA signature. The expression levels of all the genes significantly upregulated in patients with PAD and that of the miRNAs of the PAD-specific miRNA signature are inversely correlated. No correlation is observed with the genes not differentially expressed. ICAM1 indicates intercellular adhesion molecule; ITGAE6, integrin α 6; miRNA, microRNA; NFKB1, nuclear factor of κ B; PAD, peripheral arterial disease; ROC, receiver operating characteristic; TGFBR2, transforming growth factor-β receptor II; and VEGFA, vascular endothelial growth factor A.

miRNAs are potentially interesting therapeutic targets and might be used to inhibit the initiation of the atherosclerotic process.

It is also interesting to note that several of the miRNAs we found to be associated with PAD have been associated with vascular disorders in previous studies or are related to vascular physiology which also strengthens the relevance of the miRNA signature we identified. MiR-15b has been associated with apoptosis and angiogenesis in myocardial infarction.⁴² MiR-27b has been identified as a proangiogenic miRNA,⁴³ regulating angiogenesis through the angiogenic inhibitor semaphorin 6A.⁴⁴ MiR-126 is known to regulate angiogenic signaling and vascular integrity,⁴⁵ and miR-16 has been found to play a role in angiogenesis by reducing the proliferation, migration, and angiogenic capacity of endothelial cells.⁴⁶ Similarly, the pathway enrichment analysis of target genes identified pathways associated with vascular disease. Noteworthy, several of these pathways have also been found by Masud et al¹² in their whole-genome expression profiling in peripheral blood mononuclear cells from patients with PAD.

The miRNAs we identified here are mainly downregulated. We could hypothesize that these miRNAs have been transferred to the cells of the arterial wall. Recently, Vickers et al⁴⁷ have demonstrated that high-density lipoproteins transport endogenous miRNAs and deliver them to recipient cells to play functional roles. However, although they are expressed in arteries, these miRNAs have not been found to be differentially expressed in atherosclerotic plaques compared with nonatherosclerotic left internal thoracic arteries.⁴⁸ This suggests that the depletion of these miRNAs in peripheral blood cells cannot be explained by their translocation to atherosclerotic plaque and damaged vessels.

We are fully aware of the small sample size of our cohort. One may argue that some positive miRNAs may have not been detected in these conditions, which we agree with. However, the main objective of our study was not necessarily to uncover an exhaustive list of miRNAs implicated in the atherosclerotic process but was to identify the top most robust miRNAs that can be further investigated for potential translation into clinical practice. For that purpose, small cohorts are usually sufficient to detect genes with the largest contribution to diseases. The validation of these results in 2 technically independent sample sets is regarded as a good method to increase the statistical power and accuracy of the results and confirm the *P* values. However, although our strategy was powerful to detect significantly dysregulated miRNAs, the small sample size does not allow us to take comorbidity factors into account. To circumvent that problem, we chose controls and patient groups that were adjusted for most known covariates involved in atherosclerosis. Further work in large cohorts of patients are now being undertaken to assess the relationship between these covariates and the miRNA expression level. Further work will also be necessary to determine whether these miRNAs are sensitive enough to differentiate the subgroups of patients with atherosclerosis such as patients with PAD, coronary artery diseases, or carotid disease. Altogether, these results further highlight the significance of the role of miRNAs in PAD pathophysiology and their potential use as diagnostic or therapeutic targets.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Peripheral arterial disease is a condition caused by atherosclerosis of the arteries diminishing blood supply to the lower limbs. MicroRNAs are short noncoding RNAs involved in gene regulation. This study has identified a 12-microRNA peripheral arterial disease–specific signature, which includes let 7e, miR-15b, -16, -20b, -25, -26b, -27b, -28-5p, -126, -195, -335, and -363. Each of these 12 microRNAs exhibited good diagnostic value, and their validated targets identified several signaling pathways relevant to vascular disorders. These results highlight the potential of microRNAs for the diagnosis of peripheral arterial disease and provide further insight into the molecular mechanisms leading to the development of peripheral arterial disease, with the potential for future therapeutic targets.