The role of 14q32 microRNAs in vascular remodelling

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Chapter 8

Upregulation of 14q32 microRNAs in human subcutaneous adipose tissue samples of patients with critical limb ischemia undergoing major amputation

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Abstract

Objective: In recent years, it has become clear that adipose tissue, both subcutaneous adipose tissue (SAT) as well as perivascular adipose tissue (PVAT), is a major contributor to the development and progression of peripheral arterial disease. We aimed to identify suitable microRNAs biomarkers to identify severe critical limb ischemia (CLI) patients at risk of major amputation.

Methods: SAT and PVAT samples were collected from patients undergoing major amputation because of severe CLI. As controls, SAT was collected from patients that underwent elective knee-replacement. Multiplex qPCRs, followed by individual qPCRs, were performed to determine differential microRNA expression between CLI and control samples. Receiver operating characteristic (ROC) curve analyses were performed to determine sensitivity and specificity for differentially expressed microRNAs.

Results: Multiplex qPCR analyses demonstrated global downregulation of microRNA expression in SAT of CLI patients compared to controls. Eight microRNAs however, of which six belong to a single microRNA gene cluster (14q32), were upregulated in CLI patients. Using individual qPCRs, we confirmed significant upregulation of 14q32 microRNAs miR-127, miR-134, miR-370, miR-376c, miR-411 and miR-539 in SAT of CLI patients compared to controls. ROC curve analyses showed an area under the curve of greater than 0.96 for all microRNAs in the studied population, which was highly significant ($P<0.01$ for all microRNAs). Finally, upregulation of 14q32 microRNAs in SAT and in PVAT of CLI patients was validated in a second independent study population.

Conclusions: Six 14q32 microRNAs are significantly upregulated in SAT and PVAT of patients with CLI. We show that 14q32 microRNAs miR-127, miR-134, miR-370, miR-376c, miR-411 and miR-539 in SAT are promising biomarkers to identify CLI patients who are at risk of major amputation.

Introduction

Peripheral arterial disease (PAD) due to atherosclerosis limits blood flow towards the lower extremities[1]. In a small portion of the patient population, PAD evolves to critical limb ischemia (CLI). CLI is characterized by rest pain, ischemic ulceration and/or gangrene of the lower limb. One year survival for CLI patients is only 75% and 30 to 40% will have undergone amputation within three years[2-4]. Aggressive medical management reduces the risk of progression to CLI, but there is to date no reliable biomarker to predict PAD development, progression and outcome, which could be useful in treatment choices for this group of patients.

Because of their important regulatory role, microRNAs are suitable biomarkers for the diagnosis and prognosis of cardiovascular diseases, including acute myocardial infarction, heart failure, diabetes mellitus and PAD[5-12]. MicroRNAs are a class of non-coding RNA molecules (~22 nucleotides long) that regulate the expression of their target genes at the messenger RNA (mRNA) level. In addition, each microRNA has up to several hundred target genes, potentially regulating as many biological processes. Important for biomarkers, the stability of microRNAs has been shown to be exceptionally high, as their small size protects them from endogenous RNase activity[13].

Cardiovascular morbidity and mortality has been linked to obesity[14]. Generally, obesity increases the risk for the development cardiovascular disease. However, the 'obesity paradox' has also been described, where obesity is associated with a more favourable prognosis compared to non-obese patients in for example heart failure patients and patients with coronary heart disease[15-17]. In addition to the classical roles in energy storage and thermoregulation, adipose tissue is nowadays considered an endocrine organ that secretes inflammatory mediators and adipokines[18]. Variations in body fat distribution, but also the inflammatory status and metabolic profile of these adipose tissue depots, can contribute to the risk of cardiovascular disease [19-21]. For example, subcutaneous adipose tissue of patients with CLI and patients with metabolic syndrome has been described to display a more pro-inflammatory phenotype, with increased expression of pro-inflammatory cytokines IL-6 and IL-8 but also of PAI-1, leptin and resistin, compared to controls[22, 23]. Thus, the composition of the adipose tissue may be as important as the quantity in patients with cardiovascular disease.

Perivascular adipose tissue, which lines the blood vessels, has both endocrine and paracrine effects on the vasculature through secretion of cytokines and adipokines. Perivascular adipose tissue has received increasing attention in vascular biology as it has been shown to play a role in type 2 diabetes and cardiovascular disease[24].

In this study, we examined the expression of microRNAs in subcutaneous adipose tissue (SAT) of CLI patients that underwent major amputation versus "healthy" control patients that underwent elective orthopaedic surgery. In addition, we looked at microRNA expression in perivascular adipose tissue (PVAT) of CLI patients. The aim of this study was to identify microRNAs that can be used as suitable biomarkers to identify patients with severe CLI who are at risk of major amputation. On the long term, the identified microRNAs may also provide insights into the complex interplay between adipose and vascular organs in health and disease.

Chapter 8

Methods

Patient Population

Boston study population. This study was conducted in accordance with the Declaration of Helsinki. The institutional review board (IRB) of the Brigham and Women's Hospital (Boston) approved the study protocol. All subjects were recruited at the department of surgery of the Brigham and Women's Hospital in Boston (MA, USA) and provided written informed consent (when applicable) before participating in the study.

SAT was collected from patients as described previously[22]. In brief, patients undergoing lower extremity major amputation (below knee or above knee) due to unreconstructable CLI (n=18) or elective orthopaedic total knee replacement (n=18) were prospectively identified via procedures approved by the local IRB. Patients in the amputation group were enrolled under an IRB approval that allowed us to collect de-identified medical information and tissue from the amputated limb without informed consent. Patients in the control group all underwent elective knee replacement for osteoarthritis. Informed consent was obtained from the control elective orthopaedic group.

Leiden study population. This study was conducted in accordance with the Declaration of Helsinki. The institutional medical ethics committee of the Leiden University Medical Center approved the study protocol (P12.265). Patients (n=6) undergoing lower extremity major amputation (below or above the knee) were recruited at the department of surgery of the Leiden University Medical Center and provided written informed consent before participating in the study. SAT and PVAT was collected from patients undergoing amputation due to untreatable critical limb. SAT and PVAT samples of these patients were de-identified and only age and gender of these patients were documented.

Subcutaneous adipose tissue collection, storage and RNA isolation

All samples were collected in the operation room by trained surgeons. SAT (~2 g) and PVAT (~50 to 100 mg; only in the Leiden study population) were collected from the amputated limb or from the operated knee of patients and immediately flash frozen in liquid nitrogen. Adipose tissue samples were stored at -80°C in RNAse free vials until the time of analysis. For RNA isolation, adipose tissue samples were homogenized by grounding using a Pellet Pestle Cordless Motor (Kimble Chase Life Science). RNA was isolated from homogenized adipose tissue using a standard TRIzol-chloroform extraction protocol. RNA concentration and purity were examined by nanodrop (Nanodrop® Technologies).

Multiplex rt/qPCR

Expression profiling of 384 different microRNAs was conducted by rt/qPCR multiplex assays. From the Boston study population, SAT samples used for multiplex rt/qPCR analyses were randomly selected from 6 out of the 18 patients that underwent amputation due to CLI (amputation samples) and 6 out of the 18 patients undergoing knee replacement (controls). Isolated RNA was reverse transcribed using Megaplex RT primers (Pool A, Life Technologies). Taqman® Universal PCR Mastermix (Life Technologies) was used to prepare the PCR reaction mix with diluted RT product. 384-well microfluidic cards (Taqman® MicroRNA Human Array A, Life technologies) were loaded with the PCR master mix. Each microRNA was assayed once by qPCR on the human panel A array cards. Amplification was performed on the Viia7 system (Applied Biosystems), using the array card block. Amplification curves were analysed using the AB software. All data were normalized against snRNA-U6 (RNU-6B). The heatmap was generated using heatmap.3 function of GMD library of R language.

Individual microRNA rt/qPCR

MicroRNA quantification was performed on all SAT samples of the Boston study population (n=18 per group) and all SAT and PVAT samples (n=6) of the Leiden study population. MicroRNA rt/qPCRs were performed according to manufacturer's protocol using individual Taqman® miR assays for miR-127-3p, miR-134-5p, miR-370-3p, miR-376c-3p, miR-411-5p and miR-539-5p (Life Technologies). Rt/qPCRs were run on the Viia7 system (Applied Biosystems). Normalization of data was performed using stably expressed endogenous control snRNA-U6 (RNU-6B).

Statistical analyses

Values are expressed as mean ± standard deviation (SD) for multiplex rt/qPCR data and for individual microRNA rt/qPCR data. Statistically significant outliers in individual microRNA rt/qPCR data were identified using a Grubb's test and subsequently excluded. Unpaired student's t-tests were used to compare groups with normal distribution and the Mann-Whitney U test was performed for data with a non-normal distribution. Receiver Operator Characteristic (ROC) Curves were analysed to determine sensitivity and specificity for each microRNA. For the patient characteristics, Student t-Test or Mann-Whitney Rank Sum Test was performed on continuous variables and Chi-square or Fisher Exact Test on categorical variables. A P-value <0.05 was considered statistically significant.

Results

Patient characteristics

Adipose samples were collected from 24 patients who underwent lower limb amputation due to severe CLI and 18 control patients who underwent knee replacement surgery. The mean age of the patients included in the Boston study population was 67.8 years for the CLI group and 60.2 years for the control group. The percentage of females was 16.7% in the CLI group compared to 50% in the control group (P=0.075). CLI patients in the Boston cohort had a greater prevalence of diabetes mellitus, coronary artery disease, chronic heart failure, renal disease and pulmonary disease and had an overall lower BMI compared to control patients. In addition, statin and insulin use was higher in these patients. Further patient characteristics can be found in Table I. For the Leiden study population, only age and gender were documented. In these patients, the mean age was 70 years and 16.7% were female (Table I).

Boston study population	**Amputation (%) (n=18)**	**Control (%) (n=18)**	***P* value**
Age (years), mean ± SD	67.8 ± 15.7	60.2 ± 13.9	0.133
Race			
White:	10 (55.56)	12 (66.67)	
Black:	5 (27.78)	4 (22.2)	0.782
Hispanic:	3(16.67)	2 (11.1)	
Female	3 (16.67)	9 (50)	0.075
BMI (kg/m^2)	25.0 ± 5.3	34.0 ± 8.0	**<0.001**
Diabetes Mellitus	14 (77.78)	2 (11.1)	**<0.001**
Hypertension	16 (88.89)	12 (66.67)	0.228
Hyperlipidemia	13 (72.22)	7 (38.89)	0.094
CAD	12 (66.67)	1 (5.56)	**<0.001**
CHF	6 (33.33)	0 (0.00)	**0.019**
CVA	4 (22.22)	0 (0.00)	0.104
Renal disease	9 (50)	0 (0.00)	**0.001**
Pulmonary disease	5 (27.78)	0 (0.00)	**0.045**
Smoking history	11 (61.11)	6 (33.33)	0.182
Antiplatelet therapy	14 (77.78)	11 (61.11)	0.469
Warfarin	4 (22.22)	10 (55.56)	0.087
Calcium channel blocker	7 (38.89)	1 (5.56)	**0.041**
Beta blocker	13 (72.22)	1 (5.56)	**<0.001**
ACE inhibitor/ARB	6 (33.33)	6 (33.33)	0.724
Statin	14 (77.78)	6 (33.33)	**0.019**
Insulin	13 (72.22)	1 (5.56)	**<0.001**
NSAID	13 (72.22)	16	0.402
Steroid	2 (11.11)	1 (5.56)	1.000

Leiden study population (n=6)	**Amputation (%)**
Age	70 ± 11.6
Female	1 (16.67)

Table I. Patient characteristics for the Boston and Leiden study populations

MicroRNA expression profiles in subcutaneous adipose tissues

The expression profiles of ~384 microRNAs were assessed in SAT samples of six CLI patients and in SAT samples of six control patients of the Boston study population. Figure 1 shows a heat map of the 40 microRNAs with the highest differential expression between CLI and control samples. Interestingly,

most microRNAs were downregulated in SAT samples of CLI patients, whereas only a subset of microRNAs was upregulated in these patients compared to the control samples (Figure 2). Increased expression of miR-127, miR-134, miR-370, miR-376c, miR-411, miR-539, miR-886-3p and miR-146b was observed in CLI samples compared to samples of the control group. Notably, six out of eight upregulated microRNAs, miR-127, miR-134, miR-370, miR-376c, miR-411 and miR-539, belong to a single microRNA gene cluster, the 14q32 microRNA cluster.

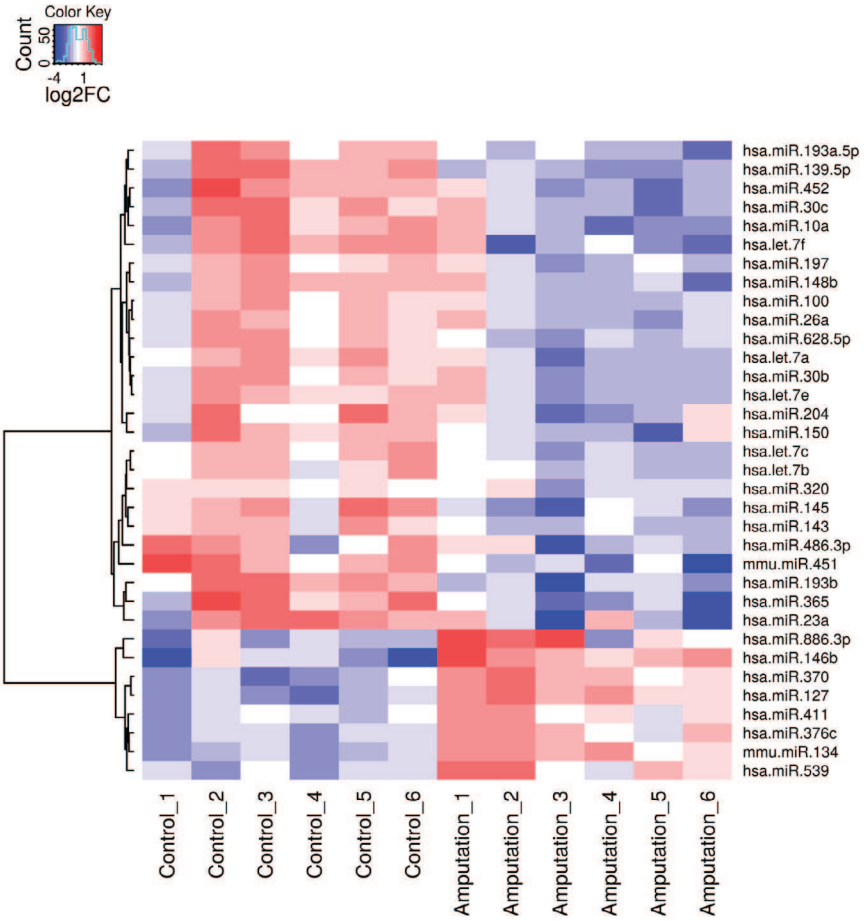

Figure 1. Heat map of differentially expressed microRNAs. MicroRNA expression of control (control_1 to control_6) and CLI (amputation_1 to amputation_6) samples are shown. Each row represents one microRNA and each column represents one sample. The colour scale shows the microRNA expression fold change relative to the average expression of the microRNA across all samples. Red colour indicates an expression level above the mean and blue colour represents expression below the mean.

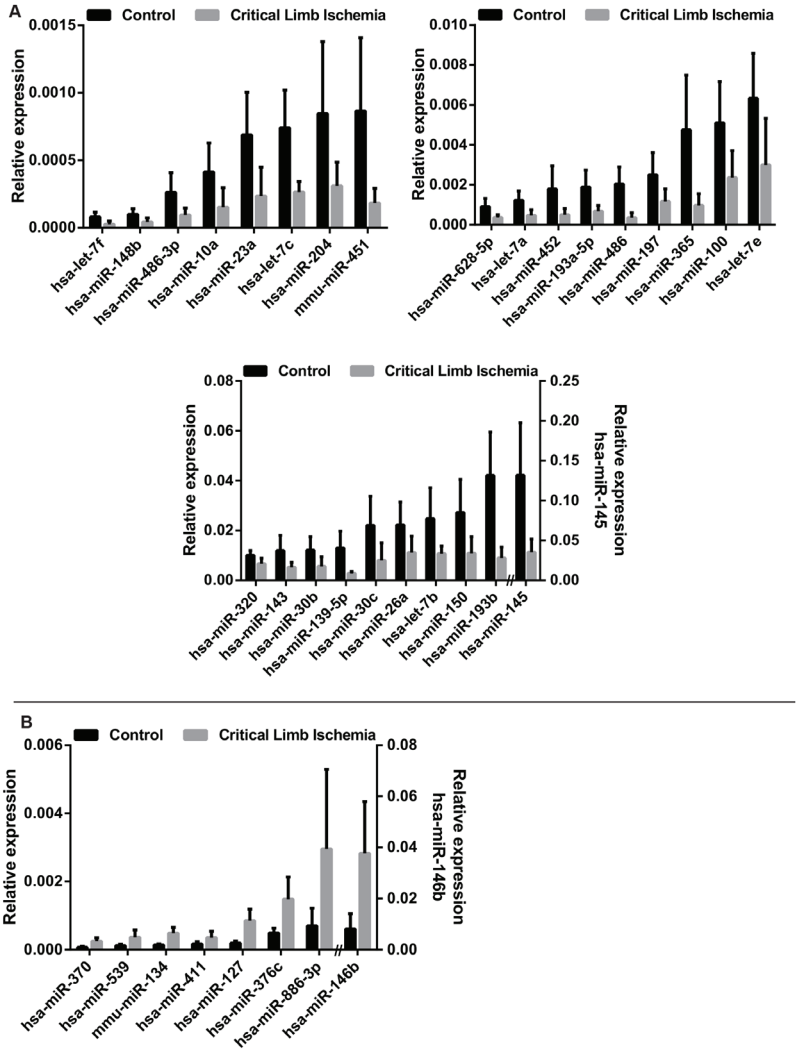

Figure 2. Differential microRNA expression between control and amputation patients. MicroRNA expression was determined by multiplex rt/qPCR in subcutaneous adipose tissue samples of control and CLI patients that underwent major amputation (n=6 patients per group) of the Boston study population. (A) Most microRNAs are downregulated in CLI samples compared to controls. (B) A subset of microRNAs is upregulated in CLI samples compared to control samples. Data are shown as mean ± stdev.

Validation of differential 14q32 microRNA expression

Previously, we have shown that inhibition of several 14q32 microRNAs has positive effects on blood flow recovery in a mouse model of CLI[25]. We performed individual microRNA rt/qPCRs to confirm the differential expression of 14q32 microRNAs miR-127, miR-134, miR-370, miR-376c, miR-411 and miR-539 in all SAT samples of CLI and control groups in the Boston study population. We confirmed significantly elevated expression levels of miR-127, miR-134, miR-370, miR-376c, miR-411 and miR-539 in adipose tissue of patients that underwent major amputation due to severe CLI (Figure 3). Expression of miR-127, miR-370 and miR-539 was approximately four-fold greater in CLI patients,

whereas miR-134, miR-376c and miR-411 showed over two-fold greater expression in these patients compared to controls.

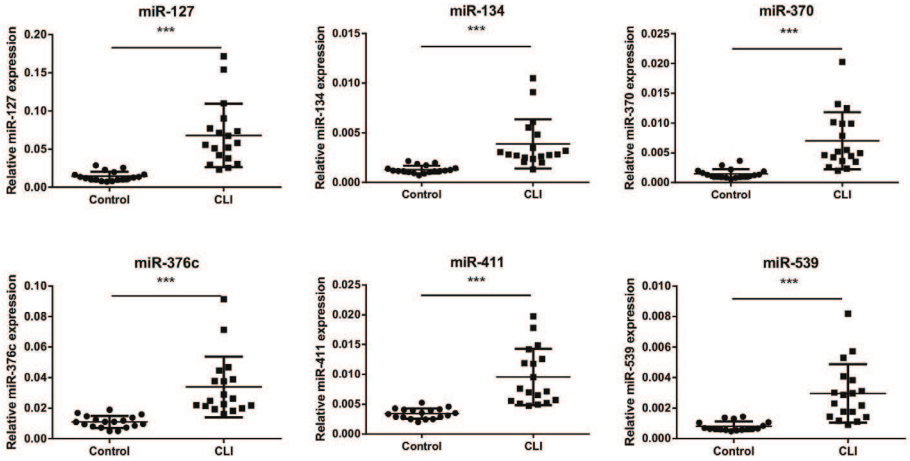

Figure 3. Upregulation of 14q32 microRNAs in amputation patients. Individual rt/qPCR measurements were performed to determine 14q32 microRNA expression in SAT samples of CLI and control samples (n=18 patients per group) in the Boston study population. Data are shown as mean ± stdev. ***P < 0.001

14q32 microRNAs as biomarkers for risk of limb loss

To determine the ability of each upregulated microRNA to predict the CLI in patients, we performed Receiver Operator Characteric (ROC) curve analysis. Using this method, all analysed 14q32 microRNAs significantly predicted CLI (P<0.01 for all microRNAs). Area under the curve (AUC) was greater than 0.96 for all examined microRNAs (Figure 4).

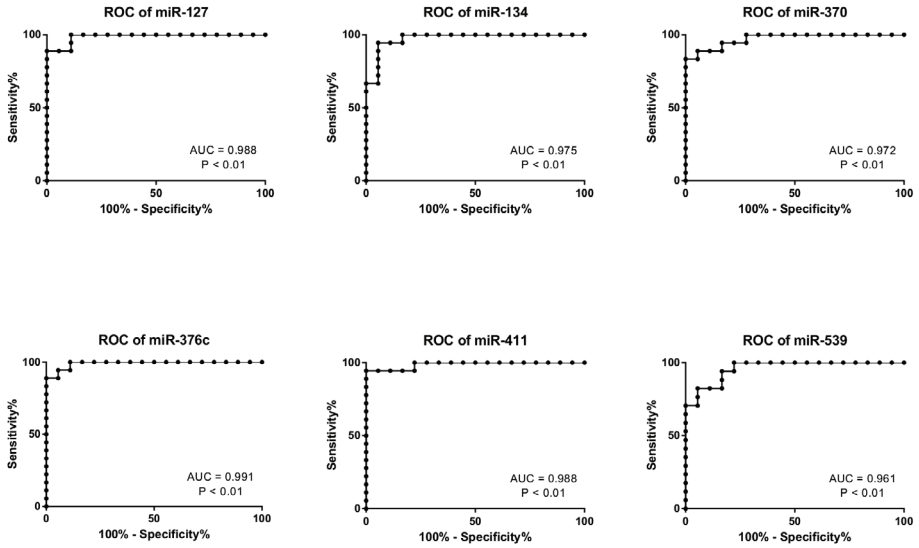

Figure 4. Diagnostic potential of 14q32 microRNAs. Receiver Operation Characteristic (ROC) curve analysis of individual microRNAs (miR-127, miR-134, miR-370, miR-376c, miR-411 and miR-539) to discriminate between CLI and control patients in the Boston study population.

Confirmation of 14q32 microRNA upregulation in a second study population

In order to validate whether upregulation of 14q32 microRNAs in SAT samples of CLI patients is independent of the studied patient population and demographics, we measured expression of 14q32 microRNAs in a second study population of patients (the Leiden study population) that underwent major lower limb amputation due to CLI. In addition, expression of 14q32 microRNAs was measured in PVAT of these patients. Similarly to the Boston study cohort, 14q32 microRNAs miR-127, miR-134, miR-370, miR-376c, miR-411 and miR-539 were significantly upregulated in SAT samples of CLI patients compared to controls (Figure 5A). Expression of 14q32 microRNAs was also upregulated in PVAT samples of these patients (Figure 5A). ROC curve analysis conducted on this subset of amputation samples from the Leiden study cohort confirmed that elevated expression of miR-127, miR-134, miR-370, miR-376c, miR-411 and miR-539 in SAT is a significant predictor of CLI (Figure 5B). All microRNAs showed an AUC of greater than 0.78 (Figure 5B).

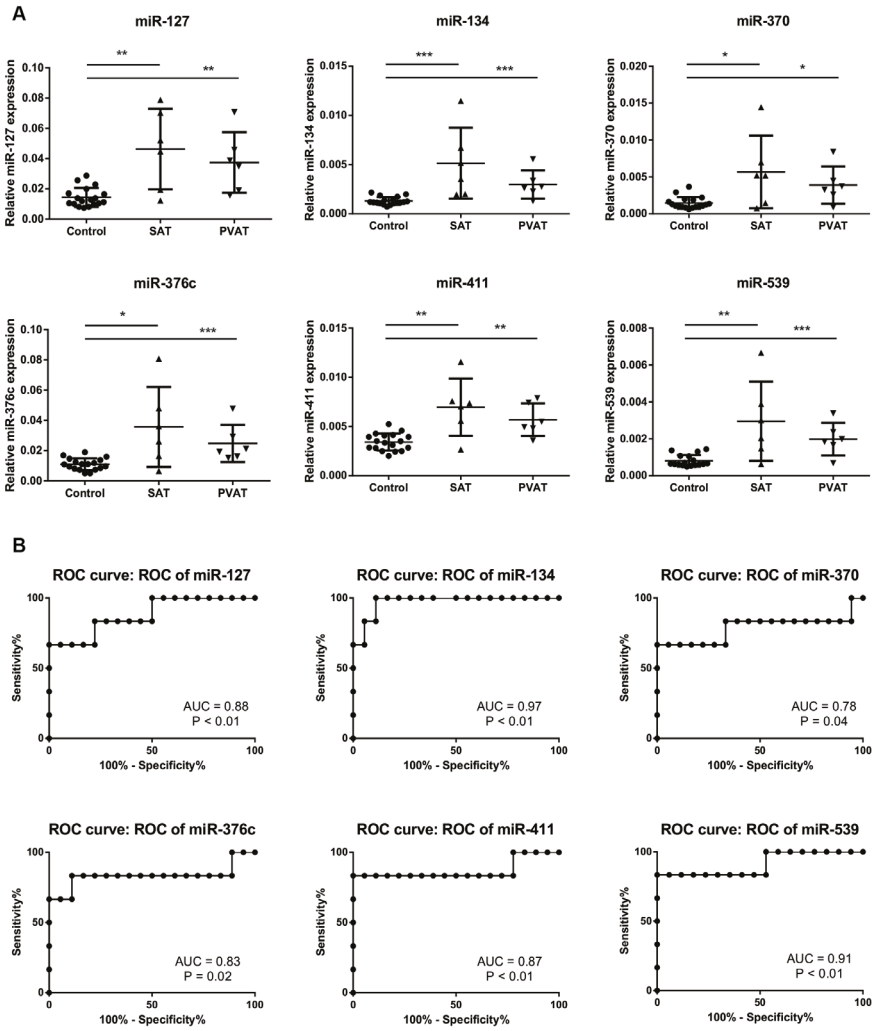

Figure 5. 14q32 microRNA upregulation in the Leiden study population. Rt/qPCR was performed on SAT and PVAT samples of CLI patients (n=6 patients) from the Leiden study population. (A) Upregulation of 14q32 microRNAs was observed in both SAT and PVAT of CLI patients compared to controls. (B) ROC curve analysis was performed to confirm the discriminative power of 14q32 microRNAs to identify CLI patients in this second study population. Data are shown as mean ± stdev. *P < 0.05; **P< 0.01; ***P < 0.001.

Discussion

The present study shows upregulation of 14q32 microRNAs miR-370, miR-539, miR-134, miR-411, miR-127 and miR-376c in the SAT of CLI patients that underwent major amputation. This study demonstrates that expression of these microRNAs in SAT may be used as biomarkers to identify CLI patients who are at risk of major amputation. These findings were confirmed in a second study population.

Previous studies have shown that SAT of CLI patients that underwent major amputation displays a distinct pro-inflammatory signature compared to SAT samples of control patients[22]. This dysregulation of inflammatory mediators was also observed in adipose tissue of patients with metabolic syndrome[23]. Here, we investigated differential expression of microRNAs in SAT of amputated CLI patients compared to control patients. Although generally microRNA expression in SAT was downregulated in CLI patients, a small group of microRNAs was upregulated in these patients. Six out of the eight upregulated microRNAs belong to the 14q32 microRNA gene cluster. Previously, we have shown involvement of several 14q32 microRNAs in post-ischemic neovascularization as well as atherosclerotic plaque formation and stability[25, 26]. Of the upregulated microRNAs reported here, several have also been implicated within cardiovascular disease and lipid metabolism. For example, miR-370 was reported to control expression of carnitine palmitoyltransferase 1A (Cpt1α) expression and fatty acid β-oxidation in liver cells. In addition, miR-370, via miR-122, controlled sterol-regulatory element binding protein 1c (SREBP-1c) and diacylglycerol acyltransferase-2 (DGAT2) expression and lipid metabolism[27, 28]. MiR-134 was shown to target angiopoietin-like 4 (ANGPTL4) and via this mechanism regulated lipoprotein lipase activity and ultimately oxLDL uptake by THP-1 macrophages[29]. Moreover, circulating miR-134 was significantly upregulated and correlated with coronary artery calcifications in patients with obstructive coronary disease[30]. Another study demonstrated elevated expression of both miR-134 and miR-370 in peripheral blood mononuclear cells (PBMCs) of patients with unstable angina pectoris compared to stable patients, suggesting that these microRNAs could be used to identify patients at risk for acute coronary syndromes[31]. Circulating miR-411 was differentially expressed in men with an abdominal aortic aneurysm compared to healthy controls, as well as in men with PAD[32]. Expression of miR-127 was elevated in symptomatic versus asymptomatic carotid plaques of patients undergoing carotid endarterectomy for atherosclerotic stenosis[33]. In most studies discussed here, microRNA expression was upregulated in patients compared to controls. These findings are in line with our data, where we show upregulation of 14q32 microRNAs miR-127, miR-134, miR-370, miR-376c, miR-411 and miR-539 in SAT samples of CLI patients at risk of amputation due to CLI. To our knowledge, there is no direct information linking miR-376c and miR-539 to PAD or CLI yet.

Most studies where microRNAs are evaluated as potential biomarker are based on the measurement of these microRNAs in serum or plasma. Rather than circulating plasmatic, we show here that local microRNA expression in adipose tissue can be used as biomarker. This provides direct local information on microRNA expression, whereas microRNAs detected in serum or plasma provides systemic

information and does not give information on the source of these microRNAs. The tight interaction between both the quantity and composition of adipose tissue and cardiovascular disease development and progression would require local evaluation of microRNA expression to accurately predict disease outcome.

Perivascular adipose tissue, which lines the blood vessels, has been shown to have local effects on the vasculature and influences both vascular health and disease[34]. Compared to other adipose tissue depots, PVAT has been reported to display a distinct (inflammatory) phenotype[35-38]. Therefore, we were also interested whether PVAT harvested from CLI patients that underwent major amputation would display a different microRNA signature than SAT of these patients. In this study, the increased expression of 14q32 microRNAs miR-127, miR-134, miR-370, miR-376c, miR-411 and miR-539 was comparable in PVAT and SAT samples of amputation patients. The increased expression of 14q32 microRNAs in PVAT of CLI patients not only supports for their role as biomarker, but also suggests an active role for 14q32 microRNAs in the interplay between PVAT and the vasculature.

Clinical Implications and Study Limitations

Although the sample size used for this study was relatively small and amputation and control cohorts were not perfectly matched for sex and age, we were able to identify several microRNAs that could be used to identify CLI patients at risk of amputation, in two different study populations. In a previous study, we also demonstrated upregulation of 14q32 microRNAs upon ischemia in a mouse model for CLI[25]. It should be noted that although we observe a profound difference between severe CLI patients that underwent an amputation and controls, we cannot exclude the possibility that these microRNAs are also upregulated in CLI patients which are not at risk of amputation. Future studies will have to investigate whether these microRNAs can be used to discriminate between patients with CLI which are at a high risk of amputation and patients which are treatable for CLI.

Conflict of interest

None declared.

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