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Circulating microRNAs and in-stent restenosis with peripheral artery disease

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Kurzfassung

Hintergrund. Die folgende Studie befasst sich mit dem Zusammenhang von zirkulierenden microRNA (miR) im Blut von Patienten nach einer Stent-Legung aufgrund einer vorher bekannten peripheren arteriellen Verschlusskrankheit (PVK) und dem Risiko einer wiederauftretenden Thrombose und Restenose. Der interventionelle Eingriff der Angioplastie dient der Aufweitung verengter bzw. verschlossener Gefäße und anschließendem Einsetzen des Stents, zum Offenhalten des Gefäßes. Die In-Stent-Restenose ist die, am häufigsten vorkommende, Komplikation bei diesem Verfahren. MiRs sind nicht-codierende, kleine RNAs, welche die Genexpression auf der posttranslationalen Ebene regulieren, und in der Pathogenese der Atherosklerose und Restenose impliziert wurden.

Methoden. Die Kausalität zwischen der zirkulierenden Menge der untersuchten miRs und dem Auftreten von kardiovaskulären Ereignissen, wie Herzinfarkt, Schlaganfall oder transitorische ischämische Attacke, gilt hier als primärer Endpunkt der Studie. Zur untersuchten Population gehören 62 Patienten mit bekannter PAVK und einer Stent-Implantation in der femoral Arterie. Die untersuchten miRs wurden aus dem Plasma der Patienten isoliert und mittels real-time PCR quantifiziert.

Resultate. Von den 11 untersuchten miRs, haben sich miR-195 und miR-92a als prognostizierend herausgestellt, in Betracht auf die Vorhersage von Ereignissen in einem Zeitraum von 2 Jahren nach einer Angioplastie mit Stent-Implantierung.

Zusammenfassung. miR-195 and miR-92a konnten in dieser Studie als potentielle Biomarker identifiziert werden, welche leicht quantifizierbar sind und Teil der Risikostratifizierung, bei PAVK-Patienten nach einer Stent-Setzung, werden könnten.

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Abstract

Background. The following study examined a relation between circulating microRNA (miR) in blood samples of peripheral arterial disease (PAD) patients with an in-stent implantation and the risk of restenosis. The interventional procedure provides the expansion of the narrowed or closed blood vessel and an affiliating onset of a medical implant (stent) which is used to maintain the vessel open for blood flow. Restenosis is the most common complication of the procedure and so a particular emphasis is placed on these miRNAs to provide future development of less invasive and faster diagnostic methods. MicroRNAs (miRNAs) are small, non-coding RNAs, which regulate gene expression on the post-translational level.

Methods. The prognostic value of circulating levels of the examined miRs in relation with the occurrence of cardiovascular events, such as heart failure, stroke or transient ischemic attack, is the primary endpoint of the study. The population of 62 Patients with PAD had an in-stent implantation at or below the inguinal ligament. The circulating miRs were isolated from blood plasma, reverse-transcribed and quantified using the real-time-PCR.

Results. Out of the eleven investigated miRs, miR-195 and miR-92a independently predicted 2-year outcomes in patients after stenting and angioplasty. Circulating levels of miR-195 and miR-92a were lower in patients with adverse atherothrombotic events during the follow-up as compared to patients without events.

Conclusion. miR-195 and miR-92a could become an important biomarker, which is easy to assess and can be used for risk stratification for patients after endovascular revascularization procedures.

Inhaltsverzeichnis

Introduction

Peripheral arterial disease (PAD)

The global burden of PAD is rising constantly, with a substantial growth of morbidity and mortality in high- as well as in middle- and low-income countries. The atherosclerotic occlusive disease, which underlies PAD, is mostly associated with an exercise limitation in the broad majority of patients.[1]

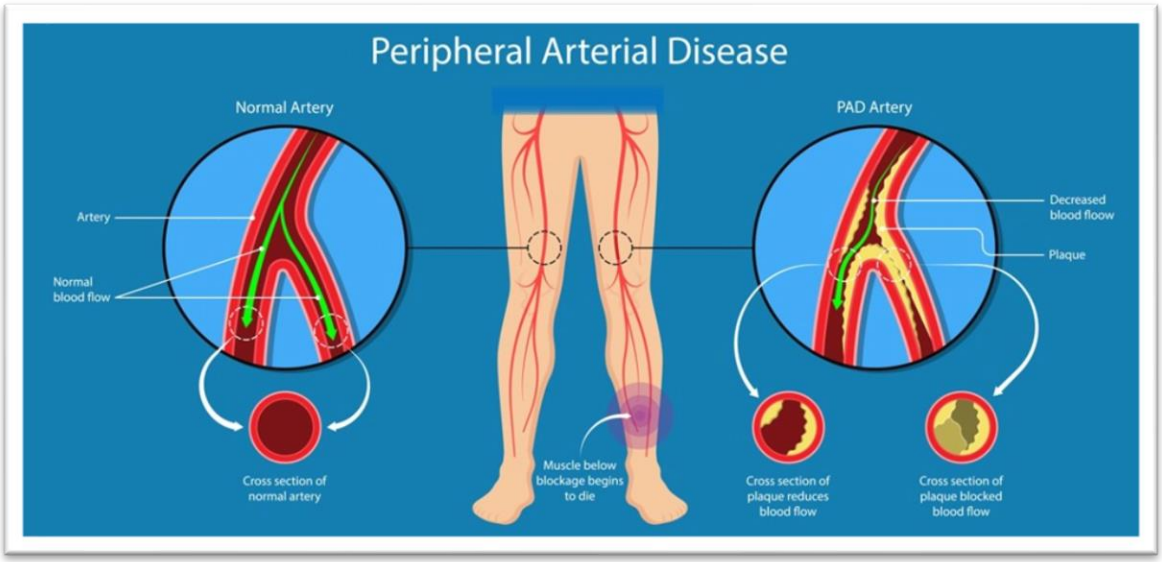

Figure 1 *Pathogenesis of Peripheral Arterial Disease (Quelle: 2021 Appalachian Regional Healthcare System; -slow https://apprhs.org/peripheral-arterial-disease-an-under-recognized -burning-emergency/)*

Like shown in Figure 1, arteries that become narrowed or completely blocked, can cause symptoms like leg pain while walking as well as at rest, incurable wounds and possibly tissue loss.

Several studies have already tried to gain insight into the molecular mechanism playing the key role in PAD onset, as well as in progression and prognosis. [2]

Peripheral artery disease (PAD) describes the impairment of blood flow to the extremities. It is an independent risk factor for both myocardial infarction and stroke. The major cause for PAD is atherosclerotic occlusive disease.[3] PAD is one of the most often overlooked diseases. Compared to patients with coronary artery disease, PAD patients are less likely to receive medical therapy. [4] The presence of symptoms rely on many things, especially on the metabolic demands of the ischaemic tissue and the degree of collateral circulation, as well on the size and location of the affected artery. [3]

Epidemiology and Diagnosis

PAD patients have similar cardiovascular outcomes to patients with ischemic stroke or coronary artery disease, with a risk of myocardial infraction, stroke or vascular death >5% per year. [1] One of the markers used in epidemiological studies is intermittent claudication, [5] which is defined by buttocks-pain, pain in thighs or calves that occurs while walking and which is relieved by standing still. These symptoms are manifested only at 10 % to 30 % of patients with PAD. [4] Less common manifestations are ischemic rest pain and ischemic ulcerations. [1]

In spite the efforts to improve the lifestyle and the use of new pharmacologic approaches reducing plasma cholesterol concentrations, cardiovascular disease (CVD) is still the principal cause of death in Europe, the United States and parts of Asia. [6] Demographic factors such as age, sex and geographic area are essential for estimation of cardiovascular risk. [5] The prevalence is even greater in individuals attending general medicine practise. In which about 20 to 30 % of patients age 50 years or older have PAD.[7]

Fortaine´s and Rutherfords´s Classification of PAD

In 1954 the first classification system, the Fontaine´s classification was published and still it maintains a popular way to stage PAD.[3][8] According to their clinical presentation the patients are graded into four stages (Figure 2.).[3][8] Fontaine´s Classification is only based on clinical symptoms, excluding other diagnostic tests. So it is usually not used in patients care, but mostly in clinical research. [8] In more recent years a similar classification was developed by Rutherford [3] in 1986 with revision in 1997 [8], which has the medical benefit of including haemodynamic data, helping to assure that any tissue loss or rest pain is closely related to PAD. [3] PAD by Rutherford is classified into acute and chronic limb ischemia, distinguishing that each presentation requires different treatment algorithms. This classification includes objective findings, such like arterial brachial indices (ABI), Doppler and pulse volume recordings. [8] Both above-mentioned classifications are used for clinical setting as well as for research purposes. [8]

A directed questioning and examination of symptoms in population with a high risk, such as persons older the 65 or person 50 years old or older with a history of symptoms or comorbidities like diabetes, exertional leg pain or non-healing extremity wounds and the ABI screening is highly recommended by the American College of Cardiology and the American Heart Association. [9] As shown in figure 2 the two classifications differentiate between four stages (Fontaine´s Classification) and seven stages (Rutherford´s Classification) for PAD.

Fontaine's		Rutherford's	
Stage	Clinical presentation	Stage	Clinical presentation
I	Asymptomatic	0	Asymptomatic
II	Intermittent claudication	1	Mild claudication
	IIa: on walking >200 m	2	Moderate claudication
	IIb: on walking <200 m	3	Severe claudication
III	Rest pain	4	Rest pain
IV	Ulceration or gangrene	5	Minor ischaemic ulceration
		6	Severe ischaemic ulcers or frank gangr

Figure 2 *Comparing Fontaine´s Classification to Rutherford´s classification (Quelle: Peripheral arterial disease: a literature review, P. Abdulhannan, D.A.Russell, S. Homer-Vanniasinkam, published 2012, British medical bulletin*[3]*)*

A low ABI, specifically less than 0.9 is an independent predictor of future cardiovascular events and is associated with a two- to fourfold relative risk increase. [9] Studies that tracked coronary heart diseases and measured ABI at baseline showed that a low ABI was specific (92.7%) for prognosticating incident coronary heart disease. [9]

The objective, non-invasive [10] method for diagnosis, for example the measurement of Doppler systolic ankle pressure are more precise and are used by many studies. The calculated ABI can be used to define the PAD progress.[5] To calculate the ABI for each leg the higher of the two pressures at the ankle is taken and divided by the higher of the two brachial pressures. As shown in the following table a normal ABI is located in the range between 1.0 to 1.2. An abnormally low ABI value is representative for atherosclerosis in the leg [10] and with a calculated ABI ≤ 0.9 PAD is diagnosed. [4] (Figure 3/4)

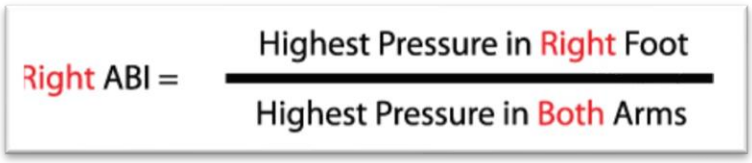

Figure 3 *Calculating ABI (Quelle: 2021 Stanford Medicine 25 - https://stanfordmedicine25.stanford.edu/the25/ankle-brachial-index.html)*

ABI Value	Interpretation	Recommendation
Greater than 1.4	Calcification / Vessel Hardening	Refer to vascular specialist
1.0 - 1.4	Normal	None
0.9 - 1.0	Acceptable	
0.8 - 0.9	Some Arterial Disease	Treat risk factors
0.5 - 0.8	Moderate Arterial Disease	Refer to vascular specialist
Less then 0.5	Severe Arterial Disease	Refer to vascular specialist

Stanford Medicine 25

Figure 4 *ABI Values and the interpretations and recommendations for the calculated value (Quelle: 2021, Stanford Medicine 25 - https://stanfordmedicine25.stanford.edu/the25/ankle-brachial-index.html)*

The diagnosis of the clinical syndromes caused by atherosclerosis are usually based on imaging tests to visualize atherosclerosis or to document target organ ischaemia. [11] Each imaging method addresses a specific clinical scenario. For non-invasive investigations typically used in PAD are ultrasonography and CT angiography. [11]

In various areas more invasive procedures, like invasive angiography, intravascular ultrasonography and optical coherence tomography, are used to guide interventional therapies. [11] Positron emission tomography (PET) and magnetic resonance imaging (MRI) are technologies that tend to have limitations when it comes to evaluating atherosclerosis. [11]

The angiography is considered the gold standard for determining PAD. [10] However, because angiography is an invasive procedure, non-invasive procedures such as CTA or MRA can be used to confirm and localize suspected PAD. [3] Both techniques are similar in terms of diagnostic accuracy, ease of use and clinical outcome. [3] When compared to angiography ABI can detect hemodynamically significant lesions with a specificity of 95 to 100% and a sensitivity of 80 to 95%. [7]

Risk Factors for PAD and atherosclerosis

Gender

PAD prevalence, either symptomatic or asymptomatic is minimally grater in men than women, especially in the younger age groups. In patients with intermittent claudication the ratio is 1:1 (men to women) or 2:1 depending on the study. In some studies, this ratio increases to 3:1 in more fatal stages of the disease, like chronic limb ischemia (CLI). Many other studies have shown a more equal distribution between men and women or even a predomination of women with CLI. [12]

Age

The incidence and prevalence of PAD increase significantly with increasing age. [12] Diabetics or smokers aged under 55 years and anyone else aged under 65 years should have an ABI performed to diagnose an existing or impeding PAD. [4]

Smoking

The first time the relationship between smoking and PAD has been recognized was in 1911, when Erb reported that intermittent claudication (IC) was more common among smokers than among non-smokers (3:1). [12] Therefor patients with IC were encouraged to decrease or even eliminate cigarette smoking. The association between smoking and PAD may be stronger than between smoking and coronary artery disease (CAD). In smokers PAD is diagnosed approximately ten years earlier than in non-smokers. [12]

To improve functional capacity, prevent the progression of PAD and reduce the risk of bypass failure, tobacco cessation is one of the major pillars of treatment. [4]

Diabetes mellitus

The association between diabetes mellitus and PAD was shown in many studies. They show that IC is twice as common among diabetes mellitus patients then patients without diabetes mellitus as a comorbidity. [12] For patients with diabetes an increase of haemoglobin A1c for 1 % the risk of PAD increases for 26%. The growing evidence in the last few decades, has shown that insulin resistance plays an important role in cardiovascular risk factors, such as hyperglycaemia, dyslipidaemia, hypertension and obesity. In patients with diabetes PAD is more aggressive then in nondiabetic-patients, with early involvement of large vessels combined with distal symmetrical neuropathy. [12] However, there is a contribution by sensory neuropathy and a deceased resistance to infections. [12] The American Diabetes Association recommends annual PAD-screening for diabetic patients with PAD. [7]

Hypertension

The endothelium is damaged by increasing hemodynamic pressure caused by hypertension. Hypertension can also lead to an increased permeability of the arterial walls for lipoproteins .[13] Undoubtedly hypertension is associated with all forms of cardiovascular disease. But the relative risk for developing PAD is higher for diabetes or smoking than hypertension. [12]

Dyslipidemia

A fasting cholesterol level that is greater than 7 mmol/L (270 mg/dL) is related to an increased incidence of intermittent claudication (IC). However, the ratio of total to high-density lipoprotein (HDL) cholesterol was the most significant predictor of occurrence of PAD in the Farmingham study. [12]

The cholesterol homeostasis is substantial for the cellular physiology and eventually altered cholesterol levels are related to metabolic diseases. [14] In the blood circulation cholesterol is carried by lipoproteins, the low-density lipoprotein (LDL), that delivers and the high-density lipoprotein (HDL) that removes cholesterol and mediate the cholesterol homeostasis. Through the imbalances cellular cholesterol accumulate and promote atherosclerosis. [14]

Although some studies failed to confirm that total cholesterol is a meaningful independent risk factor for PAD, some studies have also shown that these factors are associated. There is still evidence that show that medical treating of hyperlipidaemia reduces the progression of PAD but also the incidence of IC. [12]

Inflammatory markers

As shown by several studies coronary and peripheral atherosclerosis are inflammatory diseases.[15] Atherosclerotic lesions onset principally in medium-sized and large elastic and muscular arteries. However they can lead to ischemia of the brain, heart or extremities.[6] . [15]

The mismatch between oxygen demand and the delivery of oxygen during exercise in PAD-patients induces an inflammatory response. [1] It leads to an increasing plasma levels of inflammatory mediators, including acid-reactive substances (which are by-products of lipid peroxidation), thromboxane, tumour necrosis factor- α, interleukin-8,

vascular cell adhesion molecule-1, soluble intercellular adhesive molecule-1, van Willebrand factor, E-selectin and thrombomodulin. [1]

Some recent studies confirmed that C-reactive protein (CRP) was raised in subjects, who were asymptomatic but developed PAD in the subsequent five years. [12]

Hyperviscosity and hypercoagulable states

Several studies reported higher levels of haematocrit and hyperviscosity in patients with PAD, possibly as a consequence of smoking. Hyperviscosity, as well as hypercoagulability have been shown to be risk factors or markers for poor prognosis. [12]

Hyperhomocysteinemia

Compared to the general population, in which the prevalence of hyperhomocysteinemia is at 1 %, the prevalence of hyperhomocysteinemia is high in the population with vascular disease. [12] Studies show that about 30 % of young PAD patients have reported hyper homocysteinemia. Hyperhomocysteinemia may be a higher risk factor for PAD than for CAD. It has been proven by several studies that hyperhomocysteinemia might be an independent risk factor for atherosclerosis. [12] Homozygous defects in necessary enzymes for the homocysteine metabolism, for example cystathionine beta synthase or methylenetetrahydrofolate reductase, lead to high plasma homocysteine concentrations. Patients with such defects have severe atherosclerosis, starting in childhood and might have their first myocardial infraction at the age of 20 years. [6]

Therapy

The medical treatment of PAD should be managed like a CAD risk equivalent in symptomatic and in asymptomatic patients. Most important in PAD treatment is smoking cessation, reaching a goal LDL level <55mg per dL, exercise and antiplatelet therapy with clopidogrel or aspirin (75 to 325 mg) daily, even though neither clopidogrel nor aspirin improve IC. In order to reduce the risk for myocardial infarction, vascular death or stroke, both, clopidogrel and aspirin are considered safe and effective. [9]

Major areas for lifestyle management of PAD is supervised exercise therapy, smoking cessation to slow the progression and to reduce the risk of amputation, graft occlusion and mortality. [3]

The cornerstone for risk management of cardiovascular diseases is the statin therapy, which can promote the regression and stabilization of atherosclerotic lesions. [16] The heart Protection Study show that simvastatin reduces stroke (by 27%), coronary artery events (by 24%) and vascular mortality (by 17%).[3] All patients with a higher cholesterol level then 3mmol/l should be treated with statins. [3] Hypertension and diabetic control are not negligible for patients in order to modify the risk factors for reducing consequences of PAD. [3]

Many early experimental studies focussed mainly on angiogenic protein, like vascular endothelial growth factor (VEGF) (for the purpose of promoting angiogenesis. Some animal studies show promising results for a therapeutic potential of angiogenic strategies for the medical treatment of PAD. [2] Despite the fact that the gene therapy had encouraged experimental results, it so far failed in clinical trials. [2]

Endovascular techniques like balloon angioplasty, stents and plaque debulking procedures are treatment options for patients with lower extremity ischemia. To treat late graft thrombosis the most important aim is to remove the clot and to correct the lesion that caused the thrombosis. [12]

Early years of performing balloon angioplasty alone, the restenosis occurrence was around 40%. Following the introduction of bare metal stents, the restenosis risk rate reduced to 25%. [17] These stents perform the mechanical vessel wall support, and the clinical outcome substantially improved, reducing the risk of in-stent restenosis (ISR). [17]

Restenosis

Restenosis is defined as the developing of a luminal narrowing after a previous percutaneous coronary intervention (PCI). Usually, it occurs within months after a PCI and represents a response of the vessel to the injury. Risk factors, such as diabetes mellitus, hypertension and others can increase the rate of restenosis.[17]

Several studies report an incidence range between 5 % to 22% after carotid endarterectomy (CEA) and the in-stent restenosis is ranging between 2.7 % up to 33 %. [18] However drug-eluting stents reduced restenosis rates, the risk of later stent thrombosis, which has been shown by large observational studies as well as by randomized clinical trials. [19]

Proportional to the degree of injury, the neointimal proliferation in arteries is a response, [17] leading to reendothelialization. [20] Carotid restenosis is a well-known threat and can represent complications of the long-term and short-term recovery from the carotid injury. [18]

Regular monitoring of the circulating levels of inflammatory proteins, cytokines and endothelial regeneration is recommended in the management of preventing restenosis. [20]

Restenosis can be considered as a profuse wound healing reaction or as maladaptive artery response to the induced trauma during revascularization.[18] The results of the following two processes: the neointimal hyperplasia, the thickening of the tunica intima and the vascular remodelling that leads to a change in the vessel size and then again derives from the contribution of the different phenomena, especially the extracellular matrix synthesis, the neoadventitia formation at the injury and the elastic recoil. The trigger for neointimal hyperplasia is the denudation during angioplasty or the endothelial damage.[18] At the same time, it is mediated by the cascade of inflammatory regulators, including chemotactic and mitogenic factors and free oxygen radicals.[18]

Due to the reorganization of constituent elements of the vascular wall the vascular remodelling leads to vessel enlargement or constriction, which leads to a lumen narrowing. [18] During the inflammatory reaction to injury proteolytic enzymes are released and initiate the constrictive process, which furthermore leads to initial degeneration of the smooth muscle cells and new matrix deposition.[18] Vascular cells re-express or up-regulate contractile protein, which also leads to constrictive remodelling. The elastic recoil causes a decrease in dimensions of the vessel and together with mural thrombosis in the pressing phase of overstretch injury, supplying a contribution to acute lumen loss. [18]

A combination of several events lead to luminal narrowing and factors that orchestrate the stages in this process lead to new targets for possible therapeutic interventions. [21]

The atherosclerotic process

The disease of vascular intima, which can involve in the whole vascular system, including aorta and coronary arteries, is characterized by intimal plaques. [13]

The healthy artery wall is structured into three layers. The adventitia, the outermost layer, which contains nerve endings, mast cells and vasa vasorum (micro vessels nourishing the outer layer of the media). The tunica media contains smooth muscle cells and a well-organized extracellular matrix consisting of elastin, collagen and other macromolecules. The atherosclerotic plaque is formed in in the innermost layer, which is called the intima. [11]

Cell biology of atherosclerosis in 1960s and 1970s was focused on smooth muscle cell proliferation as the main focus for atherosclerotic plaques. . [22] Nowadays the role of mononuclear phagocyte as an effector has developed, with characterized macrophage-derived mediators, like cytokines. This concept based on the dynamic interplay between vascular cells and mononuclear phagocytes during atherogenesis, has become generally accepted. [22]

Caused by the luminal stenosis through narrowing or to thrombotic obstruction, symptoms related to a reduction in blood flow arise. Other symptoms like angina pectoris can be caused by ischemia due to stenotic lesions and increase myocardial oxygen damage while physical activities. [11] Clinical presentation of atherosclerosis can either be acute or chronic and depending on the vascular territory involved it differs significant. Most cases of PAD remain asymptomatic for decades because of the diffuse

and slow progression. [11] In some arias, like the renal arteries the typical presentation is a long-developing, chronic syndrome (such as progressive renovascular hypertension or/and deterioration renal function subordinated to renal artery stenosis).In other vascular territories, such as the coronary arteries, both acute and chronic presentations (stable angina pectoris) commonly arise. [11] Atherosclerotic plaque rupture and thrombosis can manifest as acute coronary syndrome (ACS), stroke or myocardial infraction, which are the most common forms. [23]

The atherogenesis can be divided in several parts over time: early lesions and the formation of the fatty streaks, the atheroma formation and progression of the atherosclerotic plaque. [13]

The low-density lipoproteins are permitted to subendothelial accumulation, which is triggered by structural alterations and endothelial dysfunction, including the absence of a solid luminal elastin layer and the exposure of proteoglycans. [23] The high bioavailable level of homeostatic mediators such as NO is one of the hallmarks of an endothelial dysfunction. [24] Inflammatory cells release substances such as reactive oxygen species and enzymes (myeloperoxidase or lipoxygenase) for oxidative modification (oxLDL).[23]

Apolipoprotein B100 (ApoB100) which contains low-density lipoprotein (LDL) for circulation through the blood flow, is binding to the negatively charged matrix proteoglycans, which leads to retention of LDL particles in the intima. As a result the plaque becomes susceptible for oxidative modification. [23]

This oxidation indicated the very first step of atherosclerosis in cardiovascular diseases. [13] These oxLDL and oxidized lipids trigger an expression of molecules for adhesion and endothelial cells secrete chemokines. Together with the deposited platelet-derived chemokines, they drive intimal immune cell infiltration. [23]

Activated endothelial cells express adhesion molecules which can bind mononuclear cells. Once adherent to the endothelial surface the mononuclear cells enter the intima by receiving chemoattractant signals, like the monocyte chemoattractant protein1, which is contributed as an important one in this process. [22] Chemokines induced by INF-γ (interferon-γ) are recruiting T lymphocytes selectively to the emergent atherosclerotic plaque. Once the monocytes are resident in the intima they mature into macrophages, and express receptors that are necessary for the uptake of modified lipoproteins and consequently the formation of foam cells. [22]

These mediators and the involved cells are depicted in figure 5.

The early “fatty-streak” lesions consist mostly of monocyte-derived macrophage-like foam cells, which are loaded with lipids.[23] Adhesion molecules expressed by endothelial cells, such as vascular cell adhesion molecule-1, bind monocytes and T lymphocytes that congregate in the early plaque. [22]

The regions that are highly infiltrated by inflammatory cells are called ´shoulder´ region of the plaque. Inflammatory cells produce proinflammatory mediators and enzymes that contribute to continuous inflammation of advanced plaques. [23]

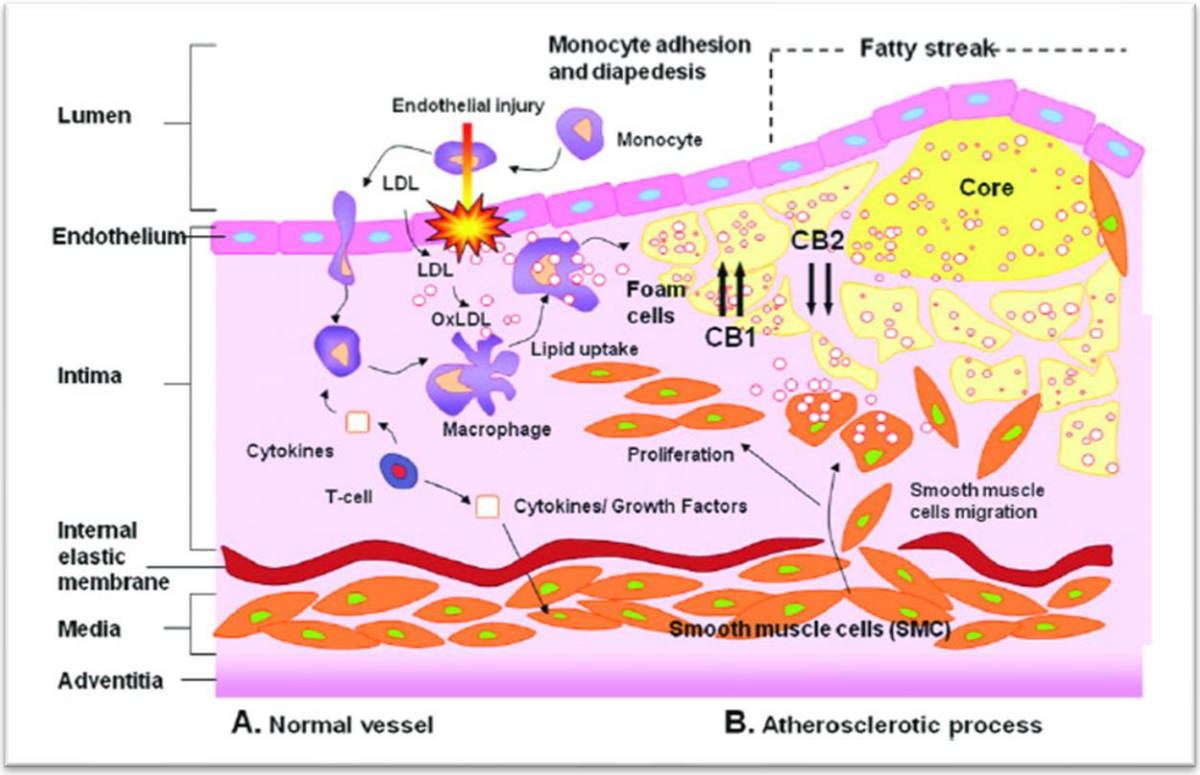

Figure 5 *normal artery compared to artery with atherosclerotic process (Quelle: M. Zubrzycki, A. Liebold,M. Zubrzycka in Journal of Physiology and pharmacology 2014, 65, 2, page 183-191)* [20]

A normal artery, shown in figure 5 – consisting of a endothelium (one cell thick layer), muscular membrane and adventitial membrane (B) due to the atherosclerotic process an accumulation of 1) inflammatory cells – monocytes transform to macrophages 2) LDL – low density lipo proteins 3) smooth muscle cells, which transformed and produce elements of connective tissue – especially between endothelial cells and the muscular layer of large arteries. [20] Macrophages that phagocyte LDL (foam cells) die and the inner content (lipids) accumulate in the extracellular space, forming the plaques lipid core. The connective tissue that surrounds the nucleus is the predominant component and builds a plaque-coating layer.[20]

One of the factors found in the plaque is the M-CSF (macrophage colony-stimulating factor) that mediate macrophage maturation. [22] A wide variety of other inflammatory mediators participate in the different phases of atherogenesis, namely the initiation, progression and the clinical complications leading to disease manifestation. [22] The inflammatory mediator ligand CD154 (binding CD40) was identified as a disease-relevant activator of the potent procoagulant tissue factor expression in human macrophages. [22]

The thrombogenicity of the plaque, as well as the plaques fibrous cap integrity are regulated by inflammatory processes. [22] Macrophages predominate numerically but T lymphocytes are also localized within lesions and may have either negative or positive effects atherosclerotic plaque growth and evolution. [11]

Atherosclerotic plaques, once established, progress by continued accumulation of more lipids and lipid-engorged cells. [11] Furthermore, components of atherosclerotic plaque drain from lesions and reach adjoining lymph nodes, where T and B cells utilise as antigens. Other components of the plaque, such as local produces cytokines, regulating immune response in these lymph nodes. [11]

During progression, some atherosclerotic plaques develop parts of calcification. This accumulation of calcium mineral in atheromata results from disrupted clearance and dysregulation of deposition. [11] Microscopic or patchy calcification are associated with mechanical instability of plaques and may advance the tendency of ruptures, as well as provoke thrombosis. Larger accumulations may be associated with a lower probability to trigger a thrombotic event [11], as shown in the following figure 6.

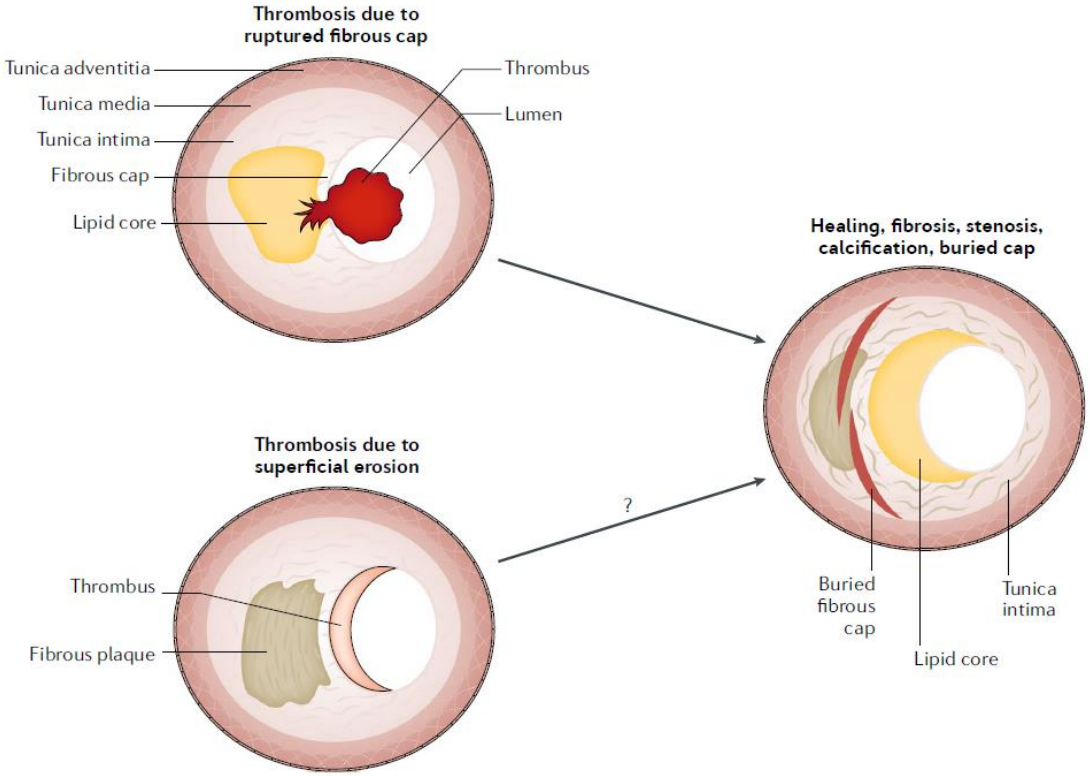

Figure 6 *Atheroma complication – The fibrous cap fractures and allows mediators of the coagulation accessing core and triggering thrombosis. As described in the chapter above* [11]

The necrotic core is formed by the death of the foam cells [22] and therefor by accumulation of cell debris and cholesterol crystals. [23] The fibro atheromatous plaques are covered by composition of collagen and SMCs (smooth muscle cells), the fibrous cap. [23]

miRNA – a small review about

Containing 19 to 26 nucleotide, miRs are short and noncoding RNAs , involved in regulation of human gene expression[26] at the post-transcriptional level. [14] They are stable in the bloodstream [27], bind to mRNA, using a conserved around 7 to 8 nucleotide long seed sequence and inhibit protein synthesis by blocking mRNA translation. [26][14] These small miRNA sequences seem to be conserved among microorganisms, plants and animals, suggesting their crucial role in posttranscriptional regulation of protein synthesis.[26] In 1993 the first miRs were identified in Caenorhabditis elegans, and nearly a decade later their functional role in human diseases were evaluated. [14]

Different miRs have been discovered to be an important part of the modulation of angiogenesis.[4] and some miRs have been already identified as potential novel tools for an individual and personalized health care for patients suffering from heart diseases. [27]

As documented by several studies, miRs can be detected in circulating blood. Because of their presence in plasma and serum in a remarkable stable form, which withstands even repeated freezing and thawing, miRs could serve as possible biomarkers for several diseases.. [28]

MiR-based therapeutics are in preclinical development and two have currently reached clinical trials. [14] One of these two: Miravirsen – which is a locked nucleic acid, that is directed against miR-122. Miravirsen targets hepatitis C virus RNA and leads to long-lasting hepatitis C virus suppression. Another miRNA therapeutic is a double stranded miRNA mimic (miR-34), which inhibits multiple oncogenic pathways as a tumor suppressor and stimulated antitumor immune responses. [14]

mRNA and cardiovascular disease

By discovering that miRplay a key role in the fine regulation of cardiovascular pathophysiologic processes, a further layer of regulators was unveiled. [17]

For an objectively monitoring of proper organ functions or for detecting ongoing pathological processes biological markers (biomarkers) are essential. A good biomarker should correlate with severity of disease and enable a reliable estimation of prognosis. [2] By assessing miR expression in the circulation of PAD-patients, potential biomarkers could be yielded for diagnosis of PAD, for prediction of future PAD risk and as potential future treatment pathways for PAD.[26]

MiR are modulating several aspects of cardiovascular disease, such as the vascular response to injury, among others. Their important role in cell-to-cell communications, for example between vascular smooth cells (VSMCs) and endothelial cells (ECs) within the vessel wall, but also communication with other cell populations (monocytes, pericytes and platelets) are mediated by miR. [17]

Because of very low concentrations of miRNA circulating in plasma, gene expression studies faced technical limitations. [26] That makes it hard to translate the use of plasma miRs or to standardize in clinical context, where samples from different origins and unsteady qualities need to be processed. As shown in previous studies specific mR expression signatures were found in peripheral blood of patients with CAD (coronary artery disease) compared to healthy individuals. . [26]

Circulating miRNAs as biomarkers conduct research and some were already related as potential biomarkers of ISR (in-stent restenosis). Studies performed on non-ISR patients, on ISR-patients and on a healthy control-population shown the dysregulation in IRS-patients in levels of miR-21, miR-145, miR-143 and miR-100. [17] Increasing expression levels of miR-143 and miR-145, which are initiated by cytokines transforming growth factor- β and bone morphogenetic protein and induce contractile genes expression. [17] Another miR which is upregulated after vascular injury is miR-424, and this miR is involved in the modulation of VSMCs proliferation (G1/s transition). [17] The involvement of the miRs stimulating or inhibiting the proliferation of VSMC is shown in following picture.

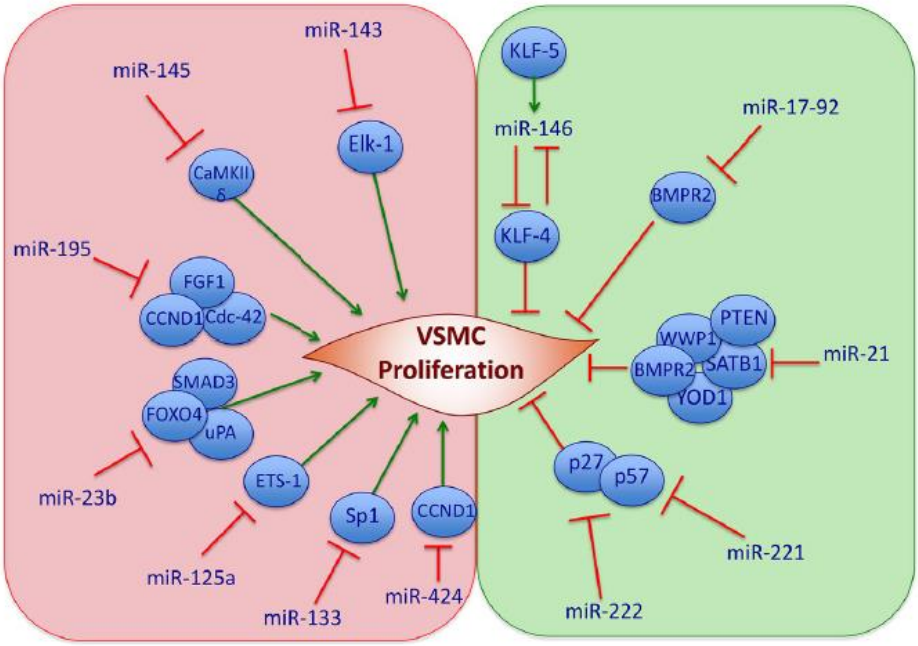

Figure 7 *Representing most relevant miRs that are modulating proliferation of vascular smooth muscle cells. The green area indicates miRs, which are promoting the synthetic phenotypes of VSMCs, and the red area indicates miRs inhibiting this process*

BMPR2: bone morphogenetic protein receptor type II, FOX04: Forkhead box protein O4, Cdc-42: cell division control protein, KLF-4: Kruppel-like factor, SMAD3: small mother against decapentaplegic, PTEN: phosphatase and tensin homolog, uPA: urokinase-type plasminogen activator, NEDD4: like E3 ubiquitin-protein ligase and WWP1 [42]

Higher expressions of miR-143 and miR-145 were shown in the smooth muscle cells. The miR-133 and miR-1 are enriched in cardiomyocytes and maintain myogenesis, hypertrophy and cardiac development. [29] The two miRs known as cardiac specific miRs, miR-208a and miR-208b are expressed by introns of myosis heavy chains and both are integral parts of stress-dependent cardiac growth and gene expression. [28]

The different types of miRs identified in endothelial cells have been classified as stimulating (let-7f, miR-27b, miR-130a) or inhibiting (miR-221 and miR-222) angiogenesis in vitro. [28]

Further studies show that a knockdown of miR-221 and miR-222 supresses VSMC (vascular smooth muscle cells) - proliferation (in vivo) and reduces neointimal lesion formation after angioplasty. [2] Especially miR-126 was highly concentrated in endothelial cells and seems to be essential for vascular development. Members of the miR17-92 cluster have been shown as modulators of the angiogenesis. (Figure 7, page 33) [28]

In PAD patients with atherosclerosis obliterans, samples have shown increased levels of miR-21, miR-27b and miR-130a and decreased levels of miR-221 and miR-222. A far larger size of individuals were analysed in revealing the level of circulating miR-126 in patients with diabetes. These confirmed the relation of the downregulation of miR-126 in patients with diabetes. [30]

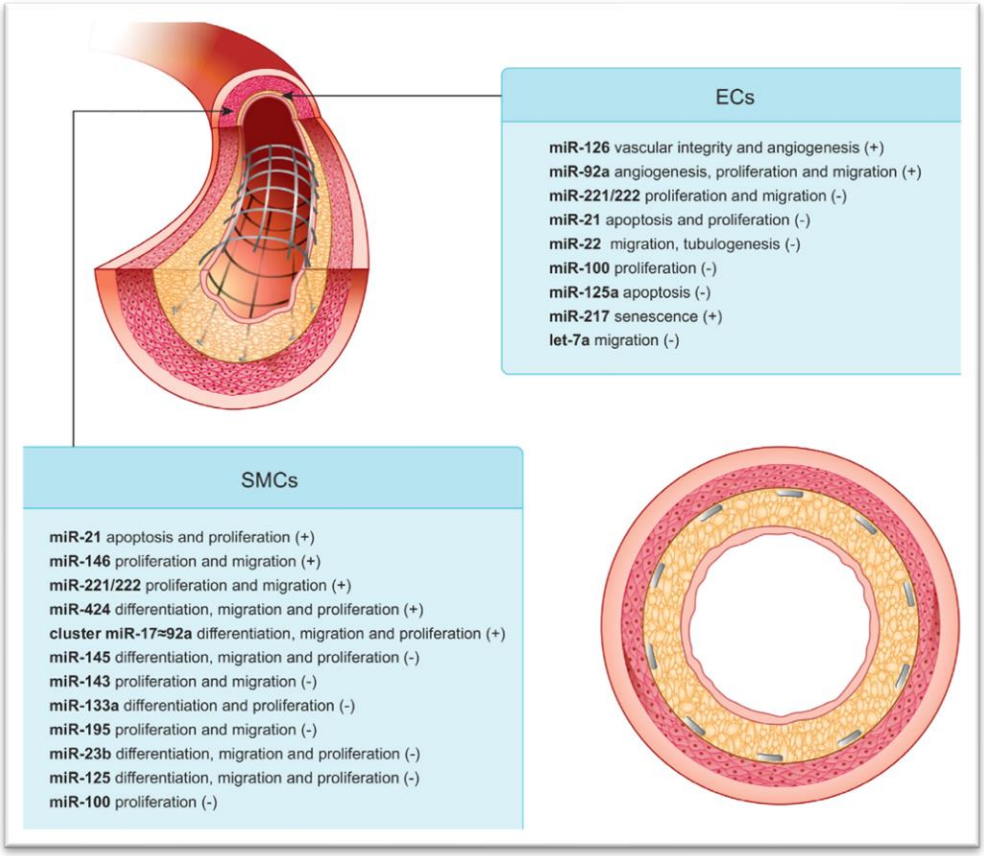

Figure 8 *miRNAs involved in cascular response to injury (Quelle: by guest on October 15, 2016 http://circres.ahajournals.org/) From John et al. Cirres 2016 (27) [17]*

Left panel: longitudinal section of an artery after a stent implantation, at the adluminal side (shadowed in yellow) a neointimal layer is showed. [17]

Left Box: a list of the miRNAs, that are most relevant for modulation of the vascular smooth muscle cells´ biology [17]

Lower right part: cross-sectional view of the same artery [17]

Right box: a list of miRNAs, that are most relevant for modulation of the endothelial cell´ biology [17]

(+) indicate induction and (-) indicate inhibition of a specific phenotype/biological effect [17]

Aims of the thesis

The aim of this diploma thesis was to compare the expression of 9 circulating miRs potentially associated with in-stent restenosis, namely miR-17, miR-21, miR-92a, miR-126, miR-195, miR-221, miR-222, miR-223 and miR-424 in patients PAD undergoing peripheral angioplasty with stent implantation.

The primary endpoint was defined as the composite of the first occurrence of non-fatal myocardial infarction, non-fatal stroke or transient ischemic attack (TIA), cardiovascular death and recurrent symptoms of PAD, i.e. claudication or critical limb ischemia, due to >80% target vessel restenosis within two years after peripheral angioplasty.

Patients und Methods

Study Population

The study population consisted of 62 consecutive patients, each of them admitted to the Department of Internal Medicine II, Division of Angiology at the Medical University of Vienna. The patients required interventional treatment with peripheral angioplasty with stent implantation between 2008 and 2010. Patients had intermitted claudication, which was classified as Rutherfort stage of PAD 2 and 3. The grade of stenosis or occlusion in the femoropopliteal segment has been confirmed by Duplex sonography. Each individual patient received long-term aspirin therapy (100mg/day) and clopidogrel (75mg /day) for three months after angioplasty and stenting with bare-metal nitinol stents. The clinical follow-up was performed one and two years after percutaneous intervention.[31]

Exclusion criteria were a known intolerance to aspirin and clopidogrel (gastrointestinal bleeding or allergic reactions), a therapy with vitamin K antagonists (such as warfarin, acenocoumarol, phenprocoumon), medical treatment with dipyridamole, ticlopidine or nonsteroidal anti-inflammatory drugs, malignant paraproteinemias, a personal or family history of bleeding disorder, myeoloproliferative disorder, thrombocytopenia induced by heparin, hepatic failure, a significant surgical procedure within one week before study inclusion, already known qualitative defects in thrombocyte function, a platelet count under 100.000 or more than 450.000 per microliter and a haematocrit under 30%. The Ethics Committee of the Medical University of Vienna approved the study protocol in compliance with the Declaration of Helsinki. A written informed consent was obtained from all study participants. [31]

MicroRNA preparation and quantification

Total RNA, which includes small RNAs were extracted from 200ml serum using miRNeasy Serum/Plasma Kit and the robotic workstation for semi-automated purification of RNA (QIAcube/Quiagen). To normalize for extraction variation between all samples synthetic *Caernorhabditis elegans* (cel-miR-39, Quiagen) was used as spike-in control. For reversed transcription reaction a fixed volume of 9 µL RNA was used and performed using miScript II RT Kit (Quiagen). The obtained cDNA (complementary DNA) was diluted 5-fold and added to quantitative real-time PCR (qPCR). The prepared plates for qPCR were performed by the automatic pipetting device (GeneTheatre, Germany, Analytik Jena AG), in order to minimize sample-to-sample variation of the Reagans. 2µL cDNA, miScript SYBR Green PCR Kit (Qiagen) and miScript primer assays (Qiagen) were used for the qPCR. Quantitative real-time PCR was performed on LightCycler® 480 (Roche, Switzerland).

All Ct-values larger than 35 were considered negative calls. In order to calculate and normalize the concentration of each miR/µL serum the spike-in control (cel-miR-39) was used.

miRNAeasy Serum/Plasma Kit

The miRNeasy Serum/Plasma Kit is a combination of phenol/guanidine-based lysis of the samples and silica-membrane-based purification of RNA. QIAzol Lysis Reagent is a monophasic solution of phenol and guanidine thiocyanate. It is designed for denaturation of protein complexes and RNases and for facilitating lysis, as well as

removing most of the residual DNA and proteins from the lysate by an organic extraction. [32]

QIAzol Lysis Reagent is added to plasma samples and then chloroform is added. After centrifugation the lysate is separated into aqueous and organic phase. The upper, aqueous phase contains the RNA, DNA partitions to the interphase and proteins stay to the lower, organic phase or the interface. [32]

Now the upper, aqueous phase was extracted and ethanol was added so appropriate binding conditions are provided for all RNA molecules. Then the samples are transferred to the RNeasy MiniElute spin column, and after centrifugation the total RNA binds to the membrane. Phenol and other contaminants can now be efficiently washed away. The eluate contains the high-quality RNA diluted in RNase-free water. [32]

Procedure

The frozen samples were thawed and 200 µl serum was taken for RNA isolation. Serum was mixed with 5 volumes of QIAzol Lysis Reagent (1000µl) by vortexing and the tube was finally placed at room temperature (23°C) for 5 min.

The next step was to take 4 µl Serum/Plasma Spike-In Control diluted at 1.6 x 108 copies/µl working solution and to add it to the samples and to mix it gently by pipetting the liquid up and down.

The mixed tube was places on the benchtop at room temperature (23°C) for three to four minutes and then places in the centrifuge. After centrifuging for 15 minutes at 12,000 x g at a temperature of 4°C the samples separated into three phases. The upper aqueous phase, containing the RNA, the interphase, which is white and contains DNA partitions, and the lower phase, which is the organic phase containing the proteins.

Now 600 µl of the upper aqueous phase was transferred to a new collection tube, thereby avoiding the transfer of any interphase material.

Following steps were performed by a Qiacube robotic workstation for semi-automated purification of RNA. 900 µl ethanol 96% was added and mixed carefully by pipetting up and down several times. Further up to 700 µl sample was pipetted into a RNeasy MinElute spin column, centrifuged for 15 seconds at ≥8000 x g (≥10,000 rpm) at room temperature (22°C). The flow-through was discarded.

In order to wash the sample, we repeated 3 steps in the centrifuge for 15 seconds and discarded the flow-through again. First with 700 µl Buffer RWT at ≥8000 x g (≥10,000 rpm) for 15 seconds. Second 500 µl Buffer RPE at ≥8000 x g (≥10,000 rpm) for 15 seconds. And last 500 µl ethanol (80%) was added onto RNeasy MinElute spin column and centrifuged at ≥8000 x g (≥10,000 rpm) for 2 minutes to wash the spin column membrane. To dry the membrane the spin column it was placed into a new collection tube and centrifuged with open lid for 5 minutes at full speed. Then discard the collection tube with the flow-through. After placing the RNeasy MinElute Spin column into a new collection tube, 14 µl RNase-free water was added directly to the middle of the membrane and centrifuged at full speed for 1 minute to elute the RNA.

miScript II RT Kit (Reverse Transcription)

RNA samples were thawed on ice and 10x miScript Nucleics Mix, RNase-free water and 5x miScript HiSpec Buffer were thawed at room temperature: (15-25°C).

The master mix for reverse-transcription was prepared on ice as follows:

5x miScript HiSpec Buffer	*2 µl*
10x miScript Nucleics Mix	*1 µl*
RNase-free water	*4 µl*
MiScript Reverse Transcriptase Mix	*1 µl*
Template RNA	*2 µl*

The master mix was mixed gently and stored on ice. After adding 8 µl master mix and 2 µl template RNA the tubes were mixed, centrifuged and stored on ice.

The samples were then Incubated samples at 37°C for 60 minutes, and then at 95°C for 5 minutes to inactivate miScript Reverse Transcriptase Mix and then place it on ice.

RT for Spike-in Standard Curve

Equipment and Reagents

5x miScript HiSpec Buffer	*4 µl*
10x miScript Nucleics Mix	*2 µl*
RNase-free water	*7 µl*
MiScript Reverse Transcriptase Mix	*2 µl*
RNA HUVEC	*2 µl*
Spike-in from stock with 7x108 copies	*3 µl*

The master mix was mixed gently and stored on ice.

Using the Program: MiScript RT

1. Incubated samples at 37°C for 60 minutes.
2. Incubated samples at 95°C for 5 minutes to inactivate miScript Reverse Transcriptase Mix and then place it on ice.

Dilution series of spike-in control:

TUBE 1	*20 µl cDNA with 1x108 copies + 20 µl H2O*	*5x108*
TUBE 2	*5 µl Tube 1 + 45 µl H2O*	*5x107*
TUBE 3	*5 µl Tube 2 + 45 µl H2O*	*5x106*
TUBE 4	*5 µl Tube 3 + 45 µl H2O*	*5x105*
TUBE 5	*5 µl Tube 4 + 45 µl H2O*	*5x104*
TUBE 6	*5 µl Tube 5 + 45 µl H2O*	*5x103*
TUBE 7	*5 µl Tube 6 + 45 µl H2O*	*5x102*
TUBE 8	*50 µl H2O*	*5x10*

MiScript® SYBR® Green PCR Kit used with miScript miRNA PCR Arrays

Material

2xQuantiTect® SYBR Green PCR Master Mix
10x miScript Universal Primer
RNase-free water
RT2 PCR Array Loading Reservoir
Optical adhesive film
384-well plate

The reagents (QuantiTect® SYBR Green PCR Master Mix, miScript Universal Primer, RNase-free water) were thawed at room temperature (24°C) and after mixing the individual solutions the master mix according to the table was prepared and contained everything except the template cDNA.

Reaction setup for real-time PCR in following table:

Component	*Volume /reaction (384-well)*
2xQuantiTect® SYBR Green PCR Master Mix	*5 µl*
10x miScript universal Primer	*1 µl*
1 µL 10x specific miScript primer assay	*1 µl*
RNase-free water	*1 µl*
Template cDNA (added in the following step)	*2 µl*
Total volume	*10 µl*

GeneTheater

Template cDNA was dispensed to the 384-well plate using GeneTheatre (Germany, Analytik Jena AG). In the following step the appropriate volumes were dispensed into the plate wells containing template cDNA. After that the plate was carefully and tightly sealed with Rotor-Disc Heat-Sealing Film. Before putting the well into the real-time cycler, the well was centrifuged for 1 minute at 1000 g at room temperature (23°C), to remove bubbles made by pipetting.

The real-time cycler is programmed according to the following table. The well was placed and the cycling program started.

Step	Time	Temperature
PCR initial activation step	15 minutes	95°C
3 -step cycling		
Denaturation	15 seconds	94°C
Annealing	30 seconds	55°C
Extension	30 seconds	70°C
Cycle number	40 cycles	

The HotStarTaq DNA Polymerase is activated by the heating step which takes 15 minutes. During the extension the fluorescence data is collected. Depending on the amount of templates cDNA and the abundance of the target the cycle number may vary.

The C_T values for miRNeasy Serum/Plasma Spike-In Control from each reaction 4 extracted to generate a standard curve by plotting the log copy number Spike-In Control used in every PCR against mean C_T value.

Statistical Analyses

All summarized categorical variables are shown as counts and percentages. In order to be compared the appropriated χ2-test or Fisher´s exact test is used. Variables that are continuous are expressed as median and interquartile range (IQR). These variables are compared by the t-Test or the Mann-Whitney U test, which is used in cases of non-normal distribution.

By correlation with survival variable using Cutoff Finder´s significance (http://molpath.charite.de/cutoff) the optimal miRs cutoff values were established. The point within the most significant log-rank test split is defined as the optimal cut-off. In order to compare time-dependent differential power of circulating miRs Kaplan-Meier failure plots were created for groups above and below the cut off value according to miRs expression. P-values smaller than 0.05 were defined as bilateral and are statistically significant. Finally for all statistical analyses STATA version 12 (StataCorp LLC, College Station, TX, USA) and SPSS 22.0 (IBM Corporation, Armonk, NY, USA) were used. [31]

Results

Following table (table 1) contains the clinical characteristics of the study population. The median age of the population was 64.5 years (interquartile rage: 58 to 73.2), 36 patients were male which make up 58.1% and the median BMI was 26.6 (interquartile rage: 23.9-29.1). The most frequent PAD-related comorbidities were hypertension with 93.5%, following hypercholesterolemia with 95.2%, both were present in almost all study subjects. A total of 26 patients (41.9%) were actively smoking and 24 patients (38.7%) had diabetes mellitus. In 19 patients, which is 30.6%, coronary artery disease and cerebrovascular disease were present and in 16 patients, which is 25.6%, respectively.

Table 1
Baseline chinical characteristics

	Overall (n=62)	**Primary endpoint (n=26)**	**No primary endpoint (n=36)**
Demographics			
Age	54.5 (58-73.2)	66 (58-76.5)	63.5 (58-69.5)
Sex (MALE)	36 (58.1)	12 (46.2)	24 (66.7)
BMI	26.6 (23.9-29.1)	26 (24.1-28.2)	27.4 (23.7-29.9)
Active smoking	26 (41.9)	8 (30.8)	18 (50)
Comorbidities			
Hypercholesterolemia	59 (95.2)	23 (88.5)	36 (100)
Hypertension	58 (93.5)	25 (96.2)	33 (91.7)
Diabetes mellitus	24 (38.7)	11 (42.3)	13 (36.1)
CAD	19 (30.6)	7 (26.9)	12 (33.3)
CVD	16 (25.8)	5 (19.2)	11 (30.6)
Previous MI	13 (21)	4 (15.4)	9 (25)
Previous TIA/Stroke	9 (14.5)	2 (7.7)	7 (19.4)
Labratory parameter			
Triglycerides	146 (110.2-193.5)	125 (99.5-182)	160.5 (129-257)
Cholesterol	184.5 (150.5-215.5)	172 (142.5-205.2)	204 (165.6-238.5)
LDL	95 (70.3-123.5)	93.2 (67.7-119)	105.6 (75-139.2)
HDL	48 (40-56.5)	47.5 (42.2-54.2)	49 (40-63)
Lipoprotein a	37.5 (14.5-93.7)	39.5 (18.2-84.7)	31 (12.2-96)
hs-CRP	1.1 (0.4-1.8)	0.8 (0.2-3.8)	1.1 (0.7-1.8)

Creatinine	1.1 (0.9-1.2)	1 (0.9-1.2)	1 (0.9-1.1)

Prior medication

	Overall (n=62)	**Primary endpoint (n=26)**	**No primary endpoint (n=36)**
ASA	62 (100)	26 (100)	36 (100)
Clopidogrel	62 (100)	26 (100)	36 (100)
Statin	56 (90.3)	22 (84.6)	34 (94.4)
ACE-Inhibitors/ARB	56 (90.3)	24 (92.3)	32 (88.9)
Beta blockers	40 (64.5)	16 (61.5)	24 (66.7)
Calcium channel blockers	25 (40.3)	12 (46.2)	13 (36.1)
Alpha blockers	5 (8.1)	3 (11.5)	2 (5.6)
Diuretics	24 (38.7)	10(38.5)	14 (38.9)
Oral anti-diabetic-medication	20 (32.2)	8 (30.8)	12 (33.3)

The primary endpoint, defined as the composite of the first occurrence of one of the following events: non-fatal myocardial infraction, non-fatal transient ischemic attack (TIA) or stroke, cardiovascular death, and recurring symptoms of PAD, i.e. claudicatio intermittens or clinical limb ischemia, due to over 80% target vessel restenosis within two years after the peripheral angioplasty.

In 26 patients (41.9%) the primary endpoint occurred within two years. One of these patients suffered non-fatal myocardial infraction, two of the patients had stroke or TIA and 23 patients had recurrent symptoms of PAD because of more than 80% target-vessel restenosis or re-occlusion. From 23 patients with recurrent symptoms, 91.3%, which is 21 patients, underwent target vessel revascularization (secondary endpoint).

The concentrations of circulating miRs were determined like described in the chapter materials and methods.

The highest expression was observed for platelet-specific miR-223 (40,604 Copies/µL, IQR: 22,127-66,277). The circulating levels of miR-143 were detectable in 27 patients (43,5%) and miR-145 in 26 patients (21,9%) at very low expression levels (miR-143: IQR: 9-21; 13 copies/µL; miR:143: IQR: 8-19; 12 copies/µL). This leads to the conclusion that these two circulating miRs may not be eligible as biomarkers for the risk stratification for patients having PAD and were therefore, miR-143 and miR-145 were excluded from further analysis.

The Mann-Whitney U test was used to compare the circulating concentrations of the analyzed population divided into groups with and without primary endpoint during the follow-up. . Concentrations are given in log2 copies / µl.

Figure 9 depicts the expression levels of the nine miRs by box-and-whisker diagrams Circulating levels of miR-92a and miR-195 were significantly lower in patients with primary endpoint during the follow-up as compared to patients without events (miR-92a 3577 copies/µl, interquartile range (IQR) 1061 – 6223, vs. 5705 copies/µl, IQR 3256 – 9444, ρ = 0.05; miR-195: 291 copies/µl, IQR 101 – 798, vs. 797 copies/µl, IQR 372 – 1616, ρ = 0.0038). Circulating levels of miR-17, miR-21, miR-126, miR-221, miR-222, miR-223 and miR-424 were similar in both groups of patients.

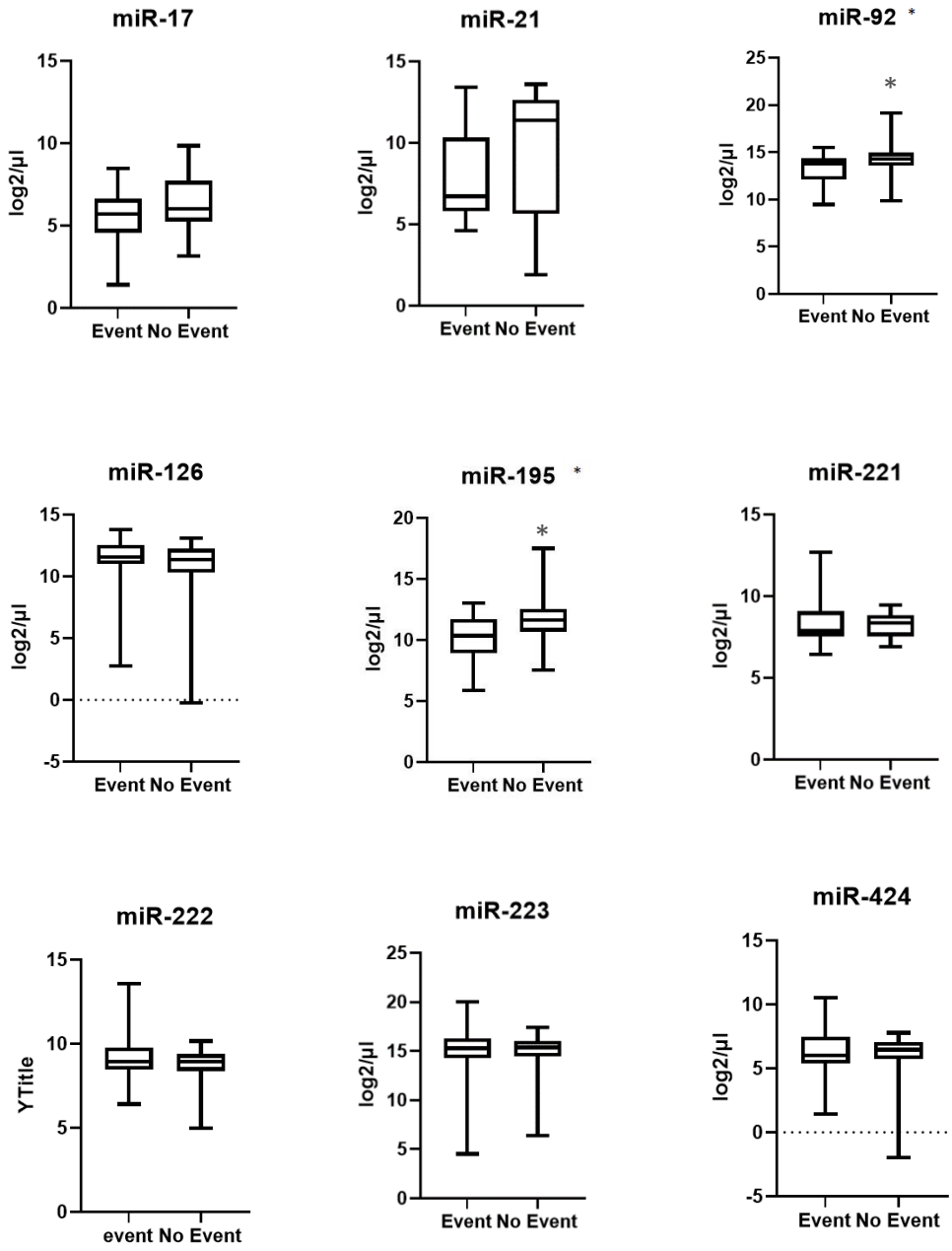

Figure 9 Circulating levels **of** miR-17, miR-21, miR-92a, miR-126, miR-195, miR-221, miR-222, miR-223, miR-424 *in patient*s *with PAD*. *Levels (log 2 copies/µl) of investigated miR in patients with or without an event in the following 2 years follow up. Statistical significance was calculated by Mann-Whitney U test and values of p ≤ 0.05 were considered significant. *p<0.005.*

Determined as the optional cut-off values for the primary endpoint were 263.3 copies/µl for miR-195 and 2167 copies/µL for miR-92a. In 48 patients (77.4%) levels of circulating miR-92a and in 47 patients (75.8%) levels of miR-195 were above the optimal cut-off. In patients with miR-92a and miR-195 concentrations below cut-off level the risk of occurrence of the primary endpoint was significantly higher (miR-92a: log rank: ρ = 0.0018; miR-195: log rank: ρ = 0.0008), compared to patients with higher circulating levels of miR-92a and miR.195 (Figure 10 and 11, respectively)

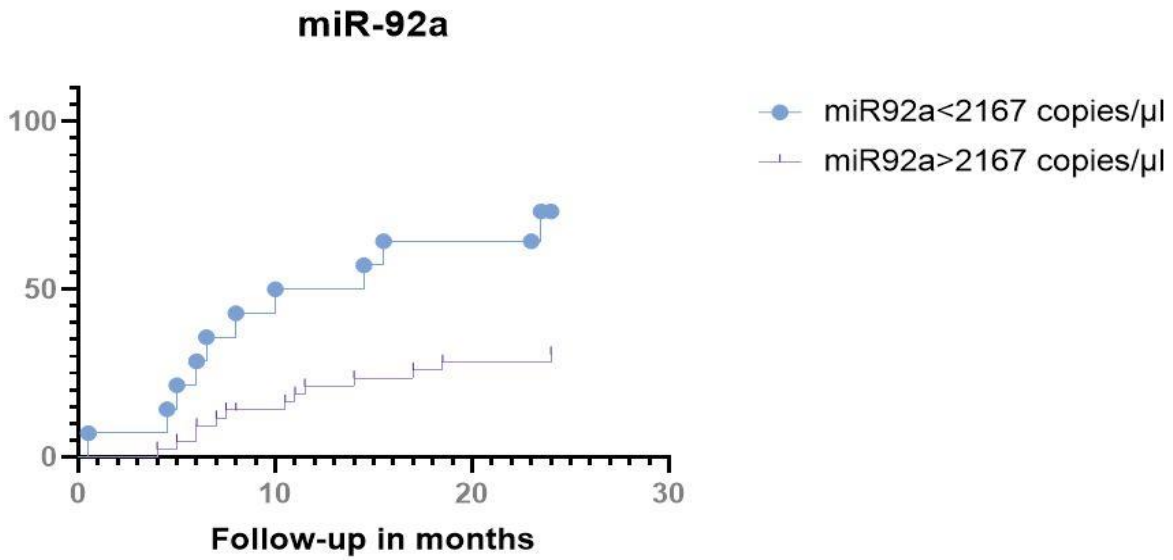

Figure 10 Kaplan Maier for the cumulative incidence of adverse ischemic events (primary endpoint) in patients with circulating levels of miR-92a below (blue line with dots) and above (dark blue line) the calculated cut-off value of 2167 copies/µl

The light blue lines with dots is showing the group with circulating levels of miR-92a above 2167 copies/µl. The darker blue line with no dots is showing the group with circulating levels of miR-92a below 2167 copies/µl.

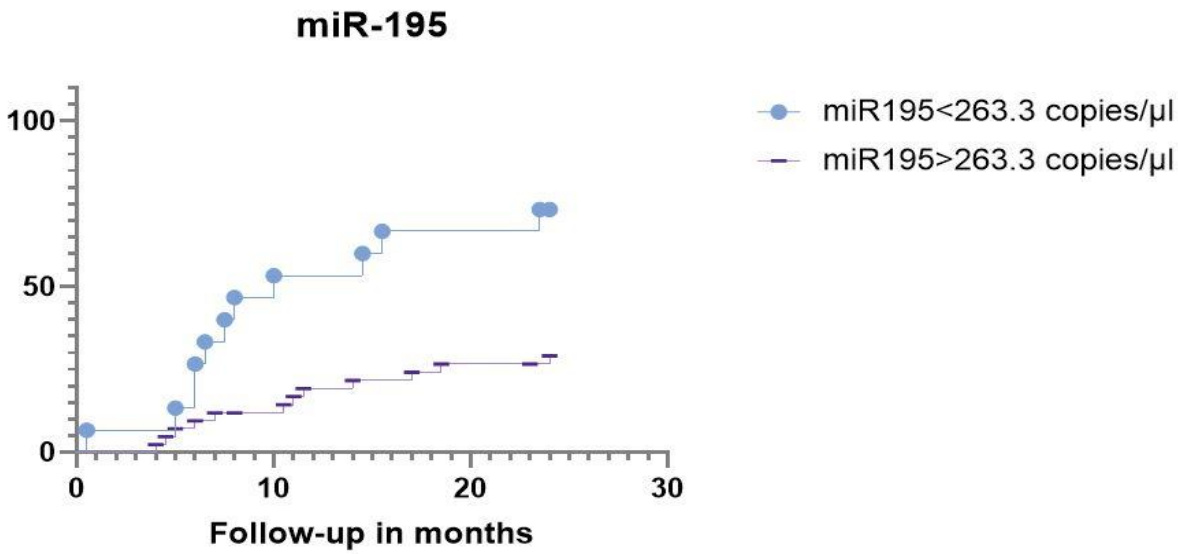

Figure 11 Kaplan Maier for the cumulative incidence of adverse ischemic events (primary endpoint) in patients with circulating levels of miR-195a below (blue line with dots) and above (dark blue line) the calculated cut-off value of 263.3 copies/µl

The light blue lines with dots is showing the group with circulating levels of miR-195 above 263.3 copies/µl. The darker blue line with no dots is showing the group with circulating levels of miR-195 below 263.3 copies/µl.

Discussion

The study performed to investigate circulating miRs and their prognostic value for adverse ischemic events and target vessel revascularization in PAD-patients with peripheral angioplasty and stent implantation. In our research we identified miR-195 and miR-92a as strong predictor of adverse atherothrombotic incidences and target vessel revascularization (TVR) after angioplasty and stenting in PAD patients.

It is unusual for PAD to manifest in the age under 55 years, it increases sharply with increasing age and affects about 8 to 10% individuals aged under 65 years and up to 20% of individuals in the age under 80 years.[10] A current or former smoking history, diabetes (regardless of whether it was type 1 or 2), hyperlipidaemia (a total cholesterol concentration of 240 mg/dL or more), a LDL (low-density lipoprotein) cholesterol concentration (160mg/dL or more), a HDL (high-density lipoprotein) concentration (35 mg/dL or less), triglyceride concentration of 200mg/dL or more, a total cholesterol to HDL ration of 5.0 or more are defined as main risk factors for atherosclerosis and further events. [33]

The rate of restenosis can largely vary and also depends on several clinical, anatomical and procedural variables.[17] Studies evaluate restenosis several times between one and 36 months post intervention. [18]

Because in-stent restenosis is the most frequent complication after stent implantation, there is need to identify new biomarkers and furthermore to help improve risk stratification and management of patients.

We therefore focused especially on circulating miRs, which are part of the regulation of smooth muscle cell (SMC) proliferation and migration. Additionally, miRs that are related to platelet activation and endothelial dysfunction were explored, both processes well known as contributors in stent thrombosis and in-stent restenosis. [31]

As shown below (Figure 12) miR-223 is counter regulated between cardiovascular and metabolic diseases, and as shown in a study in 2017 no overlap in the pictured miRs was found between these two diseases, allowing differentiation between cardiovascular and metabolic disorders. [34] MiR-223 was first subsequently identified in the heamatopoietic system, expressed in the myeloid compartment. It has also been considered as an adjuster of the inflammatory response and the granulocyte production. [35] Therefore, many studies had been investigating the possible involvement of miR-223 in cardiovascular pathophysiology and their significant expression in human hearts [35] and is well known to be a platelet-enriched specific myeloid miRNA. [35]

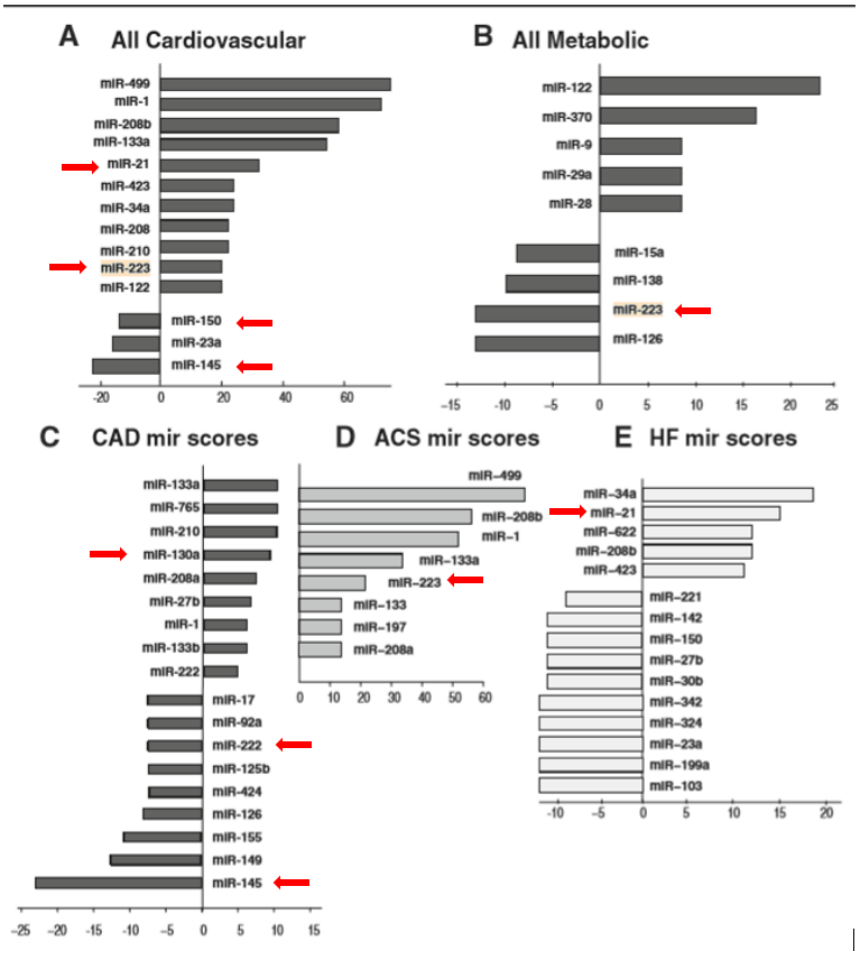

Figure 12 miRNAs involved in cascular response to injury (Quelle: Šatrauskienė et al. BMC Cardiovascular Disorders (2017) 17:173 DOI 10.1186/s12872-017-0609-z) [34]

Nevertheless, circulating levels of miR-223 were not associated with 2-year outcomes of PAD-patients after endovascular revascularization. In the present study all PAD patients had the same antiplatelet medical treatment, this similar state of platelet activation must be reflected in constant levels of circulating miR-223 throughout the whole study population. We performed the quantification of the investigated miRs in serum samples

and higher levels of miR-223 may be the result of the clotting process and platelet activation after blood sampling.

The vascular endothelial growth factor A (VEGF-A) may be evidenced to have high positive scores in cardiac vascular diseases, indicating a highly probable upregulation and an increase in neo-angiogenesis. [34] The disease specific downregulation of miR-145 and a regulation of different other miRs, like miR-15b, miR-17, miR-21, miR-126, miR-150 and miR-195. This increased neo-angiogenesis has been correlated positively with the formation of the atherosclerotic plaque. The negative scores in ACS and hearth failure (HF) suggests reduction of angiogenesis.[34]

MiR-17 and miR-126 (17 to 92 cluster), and miR-130a, miR-221 (members of the let-7 family) are highly expressed in endothelial cells in the vascular wall. [28] Meanwhile results of our study didn't show any significant association between miR-126 in any outcome in our study population.

Publications published 2009 highlighted the importance of miR-143/45 in the regulation of smooth muscle cell phenotypes. [36] The results showed the high expression of miR-143 and miR-145 in contractile smooth muscle. They also established the reduction in proliferative conditions. The genetic loss of these miRs in mice substantiate a decrease in smooth muscle stress fibre formation and caused an increase in rough endoplasmic reticulum. [36] Furthermore, these two are indicators a more synthetic and less differentiated phenotype. A study in 2015 showed the induction of miR-145 in smooth

muscle cells by endothelial signalling, the regulation of TNF-β receptor II (TGFBR2) expression and consequently supresses the matrix gene expression. This may have effects in disease progression, whereas the suppressed matrix synthesis could be used to weaken fibrosis in numerous tissues. [36] In this study the miR-143 and miR-145 observed the lowest circulating levels. Even through a study performed in 2014 proposed these two miRs as potential biomarkers for in-stent restenosis[37], in our results it has been shown that both miR-143 and miR-145 were either undetectable in almost 50% of the examined patients or showed marginal detection levels. This leads to the conclusion that these two circulating miRs may not be eligible as biomarkers for the risk stratification for patients having PAD.

Out of the eleven in this study investigated miRs, miR-92a and miR-195 were independently prognosticating 2-year outcomes in examined patients after stenting and angioplasty.

Couple of studies performed suggest that miR-195 may play a highly important role in adverse cardiac remodelling. [38]

MiR-195 is conserved among different species and their function is most likely similarly comparable, not only in reducing inflammation and cell proliferation, but also in inhibiting neointimal formation after injury. [39] A study performed on rat carotid arteries in 2012 show an 80% lower expression level of endogenous miR-195 in injured arteries, compared to uninjured arteries. [39]

Documentations elucidate that miR-195 regulate VSMC proliferation, migration and production of proinflammatory biomarkers. [39] This information may lead to the conclusion, that circulating levels of miR-195 incorporate essential pathophysiological processes for restenosis, especially inflammation and the smooth muscle cell´s phenotype. As a result, miR-195 constitute a prognostic and not only a diagnostic marker, and therefore improve risk management of PAD patients and provide pathophysiological insight.

As evidenced in studies before the expression of miRNA-92a is localised in endothelial cells in the aortic arch and induces a proatherogenic response and endothelial dysfunction. [40] This endothelial specificity was shown to be around 15-fold higher than in vascular smooth muscle cells and macrophages. In vivo studies on inhibiting miR-92a showed atheroprotective effects in mouse model of atherosclerosis.[41] PAD patients in the present study show high expression levels of circulating miRNA-92a, which results from the advanced stage of atherosclerosis. Nevertheless, lower levels of miR-92a were related to an increasing risk of the primary endpoint in our tested study population. We observed significantly lower levels of circulating miR-92a in patients having hypertension. Hypertension is one of the most important risk factors for atherothrombotic events, and could have contributed to increased restenosis rate in patients with lower circulating miRNA-92a expression.

Regarding to the several findings miR-21 represents an activating proliferative modulator of vessel smooth muscle cells and has negative affection to neointimal lesion formation. [2] Both miR-21 and miR-126 that was previously specified to be associated with platelet activation had much lower circulating levels when compared to miR-223.

Several limitations of the present study are:

Based on current literature about the present investigations of the coherence of circulating levels of miRs in PAD patients and the prognostic values of these miRs we investigated here 11 different miRs. However, many other miRs, which were not part of the trial, may have prognostic value in this setting.

Another limitation is the small sample size used in this study.

Furthermore, it is to be noted, that for normalization a cel-miR-39 spike in control was used, as stated before. At the moment, there are no generally valid and accepted standards for normalizations [31]. Using different normalization approaches may result in different normalized data.

In conclusion, circulating miR_195 and miR-92a predict adverse ischemic events and TVR in PAD patients undergoing an angioplasty through the infrainguinal artery with stent implantation. As a result, miR-195 and miR-92a could become an important biomarker, which is easy to assess and can be used for risk stratification for patients after endovascular revascularization procedures.

*"Ich habe mich bemüht, sämtliche Inhaber*innen der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir."*

List of figures

List of abbreviations

ABI – arterial brachial indices

ACE-Inhibitors – angiotensin converting enzyme inhibitors

ACS – acute coronary syndrome

ApoB100 – apolipoprotein B100

ASA – acetylsalicylic acid

BMI – body mass index

CAD – coronary artery disease

CAS – carotid angioplasty stenting

cDNA – complementary DNA

CEA – carotid endarterectomy

CLI – chronic limb ischemia

CRP – c-reactive protein

CTA – computed tomography angiography

CVD – cardiovascular disease

DM – diabetes mellitus

EC – endothelial cell

HDL – high density lipoprotein

HF – hearth failure

HR – hazard ratio

IC – intermittent claudication

INF- γ – interferon γ

ISR – intrastent restenosis

LDL – low density lipoprotein

M-CSF – macrophage colony-stimulating factor

MI – myocardial infarction

miR – micro RNA

MRI – magnetic resonance imaging

oxLDL – oxidative modification of low density lipoprotein

PAD – peripheral artery disease

PCI – percutaneous coronary intervention

PET – positron emission tomography

PTA – percutaneous transluminal angioplasty

PVK – periphere arterielle Verschlusskrankheit

qPCR – quantative real-time polymerase chain reaction

RNA – ribonucleic acid

SD – standard deviation

TIA – transient ischemic attack

TNF – tumor necrosis factor

TVR – target vessel revascularization

VEGF – vascular endothelial growth factor

VSMCs – vascular smooth cells

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