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Review Article

Peripheral artery disease, redox signaling, oxidative stress – Basic and clinical aspects

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ABSTRACT

Reactive oxygen and nitrogen species (ROS and RNS, e.g. H_2O_2, nitric oxide) confer redox regulation of essential cellular signaling pathways such as cell differentiation, proliferation, migration and apoptosis. At higher concentrations, ROS and RNS lead to oxidative stress and oxidative damage of biomolecules (e.g. via formation of peroxynitrite, fenton chemistry). Peripheral artery disease (PAD) is characterized by severe ischemic conditions in the periphery leading to intermittent claudication and critical limb ischemia (end stage). It is well known that redox biology and oxidative stress play an important role in this setting. We here discuss the major pathways of oxidative stress and redox signaling underlying the disease progression with special emphasis on the contribution of inflammatory processes. We also highlight therapeutic strategies comprising pharmacological (e.g. statins, angiotensin-converting enzyme inhibitors, phosphodiesterase inhibition) and non-pharmacological (e.g. exercise) interventions. Both of these strategies induce potent indirect antioxidant and anti-inflammatory mechanisms that may contribute to an improvement of PAD associated complications and disease progression by removing excess formation of ROS and RNS (e.g. by ameliorating primary complications such as hyperlipidemia and hypertension) as well as the normalization of the inflammatory phenotype suppressing the progression of atherosclerosis.

1. Introduction

1.1. Redox regulation versus oxidative stress

There is ample evidence that many diseases and drug-induced complications are associated with or even based on increased levels of reactive oxygen and nitrogen species (ROS and RNS), so-called oxidative stress [1,2]. In contrast, redox regulation is the process in which ROS and RNS act as signaling molecules by reversible redox modifications in enzymes that affect cellular processes (e.g. S-nitrosation of caspase-3 to control apoptosis [3], NFκB activation via thiol oxidation mediated IκB degradation [4]) [5,6]. Chronic oxidative stress conditions will lead to the accumulation of posttranslational oxidative modifications in biomolecules (e.g. protein carbonylation or aldehyde/ketone adducts, nitration and sulfoxidation, DNA lesions such as 8-oxo-dG) and interference with physiological redox signaling (e.g. impaired H_2O_2 signaling in essential cellular processes) [7,8]. Many cardiovascular, neurodegenerative, and inflammatory diseases as well as cancer are associated or even triggered by oxidative stress [9–13] and there is at least strong clinical evidence that the severity of these diseases in most cases correlates with the levels of established redox biomarkers (reviewed in a critical position paper [14]), although the causal role of oxidative stress in the clinical setting remains under heavy debate (for contra see [15], for pro see [1,16]).

Despite the above mentioned oxidative stress concept in disease progression most large scale clinical studies on the efficacy of orally-administrated antioxidants (namely vitamins) in patients turned out neutral (e.g. HOPE, HOPE-TOO; for review see [1,17,18]) or even showed negative outcome (e.g. for vitamin E) [17,19–21]. These disappointing results are contrasted by numerous small cohort studies with acute (short-term) and/or high dose administration (e.g. via infusion) of antioxidants showing positive outcome in various diseases (reviewed in [1,17,22]). The reasons for this obvious discrepancy were reviewed in very detail and most probably comprise that systemic therapy with non-specific antioxidants interferes with essential physiological redox signaling pathways affecting cell differentiation, prolif-

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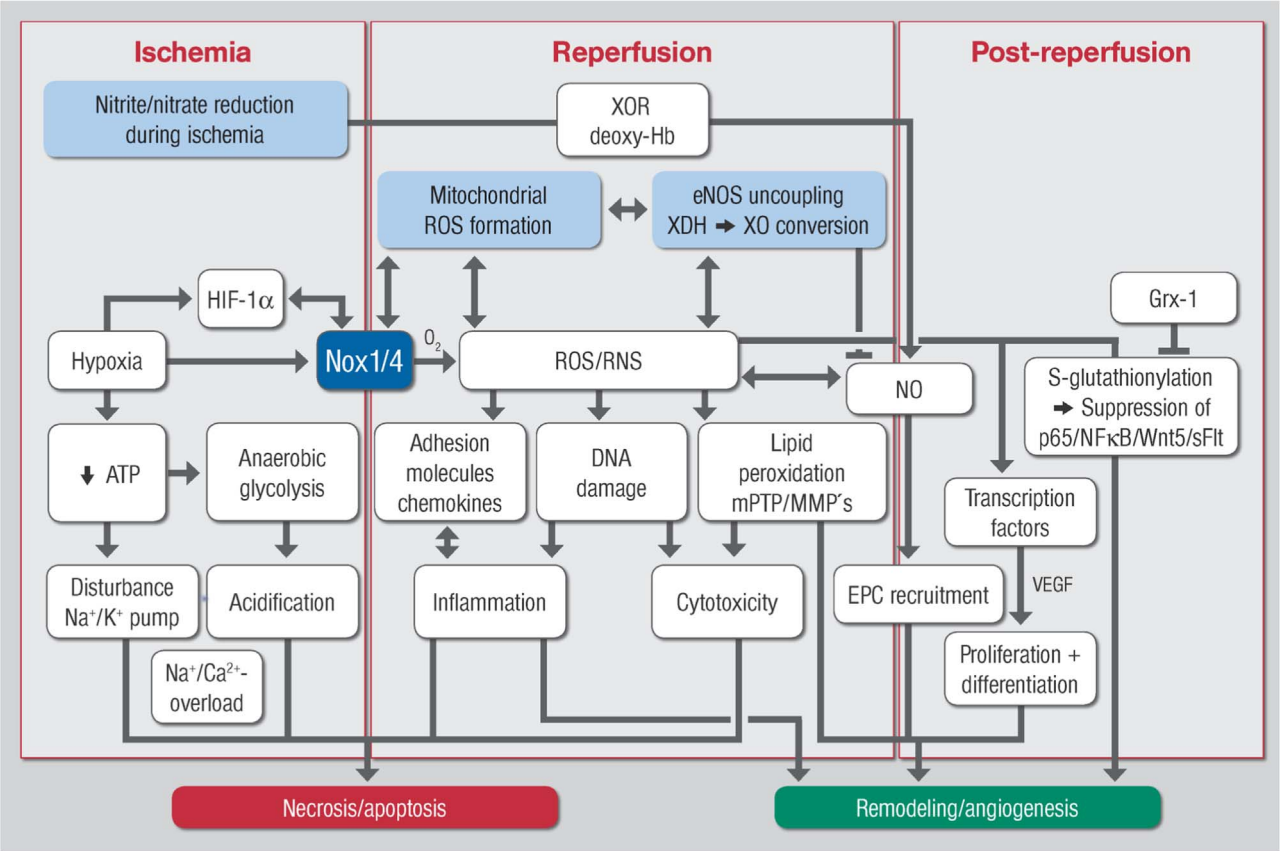

Fig. 1. The three phases of ischemia reperfusion (I/R) damage and recovery that can be also applied to disease mechanisms of peripheral artery disease. The major redox-regulated pathways or ROS sources are illustrated in the scheme. Major players are HIF-1α, NOX1/4, mitochondria, eNOS, XO, Grx-1 either promoting or preventing necrosis/apoptosis, oxidative stress, inflammation and remodeling/angiogenesis. For detailed explanation see main text. Modified from [37]. Abbreviations: HIF-1α, hypoxia-inducible factor 1α; NOX1/4, NADPH oxidase isoform 1 or 4; XOR, xanthine oxidoreductase; deoxy-Hb, deoxy-hemoglobin; eNOS, endothelial nitric oxide synthase; XDH, xanthine dehydrogenase; XO, xanthine oxidase; mPTP, mitochondrial permeability transition pore; MMPs, matrix-metalloproteinases; EPC, endothelial progenitor cells; Grx-1, glutaredoxin-1; VEGF, vascular endothelial growth factor.

eration, migration and apoptosis, as well as life-essential stress adaptation pathways (e.g. ischemic preconditioning-like pathways, Nrf2/heme oxygenase-1 antioxidant pathway) [1,17,18].

In the subsequent chapters we will discuss the role of redox signaling and oxidative stress in ischemia/reperfusion damage in general (critically reviewed in [23]) and in peripheral artery disease (PAD) in particular, also largely based on our own clinical observations [24–27]. As early as 1977 it was proposed that autoxidation processes (loss of endogenous antioxidants) might be involved in the pathophysiology of PAD in humans [28]. According to recent state-of-the art studies and reviews, markers of oxidative damage (e.g. 4-hydroxynonenal and protein carbonyls) increased with clinical stage of disease, blood flow limitation in the ischemic leg, and reduced myofiber cross-sectional area and oxidative stress was therefore proposed as possible cause of PAD in humans [29–31]. The underlying pathology of PAD comprises ischemia reperfusion and chronic inflammation, both processes that were shown in vivo and ex vivo to be redox regulated [32].

1.2. Redox regulation and oxidative stress in ischemia/reperfusion

Ischemia/reperfusion injury (IRI) is a main feature of various cardiovascular diseases like myocardial infarction, stroke and PAD [33,34]. IRI begins with occlusion of a vessel followed by interruption of the blood flow and temporary lack of oxygen and nutrients. Oxidative phosphorylation is disturbed and generation of adenosine triphosphate (ATP) reduced. Inactivity of the Na^+/K^+ pump results in intracellular calcium overload causing apoptotic and necrotic cell death (as reviewed in [35]). This initial “oxygen poor” phase is followed by reperfusion and recovery of demanded oxygen supply to the cells. However, this phase is accompanied by an increase in ROS formation, which leads to opening of mitochondrial permeability pore (mPTP), lipid peroxidation, DNA damage and triggers inflammatory processes (release of cytokines and upregulation of adhesion molecules) (as reviewed in [36,37]). At this time oxygen can be seen as a “double-edged sword” [38].

Later on in IRI and also permanent ischemia, ROS are essential signal molecules responsible for proliferation of smooth muscle cells and for angiogenesis mediated by vascular endothelial growth factor (VEGF) or hypoxia-inducible factor (HIF-1) [39]. HIF-1 plays a central role in cellular oxygen sensing with a close crosstalk with mitochondrial sensing of hypoxia [40]. It has been shown in cultured human endothelial cells that a decrease of ROS by use of antioxidants (N-acetylcysteine), pharmacological inhibition or genetic knock-down of NADPH oxidase reduces VEGF-mediated downstream activity and neovascularization [41,42]. Mice lacking Nox4 showed impaired recovery of blood flow and angiogenesis after femoral artery ligation [43]. Besides ROS, also RNS like peroxynitrite are involved in IRI and chronic ischemia. eNOS-derived nitric oxide (˙NO) mediates vasorelaxation and anti-proliferative properties under physiologic conditions (as reviewed in [22]). However, during IRI, eNOS uncoupling due to increased oxidative stress and hypoxia leads to formation of ROS instead of ˙NO by the enzyme. Reduced ˙NO bioavailability and increased superoxide/peroxynitrite levels follow, which causes cell damage and impaired vascular function (as reviewed in [22,36]). In this case ROS stimulate further ROS production by uncoupling of eNOS. The latter mechanism is similar to mitochondria-derived oxidative stress. Mitochondria have been described as major sources of oxidative stress in IRI [44]. Known from animal studies and cultured human cells,

cytosolic ROS can trigger uncoupling of the mitochondrial respiration chain, which in turn can be released via an opened mPTP to the cytosol with further increase of cytosolic oxidative stress as a consequence (for review see [36,45,46]). Among the adverse effects of superoxide, hydrogen peroxide and peroxynitrite in IRI are the increase in platelet activity and microvascular constriction/occlusion (e.g. by oxidative inhibition of soluble guanylyl cyclase in rat aorta [47,48], nitration/inactivation of prostacyclin synthase in cultured endothelial cells [22,49] and activation of cyclooxygenase via increased peroxide tone in cultured vascular smooth muscle cells [36,50]) (as reviewed in [51]), the two major complications in IRI in general and patients with PAD in particular.

In fact ROS play a Janus-faced role in IRI and permanent ischemia. On the one hand they are toxic and lead to cell death, on the other, ROS as signaling molecules mediate important compensatory mechanisms to recover perfusion and blood supply (Fig. 1). In the first phase of ischemia the loss of ATP production, calcium overload and drop in pH leads to cell death via necrosis and apoptosis. Hypoxia activates the transcription factor hypoxia-inducible factor-1α (HIF-1α), which together with calcium overload activates NADPH oxidases. Likewise, NADPH oxidases and ROS in general stabilize HIF-1α as studied in pulmonary vessels from patients with pulmonary vasculopathy [52,53]. In the ischemic phase the reduction of inorganic nitrite via xanthine oxidoreductase (XOR) and deoxy-hemoglobin/myoglobin (deoxy-Hb/Mb) may provide nitric oxide ($^{\bullet}$NO) to improve the perfusion to overcome hypoxia as studied in human forearm [54–58]. Due to altered mitochondrial metabolism reduction of inorganic nitrite may serve also as a source of $^{\bullet}$NO in hypoxic mitochondria (as reviewed in [59]). Under normoxic conditions also bacteria in the gastrointestinal tract [60] as well as immune cells such as macrophages (studied in mice) [61,62] may contribute to the bioactivation of inorganic nitrite and nitrate. $^{\bullet}$NO contributes to the stabilization of HIF-1α [63] but, via S-nitros(yl)ation, may also protect critical thiol-dependent enzymes from oxidative inactivation during the reperfusion phase [59]. In the second phase, upon reperfusion, activated NADPH oxidases and hypoxia-adapted mitochondrial respiratory chain generate huge amounts of superoxide and hydrogen peroxide, where NADPH oxidases and mitochondria can stimulate ROS formation from each other, which was studied in pulmonary artery smooth muscle cells [64], leading to conversion of xanthine dehydrogenase (XDH) to ROS-producing xanthine oxidase (XO) and to endothelial nitric oxide synthase (eNOS) uncoupling (as reviewed in [65,66]). Thereby, reoxygenation leads to increased oxidative stress and diminished $^{\bullet}$NO levels, which triggers the expression of adhesion molecules, production of chemokines, oxidative DNA damage, lipid peroxidation, activation of matrix metallo-proteinases (MMPs) with increased inflammation and cytotoxicity with mostly detrimental effects for the cell (necrosis and apoptosis). However, in the third phase of remodeling and angiogenesis (post-reperfusion) certain subsets of immune cells and MMPs are required to repair the tissue damage from I/R phases. $^{\bullet}$NO stimulates recruitment of endothelial progenitor cells (EPCs) to areas with defects, ROS/RNS activate transcription factor that initiate remodeling and angiogenesis via vascular endothelial growth factor (VEGF). Certain levels of ROS and RNS are required for the regulation of pro- and anti-inflammatory pathways (e.g. for the suppression of the antiangiogenic p65/NFκB/Wnt5/sFlt pathway by S-glutathionylation but also for the activation NFκB-dependent pathways that are needed for the activation of repair-associated inflammatory pathways), e.g. as produced by HIF-1α regulated Nox2 as studied in human umbilical vein endothelial cells (HUVECS) [67]. The mitochondrial permeability transition pore (mPTP) plays an essential role during ischemia/reperfusion mediated necrosis and mostly apoptosis but, by transient, repeated opening/closing cycles can also confer long-term antioxidant protection by induction of antioxidant defense enzymes (the concept underlying ischemic pre- and post-conditioning [68,69]).

The recent publication by Granger and Kvietys provides an excellent overview on the contribution of the major ROS sources xanthine oxidase, mitochondria, NADPH oxidases and uncoupled eNOS in ischemia/reperfusion damage and also discusses the evidence for a vital crosstalk between these different ROS sources in hypoxia/reoxygenation [70]. As studied in mice, the recovery from ischemia/reperfusion damage is largely redox regulated by thiol oxidation (disulfide), S-glutathionylation and glutaredoxin networks [71].

2. What is peripheral artery disease? Definition and clinical importance

PAD is defined as obstructive disease of non-cardiac arterial vessels mostly affecting arteries of lower extremities [72,73]. The most common cause of PAD is atherosclerosis.

Based on current estimations about 200 million people worldwide suffer from PAD [74]. Thus PAD is one of the main manifestations of atherosclerosis. The prevalence of PAD in the general population is 3–10% [75,76], which is increased at the age of 70 years to 15–20% [77,78]. The cardiovascular mortality within 5 years after diagnosis of stable PAD is 11–23% [79]. The risk of major amputation is less than 2% for patients with stable PAD, whereas the risk of major amputation within the next 12 months is increased to almost 50% in patients with critical limb ischemia [80]. The most likely reason for the increased cardiovascular mortality of PAD patients is the fact that they also suffer from severe atherosclerosis in other (e.g. coronary) vascular territories [79,81]. Therefore patients with PAD are at high risk of cardiovascular events such as myocardial infarction or stroke [82].

Accordingly, the most important risk factors for the development and progression of PAD are similar to those for atherosclerosis in general: smoking, diabetes, hypertension and hypercholesterolemia. The strongest associations are observed for smoking and diabetes [80]. Moreover the location of atherosclerotic manifestation in the arterial vessel tree differs according to main risk factor profile. Patients with diabetes suffer more often from occlusion of the lower limb arteries while smokers develop mostly stenotic disease of the iliac or femoral arteries [74].

As atherosclerosis is an inflammatory disease it is not surprising that markers of inflammation such as the acute phase proteins C reactive protein and fibrinogen or pro-inflammatory cytokines like interleukin-6 (IL-6) and interleukin-18 (IL-18) are associated with increasing burden of atherosclerosis [83]. In particular patients with elevated inflammatory markers and PAD are at high risk for cardiovascular events such as myocardial infarction, stroke or cardiovascular death.

Clinical symptoms of PAD are defined by the “Fontaine” and “Rutherford” Classification (Table 1). The typical clinical symptom of stable PAD is walking pain defined as “intermittent claudication” (Rutherford category 1, 2 or 3, Fontaine category II). The most severe clinical manifestation of PAD is critical limb ischemia characterized by

Table 1.
Clinical characterization of peripheral artery disease according to the Fontaine and Rutherford classification. Modified from [80,84].

Fontaine		Rutherford	
Category	Clinical symptoms	Category	Clinical symptoms
I	Asymptomatic	0	Asymptomatic
IIa	Pain-free walking distance > 200 m	1	Mild symptoms of intermittent claudication
IIb	Pain-free walking distance < 200 m	2	Moderate symptoms of intermittent claudication
		3	Severe symptoms of intermittent claudication
III	Pain at rest	4	Pain at rest
IV	Ischemic ulcer or gangrene	5	Lesions with minor tissue loss
		6	Ischemic ulcer or gangrene

rest pain or tissue loss (Rutherford category 4, 5 or 6, Fontaine III or IV) [84]. In particular, patients with diabetes develop PAD with peripheral ulcers or gangrene.

The easiest way to detect PAD independently from clinical symptoms is to measure ankle pressure using a blood pressure cuff and a Doppler probe. Ankle-brachial index (ABI) is calculated as relation between systolic ankle and arm pressure [85,86]. Usually an ankle-brachial index of 0.9 or less is defined as PAD. However, PAD is often overlooked because many patients have no clinical symptoms although thy have a low ankle brachial index [77]. Nevertheless, asymptomatic patients are at increased risk for cardiovascular events, too. Therefore, screening for PAD by alternative methods is also important.

Although patients with PAD are at high cardiovascular risk, the awareness is very low. In fact patients with PAD are often undertreated in regard to consequent risk factor control compared to patients with manifestations of atherosclerosis in other arterial territories such as coronary artery disease [82].

3. Peripheral artery disease, redox signaling, oxidative stress

3.1. Evidence from animal models

In the past decades PAD has been investigated in several animal models. Besides thrombo-embolic models, acute ligation and excision models are mostly used for investigation of PAD in mice or rats. The severity of experimental hind limb ischemia (HLI) depends on the location and length of arterial occlusion. For example ligation of the femoral artery (FA) alone results only in mild ischemia, since blood flow persists via collateral vessels. On the other hand, ligation and excision of the complete FA and its side branches results in severe ischemia with necrosis of the toe. Inhomogeneity of animal models and surgical procedures limit the reliability and translation of preclinical results in human PAD [87], although a recent report shows that the used animal model of critical limb ischemia (by unilateral femoral and iliac ligatures) shares key features with the human pathology including increased muscular ROS formation [88]. Importantly in this latter study ROS formation was measured by the gold standard technique, electron paramagnetic resonance spectroscopy and also correlated with dihydroethidium staining and expression levels of antioxidant enzymes.

It is well established that smoking and diabetes mellitus, main risk factors for PAD, promote oxidative stress and reduce $^{\bullet}$NO bioactivity, which enhances inflammatory pathways (studied in humans and HUVECs) [89,90]. In PAD, neovascularization constitutes an important adaptive mechanism against ischemia, in which vascular endothelial growth factor (VEGF) plays a predominant role (as reviewed in [91]). The function of VEGF in angiogenesis largely depends on endothelium-derived $^{\bullet}$NO, which also has been identified as a mediator of endothelial cell migration and mobilization of endothelial progenitor cells (EPC) from the bone marrow of mice [89,92]. Reduced bioavailability of $^{\bullet}$NO by direct ROS-mediated break-down or oxidative uncoupling of eNOS disturbs this pathway. Nevertheless, it cannot be generalized that ROS formation leads to cellular toxicity and impairs angiogenesis, because low levels of hydrogen peroxide and superoxide can serve as or are even essential (e.g. Nox2, Nox4) as intracellular messengers and can promote angiogenesis and reduce tissue injury. This is also supported by an elegant study in transgenic mice overexpressing glutaredoxin-1 (Grx-1) [93]. In these mice oxidatively induced S-glutathionylation, the essential stimulus of angiogenesis/revascularization and recovery from hind limb ischemia, is removed continuously. In Grx-1 transgenic mice the suppression of the p65/NFκB/Wnt5/sFlt pathway is not suppressed sufficiently by S-glutathionylation of p65 leading to increased sFlt expression and reduced angiogenesis via VEGF. Likewise, increased S-glutathionylation in Grx-1 knockout mice stabilizes HIF-1α and promotes angiogenesis [94].

In support of this, Bir et. al. showed by an animal model of compromised glutathione synthesis and reduced antioxidant capacity ($Gclm^{+/-}$ mutant mice) that partly preserved glutathione synthesis, with slightly increased oxidative stress leads to improved blood flow and angiogenesis, which was accompanied by increased VEGF levels [95]. Higher ROS levels, however, induced by complete loss of glutathione synthesis showed no beneficial effects ($Gclm^{-/-}$ mutant mice) [95]. Furthermore, it has been shown that genetic deletion of NADPH oxidase isoform 2 (Nox2), a main source of ROS in the vascular wall, reduces flow recovery and capillary density in hind limb ischemia of Nox2 knock-out mice [96,97]. Also Nox4-derived H_2O_2 was shown to be necessary for angiogenesis in mice. Deficiency in Nox4 led to reduced eNOS expression, $^{\bullet}$NO production and heme oxygenase-1 (HO-1) expression, accompanied by apoptosis and inflammatory activation [43]. However, conditions of increased oxidative stress, as evident in diabetes, genetic deletion of antioxidant proteins or smoking, are deleterious for neovascularization after hind limb ischemia. Accordingly Nox2 knock-out mice exposed to cigarette smoke were protected against the detrimental effects of hind limb ischemia. The latter was accompanied by improved VEGF/$^{\bullet}$NO signaling and preserved eNOS expression in ischemic tissue [98]. Diabetes in mice leads to reduced eNOS expression and EPC migration in response to VEGF stimulation, and furthermore to increased 3-nitrotyrosine levels [99]. Similar findings were made in copper-zinc superoxide dismutase-deficient mice [100]. Likewise, mesenchymal stem cells transplanted from diabetic db/db mice were less potent to prevent the hind limb ischemia-dependent damage than those cells from wild type animals, but their protective potential was improved by prior Nox4 siRNA or N-acetylcholine treatment [101]. In ischemic hind limbs of eNOS knock-out mice and more severe in placental growth factor (PlGF)/eNOS double knock-out mice, capillary density was reduced accompanied by increased macrophage infiltration, iNOS expression and 3-nitrotyrosine positive proteins [102]. As studied in mice, the loss of eNOS activity during IRI in general and HLI in particular is eventually compensated by increased biosynthesis of $^{\bullet}$NO from inorganic sources such as nitrate and nitrite (by bacterial systems or xanthine oxidoreductase and deoxy-Hb), which are obviously even more efficient in ischemic tissues [57,103].

Besides cytosolic ROS formation also other ROS sources are involved in the pathogenesis of PAD. Knock-out of $p66^{SchA}$, a redox enzyme localized in the mitochondria that generates mitochondrial ROS as signaling molecules for apoptosis, led to faster regeneration of muscle fibers and lower oxidative stress during ischemia compared to control animals. The importance of mitochondrial ROS formation goes in line with recent findings of Ryan et al. who demonstrated that mitochondria-targeted overexpression of the antioxidant enzyme catalase in mice prevents high fat diet-induced ischemic limb necrosis, myopathy and mitochondrial dysfunction, but did not alter hind limb blood flow [104]. Another study revealed mitochondrial dysfunction (impaired respiration at complexes I, III and IV) and oxidative stress as revealed by increased protein carbonyl and 4-hydroxynonenal content as well as impaired activity of manganese superoxide dismutase (SOD2) in mice with hindlimb ischemia by ligation/division of the common femoral and iliac arteries [105]. In a model of hind limb ischemia in diabetic ob/ob mice it was shown that unacetylated ghrelin rescues miR-126 expression that controls sirtuin-1 and manganese superoxide dismutase (SOD2), all of which improved the oxidative stress levels in these animals and recovery from hind limb ischemia [106]. This also emphasizes the potential clinical impact of targeting redox signaling and oxidative stress in avoiding limb loss in PAD and diabetic leg patients.

3.2. Evidence from clinical studies

Patients with PAD are limited by walking distance and can often not participate in daily activities. Therefore, patients with PAD are at increased risk for social isolation and depression. Reduced physical activity is followed by higher risk for obesity, arterial hypertension, dyslipidemia and diabetes which are all also risk factors for endothelial

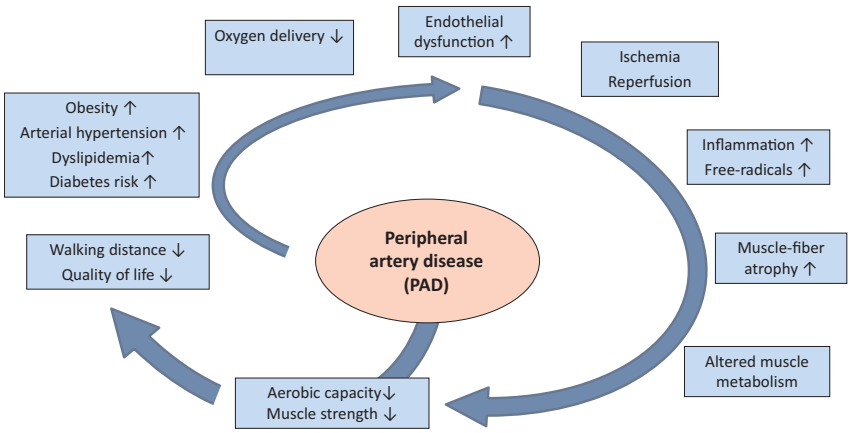

Fig. 2. Pathophysiological disabilities associated with peripheral artery disease. Modified from [107,108].

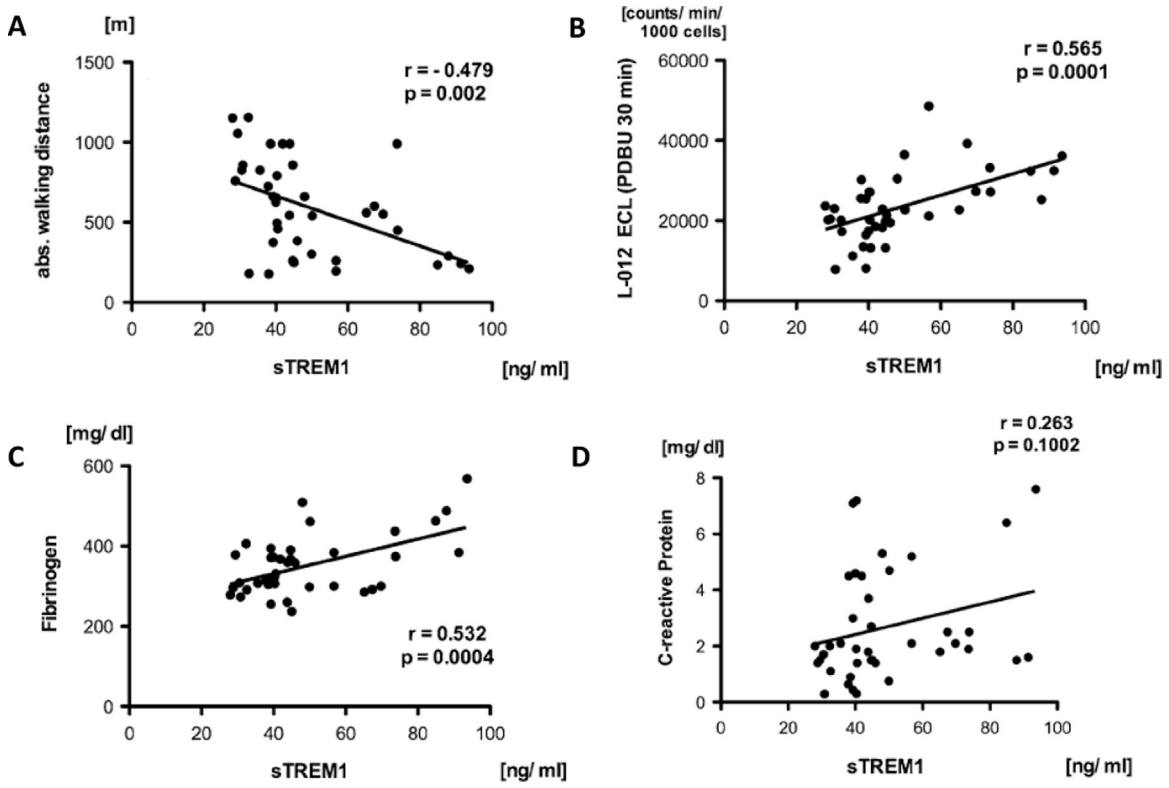

Fig. 3. Correlation of the marker of inflammation soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) with oxidative stress and walking distance in PAD patients with intermittent claudication. We found an inverse correlation for sTREM-1 with the absolute ($r = -0.479$; $p = 0.002$) (A), a direct correlation with whole blood oxidative burst after phorbol ester stimulation ($r = 0.565$; $p = 0.0001$) (B) and the general markers of inflammation fibrinogen ($r = 0.532$; $p = 0.0004$) (C) and CRP ($r = 0.263$; $p = 0.1002$) (D). Number of patients was $n = 40$. Adapted from [25]. With permission of Springer-Verlag Berlin Heidelberg. Copyright © 2015.

dysfunction and atherosclerosis (Fig. 2) [107,108]. Because of limited blood flow, nourishment of muscle cells is reduced and muscle metabolism is impaired. In particular in the clinical stage of intermittent claudication the alternation between repeated ischemia and reperfusion triggers inflammation and oxidative stress [26,27,109–111]. The risk of PAD was significantly increased in individuals with higher oxidized phospholipids [110] and lipid peroxidation products such as malondialdehyde and oxidized low-density lipoproteins (LDL) [112], which are established biomarkers of oxidative stress. This observation was further supported by increased levels of autoantibodies against oxidized LDL in patients with early-onset of peripheral artery disease [113]. Serum levels of S-glutathionylated proteins are a sensitive risk-marker for atherosclerosis in patient collectives of PAD and negatively correlated with the ankle-brachial index [114]. Among the reported markers of PAD there are several associated with oxidative stress and inflammation [31].

We observed these effects under clinical conditions in patients with PAD. In the MonoxGo project patients with intermittent claudication, critical limb ischemia and healthy controls were compared in regard to the relation of pro-inflammatory mononuclear cells with markers of oxidative stress. We found an increased number of pro-inflammatory monocytes in patients with intermittent claudication [27]. In contrast, patients with critical limb ischemia showed a reduced expression of pro-inflammatory cells. Possibly, an adequate regulation of inflammatory cells in this advanced stage of the disease is altered due to the chronic ischemic milieu. This goes in line with the finding, that cells from patients with critical limb ischemia showed significant higher production of reactive oxygen and nitrogen species (RONS) measured

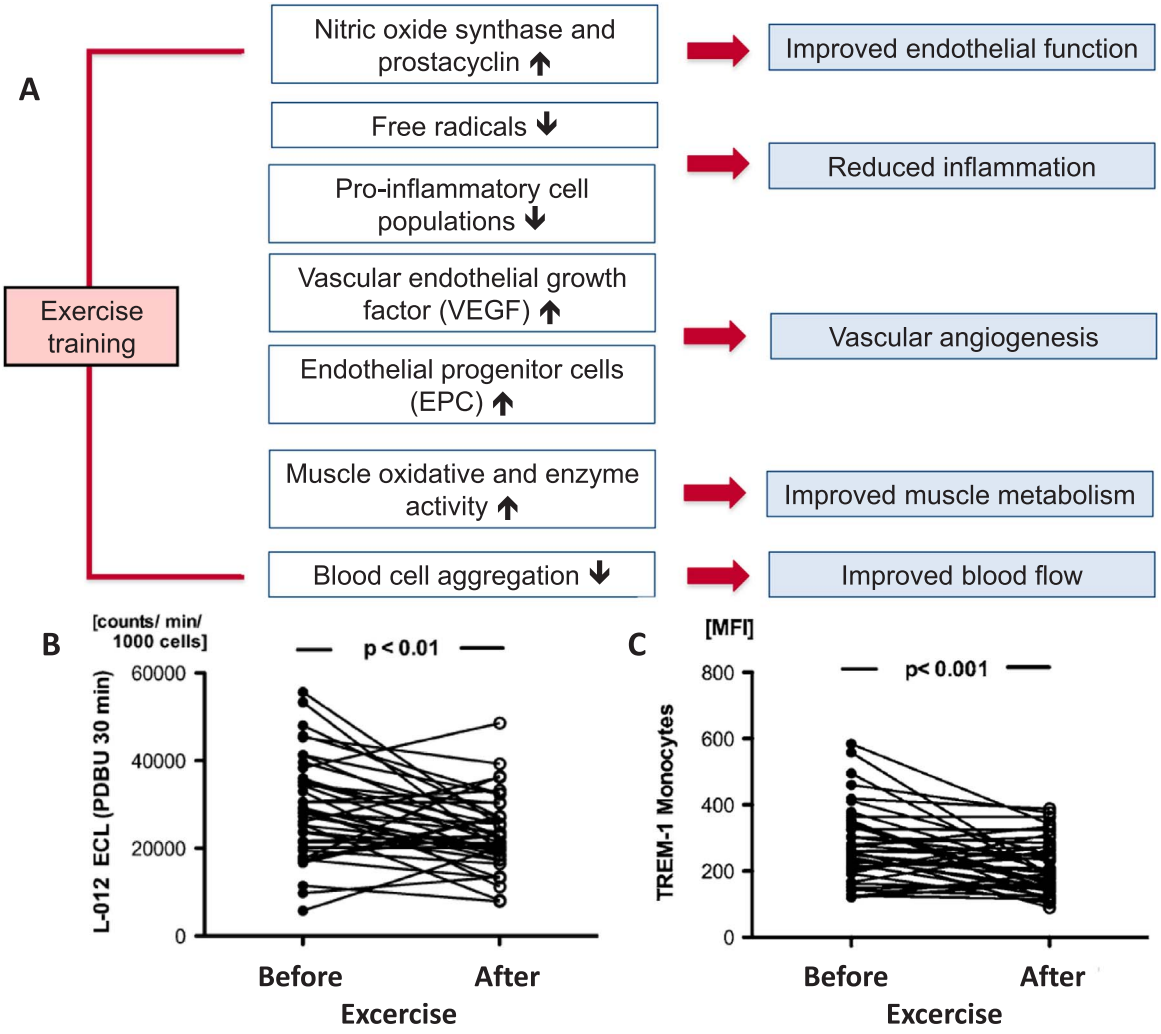

Fig. 4. Positive effects of exercise training on peripheral perfusion in patients with intermittent claudication. (A) Summary of potential beneficial effects of exercise training on PAD. Modified from [108,110,116]. With permission of Massachusetts Medical Society. Copyright © 2002. (B, C) Original data on decreased oxidative burst in phorbol ester stimulated whole blood (measured by L-012 ECL) and expression of the pro-inflammatory molecule sTREM-1 on monocytes of PAD patients with intermittent claudication after exercise. Number of patients was n = 40.Adapted from [25]. With permission of Springer-Verlag Berlin Heidelberg. Copyright © 2015.

by L-012 (luminol analogue) chemiluminescence at baseline and after stimulation by phorbol ester [26]. In patients with intermittent claudication, we observed a highly significant inverse correlation between the marker of inflammation triggering receptor expressed on myeloid cells-1 (TREM-1) with the absolute as well as the pain-free walking distance, a positive correlation with whole blood oxidative burst (measured by L-012 chemiluminescence) and fibrinogen, representing a general marker of inflammation, whereas for C-reactive protein (CRP) only a trend for a relationship was observed (Fig. 3) [25].

One of the most important mechanisms to prevent or resolve muscle ischemia is the stimulation of collateral vessel growth. Collateral growth underlies multiple pathophysiological mechanisms. To understand these causal relations is the key to develop new therapeutic strategies. In this regard, shear stress seems to be of importance. Arteries react to shear stress with diameter growth and endothelial remodeling [115], in addition to an up-regulation of adhesion molecules on the endothelial surface.

Under stable conditions such as in patients with intermittent claudication, blood flow is increased in the affected limb and may contribute to the resolution of tissue damage. In critical limb ischemia these compensatory responses to ischemia are ineffective [31,116]. In consequence, ongoing insufficient tissue perfusion leads to chronic inflammation and oxidative stress. Under this pathophysiological hypothesis, we evaluated in particular patients with critical limb ischemia with regard to mononuclear cells potentially involved in angiogenesis [117]. For these patients we found a strong association of inflammatory markers (C-reactive protein [CRP], fibrinogen) with a reduced proportion of pro-angiogenic Tie-2 expressing monocytes (TEM) or endothelial progenitor cells (EPC). Accordingly, we hypothesize that a severe chronic inflammation can have negative effects on potential pro-angiogenic cells and thus inhibit repair mechanism in ischemic tissue. In conclusion, it seems very important to react in earlier stages of the disease when protective mechanism can still be stimulated.

3.3. Therapy of peripheral artery occlusive disease

Patients with PAD are at increased risk for cardiovascular events. Therefore, therapeutic strategies for risk reduction are crucial in PAD patients [118]. In particular statins, ACE-inhibitors are highly effective to improve cardiovascular prognosis in PAD patients, most probably by reducing primary PAD-associated complications such as hypertension, inflammation and hyperlipidemia, all of which confers antioxidant, anti-inflammatory and anti-atherosclerotic effects [119,120]. In support of the importance of $^{\bullet}$NO in the therapeutic concepts, and in line with the mentioned benefits of $^{\bullet}$NO from inorganic nitrite and nitrate sources in ischemia/reperfusion (see Section 1.2 and Fig. 1), also inorganic nitrate therapy increased the pain-free walking distance of

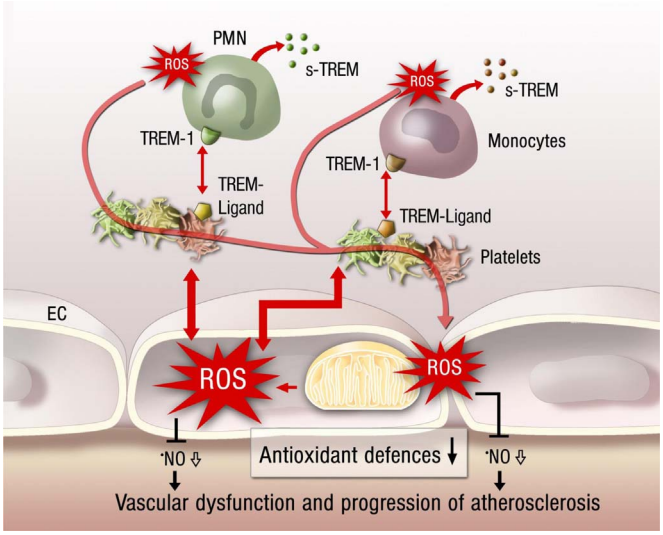

Fig. 5. Proposed hypothesis on the role of oxidative stress, neutrophils (PMN) and monocytes in peripheral artery disease (PAD). In PAD patients platelets are strongly activated. Also markers of inflammation like triggering receptor expressed on myeloid cells-1 (TREM-1) were largely increased in plasma of PAD patients. PMN and monocytes are stimulated via Interaction of TREM-ligand with TREM-1, which leads to an increased production of ROS. This will further increase endothelial ROS formation, possibly via NADPH oxidases, which then might activate even more platelets, thus leading to a positive feedback mechanism. The increased burden of vascular ROS will potentially also inhibit endothelial nitric oxide (˙NO) formation, which will ultimately lead to vascular dysfunction and progression of atherosclerosis. Whether mitochondrial ROS are involved in immune cell activation and subsequent tissue damage by infiltrating immune cells is likely but remains to be demonstrated in patients with PAD. sTREM being shedded by PMN and monocytes might further stimulate chronic inflammation or act as a regulatory molecule to regulate the response of the innate immune system to the inflammatory stimulus in atherosclerosis. The scheme summarizes our previous findings [26,27].

claudicants [121]. There are different therapeutic strategies to treat PAD aimed to bring back blood to ischemic tissue. One strategy is to re-open arteries by endovascular intervention or to bypass occluded arteries by venous or synthetic grafts [122]. After successful revascularization, wound healing and pain-free walking distance are improved. This has of course positive effects on inflammation and oxidative stress [123]. Nevertheless, one still has to assume future vessel restenosis or graft occlusion after interventional therapies.

It is of current interest to develop strategies to improve collateral vessel growth in PAD patients. A number of studies investigated gene therapies or cell-based therapies to provoke new vessel formation, but most of these could not satisfy the expectations [84]. Neither direct antioxidant therapies with modern antioxidant concepts such as NADPH oxidase inhibitors or activators of intrinsic antioxidant pathways such as Nrf2 activators nor direct anti-inflammatory therapy by monoclonal antibodies against chemokines have been investigated so far in human PAD. However, experimental and human observational data suggest that antioxidant and anti-inflammatory strategies may be successful in PAD patients (see preceding chapters) [124,125]. Endovascular therapy improved blood flow, walking distance and oxidative stress parameters in patients with PAD [123]. Recently, it was shown that dark chocolate improves the walking distance and ˙NO production and decrease isoprostane levels in PAD patients, most likely by effects of antioxidant components of dark chocolate on NADPH oxidase activity [126], as previously shown for the improvement of endothelial function by flow-mediated dilation measurements [127]. Likewise, supplementation with N-acetylcysteine could be a candidate for antioxidant therapy of PAD patients since this drug was shown to improve human coronary and peripheral endothelium-dependent vasodilation [128].

In contrast the “natural” way to improve collateralization by exercise training seems to be more successful in particular for patients with intermittent claudication. Exercise has multiple positive effects for the limb perfusion but also confers systemic beneficial effects (Fig. 4) [107,108,116]. In general, physical activity has been shown to greatly improve cardiovascular function, in part through improved bioavailability of ˙NO, enhanced endogenous antioxidant defense and a lowering of the expression of enzymes involved in ROS production [129]. We reported decreased oxidative burst and sTREM expression in leukocytes of PAD patients with intermittent claudication after exercise training [25].

To evaluate the effects of exercise training on systemic inflammation and oxidative stress we asked 40 patients with intermittent claudication to perform a home-based exercise training program for a mean duration of 12 months. In this setting we observed an improvement of the inflammatory phenotypes of mononuclear cell populations, reduction of systemic inflammatory markers and reduction of ROS production [25]. These changes were associated with an improvement of walking distance.

Previous studies consistently show better effects on pain-free and maximal walking distance if exercise training is performed under

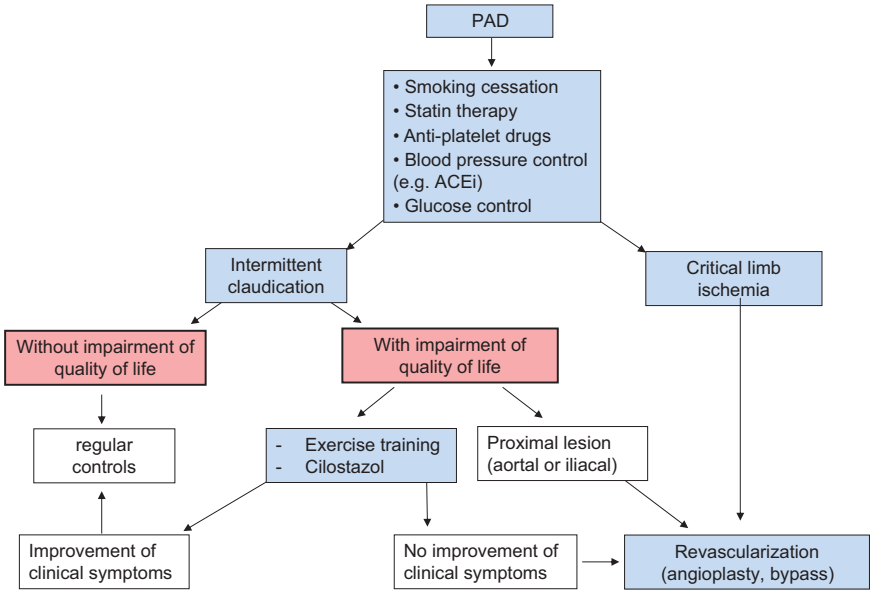

Fig. 6. Proposed treatment regimen for patients with PAD. Modified from [80].

standardized conditions [80]. Therefore we were interested in the effects of a supervised exercise training on angiogenic cells in comparison to a home-based non-supervised exercise training [24]. In total 20 patients with intermittent claudication performed supervised exercise training and 20 patients performed non-supervised home-based training. We found positive effects in both training groups on circulating angiogenic progenitor cells and pro-angiogenic Tie-2 expressing monocytes (TEM). However, the supervised form of exercise training was more efficient than the home-based form in regard to individual improvement of the walking distance or amelioration of inflammation or VEGF [24,117].

In the case that exercise training is not possible or improvement of walking distance is not satisfactory, some drugs can improve walking distance too [118]. One of these drugs is cilostazol, which causes peripheral vasodilatation and inhibits platelet aggregation. In addition several studies showed reduction of restenosis after peripheral or coronary interventions [130]. However, another current study did not find effects of cilostazol on systemic markers for oxidative stress in a small population of PAD patients [131].

4. Conclusions and clinical implications

Oxidative stress and redox regulation play an important role in peripheral artery disease (PAD). ROS and RNS production largely depends on the pro-inflammatory phenotype of monocytes and myeloid dendritic cells in patients with PAD [27]. Increased oxidative stress as well as the pro-inflammatory phenotype (as envisaged by higher serum levels of sTREM) in patients with critical limb ischemia and intermittent claudication, walking less than 300 m in a treadmill test, are obviously the primary causes of endothelial dysfunction, which will further contribute to progression of atherosclerosis (Fig. 5) [26]. Endothelial dysfunction is an independent risk factor for mortality in claudicants [132].

Home-based exercise training for a mean time of 12 months reduced expression of several inflammatory surface molecules on monocytes, dendritic cells and neutrophils in PAD patients as well as serological markers of inflammation, paralleled by an increased pain-free and absolute walking distance and reduced oxidative burst in whole blood [25]. In a follow-up study we elucidated that supervised exercise training was more efficient in improving the inflammatory status of PAD patients and their walking distance than non-supervised exercise training, although any form of physical activity had beneficial effects [24]. Further studies on the role of inflammation and oxidative stress in PAD in particular, as well as in other atherosclerotic diseases in general, are necessary. From a therapeutic point of view, special emphasis should be laid on the non-pharmacological intervention in form of (home-based) exercise training [133], which turned out to provide highly beneficial effects on the quality of life of PAD patients and the progression of the disease and should be applied besides other established cardiovascular pharmacological therapies (e.g. anti-atherosclerotic, antihypertensive and anti-inflammatory drugs like statins, angiotensin converting enzyme inhibitors and AT1-receptor blockers [134,135] or cilostazol [118,136,137], also conferring indirect antioxidant effects [138]). A reasonable treatment regimen for PAD is shown in Fig. 6.

Exercise training in general is highly beneficial for the prognosis in most cardiovascular diseases, including PAD, by decreasing oxidative stress and inflammation (for review see [139]). However, the prognostic value of supervised exercise training or direct anti-inflammatory and antioxidant therapy (e.g. by monoclonal antibodies against chemokines or modern antioxidant strategies like NADPH oxidase inhibitors or Nrf2 activators) on cardiovascular mortality of PAD patients remains to be established by future clinical studies. A combined antioxidant, anti-atherosclerotic and anti-inflammatory therapy to target the multifactorial pathology of PAD was suggested in a recent review article [118] and epidemiological human studies showed that PAD patients with higher physical activity during daily life have reduced mortality and cardiovascular events compared with PAD patients with the lowest physical activity [140].

Conflict of interest

None.

Acknowledgments

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