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ORIGINAL PAPER

Change of walking distance in intermittent claudication: impact on inflammation, oxidative stress and mononuclear cells: a pilot study

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Abstract
Background Atherosclerosis is a chronic inflammatory process involving the immune system and formation of reactive oxygen species (ROS). We investigated changes of mononuclear blood cells and ROS production in relation to the walking distance of patients with intermittent claudication during home-based exercise training.
Methods Forty patients with intermittent claudication were asked to perform a home-based exercise training for a mean time of 12 months. ROS formation was measured using the luminol analogue L-012. Peripheral blood leucocytes [monocytes, polymorphonuclear neutrophils (PMN) and dendritic cells (DC)] were analysed by flow cytometry and analysed for the expression of major inflammatory surface molecules.
Results At follow-up, patients showed an increased walking distance and reduced ROS production upon stimulation with a phorbol ester derivative (PDBu) ($p < 0.01$). Monocytes changed their inflammatory phenotype towards an increased anti-inflammatory $CD14^{++}$-$CD16^{-}$ subpopulation ($p < 0.0001$). Adhesion molecules CD11b, CD11c and TREM-1 on monocytes and PMN decreased (all $p < 0.01$). On DC expression of HLA-DR, CD86 or CD40 decreased at follow-up. Inflammatory markers like fibrinogen, C-reactive protein or soluble TREM-1 (sTREM-1) decreased over the observation period. Finally, we found a close relation of sTREM-1 with the walking distance, fibrinogen and ROS production.
Conclusions We observed an amelioration of the proinflammatory phenotype on monocytes, DC and PMN, as well as a reduced ROS production in PAD patients under home-based exercise, paralleled by an increased walking distance. Our data suggest that a reduced inflammatory state might be achieved by regular walking exercise, possibly in a dimension proportionately to changes in walking distance.

Keywords ROS · PMN · Monocytes · Dendritic cells · Peripheral arterial disease · Exercise training

Abbreviations

ACE	Angiotensin converting enzyme
AT_1	Angiotensin receptor 1
BMT	Best medical treatment
CAD	Coronary artery disease
DC	Dendritic cells
ECL	Enhanced chemiluminescence
FSC/SSC	Forward scatter/sideward scatter
EPC	Endothelial progenitor cells
IC	Intermittent claudication
IMT	Intima-media thickness
LV-EF	Left ventricular ejection fraction
mDC	Myeloid DC
MFI	Mean fluorescence intensity
NOX	NADPH oxidase
PAD	Peripheral arterial disease

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PBMC	Peripheral blood mononuclear cells
pDC	Plasmacytoid DC
PDBu	Phorbol 12,13-dibutyrate
PMN	Polymorphonuclear neutrophils
PTA	Percutaneous transluminal angioplasty
ROS	Reactive oxygen species
sTREM	Soluble TREM
TREM	Triggering receptor expressed on myeloid cells

Introduction

Patients with peripheral arterial disease (PAD) have a high cardiovascular mortality [1], due to a high prevalence of further atherosclerotic manifestations in the coronary and cerebral circulation [2, 3]. Therefore, PAD can be considered to be a marker of advanced vascular disease.

Being a chronic inflammatory disease, atherogenic processes involve an inflammatory response of the innate and adaptive immune system. In the early stages of atherosclerosis, polymorphonuclear granulocytes (PMN) and monocytes, attracted by divers proinflammatory stimuli, enter lesion-prone arterial sites [4], where they promote further inflammation and development of atherosclerotic lesions. Regulation of these inflammatory processes is performed by dendritic cells (DC), being present in the atherosclerotic plaque [5]. Interest in these circulating blood cells grows, in order to use them as significant biomarkers [6]. Evidence from human and murine studies are reviewed in [7].

Inflammation plays a central role in atherosclerosis [8], leading to production of reactive oxygen species (ROS) [9], thus causing endothelial dysfunction and further aggravation of atherosclerosis. In this regard, especially PAD, representing a generalised form of atherosclerosis, is accounted for a high inflammatory status [10], explaining the high cardiovascular mortality rate [1].

Therefore, it is essential to treat PAD patients consequently as early as possible in dependence of each patient's disease state. According to international guidelines, for patients in Fontaine state I or II A/B (Rutherford 1–3), a conservative treatment with best medical treatment and exercise training is recommended [11, 12]. The exercise training is most effective under professional supervision; however, in the absence of a structured and supervised exercise program, a non-supervised home-based exercise training is recommended, although inferior to the supervised training [13]. It is well known that physical training has a positive effect on inflammation, immune cells and atherosclerosis. Part of the responsible mechanisms is related to the mobilisation of endothelial progenitor cells (EPC) stimulated by either moderate [14–16] or intensive, strenuous exercise [17, 18] (for review see [19]).

However, little is known about the influence of exercise training on immune cells in PAD patients. The aim of the present pilot study is to analyse any change in the proinflammatory phenotype of PMN, monocytes and DC by a home-based exercise training, and its effect on oxidative stress. We hypothesise that an increased capability to walk leads to an amelioration of the inflammation, resulting in a less proatherogenic phenotype of PMN, monocytes or DC as well as a reduced ROS production.

Materials and methods

Study population

The present study was designed as a pilot study and carried out in accordance with the principles outlined in the Declaration of Helsinki and was approved by the Ethics Committee of the University of Mainz and the State of Rheinland-Pfalz, Germany. Participation was voluntary. Each participant gave written informed consent. Due to the pilot study design, it was not possible to calculate the sample size. We enrolled 40 patients with known PAD, admitted to the Medical Department of the Johannes Gutenberg University Mainz with intermittent claudication (IC) (Fontaine Stage II A/B). Mean follow-up time was 12 ± 1.41 months. Patients with known cancer, autoimmune disease, chronic inflammatory conditions, orthopaedic maladies (chronic arthrosis of hip or knee, spinal canal stenosis) not allowing them to train properly, age under 18 or pregnancy were excluded from the study. Patients who had received antihypertensive treatment or who had received a diagnosis of hypertension (blood pressure above 140/90 mmHg) were considered to have arterial hypertension. Smoking was classified as current smoking, past smoking (stopped between >4 weeks and <40 years ago) or never smoking. Diabetes mellitus was diagnosed in patients who had previously undergone dietary treatment or had received oral antidiabetic or insulin medication or who had a current fasting blood glucose level >125 mg/dl. Family history of premature atherosclerosis was attributed to patients with a documented case of PAD, atherosclerotic stroke or coronary artery disease (CAD) in a first-degree relative before the age of 65 years. Hyperlipoproteinemia was diagnosed in patients who had been given lipid-lowering medication or had a history of cholesterol levels >240 mg/dl. The age median of the study group was 70 years [64; 73]. The distribution of medication taken by the study participants and their cardiovascular risk factors are given in Tables 1 and 2, respectively.

Table 1 Medication of the study population

	Admission ($n = 40$)	Follow-up ($n = 40$)	p value
Aspirin [100 mg] (%)	31 (77.5 %)	31 (77.5 %)	1.0
Clopidogrel [75 mg] (%)	10 (25 %)	2 (25 %)	**0.03**
Vit. K+ antagonists (%)	5 (12.5 %)	5 (12.5 %)	1.0
β-Blockers (%)	28 (70 %)	27 (67.5 %)	0.81
Metoprolol (%)	7 (17.5 %)	8 (20 %)	0.77
[mg]	61.1 ± 23.2	59.4 ± 22	0.89
Nebivolol (%)	7 (17.5 %)	6 (15 %)	0.76
[mg]	5 ± 0.0	5 ± 0.0	1.0
Bisoprolol (%)	12 (30 %)	11 (27.5 %)	0.81
[mg]	3.44 ± 1.42	4.43 ± 3.03	0.66
Carvedilol (%)	2 (5 %)	2 (5 %)	1.0
[mg]	15.6 ± 13.3	15.6 ± 13.3	1.0
Statins (%)	35 (87.5 %)	35 (87.5 %)	1.0
Simvastatin (%)	31 (77.5 %)	31 (77.5 %)	1.0
[mg]	35.66 ± 12.52	35.66 ± 12.52	0.99
Rosuvastatin (%)	2 (5 %)	2 (5 %)	1.0
[mg]	7.5 ± 3.5	7.5 ± 3.5	1.0
Pravastatin (%)	1 (2.5 %)	1 (2.5 %)	1.0
[mg]	40 ± 0	40 ± 0	1.0
Fluvastatin (%)	1 (2.5 %)	1 (2.5 %)	1.0
[mg]	20 ± 0	20 ± 0	1.0
ACE-inhibitors (%)	19 (47.5 %)	19 (47.5 %)	1.0
Ramipril (%)	15 (37.5 %)	15 (37.5 %)	1.0
[mg]	7.17 ± 2.81	7.50 ± 2.84	0.76
Lisinopril (%)	2 (5 %)	2 (5 %)	1.0
[mg]	12.5 ± 10.6	12.5 ± 10.6	1.0
Trandolapril (%)	1 (2.5 %)	1 (2.5 %)	1.0
[mg]	2 ± 0	2 ± 0	1.0
Enalapril (%)	1 (2.5 %)	1 (2.5 %)	1.0
[mg]	5 ± 0	5 ± 0	1.0
AT_1-blocker (%)	17 (42.5 %)	18 (45 %)	0.82
Losartan (%)	3 (7.5 %)	3 (7.5 %)	1.0
[mg]	50 ± 0	50 ± 0	1.0
Valsartan (%)	7 (17.5 %)	7 (17.5 %)	1.0
[mg]	160 ± 92.4	160 ± 92.4	0.95
Candesartan (%)	3 (7.5 %)	4 (10 %)	0.69
[mg]	10.67 ± 4.62	10.0 ± 4.0	1.0
Irbesartan (%)	1 (2.5 %)	1 (2.5 %)	1.0
[mg]	75 ± 0	75 ± 0	1.0
Olmesartan (%)	1 (2.5 %)	1 (2.5 %)	1.0
[mg]	20 ± 0	20 ± 0	1.0
Telmisartan (%)	2 (5 %)	2 (5 %)	1.0
[mg]	60 ± 28.3	60 ± 28.3	1.0
Ca^{2+}-channel blocker (%)	18 (45 %)	19 (47.5 %)	0.82
Amlodipin (%)	15 (37.5 %)	15 (37.5 %)	1.0
[mg]	6.83 ± 2.75	6.33 ± 2.29	0.66
Lercanidipin (%)	2 (5 %)	2 (5 %)	1.0
[mg]	10 ± 0	15 ± 7.1	0.62
Verapamil (%)	1 (2.5 %)	2 (5 %)	0.56
[mg]	180 ± 0.0	210 ± 42.4	0.5

Statistically significant changes ($p < 0.05$) are highlighted in bold

Table 2 Clinical characteristics of the study population

	Admission ($n = 40$)	Follow-up ($n = 40$)	p value
Absolute walking distance (m)	422 [226; 669]	580 [293; 825]	**<0.0001**
Pain-free walking distance (m)	208 [146; 382]	345 [247; 655]	**<0.001**
Ankle-Brachial-Index (ABI)	0.65 [0.47; 0.71]	0.72 [0.60; 0.83]	**0.03**
Intima-media thickness (IMT) (cm)	0.9 [0.70; 0.975]	0.7 [0.60; 0.87]	0.23
LV-EF (%)	55 [50; 61.3]	59 [50; 67.4]	0.38
Mean blood pressure (mmHg)	110 [105; 120]	110 [100; 115]	0.35
Fibrinogen (mg/dl)	359 [315; 412]	329 [299; 384]	**0.02**
C-reactive protein (mg/dl)	2.85 [1.43; 5.03]	1.95 [1.43; 4.30]	**0.02**
P-Selectin (ng/ml)	50.21 [45.05; 61.52]	56.19 [43.85; 63.89]	0.58
sTREM-1 (ng/ml)	42.15 [34.33; 83.16]	42.83 [38.17; 56.70]	**<0.01**
Glucose (mg/dl)	102 [89.75; 108.00]	102 [94.25; 113.50]	0.19
HbA1c (%)	6.05 [5.73; 6.38]	6.00 [5.70; 6.45]	0.87
Total cholesterol (mg/dl)	189 [166.8; 214]	188 [167; 213.8]	0.33
LDL cholesterol (mg/dl)	107 [82.5; 130.8]	98 [85.75; 120.3]	0.6
HDL cholesterol (mg/dl)	45.5 [40.25; 56.75]	47.5 [41.00; 52.75]	0.83

Values are given as median (25th percentile/75th percentile). Statistically significant changes are highlighted in bold

For the study, patients were asked to perform a home-based exercise training according to the recommendations of the present guidelines [11, 12]. In brief, the patients were informed to walk at least 30 min up to 60 min per day, at least on 3–5 days a week. They were asked to walk with an intensity to nearly reach their typical claudication sensations, then to rest for up to 5 min and repeat the same distance with a lower intensity. This protocol is in accordance with standard exercise recommendations [20]. Written information, on how to perform the home-based exercise training under self-management was handed out at the beginning of the study.

To evaluate the pain-free and absolute walking distance of each patient at the beginning of the study and at follow-up appointment, we measured their walking distances after standard protocol on a treadmill with 10 % elevation at a speed of 3.0 km/h. The same elevation and speed were used at both treadmill tests. All blood samples were drawn after overnight fasting period.

Preparation of blood samples

From each participant blood was drawn by venopuncture after a fasting period of at least 12 h, before treadmill testing. For the different analyses, whole blood samples were either heparinised or drawn in EDTA or sodium citrate. In dependence of the designated tests, the samples were transported immediately to our lab or the hospitals core facility for routine blood analysis (Institute for Clinical Chemistry and Laboratory Medicine), either on ice or at room temperature.

Measurement of ROS formation from human whole blood by chemiluminescence

The measurement of ROS formation in human whole blood with enhanced chemiluminescence (ECL) was performed under basal or stimulated conditions using the luminol analogue L-012 (8-Amino-5-Chloro-7-Phenylpyrido[3,4-d]pyridazin-1,4-(2H,3H)Dion sodium salt) (purchased from Wako Pure Chemical Industries (Japan)). To induce the oxidative burst, we used phorbol 12,13-dibutyrate (PDBu) (10 μM) (purchased from Sigma). L-012 detects all kinds of ROS and is therefore applicable to measure oxidative stress in total [21], especially in whole blood L-012 is particularly suitable for detection of O_2^-, mainly produced by NOXs [22, 23].

The method used has been described earlier [22]. In brief, venous blood was drawn into sodium citrate and immediately analysed. The blood was kept at room temperature and diluted 1:50 in Dulbecco's PBS (with 1 mM Mg^{2+}, Ca^{2+}, and without bicarbonate), and stimulators of NOX activity were added together with L-012 (100 μM). The final volume of each sample was 0.2 ml. Chemiluminescence was counted in a Centro chemiluminescence plate reader from Berthold Techn. (Bad Wildbad, Germany) at 30 min for basal ROS formation without NOX stimulators and for PDBu-stimulated condition. Chemiluminescence was expressed as counts per minute after incubation time of 30 min. The results were finally standardised by the amount of whole blood leucocytes. As quality control, we used inhibitors of NOX activity and the superoxide scavenger PEG-SOD, as we have previously reported [22].

Flow cytometry of PMN, monocytes and DC

Flow cytometric analysis was performed after standard protocol which we used in the two recent publications [24, 25] in adaption of the protocol of Della Bella [26]. In brief, PMN, monocytes and DC were identified by different gating strategies (Fig. 1a_1, b_1). The PMN and monocyte populations were identified by size and granulation in the forward scatter/sideward scatter diagram (FSC/SSC) (a_1), as well as by the marker molecules CD45, CD14 (both bd-biosciences, Heidelberg, Germany) (a_3) and CD86 (Beckman-Coulter Inc. Krefeld, Germany) (a_2). Monocytes (a_4) were analysed for CD16 (Invitrogen, Darmstadt, Germany), CD14, CD11b, CD11c (all bd-biosciences,

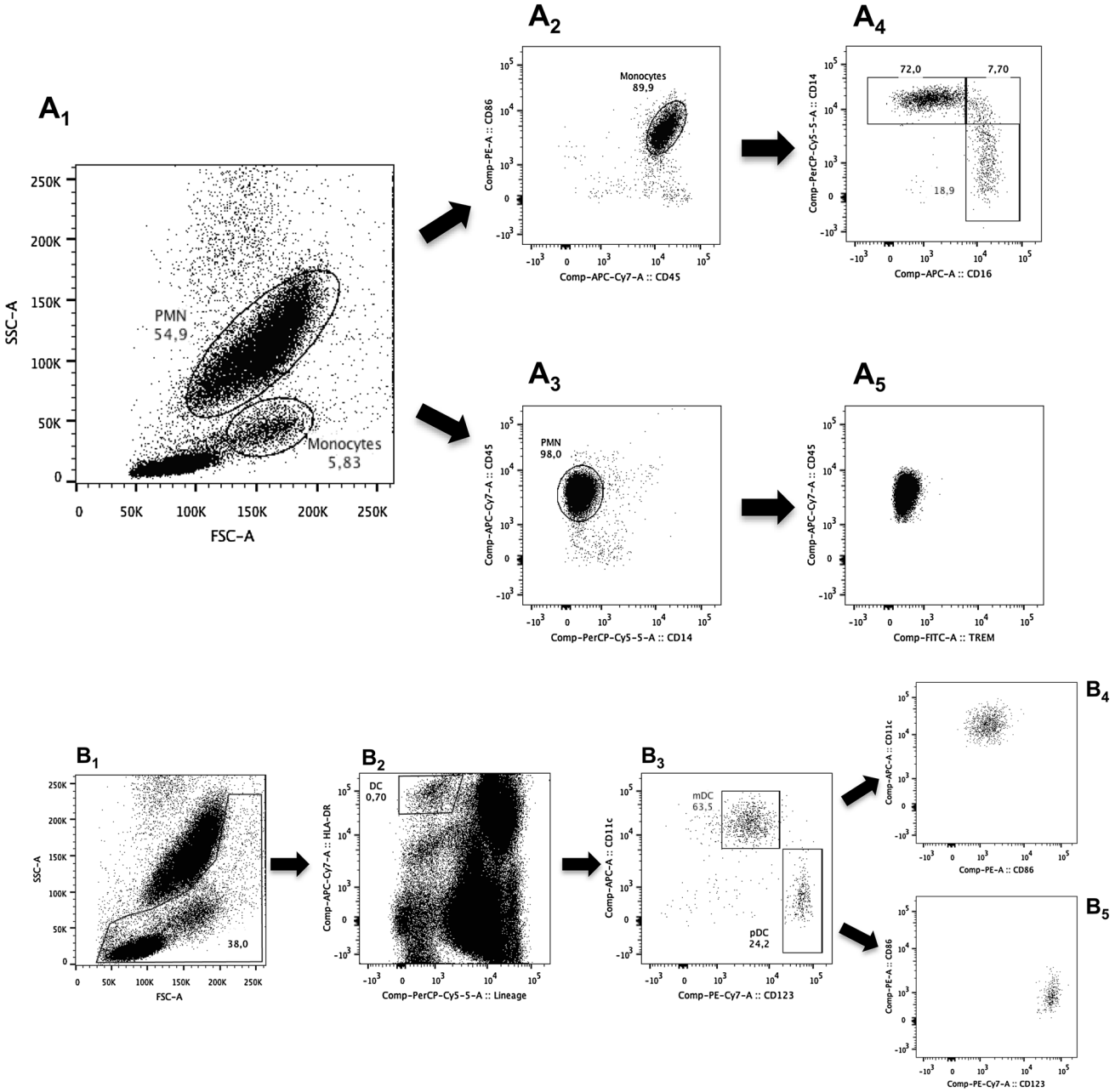

Fig. 1 Flow cytometric analysis of monocytes, PMN and DC. **a_1** The PMN and monocyte populations were identified by size and granulation in the FSC/SSC diagram, as well as by the marker molecules CD45, CD14 and CD86 for monocytes (**a_2**). PMN were identified as negative for CD14, but expressing CD45 (**a_3**). From these populations, the expression of surface molecules on monocytes (**a_4**) and PMN (**a_5**) was analysed. Similarly, the DC population was identified in the FSC/SSC diagram (**b_1**). The total DC population was identified being positive for HLA-DR and negative for an antibody cocktail of several lineage marker molecules (**b_2**). mDC and pDC were identified by CD11c and CD123, respectively (**b_3**). Each population of $CD11^+$ mDC (**b_4**) and $CD123^+$ pDC (**b_5**) was then analysed for surface molecules of interest

Heidelberg, Germany), M-DC8 (Slan-DC) (Miltenyi, Bergisch-Gladbach, Germany) and TREM-1 (clone 1C5 [27]). PMN were analysed for TREM-1, CD11b and CD11c (a_5).

The DC population was identified by the marker molecule HLA-DR (bd-biosciences, Heidelberg, Germany) and an antibody cocktail of several lineage marker molecules (CD3, CD14, CD16, CD19, CD20—all bd-biosciences, Heidelberg, Germany) (Fig. 1b_2). DC were classified as HLA-DR (+) and lineage (−). From this population, mDC and pDC were identified by CD11c and CD123 (eBiosciences, Frankfurt/Main, Germany) (b_3), respectively. Each population was then analysed for CD86 (Beckman-Coulter Inc. Krefeld, Germany) and CD83 (bd-biosciences, Heidelberg, Germany) (b_4, b_5).

For the analysis of peripheral blood monocytes, DC and PMN 250 µl of Li^+-heparinised whole venous blood/per sample was used from each participant. The samples were transferred to 5 ml FACS tubes (BD Falcon) and washed twice before erythrocytes were then lysed with BD lysing solution (bd-biosciences) for 10 min at room temperature. Cells were then stained with directly fluorochrome-conjugated antibodies. Staining conditions for each monoclonal antibody were preliminarily determined. Data are presented as percentage of positive cells and mean fluorescence intensity (MFI), corrected for background fluorescence, determined by unstained sample measurements.

Routine laboratory methods

Blood samples were drawn from each participant under standardised conditions (see above). Serum was centrifuged at 4000*g* for 10 min, immediately divided into aliquots, and frozen at −80 °C until analysis. Lipid serum levels (total, HDLand LDL Cholesterol, triglycerides), blood glucose, HbA1c, fibrinogen and cellular counts were measured immediately after transfer to the hospitals core facility for routine blood analysis (Institute for Clinical Chemistry and Laboratory Medicine) under their current standardised conditions. C-reactive protein (CRP) was determined by a highly sensitive, latex particle-enhanced immunoassay (detection range 0–20 mg/dl; Roche Diagnostics). Measurement of the soluble form of TREM-1 (sTREM-1) was performed by ELISA, as described previously [28].

Cell counts

Total leucocyte count, and PMN and monocyte count/ml, as well as total PMN and monocyte proportion were determined by routine blood work analysis. The proportion of total DC, mDC and pDC was determined by flow cytometry. To achieve the absolute DC, mDC and pDC count/ml, we set the above determined DC-proportions in relation to the total leucocyte count/ml. The mDC/pDC ratio was determined by the total mDC and pDC counts/ml.

Statistical analysis

For data management and statistical analysis, we used the Prism V5.0b statistical software package (Graphpad, San Diego, CA, USA). Data are given as median [25th; 75th percentile] for continuous variables. A value of $p < 0.05$ was considered to be significant. The two time points of the study population (admission, follow-up) were contrasted using a two-tailed paired *t* test in the case of a Gaussian distribution. If the normality test (D'Agostino) failed, we then used Wilcoxon matched pairs nonparametric test instead. Correlation of continuous data among the general study population was performed using Spearman's correlation coefficients (*r*).

Results

Clinical results after follow-up

After performed the home-based exercise program, we recalled the patients for a further treadmill test. We found that the pain-free walking distance increased significantly from 208 m at admission up to 345 m at follow-up. Similarly, the absolute walking distance increased significantly from admission to follow-up by 158 m. The ABI after training was significantly higher than at admission. Intima-media thickness (IMT) and left ventricular ejection fraction (LV-EF) did not differ between admission and follow-up. Markers of inflammation showed a significant reduction at follow-up. Next to CRP and fibrinogen, we found a decrease in sTREM-1 from admission to follow-up. P-Selectin, as a marker of platelet activation, did not differ between admission and follow-up. The analysis of the routine blood work after training revealed no differences between the two time points at admission and follow-up. During the observation period, no changes in their cardiovascular risk profile (e.g. smoking habits) or the individual medication were reported. Data are presented in Table 2.

Concerning the medication taken by the patients, we found that 31 patients received Aspirin and stayed on it. Ten patients had Clopidogrel prescribed. Of these, two reported to suffer from Aspirin intolerance. The further eight patients on Clopidogrel had a dual platelet aggregation blockade, due to coronary stenting within the preceding 12 months, thus, the dual platelet inhibition in these was possible to be terminated at the beginning of the study and continued with Aspirin monotherapy. Vit. K antagonists were taken by five patients at the beginning and

end of the study. For antihypertensive treatment, 19 patients received an ACE-inhibitor, 15 an AT_1-blocker, 28 patients had a β-blocker and 18 reported to take a Ca^{2+} channel blocker. At follow-up, the same 19 patients continued to take their predescribed ACE-inhibitor, whereas two more patients reported to take an AT_1-blocker. For the β-blocker medication, one patient changed for a Ca^{2+} channel blocker (bisoprolol for verapamil). The mean dosage mostly remained stable, except for some changes, but these were nor significant (see Table 1).

Changes in relative and absolute cell numbers after follow-up

Concerning the analysis of cell numbers, we observed no change in both the relative and total amount/ml of PBMC, monocytes, PMN or total DC, as well as in the total amount of thrombocytes/ml from admission to follow-up. However, mDC decreased significantly from admission to follow-up in both relative and absolute count/ml. In contrast, pDC did not differ in relative and absolute numbers from admission to follow-up, thus, the mDC/pDC ratio was not affected significantly (for results see Table 3).

Changes in expression of cellular surface molecules on monocytes after follow-up

The analysis of changes in the relative distribution of CD14/CD16-positive monocytes after performed the exercise training, revealed a significant increase of the classical, anti-inflammatory $CD14^{++}CD16^{-}$ monocyte subpopulation (Fig. 2a). In contrast, both proinflammatory monocyte subpopulations, the intermediate $CD14^{+}CD16^{+}$ subpopulation (Fig. 2b) and the non-classical $CD14^{+-}$ $CD16^{++}$ subpopulation (Fig. 2c), decreased significantly. Similarly, for MDC-8, being co-expressed with CD16, we observed a decrease of the proportion of MDC-8^{+} monocytes over time (Fig. 2d).

For the costimulatory surface molecule CD86, we found a significant decrease after the training period (Fig. 3a), as well as for the adhesion molecules CD11b (Fig. 3b), CD11c (Fig. 3c) and TREM-1 (Fig. 3d).

Changes in expression of cellular surface molecules on PMN after follow-up

The involvement of PMN in atherosclerosis is mainly triggered via adhesion to endothelial cells and platelets. We therefore analysed the adhesion molecules CD11b, CD11c and TREM-1 on PMN. Similar to monocytes, we observed a significant decrease of the expression of CD11b (Fig. 4a), CD11c (Fig. 4b) and TREM-1 (Fig. 4c) from admission to follow-up.

Changes in expression of cellular surface molecules on DC after follow-up

DC were analysed for typical inflammatory surface molecules, important for stimulation and co-stimulation. For both, mDC and pDC we found a significant decrease of HLA-DR and CD86 at follow-up. Furthermore on mDC, CD83, CD40 and CD80 decreased significantly, whereas on pDC only CD40 decreased at follow-up (for all results see Table 4).

Table 3 Relative and absolute distribution of cells

	Admission ($n = 40$)	Follow-up ($n = 40$)	p value
PBMC/ml	7.10×10^6 [5.66×10^6; 7.92×10^6]	6.67×10^6 [5.89×10^6; 7.96×10^6]	0.31
Monocytes (%)	6.3 [5.63; 7.38]	6.25 [5.73; 7.50]	0.54
Monocytes/ml	4.46×10^5 [3.55×10^5; 5.72×10^5]	4.26×10^5 [3.42×10^5; 5.20×10^5]	0.12
PMN (%)	64.95 [58.55; 68.18]	61.00 [56.25; 66.00]	0.19
PMN/ml	4.45×10^6 [3.47×10^6; 5.35×10^6]	4.10×10^6 [3.42×10^6; 4.87×10^6]	0.20
Total DC (%)	0.86 [0.68; 1.10]	0.83 [0.63; 1.01]	0.20
Total DC/ml	5.61×10^4 [4.77×10^4; 7.28×10^4]	5.34×10^4 [4.27×10^4; 6.51×10^4]	0.14
mDC (%)	68.55 [60.13; 75.63]	56.70 [50.93; 69.50]	**<0.001**
mDC/ml	3.82×10^3 [3.11×10^3; 5.23×10^3]	2.91×10^3 [2.37×10^3; 4.00×10^3]	**<0.01**
pDC (%)	21.70 [14.40; 30.00]	22.90 [15.15; 32.08]	0.85
pDC/ml	1.42×10^3 [0.79×10^3; 1.87×10^3]	1.28×10^3 [0.72×10^3; 1.72×10^3]	0.98
mDC/pDC ratio	3.23 [1.94; 5.79]	2.36 [1.50; 4.34]	0.26
Platelets/ml	2.58×10^8 [2.06×10^8; 3.15×10^8]	2.52×10^8 [2.02×10^8; 3.10×10^8]	0.42

Total cell counts for PBMC, platelets, monocytes, PMN, total DC/ml, mDC and pDC/ml as well as the proportion of monocytes, PMN and of mDC and pDC in relation to the total DC count/ml. Values are given as median (25th percentile/75th percentile). Statistically significant changes are highlighted in bold

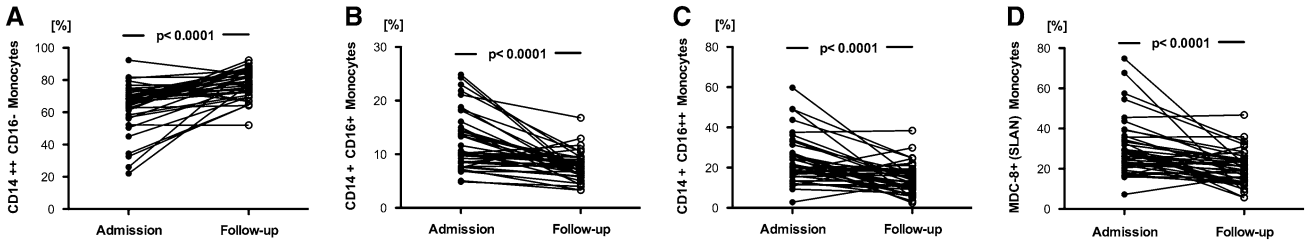

Fig. 2 Proportion of CD14 CD16 monocytes and MDC-8^{+} monocytes. **a** The classical CD14^{++}CD16^{-} monocyte subpopulation increased after 12-month exercise training (admission: 66.37 % [59.14; 72.36 %] vs. follow-up: 78.82 % [72.58; 85.80 %]; $p < 0.0001$), whereas the proinflammatory/proatherogenic **b** intermediate CD14^{+}CD16^{+} monocyte subpopulation (admission: 10.48 % [8.40; 14.88 %] vs. follow-up: 7.80 % [5.95; 9.30 %]; $p < 0.0001$) and **c** non-classical CD14^{+}CD16^{++} monocyte subpopulation decreased (admission: 21.07 % [16.67; 27.05 %] vs. follow-up: 13.16 % [9.03; 18.30 %]; $p < 0.0001$) from admission to follow-up time point. **d** Similarly, the proportion of monocytes expressing MDC-8, being co-expressed with CD16, decreased during the training period (admission: 28.05 % [21.78; 36.45 %] vs. follow-up: 19.40 % [13.00; 24.23]; $p < 0.0001$). All $n = 40$

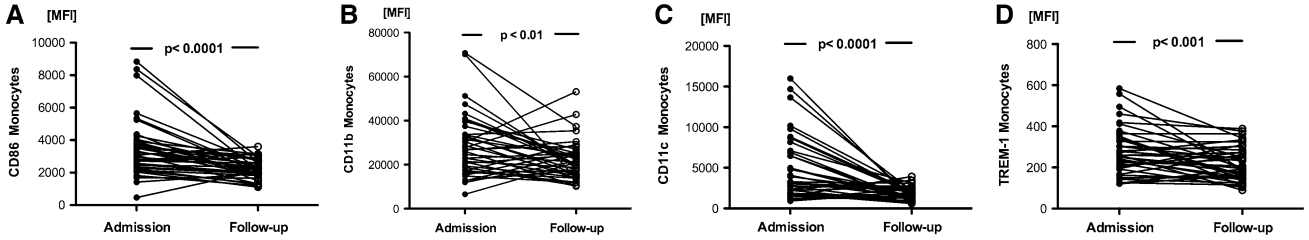

Fig. 3 Expression of proinflammatory molecules and adhesion molecules on monocytes. **a** From admission to follow-up, the MFI of CD86 decreased significantly (admission: 3262 [2380; 3916] vs. follow-up: 2170 [1934; 2655]; $p < 0.0001$), as well as the expression height of major adhesion molecules like **b** CD11b (admission: 26,816 [17,759; 36,523] vs. follow-up: 20,488 [15,147; 25,236]; $p < 0.01$), **c** CD11c (admission: 3081 [1816; 6806] vs. follow-up: 1742 [1091; 2379]; $p < 0.0001$) and **d** TREM-1 (admission: 260.5 [196.8; 347.3] vs. follow-up: 193.5 [148.8; 273.5]; $p < 0.001$). All $n = 40$

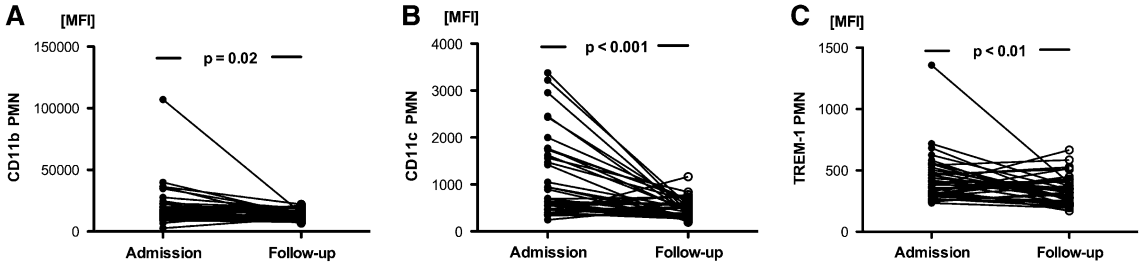

Fig. 4 Expression of adhesion molecules on PMN. Similarly to monocytes, we observed a decrease from admission to follow-up time point for the adhesion molecules **a** CD11b (admission: 15,415 [11,356; 20,047] vs. follow-up: 12,737 [8722; 15,538]; $p = 0.02$), **b** CD11c (admission: 637 [465; 1608] vs. follow-up: 467 [327; 607]; $p < 0.001$), and **c** TREM-1 (admission: 396 [310; 500] vs. follow-up: 319 [235; 405]; $p < 0.01$). All $n = 40$

ROS production after follow-up

Oxidative stress is a driving force in atherogenesis. We therefore sought to analyse changes in ROS production over the training period. At basal levels we observed no difference between admission and follow-up (Fig. 5a). NOXs stimulation with PBDu revealed a significant decrease of ROS production after 30 min from admission to follow-up (Fig. 5b).

Correlation of sTREM-1 with walking distance, ROS production and markers of inflammation

For sTREM-1, we found an inverse correlation with the absolute walking distance (Fig. 6a) and the pain-free walking distance (Fig. 6b) measured at follow-up, whereas ROS production after PDBu stimulation correlated directly with sTREM-1 (Fig. 6c). Similarly, fibrinogen correlated directly (Fig. 6d) with sTREM-1 at follow-up, but CRP did not correlate (Fig. 6e).

Table 4 Mean fluorescence intensity (MFI) for mDC and pDC

	Admission ($n = 40$)	Follow-up ($n = 40$)	p value
mDC			
HLA-DR	35,872 [25,751; 49,881]	35,872 [25,751; 49,881]	**<0.01**
CD86	966 [668; 1544]	721 [609; 900]	**<0.0001**
CD80	135 [103; 201]	98 [86; 120]	**<0.01**
CD83	130 [103; 199]	109 [89; 133]	**0.02**
CD40	109 [88;309]	89 [77; 114]	**0.02**
pDC			
HLA-DR	21,532 [17,573; 31,746]	17,133 [14,040; 22,112]	**<0.01**
CD86	545 [411; 2199]	495 [378; 650]	**0.02**
CD80	119 [98; 177]	103 [96; 118]	0.19
CD83	125 [102; 188]	112 [100; 139]	0.63
CD40	280 [210; 2081]	276 [196; 354]	**<0.01**

We determined the expression height of typical costimulatory surface molecules on mDC and pDC at admission and follow-up time point for. Values are given as median (25th percentile/75th percentile). Statistically significant changes ($p < 0.05$) are highlighted in bold

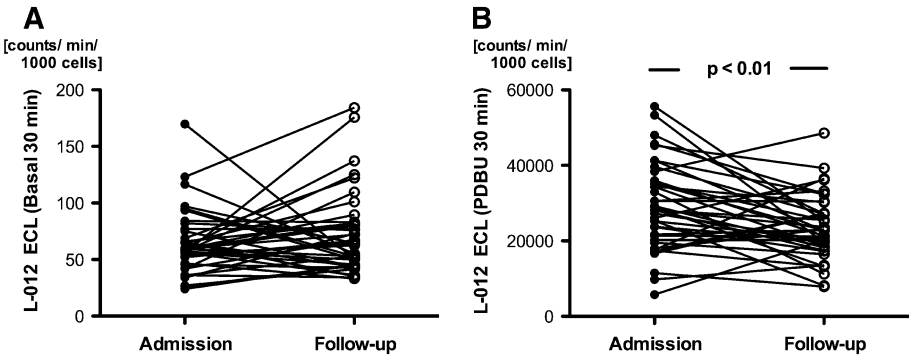

Fig. 5 Decreased ROS production at follow-up: **a** analysing the basal ROS production we did not find a difference between admission and follow-up time point (admission: 58.58 [48.00; 73.46] vs. follow-up: 65.42 [45.58; 82.92]; $p = 0.16$). However, **b** stimulation with PDBu for 30 min leads to a decrease of ROS (admission: 28,161 [20,493; 35,799] vs. follow-up: 22,704 [18,349; 27,299]; $p < 0.01$) at follow-up. All $n = 40$

Discussion

Atherosclerosis is a chronic inflammatory disease influenced by oxidative stress and inflammation [29]. Therefore, in the treatment of atherosclerotic diseases, especially of PAD, the necessity for anti-inflammatory/-oxidative strategies is highly demanded [30]. At present, concerning international guidelines, exercise training is the primary recommended therapy for PAD in the Fontaine stadium I and II [11, 12]. Preferably, a supervised exercise training is recommended if possible, though, at least a home-based, non-supervised exercise training should be performed. Recently, we have reported that patients with PAD show an increased proinflammatory phenotype on monocytes and DC [24], as well as an increased production of ROS, with sTREM-1 as an independent marker of proinflammation in PAD patients [25].

In the present pilot study, we were therefore interested whether the previously reported observations in patients with intermittent claudication might change under home-based exercise training, independent of the training protocol. We found after a mean of approximately 12-month training that an increased pain-free and absolute walking distance in comparison to the measured walking distances at the start of the study. Furthermore, the ABI increased and routine inflammatory markers like CRP and fibrinogen decreased. These results clearly show our data to be consistent with the present recommendations [11, 12] and that exercise training, even if it is non-supervised, is very successful in improving the limitations PAD patients encounter and that it might ameliorate their proinflammatory status.

At follow-up time point, we found an increase in the anti-inflammatory, classical $CD14^{++}CD16^{-}$ monocytes,

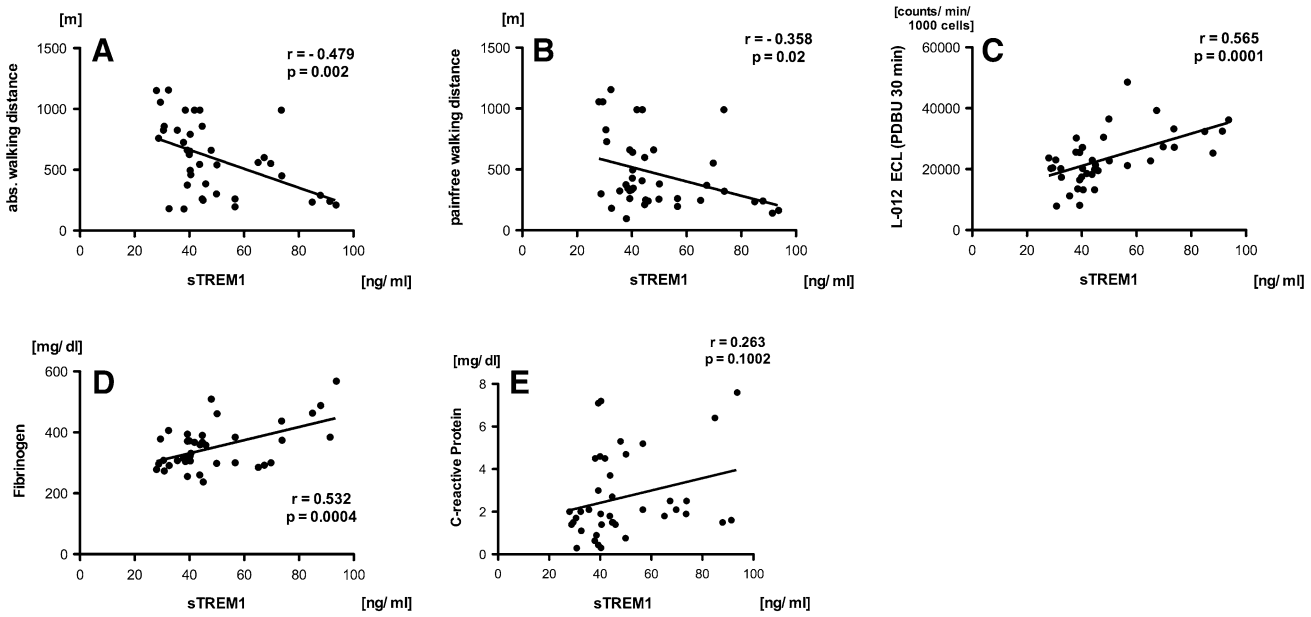

Fig. 6 Correlation of sTREM-1 with ROS walking distance, a marker of inflammation: we found an inverse correlation for sTREM-1 with the absolute ($r = -0.479$; $p = 0.002$) (**a**) and pain-free walking distance ($r = -0.358$; $p = 0.02$) (**b**). ROS production after PDBu stimulation ($r = 0.565$; $p = 0.0001$) (**c**) and fibrinogen correlated directly ($r = 0.532$; $p = 0.0004$) (**d**), but CRP did not correlate ($r = 0.263$; $p = 0.1002$) (**e**). All $n = 40$

paralleled by a decrease of the proinflammatory, intermediate $CD14^{+}CD16^{+}$, non-classical $CD14^{+}CD16^{++}$ and MDC-8^{+} monocyte subpopulation, next to the surface molecules CD86, and adhesion molecules CD11b, CD11c and TREM-1. PMN showed a decrease of these adhesion molecules after 12-month training as well. Similarly, for DC (both mDC and pDC), we found a decrease in the expression of CD86, CD40 and HLA-DR, whereas for CD80 and CD83 expression, we observed a decrease only on mDC.

It is well known that endurance training can promote the mobilisation of EPC [14–18], the basis for neoangiogenesis and therapeutic goal of exercise training in PAD patients. Nevertheless, the mechanisms on how exercise influences the immune system are complicated and not fully understood. Exercise might not only have beneficial, but also deleterious effects on our health depending whether a high or moderate intensity training was performed (for reviews see [31, 32]). Apart from a general influence on the innate and adaptive immune system [31], training can directly influence PMN and monocytes, though most studies only analysed the acute effects achieved by exercise training (i.e. ~1–2 h of training). Little is known about its influence on DC [33, 34], and any training influence on mDC or pDC has not been studied.

The reported changes by endurance training concerning the phenotype of monocytes [35, 36], were observed only immediately after a short and intense exercise period. However, little is known about long-term effects of exercise training on monocyte subpopulations and their phenotype. In this regard, Timmerman et al. [37] reported an increase in $CD14^{++}CD16^{-}$ monocytes in actively exercising individuals in contrast to those living an inactive life style. Likewise to our study population, both proinflammatory CD14/CD16 subpopulations decreased, indicating that a continuous and long-term exercise training needs to be performed in order to lower the proinflammatory monocyte subpopulations.

The influence of exercise training on PMN has scarcely been investigated. Similarly, to the expression of CD14 and CD16 on monocytes, acute and high intensity exercise training leads to an increase of CD11b [38], possibly through mobilisation of PMN with a high expression of CD11b from the marginal pool. Long-term effects on PMN and major adhesion molecules by exercise training, however, are not known.

Despite the changes of surface molecules on PMN, their ability of a respiratory burst is also influenced. Acute exercise training with a high intensity, but also long-term training, leads to a reduced oxidative burst and ROS production by PMN [39, 40]. In our study population, we found a reduced oxidative burst from whole blood after stimulation with PDBu at follow-up in comparison to the beginning of the study.

We have recently described a possible reason for an increased ROS production in PAD patients [25], by an increased expression of TREM-1 on PMN of PAD patients, hypothetically leading to an intensified interaction with

platelets, possibly enhancing their ROS production [41]. A reduced expression of TREM-1 on PMN and monocytes, might then explain the reduced ROS production that we observed in the present study, although we did not observe a decrease in P-Selectin levels, as a marker of platelet activation [41]. A possible reason for this result might be the platelet inhibition in most of the patients, since Aspirin and Clopidogrel are strong inhibitors of platelet activation (for review see [42]). Nevertheless, a decrease in TREM-1 expression suggests that exercise training leads to a reduction of the innate immune activation state found in PAD patients [25]. Next to that, we and others have identified its soluble form sTREM-1, to be an important and independent marker of chronic inflammatory conditions, such as chronic obstructive pulmonary disease (COPD) or inflammatory bowel disease (IBD) [25, 43, 44]. Our previous [25] and present data therefore indicate that sTREM-1 mirrors the disease's activity in PAD patients, especially since sTREM-1 correlates directly with PDBU-induced ROS and fibrinogen, and inversely with both the pain-free distance, as well as the absolute walking distance, and therefore might be a suitable surrogate parameter to monitor the training efficacy and the amelioration of the inflammatory status in these patients.

Limitations

The present study was designed as a pilot study since we were not certain about possible measurable changes other than routine clinical data like ABI and walking distance. We therefore did not include a study arm for supervised exercise training or analysed quality of life by questionnaires, subjects well examined and established in the treatment of patients with intermittent claudication. Due to the nature of a home-based exercise training, we cannot state how patients trained and whether all trained with a comparable intensity. Furthermore it is known, that over time more and more patients fail to attend the exercise training groups [45]. Whether this is related to a decline in motivation and whether this has occurred in our study group to a similar extend cannot be said for sure, since we have tried to reduce the amount of supervision to a minimum. Thus, another limitation of the study is not having the training performed under control of specialised sport physiologist. However, by handing out information material on how to train, we hopefully have limited the variance to an acceptable range. Whether the training performed in the present study is similarly sufficient in influencing the proinflammatory phenotype than supervised exercise training requires further studies.

The most important limitation of our study is the lack of a control group of PAD patients not training and just receiving best medical treatment (BMT). Motivation by study participation itself can improve the clinical outcome, independent of the intervention [46]. To exclude such a confounder, one had to perform a randomised prospective study. In addition, a study arm for BMT alone stands in contrast to the recommendations of the guidelines [11, 12], where a further treatment (bypass surgery, percutaneous transluminal angioplasty (PTA), supervised or non-supervised exercise training) on top of BMT is required.

The lack of tissue samples for ROS measurement has to be acknowledged as a methodological limitation of our study as well. In order to better assess our findings for their clinical relevance, data on the further clinical outcome would be preferable. Future studies to address this question need then to be performed, moreover since the relatively small number of the study patients ($n = 40$) requires the current data to be validated in other large cohorts.

Conclusions

To our best knowledge, we report here for the first time a reduced expression of several inflammatory surface molecules on monocytes, DC and PMN in PAD patients as well as serological markers of inflammation, paralleled by an increased pain-free and absolute walking distance after exercise training. Data from our pilot study bring interesting additional information on the anti-inflammatory and anti-oxidative strategies in the treatment of PAD, indicating that any exercise training should be performed, especially wherever the possibility for a supervised training group is not present. Further studies of the role of exercise training program in the progression and prognosis of PAD and other cardiovascular diseases are now needed.

Acknowledgments This paper contains in part data reported in the medical thesis of Martin Scheer.

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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