Exercise training in intermittent claudication: Effects on antioxidant genes, inflammatory mediators and proangiogenic progenitor cells

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Summary
Exercise training remains a therapy of choice in intermittent claudication (IC). However, too exhaustive exercise may cause ischaemic injury and inflammatory response. We tested the impact of three-month treadmill training and single treadmill exercise on antioxidant gene expressions, cytokine concentrations and number of marrow-derived proangiogenic progenitor cells (PPC) in the blood of IC patients. Blood samples of 12 patients were collected before and after training, before and after the single exercise. PPCs were analysed with flow cytometry, cytokine concentrations were checked with Milliplex MAP, while expression of miRNAs and mRNAs was evaluated with qRT-PCR. treadmill training improved pain-free walking time (from 144 ± 44 seconds [s] to 311 ± 134 s, p=0.02) and maximum walking time (from 578 ± 293 s to 859 ± 423 s, p=0.01) in IC patients. Before, but not after training, the single treadmill exercise increased the number of circulating CD45dimCD34+CD133-KDR+ PPCs (p=0.048), decreased expression of HMOX1 (p=0.04) in circulating leukocytes, reduced tumour necrosis factor-α (p=0.03) and tended to elevate myeloperoxidase (p=0.06) concentrations in plasma. In contrast, total plasminogen activator inhibitor-1 was decreased by single exercise only after, but not before training (p=0.02). Both before and after training the single exercise decreased monocyte chemoattractant protein (MCP)-1 (p=0.006 and p=0.03) concentration and increased SQD1 (p=0.001 and p=0.01) expression. Patients after training had also less interleukin-6 (p=0.03), but more MCP-1 (p=0.04) in the blood. In conclusion, treadmill training improves walking performance of IC patients, attenuates the single exercise-induced changes in gene expressions or PPC mobilisation, but may also lead to higher production of some proinflammatory cytokines.

Keywords
Ischaemia, oxidative stress, inflammation, microRNA, heme oxygenase-1

Introduction
Intermittent claudication (IC), which is one of the most common manifestations of peripheral occlusive arterial disease (POAD), affects 12% of adult men between the ages of 55 and 74, and is associated with 30% of total mortality (1). Risk of IC is increased by smoking, diabetes, hypertension or coronary heart disease (2). Cycles of ischaemia and reperfusion during the exercise and rest, respectively, may lead to endothelial dysfunction, the augmented activation of neutrophils (3, 4), microalbuminuria (5), increase in reactive oxygen species (ROS) (6), and subsequently aggravate the atherosclerosis. Since levels of plasma ROS scavengers are lower in IC patients (7), increase in ROS after the exercise, observed as well in healthy individuals (8), may be especially deleterious for the endothelium of claudicants. Furthermore, function and activity of immune cells, which are key players in development and progression of atherosclerosis, are regulated by microRNA, of which mir-146a and mir-155 seem to have high impact on lipid uptake, inflammatory cytokine and antioxidant response (9, 10). Finally, POAD patients have lower levels of circulating endothelial progenitor cells (CD34+KDR+ and CD34+CD133+) than healthy controls (11, 12).

Since the 1960s supervised exercise training is considered as effective and low-cost therapy for IC (11). However, still little is known about the influence of the training on the leukocyte antioxidant gene and microRNA response, while data on the mobilisation of proangiogenic progenitor cells (CD45dimCD34+CD133-KDR+) is not yet available. In this study we tested the impact of three-month treadmill training and single treadmill exercise on antioxidant gene expressions, cytokine concentrations and number of marrow-derived proangiogenic progenitor cells (PPC) in the blood of IC patients.
giogenic progenitor cells (PPCs), and plasma levels of growth factors or inflammatory mediators are inconsistent. Therefore we have assessed the number of circulating PPCs enriched in the endothelial progenitors (populations phenotyped as CD45dimCD31+CD133+, CD45dimCD34+CD133+KDR+ and CD45dimCD34+CD133+KDR−) at baseline (T0), on day 0 (D0), and after 12 weeks (D90) of training. Mobilisation in response to the exercise was evaluated at 1 (T+1h), 3 (T+3h) and 6 (T+6h) hours after the exercise. Moreover, we have measured the expression of heme oxygenase-1 (HMOX1), heme oxygenase-2 (HMOX2) and superoxide dismutase-1 (SOD1), as well as miR-146a, miR-155 and miR-200c in peripheral leukocytes, and profiled the level of 25 growth factors or inflammatory mediators at T0 and T+6h in group of IC Fontaine stage II patients treated with the treadmill training.

Methods

Exercise training

Twelve patients (8 females and 4 males) with POAD and Fontaine stage II IC were recruited for this study. Table 1 identifies characteristics of patients at D0 and D90. The research complied with the Declaration of Helsinki and was approved by the local ethics committee. Patients were provided with written informed consent for the study.

The exercise program consisted of 12 weeks of supervised, intermittent treadmill walking three days per week. During each session patients walked on the treadmill (Gait Trainer, Biodex, Shirley, NY, USA) till moderate claudication pain, then rested until claudication pain had abated and resumed walking. The intensity of claudication pain was determined on the 0 – 5 pain scale, where 1 = no pain, 2 = onset of claudication pain, 3 = mild pain, 4 = moderate pain, 5 = maximal claudication pain (12).

The walking exercises were performed at a speed of 3.2 km/hour (h) and grade that induce claudication within 3–5 minutes (min). In subsequent visits, the grade of the treadmill was increased if the patients were able to walk for 8 min or longer without reaching moderate claudication pain (13). Walking exercise time during the single session began at 30 min and progressively increased by 5 min per two weeks until a total of 55 min of exercise was accomplished.

The word ‘exercise’ is used throughout the article to refer to the single treadmill exercise, whereas the term ‘training’ applies to the three month long training program.

Walking ability

Maximal walking time (MWT), defined as the point at which patient could no longer tolerate increase in the leg pain during walking, and time to the onset of claudication (pain-free walking time, PFWT) were measured before the program and after 12 weeks. Patients performed graded treadmill test according to Gardner protocol (14), starting at 3.2 km/h at zero grade. Thereafter, with speed kept constant, the grade was increased 2% every 2 min until the maximal claudication pain.

Flow-mediated dilatation

Brachial artery flow-mediated dilatation (FMD) was measured between 7–8 a.m. after overnight fasting before the training and after three months using standard procedure (15). The brachial artery was imaged 2 cm above the ante cubital fossa using a high-resolution echo-Doppler ultrasound (Sequoia 512, Acuson, linear probe 7 MHz, Mountain View, CA, USA). The mean of three maximal end-diastolic diameter measurements was assumed to calculate FMD value.

Isolation of peripheral blood total nucleated cells and sera

Blood samples were collected in EDTA tubes at vascular outpatient clinic before (D0) and after the three month long training programme (D90). At each time point, blood samples were collected before (T0) and at 1, 3 and 6 h after the exercise (T+1h, T+3h and T+6h). Plasma was transferred to the 1.5 ml tubes and frozen following the centrifugation for 10 min at 670 g. Total nucleated cells (TNCs) were obtained from the blood samples after the ammonium chloride red blood cell lysis (0.15 M NH4Cl, 10 mM KHCO3, 0.1 mM EDTA).

Flow cytometry

Number of circulating PPCs was assessed with the flow cytometry. TNCs were immunolabelled with the fluorescently conjugated antibodies against endothelial, stem and haematopoietic markers. Circulating CD45dimCD31+CD133+ PPCs were stained with anti-CD45-FITC (BD Biosciences, San Diego, CA, USA), anti-CD31-PE (Biolegend, San Diego, CA, USA) and anti-CD133/1(AC133)-APC (Miltenyi Biotec, Auburn, CA, USA) antibodies. CD45dimCD34+CD133+KDR+ and CD45dimCD34+CD133+KDR− were assessed following the staining with anti-CD45-APC-Cy7 (Biolegend), anti-CD34-PE-Cy5 (BD Bioscience), anti-CD133/1(AC133)-PE (Miltenyi Biotec) and anti-CD309(KDR)-APC (Biolegend) antibodies. Additionally all the nucleated events were stained with Hoechst 33342 to exclude debris, platelets and non-lysed erythrocytes from the analysis. TNC suspensions in autoMACS Running Buffer (Miltenyi Biotec) supplemented with 2% foetal bovine serum (FBS, Lonza, Basel, Switzerland) were incubated with the antibodies and Hoechst 33342 for 30 min on ice, then washed and collected on LSR II flow cytometer (Becton Dickinson). Number of cells per 1 ml of peripheral blood (PB) was cal-
Table 1: Characteristics of the analysed group of peripheral arterial occlusive disease patients suffering from intermittent claudication.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Stage of training</th>
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<tbody>
<tr>
<td></td>
<td>D0</td>
</tr>
<tr>
<td>Age</td>
<td>65 ± 9</td>
</tr>
<tr>
<td>Female/Male</td>
<td>8/4</td>
</tr>
<tr>
<td>BMI</td>
<td>24.85 ± 4.35</td>
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<tr>
<td>ABI</td>
<td>0.52 ± 0.15</td>
</tr>
<tr>
<td>Active smokers</td>
<td>7/12 (11 ± 8 cigarettes /day)</td>
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<tr>
<td>Former smokers</td>
<td>4/12 (smoking for 35 ± 5 years)</td>
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</table>

**Laboratory values**

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<th>Laboratory Parameter</th>
<th>Unit</th>
<th>D0</th>
<th>D90</th>
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</thead>
<tbody>
<tr>
<td>Total cholesterol (TC)</td>
<td>mM</td>
<td>4.63 ± 1.16</td>
<td>4.39 ± 0.92</td>
</tr>
<tr>
<td>HDL</td>
<td>mM</td>
<td>1.57 ± 0.33</td>
<td>1.66 ± 0.63</td>
</tr>
<tr>
<td>LDL</td>
<td>mM</td>
<td>2.55 ± 0.96</td>
<td>2.34 ± 0.77</td>
</tr>
<tr>
<td>TG</td>
<td>mM</td>
<td>1.16 ± 0.45</td>
<td>1.27 ± 0.59</td>
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<tr>
<td>Haemocrit (%)</td>
<td></td>
<td>42.1 ± 3.9</td>
<td>41.3 ± 2.2</td>
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<tr>
<td>RBC</td>
<td>10⁶/μl</td>
<td>4.45 ± 0.34</td>
<td>4.47 ± 0.30</td>
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<tr>
<td>WBC</td>
<td>10³/μl</td>
<td>6.19 ± 1.66</td>
<td>6.01 ± 1.01</td>
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<tr>
<td>NEU</td>
<td>10⁶/μl</td>
<td>4.03 ± 1.30</td>
<td>3.75 ± 1.01</td>
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<tr>
<td>MONO</td>
<td>10³/μl</td>
<td>0.56 ± 0.18</td>
<td>0.64 ± 0.28</td>
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<tr>
<td>PLT</td>
<td>10³/μl</td>
<td>201 ± 57</td>
<td>184 ± 79</td>
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<tr>
<td>CRP</td>
<td>mg/l</td>
<td>5.04 ± 7.78</td>
<td>2.04 ± 2.57</td>
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<tr>
<td>Fibrinogen</td>
<td>g/l</td>
<td>4.07 ± 1.19</td>
<td>3.91 ± 0.95</td>
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**Treatment**

<table>
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<th>D12/D12</th>
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<tr>
<td>Statins</td>
<td></td>
</tr>
<tr>
<td>Acetylsalicylic acid</td>
<td></td>
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<td>ACE inhibitors</td>
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**Comorbidities**

<table>
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<th>Condition</th>
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<td>2/12</td>
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<tr>
<td>Asthma</td>
<td>1/12</td>
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</table>

Quantitative real-time PCR

RNA was isolated from the TNCs with the phenol-chloroform extraction. One μg of total RNA was reverse transcribed with the NCode™ VILO™ miRNA cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR was performed in the StepOnePlus system (Applied Biosystems, Foster City, CA, USA) with the specific primers, 50 ng of cDNA and SYBR Green Quantitative RT-PCR kit (Sigma-Aldrich, St. Louis, MO, USA) in the total volume of 15 μl. U6 was used as the reference for the miR-146a, miR-155 and miR-200c analysis, while β2-microglobulin was used for the HMOX1, HMOX2 and SOD1.

Multiplex immunoassays

Concentrations of plasma sE-selectin, soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1), adiponectin, total plasminogen activator inhibitor-1 (tPAI-1), matrix metalloproteinase-9 (MMP-9), myeloperoxidase (MPO) were assessed with the Milliplex MAP Cardiovascular Panel I (Millipore, Billerica, MA, USA); interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-12, IL-17, monocyte chemotactic protein-1 (MCP-1), tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), fibroblast growth factor-2 (FGF-2), Flt-3 ligand, granulocyte colony-stimulating factor (G-CSF) with the Milliplex MAP Cytokine/Chemokine Panel I, and stromal cell derived factor-1 (SDF-1)α+β, leukemia inhibitory factor (LIF), thrombopoietin (TPO), stem cell factor (SCF) with the Milliplex MAP Cytokine/Chemokine Panel II reagents (both Millipore) on the Luminex FlexMAP 3D platform, according to the vendor’s instructions.

In cell ELISA

Phosphorylation of nitric oxide synthase-3 (NOS3) Ser1177 in human aortic endothelial cells (HAoEC) in response to the IC patients’ plasma was evaluated with the in cell ELISA. Briefly, 5 x 10⁴/well of HAoECs were seeded on 96-well plate 48 h prior to the stimulation with plasma, and starved with the EBM-2 (Lonza) supplemented with 0.5% FBS 12 h before the stimulation. HAoECs were treated with 10% IC plasma in EBM-2 for 30 min, then washed with Twin Buffered Saline (TBS), fixed with 4% formaldehyde in TBS and permeabilised. Activity of internal peroxidases was quenched with 1% H₂O₂. Then, cells were blocked with 3% bovine serum albumin (BSA) + 10% goat serum in TBS and incubated overnight in 4°C with the rabbit antibody against phosphorylated NOS3 Ser1177 (Abcam, Cambridge, MA, USA). Next, cells were incubated with biotinylated goat anti-rabbit antibody (Vector Lab, Burlingame, CA, USA) and streptavidin conjugated with horseradish peroxidase (HRP) (R&D Systems, Minneapolis, MN, USA). HRP activity was measured with the colorimetric assay and normalised to the Janus Green B (Sigma-Aldrich) whole cell stain.

Statistical analysis

Results are expressed as mean ± SD unless otherwise stated. Differences in response to the exercise or trainings were assessed with the paired t-test. Statistical significance was accepted at p=0.05.
Treadmill training increases PFWT and MWT in IC patients

Treadmill training increased PWFT from 155 ± 46 s at baseline to 385 ± 164 s at day 90 (p=0.01) (Fig. 1A), and MWT from 561 ± 246 to 1045 ± 480 s (p=0.003) (Fig. 1B). Moreover, we have observed evident trend towards increase of the FMD (D0: 4.86 ± 2.27; D90: 6.05 ± 2.79; p=0.076) (Fig. 1C) after the training. Subsequently, we have checked if the improvement in the endothelial function could have resulted from the release of molecules that affect activity of nitric oxide synthetase (NOS)3. There was no change, however, in the level of NOS3 phospho-Ser1177 in HAoEC cell line in response to plasma collected from patients either after exercise or after the training (Fig. 1D).

Exercise increases number of circulating CD45\(^{dim}\)CD31\(^{-}\)CD133\(^{+}\) (A), CD45\(^{dim}\)CD34\(^{-}\)CD133\(^{+}\)KDR\(^{-}\) (B), and CD45\(^{dim}\)CD34\(^{-}\)CD133\(^{+}\)KDR\(^{-}\) (C) proangiogenic progenitor cells in response to the single exercise (T+1h, T+3h, T+6h vs. T0) and training (D90 vs. D0). D Mobilisation of CD45d\(^{im}\)CD34\(^{+}\)CD133\(^{-}\)KDR\(^{+}\) PPCs 3 h after the exercise at the beginning of training series. Values are mean ± SD.

**Results**

**Treadmill training increases PFWT and MWT in IC patients**

Treadmill training increased PWFT from 155 ± 46 seconds (s) at baseline to 385 ± 164 s at day 90 (p=0.01) (Fig. 1A), and MWT from 561 ± 246 to 1045 ± 480 s (p=0.003) (Fig. 1B). Moreover, we have observed evident trend towards increase of the FMD (D0: 4.86 ± 2.27; D90: 6.05 ± 2.79; p=0.076) (Fig. 1C) after the training. Subsequently, we have checked if the improvement in the endothelial function could have resulted from the release of molecules that affect activity of nitric oxide synthetase (NOS)3. There was no change, however, in the level of NOS3 phospho-Ser1177 in HAoEC cell line in response to plasma collected from patients either after exercise or after the training (Fig. 1D).

**Exercise increases number of circulating CD45\(^{dim}\)CD31\(^{-}\)CD133\(^{+}\) proangiogenic progenitor cells**

Various populations of bone marrow-derived stem and progenitor cells enriched for the proangiogenic potential have been shown to contribute to the vascular homeostasis. Therefore, we have evaluated number of three populations of PPCs after the exercise and treadmill training. Number of circulating CD45\(^{dim}\)CD31\(^{-}\)CD133\(^{+}\) in

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and CD45dimCD34⁺CD133⁺KDR⁺ cells did not change in response to the single exercise either on day 0 or on day 90 or in response to the training (Fig. 2A, B). Number of more differentiated population of CD45dimCD34⁺CD133⁻KDR⁺ cells increased in the whole trained group 3 h after the exercise on day 0 (T0: 20 ± 19, T+3h: 36 ± 36 cells per 1 ml of PB, p=0.048) (Fig. 2D), however, remained stable on day 90 and over the training. Interestingly, number of CD45dimCD34⁺CD133⁻KDR⁺ cells correlated positively with the ankle-brachial index (ABI) (p=0.035, Spearman R = 0.68), whereas CD45dimCD34⁺CD133⁺KDR⁺ with the MWT (p=0.017, Spearman R = 0.74) on day 90 (see Suppl. Table 1, available online at www.thrombosis-online.com).

Exercise changes the expression of antioxidant enzymes in IC patients

Walking as well as treadmill exercise in intermittent claudication result in oxygen imbalance and ischaemia-reperfusion cycles that may lead to oxidative damage of the endothelium. Therefore we have evaluated expression of antioxidant genes HMOX1, HMOX2 and SOD1 as well as miRNAs associated with regulation of endothelial function in TNCs of IC patients subjected to the training. Expression of SOD1 was enhanced 6 h after the single exercise on day 0 (T0: 0.017 ± 0.003; T+6h: 0.023 ± 0.003, p=0.0001) and on day 90 (D90: T0: 0.017 ± 0.002; T+6h: 0.022 ± 0.004, p=0.01). SOD2, SOD3, and NOS2 did not change significantly (data not shown). Noteworthy, expression of HMOX1 decreased in response to the single exercise at day 0 (T0: 0.0013 ± 0.0006; T+6h: 0.0008 ±
Treadmill training decreases plasma IL-6 but increases MCP-1

Ischaemia-reperfusion cycles in IC during walking and rest may lead to the induction of inflammatory response and so aggravate the atherosclerosis. Therefore, we have evaluated impact of single exercise and training on the plasma levels of the growth factors and mediators of inflammation. Plasma IL-6 level in IC patients was lower on D90 than on D0 (D0: 4.77 ± 5.84; D90: 3.50 ± 4.73 pg/ml, p=0.03) (Fig. 4B). Concomitantly, we have observed increase in plasma MCP-1 level (D0: 364 ± 214; D90: 622 ± 294 pg/ml, p=0.04) (Fig. 4A). Interestingly, concentration of MCP-1 decreased in response to the single exercise at D0 (T0: 364 ± 214; T+6h: 308 ± 198 pg/ml; p=0.005) (Fig. 3C) and D90 (T0: 622 ± 294; T+6h: 561 ± 287 pg/ml; p=0.03). Moreover, there was an evident trend towards increased plasma MPO level at day 0 (T0: 15.6 ± 4.0; T+6h: 22.6 ± 7.3 ng/ml; p=0.06) (Fig. 3A). Single exercise decreased total PAI-1 at D90 (T0: 118.63 ± 51.45; T+6h: 79.63 ± 24.71 ng/ml, p=0.02) (Fig. 3F) and TNF-α at D0 (T0: 10.11 ± 4.68; T+6h: 8.50 ± 3.58 pg/ml, p=0.03) (Fig. 3B). Furthermore, there was a trend towards lower level of soluble E-selectin at D90 in comparison to the D0 (D0: 26.64 ± 10.12; D90: 22.24 ± 6.86 ng/ml, p=0.06) (Suppl. Fig. 2A, available online at www.thrombosis-online.com).

Neither training nor exercise changed plasma levels of sVCAM-1, sICAM-1, adiponectin, IL-1β, IL-8, IL-10, IL-12 IL-17, VEGF, IFN-γ, SDF-1α+β, G-CSE, Flt3 ligand, FGF-2, TPO, SCF and LIF (Suppl. Fig. 2B-T, available online at www.thrombosis-online.com). Interestingly concentrations of proinflammatory IL-6 and IL-12 in plasma of patients at day 90 were positively correlated with MWT (Spearman R=0.71, p=0.027, and Spearman R=0.65, p=0.049). IL-6 correlated also with PWFT (Spearman R=0.84, p=0.004) (Suppl. Table 1, available online at www.thrombosis-online.com).

**Discussion**

IC remains one of the major problems related to the POAD. Since the effectiveness of most pharmacotherapies, except for aspirin, pentoxyfilline and dipyridamole, remains low, training is the treatment of choice to improve the quality of life of patients with IC (11). We have confirmed that treadmill training improves both PWFT and MWT in claudicant subjects. Furthermore, exercise training tended to improve endothelial function measured with the FMD what corresponds to the published data (16). Increase in FMD would probably reach the statistical significance in the larger group of patients (15). Treadmill training did not change the influence of IC patients’ plasma on the NOS3 Ser1177 phosphorylation. However, increased shear stress might have induced NOS3 activity in situ in the patients (17).

We demonstrated that number of CD45<sup>dim</sup>CD34<sup>+</sup>CD133<sup>-</sup>KDR<sup>+</sup> PPCs increased 3 h after the single exercise at the beginning of the training, but remained stable at D90. This might suggest the attenuation of ischaemic stress response induced by a single exercise after the training. PPCs, enriched for the endothelial lineage commitment can be mobilised in response to ischaemia and can incorporate to the vessels and take part in the vessel regeneration. On the other hand, increased number of circulating proangiogenic cells could have resulted from the shear stress induced differentiation of earlier progenitors (18), especially with no difference we observed in CD45<sup>CD133</sup>CD3<sup>dim</sup> bright circulating endothelial cells (CEC), which are markers of vascular damage (data not shown). Number of circulating CD3<sup>CD19</sup>CD33<sup>CD45</sup>CD133<sup>+</sup>KDR<sup>+</sup>...
tended to increase 10 min after the exercise in PAD patients who walked until the claudication-limiting symptoms occurred (19). In other study, treadmill training augmented number of circulating CD45+CD34+CD133+KDR+ cells after the six month long series of trainings. After three months, the number of the latter circulating cells tended to increase (20). Our results suggest that mobilisation of bone marrow-derived PPCs may require strong stimulus associated with the ischaemia during the intensive training. This might be the reason for the relatively weak influence of the exercise model applied in our study, which was limited to particular subpopulation of PPC.

Exhaustive and acute training increase the oxidative stress and may subsequently lead to vascular damage. In our study, single exercise increased level of SOD1 mRNA in peripheral blood TNCs both at the beginning of training and at D90. On the other hand, single exercise decreased HMOX1 expression in POPAD patients on D0, but not after training on D90. Low level and reduced induction of HO-1 results in decreased antioxidative protection of cells, including endothelial cells (21). Interestingly, treadmill exercise was shown to induce HMOX1 expression in lymphocytes in young, healthy individuals in controlled treadmill test (22). In another study, expression of HMOX1 increased after a half-marathon run in granulocytes, monocytes and lymphocytes of male trained subjects (23). We may speculate that increase in the ROS production caused by exercise could enhance the expression of antioxidant SOD1. Nevertheless, even in healthy individuals increase in lymphocyte antioxidant defence may be not sufficient to prevent oxidative damage (24). Our results suggest that efficacy of the protective mechanisms can be reduced by exercise-induced downregulation of HMOX1 in IC patients. Three-month training may improve the antioxidative response, through preventing the decrease in HMOX1. Additionally, we observed a trend toward increased expression of HMOX2 at D90, whose cytoprotective functions are similar to HMOX1.

Pro-inflammatory IL-6, one of the biomarkers associated with the PAD (25, 26), which was reported to correlate with the poorer walk performance (27), was decreased in response to treadmill training in the analysed group of IC patients. On the other hand, plasma level of MCP-1, potent monocye chemoattractant associated with the PAD (28), was increased in the same trained subjects. It is notable that inflammatory mediators, especially MCP-1, has been shown to be involved in arteriogenesis (29, 30). Interestingly, single exercise on D0 decreased plasma MCP-1. Moreover, in our group of patients there was a positive correlation between MWT or PWMT at D90 and concentration of IL-6, accompanied by a negative correlation with expression of anti-inflammatory miR-146a in TNC.

Plasma MPO is upregulated in the healthy individuals subjected to the severe exercise, e.g. marathon (31) or half-marathon (32). MPO is also a biomarker associated with higher risk of cardiovascular events in PAD patients (33). We found that plasma levels of MPO in IC subjects tended to increase in response to the single exercise at D0 but remained stable over the trainings. Lack of MPO increase after the single exercise in trained IC patients can be related to the blunted neutrophil activation and degranulation (34). Decreased level of total PAI-1 in response to the exercise after the training may suggest pro-fibrinolytic effect of the single exercise after the training. It was shown that PAD patients have disturbed fibrinolytic activity that is improved with the training (35). Finally, plasma levels of sVCAM-1, sICAM-1, adiponectin, IL-1β, IL-8, IL-10, IL-12 IL-17, VEGF, IFN-γ, SDF-1α, G-CSF, Flt3 ligand, FGF-2, TPO, SCF and LIF remained stable after the single exercise and over the series of trainings. However, levels of sVCAM-1, sICAM-1 (36), IL-1β, IL-6 (37) might have increased at peak exercise and decrease after the short rest (<1 h), while IL-8 might have decreased (38). Lack of changes in the plasma VEGF and FGF-2 is in concordance with previously published data (39).

In spite of the low number of patients analysed we have been able to detect changes in the number of circulating PPCs, antioxidant gene response and inflammatory mediators. Neither smoking nor comorbidities influenced the measured parameters. However, a larger group of the claudicant patients would be necessary to confirm the effects of exercise on the mobilisation of PPC subpopulations.

In summary, treadmill training improves walking performance of IC patients, attenuates the single exercise induced changes in gene expression profile or in the number of CD45+CD34+CD133 KDR+ PPCs, but may also result in a higher production of some inflammatory mediators.

Acknowledgements
We would like to acknowledge Dr. Ewa Zuba-Surma from the Department of Cell Biology, Jagiellonian University, Krakow, for her technical assistance with the establishment of the immunolabeling protocol for the flow cytometry. We thank Dr. Rafal Januszek, 2nd Clinics of Internal Medicine, Jagiellonian University Medical College for the brachial artery FMD assessment.

Conflicts of interest
None declared.

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