Exercise training improves in vivo endothelial repair capacity of early endothelial progenitor cells in subjects with metabolic syndrome

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Abstract: BACKGROUND: Endothelial dysfunction and injury are considered to contribute considerably to the development and progression of atherosclerosis. It has been suggested that intense exercise training can increase the number and angiogenic properties of early endothelial progenitor cells (EPCs). However, whether exercise training stimulates the capacity of early EPCs to promote repair of endothelial damage and potential underlying mechanisms remain to be determined. The present study was designed to evaluate the effects of moderate exercise training on in vivo endothelial repair capacity of early EPCs, and their nitric oxide and superoxide production as characterized by electron spin resonance spectroscopy analysis in subjects with metabolic syndrome. METHODS AND RESULTS: Twenty-four subjects with metabolic syndrome were randomized to an 8 weeks exercise training or a control group. Superoxide production and nitric oxide (NO) availability of early EPCs were characterized by using electron spin resonance (ESR) spectroscopy analysis. In vivo endothelial repair capacity of EPCs was examined by transplantation into nude mice with defined carotid endothelial injury. Endothelium-dependent, flow-mediated vasodilation was analysed using high-resolution ultrasound. Importantly, exercise training resulted in a substantially improved in vivo endothelial repair capacity of early EPCs (24.0 vs 12.7%; $p < 0.05$) and improved endothelium-dependent vasodilation. Nitric oxide production of EPCs was substantially increased after exercise training, but not in the control group. Moreover, exercise training reduced superoxide production of EPCs, which was not observed in the control group. CONCLUSIONS: The present study suggests for the first time that moderate exercise training increases nitric oxide production of early endothelial progenitor cells and reduces their superoxide production. Importantly, this is associated with a marked beneficial effect on the in vivo endothelial repair capacity of early EPCs in subjects with metabolic syndrome.

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Conflict of interest
The authors declare they have no conflicts of interest.

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Abstract

Background:
Endothelial dysfunction and injury are considered to contribute considerably to the development and progression of atherosclerosis. It has been suggested that intense exercise training can increase the number and angiogenic properties of early endothelial progenitor cells (EPCs). However, whether exercise training stimulates the capacity of early EPCs to promote repair of endothelial damage and the potential underlying mechanisms remains to be determined. The present study was designed to evaluate the effects of moderate exercise training on in vivo endothelial repair capacity of early EPCs, and their nitric oxide and superoxide production as characterized by electron spin resonance spectroscopy analysis in subjects with metabolic syndrome. Methods and results: Twenty-four subjects with metabolic syndrome were randomized to an 8 weeks exercise training or a control group. Superoxide production and nitric oxide (NO) availability of early EPCs were characterized by using electron spin resonance (ESR) spectroscopy analysis. In vivo endothelial repair capacity of EPCs was examined by transplantation into nude mice with defined carotid endothelial injury. Endothelium-dependent, flow-mediated vasodilation was analysed using high-resolution ultrasound. Importantly, exercise training resulted in a substantially improved in vivo endothelial repair capacity of early EPCs (24.0 vs 12.7%; $p<0.05$) and improved endothelium-dependent vasodilation. Nitric oxide production of EPCs was substantially increased after exercise training, but not in the control group. Moreover, exercise training reduced superoxide production of EPCs, which was not observed in the control group.

Conclusions:
The present study suggests for the first time that moderate exercise training increases nitric oxide production of early endothelial progenitor cells and reduces their superoxide production. Importantly, this is associated with a marked beneficial effect on the in vivo endothelial repair capacity of early EPCs in subjects with metabolic syndrome.

Introduction

Endothelial dysfunction and injury are thought to contribute considerably to the development and progression of atherosclerotic vascular disease.1–3 An association of a reduced number of FACS-determined endothelial progenitor cells with cardiovascular events has recently been suggested.4–6 Notably, early endothelial progenitor cells (EPCs) besides promoting neovascularisation have also been shown to promote endothelial repair mechanisms.7–10 Endothelial repair in response to early EPCs is likely to be largely mediated by paracrine effects.11,12 Obesity and a sedentary lifestyle are frequently associated with metabolic syndrome or diabetes, and these subjects have a markedly abnormal endothelial and early EPC function.7,13–15 Importantly, in subjects with metabolic syndrome and/or diabetes, nitric oxide production of early EPCs was markedly reduced and superoxide production was increased; it is likely that this played a causal role for the impaired endothelial repair capacity of early EPCs in these subjects.7,13–15 It is noteworthy that regular physical activity is inversely correlated to the risk of cardiovascular events16–20 and all-cause mortality as well as cardiovascular morbidity and mortality.16,19 Notably, exercise training has been observed to improve peripheral and coronary endothelial function. 21,22 An experimental study has suggested that exercise training increases the number of endothelial progenitor cells and their angiogenic capacity by an endothelial NO-synthase-dependent mechanism.23 Recent clinical studies have suggested that an increase of early EPC numbers after exercise training is ‘particularly observed in patients with ongoing ischaemia during exercise, i.e. with peripheral artery disease,24,25 whereas an improvement in the angiogenic capacity of early EPCs (i.e. matrigel integration capacity) was also observed in subischaemic exercise in patients with coronary disease.25 However, it remains to be elucidated if physical exercise training has an effect on the in vivo endothelial repair capacity of early endothelial progenitor cells and the potential underlying mechanisms. The aim of the present study was to examine the impact of moderate exercise training on the in vivo endothelial repair capacity of early endothelial progenitor cells in subjects with metabolic syndrome and to determine potential underlying mechanisms whereby exercise training may alter endothelial repair capacity of EPCs. In particular, nitric oxide production and superoxide production of early EPCs were determined before and after exercise training by using electron spin resonance spectroscopy analyses.

Methods

Study protocol

Subjects with metabolic syndrome (N¼24), as defined by the criteria of the AHA and National Heart, Lung, and Blood Institute Scientific Statement26 and the International Diabetes Federation Metabolic Syndrome World-wide Definition27 as well as a recent scientific statement

of the American Heart Association, 28 were randomized (1 : 1) to an exercise training group or control group, respectively. Patients with relevant peripheral vascular disease were excluded from the present study due to potential problems in maintaining the exercise protocol. The exercise-training group contained one patient with coronary heart disease and the control group contained four, but there was no symptomatic ischaemia (i.e. leg pain or chest pain) during the exercise. Patients in the exercise-training group performed regular physical exercise with a bicycle ergometer 5 times a week 30 min/day for 8 weeks. Before starting the training programme, all patients underwent a maximal ergometer test to determine the appropriate individual level of exercise intensity. The maximal ergometer test was performed at the beginning of the study and there was no significant difference in the VO2max at baseline between the two groups (21.31 ml/min/kg vs 18.6 ml/min/kg, NS). The training heart rate was defined as the heart rate at 50% (first 4 weeks) and 70% (second 4 weeks), respectively, from the maximum oxygen uptake (VO2max) during the ergometer test. During the first 4 weeks of the exercise training programme patients exercised with the heart rate observed at 50% of VO2max and the following 4 weeks at 70% of VO2max. Blood samples were obtained for isolation and characterization of early EPCs at baseline and after 8 weeks of follow-up in each group. Endothelial function was determined by high-resolution ultrasound at baseline and after 8 weeks to determine the effect of exercise on vascular function as described below. Characteristics of subjects with metabolic syndrome are shown in Table 1. Written informed consent was obtained from all participants and the study protocol was approved by the local ethics committee. The present study was registered in a public registry of clinical studies (ClinicalTrials.gov Identifier: NCT 00515476).

Isolation and cultivation of early EPCs

Early EPCs were isolated and cultured as described previously.7,11,15,29–32 Briefly, peripheral blood mononuclear cells were isolated by density gradient centrifugation with LSM 1077 Lymphocyte (PAA Laboratories Coelbe, Germany) and 107 cells were cultured on fibronectin- coated 6-well plates in endothelial cell basal medium-2 (containing 5 mmol/l glucose) supplemented with endothelial growth medium–SingleQuots exactly as indicated by the manufacturer except for hydrocortisone (Clonetics, Inc). After 4-day culture, nonadherent cells were removed by washing plates with PBS. Remaining cells were trypsinized and used for in vivo functional analysis. The FACS characterization of endothelial marker expression of these cells has been described in detail previously.7

Measurements of early EPC superoxide production

Superoxide production of early EPCs was determined by using electron spin resonance (ESR) spectroscopy and the spin trap CM-H as described elsewhere. 7,11,33,34 Early EPCs

were resuspended in 250µl PBS (37℃) and kept at 37 ℃ until use. ESR measurements were performed in 50µl glass capillaries (Brand, Germany). ESR spectra were recorded using a MiniScope ESR spectrometer (Magnettech; Berlin, Germany). Early EPC superoxide production was determined by following the oxidation of specific superoxide spin-trap, 1-hydroxy-3-methoxycarbonyl- 2,2,5,5-tetramethyl pyrrolidine HCl (CM-H). ESR instrumental settings were as follows: field sweep 108 G, microwave frequency 9.78 GHz, microwave power 20mW, modulation amplitude 2G, 4096 points resolution and receiver gain 1x105 (74 dB). From each subject two early EPC samples were analyzed and the mean value was used for further analysis.

Measurements of early EPC nitric oxide production

Nitric oxide (NO) production of early EPCs was examined by electron spin resonance (ESR) spectroscopy analysis using the spin-trap colloid Fe(DETC)2 as described elsewhere.7,32,35 Early EPCs were resuspended in 250µl of Krebs-Hepes buffer (37℃) and 250µl of colloid Fe(DETC)2 (final concentration 285µM) was added to each sample and incubated at 37℃ for 60 min. ESR spectra were recorded using a MiniScope ESR spectrometer (Magnettech). ESR instrumental settings were as follows: center-field (B0) 3280G, sweep 198G, microwave power 4 dB, amplitude modulation 8G, 4096 points resolution, sweep time 120 s and number of scans 4. Signals were quantified by measuring the total amplitude after correction of baseline and subtracting background signals. Incubations with colloid Fe(DETC)2 alone were used to correct for background signals. The mean value of two different samples of each subject was used for further analysis. In previous studies we have performed experiments that indicated that the ESR signal detected in early EPCs is NO-specific (data not shown).

Animals

The local animal research committee approved all animal protocols. NRMInu/nu athymic nude mice were used as described below.

In vivo re-endothelialization assay

In vivo endothelial repair capacity of early EPCs from subjects with metabolic syndrome was determined as described previously.7,11,15,32,36,37 In brief, male NRMInu/nu athymic nude mice, aged 8–12 weeks, were injected with human EPCs. Animals were anaesthetized with ketamine (100 mg/kg IP) and xylazine (5 mg/kg IP). Carotid artery electric injury was performed at the left common carotid artery with a bipolar microregulator (ICC50, ERBE-Elektromedizin GmbH, Tuebingen, Germany). An electric current of 2W was applied for 2 s to each mm of carotid artery over a total length of exactly 4mm with the use of a size marker parallel to the carotid artery. Early EPCs (5x105 cells) were resuspended in 100µl of pre-

warmed PBS (37℃) and transplanted 3 hours after ca rotid injury via tail vein injection with a 27-gauge needle. The same volume of PBS was injected into placebo mice. Three days after carotid injury, endothelial regeneration was evaluated by staining denuded areas with 50µl of solution containing 5% Evans blue dye via tail vein injection as described previously.38 The re-endothelialized area was calculated as the difference between the injured area and the blue-stained area by computer-assisted morphometric analysis. This model has been shown to allow accurate quantification of re-endothelialization.37

Measurement of Flow-Mediated, Endothelium-Dependent Vasodilation

Endothelium-dependent vasodilation (FDD) was examined in all participants as described elsewhere, and this measurement is very well established in our laboratory. 15,31,34,39,40 In brief, radial artery diameters were measured using high-resolution ultrasound (ASULAB). Then, an 8-min wrist arterial occlusion was performed and FDD was assessed in response to reactive hyperaemic blood flow. All measurements were recorded and two investigators unaware of the interventions subsequently analyzed vessel diameters. This method is well established in our laboratory and has an excellent reproducibility and variability.15,31,34,39,40

Statistical analysis

All data are expressed as mean±SE. Comparisons of measurements were done by Mann-Whitney U test. Data were analyzed by using GraphPad Prism 4.03TM. A value of $p<0.05$ was considered statistically significant.

Results

Early EPCs from subjects with metabolic syndrome were transplanted into nude mice with carotid injury and the re-endothelialized area was measured after 3 days by morphometric analysis (Figure 1A and B) before and after exercise training or from the control group. Importantly, the in vivo endothelial repair capacity of early EPCs was markedly increased after 8 weeks of regular exercise training, whereas no change was observed in the control group (Figure 1A). The number of cultivated early EPCs in the exercisetraining group was moderately increased after regular physical training (15.4 x106±2.5 EPCs vs 19.7 x106±2.7 EPCs; $p<0.05$), whereas no significant changes were observed in the control group (17.8 x106±4.3EPCs vs 13.3 x106±2.0 EPCs; NS).

Effect of exercise training on superoxide production

Along with other researchers, we have recently observed that an increased superoxide production of early EPCs derived from the NAD(P)H oxidase represents a potentially important mechanism limiting the in vivo endothelial repair capacity of early EPCs in patients with dia-

betes and metabolic syndrome.7,13–15 We have therefore determined the impact of regular physical exercise training on superoxide production of early EPCs as measured by electron spin resonance (ESR) spectroscopy analysis. Notably, after 8 weeks regular exercise training we observed a profound reduction of early EPC superoxide production in the exercise-training group, whereas no significant change was observed in the control group (Figure 2A and B).

Effect of exercise training on nitric oxide (NO) production

Several studies have indicated that endothelial NO synthase-derived NO production of early EPCs represents a critical determinant for their in vitro and in vivo function.7,23,41 In the present study the effect of exercise training on NO production of EPCs from subjects with metabolic syndrome was determined by using electron spin resonance (ESR) spectroscopy analysis and the spin-trap colloid Fe(DETC)2 at baseline and after 8 weeks of exercise training and in the control group. After 8 weeks of regular moderate physical exercise training the NO production was significantly increased (Figure 3) in the exercise-training group but not in the control group.

Effect of exercise on flow-dependent, endothelium-mediated vasodilation

Flow-dependent, endothelium-mediated dilation (FDD) of the radial artery in response to reactive hyperaemic blood flow after 8-min wrist arterial occlusion was measured at baseline and after 8 weeks with and without vitamin C (3 g i.v.) in subjects with metabolic syndrome. Endothelium-dependent vasodilation was substantially improved n the exercise-training group after 8 weeks of regular physical exercise, but not in the control group (Figure 4A and B). To determine, whether this may have been related to a reduction of vascular oxidant stress, the acute effect of a high dose of vitamin C (3 g i.v.) was evaluated before and after exercise training. Notably, the acute administration of the antioxidant vitamin C improved endotheliumdependent vasodilation before exercise training, an effect that was lost after 8 weeks of regular physical exercise. These observations suggest indirectly that exercise training resulted in a marked reduction of vascular oxidant stress in subjects with metabolic syndrome (Figure 4A). No significant changes were observed in the control group.

Discussion

The present study demonstrates for the first time that moderate exercise training leads to a markedly improved in vivo endothelial repair capacity of early EPCs in subjects with metabolic syndrome. Furthermore, this was associated with a substantially improved endothelium-dependent vasodilation in subjects with metabolic syndrome, which was not observed in the control group. Furthermore, the present study demonstrates that regular physical exercise

training markedly reduces superoxide production of early EPCs in subjects with metabolic syndrome, as indicated by electron spin resonance spectroscopy analyses. Moreover, we have observed an increase in NO production of early EPCs after exercise training that was not observed in the control group. The present findings provide novel evidence suggesting that regular moderate exercise training exerts a marked beneficial effect on in vivo endothelial repair capacity of early EPCs, likely, at least in part, due to a reduced oxidant stress and increased NO availability of early EPCs. Endothelial dysfunction and injury are thought to represent important mechanisms leading to development and progression of atherosclerosis and its detrimental clinical complications.1,2 Furthermore, circulating early EPCs have been shown to have the potential to promote endothelial repair mechanisms in addition to their effects on neovascularization.9,10,42 We and others have recently observed that this is proably mediated by paracrine effects, since early EPCs are largely located in the subendothelial space of the areas with endothelial injury.11,12 This concept is also supported by a recent experimental study suggesting that circulating EPCs very rarely contribute to plaque endothelium in apoE deficient mice43 Several studies have indicated that in patients with cardiovascular risk factors, particularly in subjects with metabolic syndrome and/or type-2 diabetes,13 the in vitro angiogenic activity of early EPCs is substantially impaired.7,13,14 In two recent studies, we observed a markedly impaired in vivo endothelial repair capacity of early EPCs in patients with diabetes and metabolic syndrome.7,13–15 With respect to the effect of exercise training, a previous study has reported that the number of CD34þ/KDRþcells is acutely increased after 30 minutes of acute exercise training in healthy subjects,44 whereas another study has suggested that in patients with established peripheral or coronary disease, exercise training increased the number of CD34þ/KDRþcells in particular in patients with ongoing ischaemia.24 Moreover, the angiogenic function of early EPCs was increased after exercise training in patients with coronary disease without ischaemia.25 Importantly, the present study demonstrates for the first time that regular physical exercise training restores the impaired in vivo endothelial repair capacity of early EPCs as revealed by transplantation of these cells into nude mice with endothelial injury. This was associated with a marked improvement of endothelium-dependent vasodilation in these subjects. These findings raised the question of what the potential underlying mechanisms are causing moderate exercise training to restore the in vivo endothelial repair capacity of early EPCs. Recently, we and other researchers have observed that increased oxidant stress of early EPCs results in a markedly impaired endothelial repair capacity of early EPCs in diabetic patients.7,14,45 In a previous study,7 we demonstrated that increased superoxide production derived from the NAD(P)H oxidase markedly limits endothelial repair capacity of early EPCs, probably in part due to a subsequent reduction of the NO bioavailability of early EPCs from these patients. Notably, the present study demonstrates that regular physical exercise training is efficacious

in reducing superoxide production and increasing NO availability of early EPCs from subjects with metabolic syndrome. Whereas previous observations have suggested that exercise training may reduce vascular superoxide production, 46 the present findings are to the best of our knowledge the first observations demonstrating that regular moderate exercise training can reduce superoxide and increase NO production of early EPCs. In a recent experimental study, it was observed that the endothelial repair after injury was accelerated in apoE-deficient mice, probably partly mediated by bone-marrow derived cells.47 In the present study as in previous studies,7,32 endothelial repair capacity of EPCs derived from subjects with metabolic syndrome was substantially impaired and improved by exercise training. One may speculate that the increased endothelial repair in apoE deficient mice represents a compensatory early response to endothelial injury, which may be exhausted in patients with long-standing metabolic syndrome. In summary, the present study demonstrates that regular moderate exercise training markedly improves in vivo endothelial repair capacity of early EPCs in subjects with metabolic syndrome. Our data further suggest, as indicated by electron spin resonance spectroscopy analyses, that a reduced oxidant stress and an improved NO availability of early EPCs represent important underlying mechanisms leading to a restored endothelial repair capacity of early EPCs after regular exercise training in metabolic syndrome.

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Figures

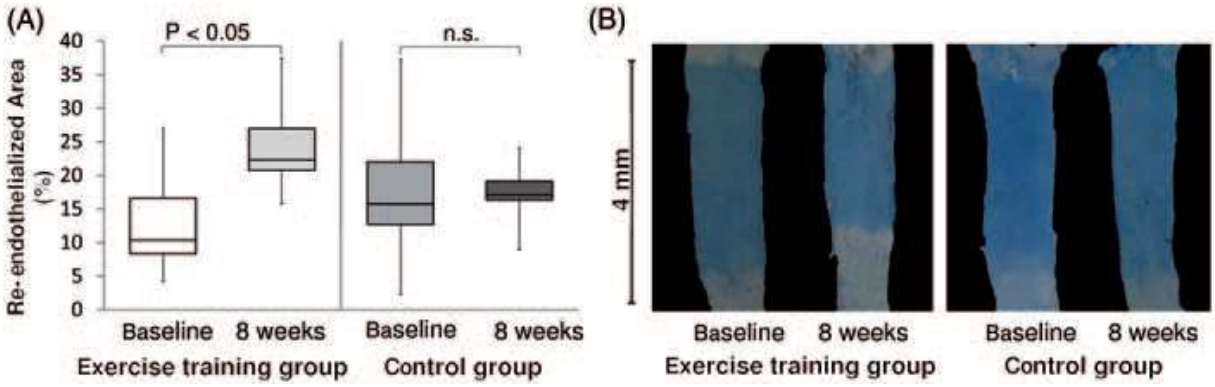

Figure 1. In vivo re-endothelialization capacity of early EPCs from subjects with metabolic syndrome (n¼24) randomised to 8 weeks of regular moderate exercise training or the control group. (**A**) The re-endothelialized area was determined at day 3 after carotid artery injury in nude mice after transplantation of early EPCs from subjects with metabolic syndrome before and after exercise. (**B**) Representative photographs of mice carotids after injury and transplantation of early EPCs from subjects with metabolic syndrome before and after exercise training or from the control group are shown. Denuded-endothelium area is stained in blue; re-endothelialized area (REA) in white.

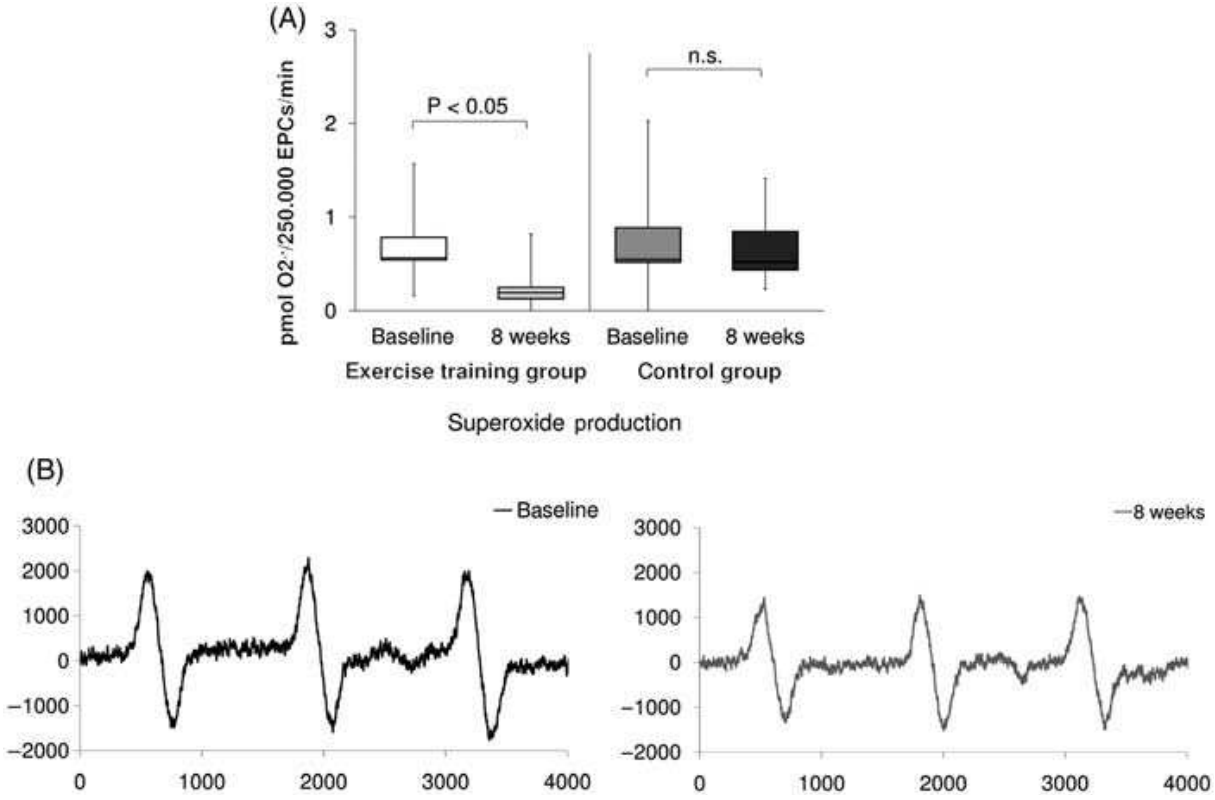

Figure 2. (**A**) Effect of exercise training on superoxide production of early EPCs from subjects with metabolic syndrome. (**B**) Electron spin resonance (ESR) spectroscopy analyses of superoxide production of early EPCs from subjects with metabolic syndrome at baseline and after 8 weeks in the exercise training group or the control group are shown. Representative ESR spectra of superoxide production of early EPCs from a subject with metabolic syndrome before and after exercise are shown.

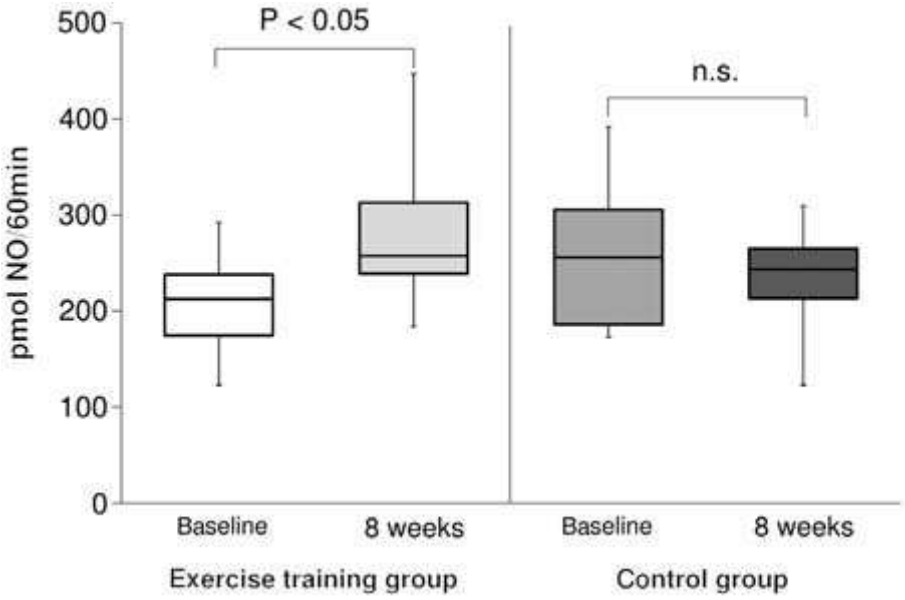

Figure 3. NO availability of early EPCs as determined by ESR spectroscopy analysis from subjects with metabolic syndrome at baseline and after 8 weeks in the exercise-training group and from the control group.

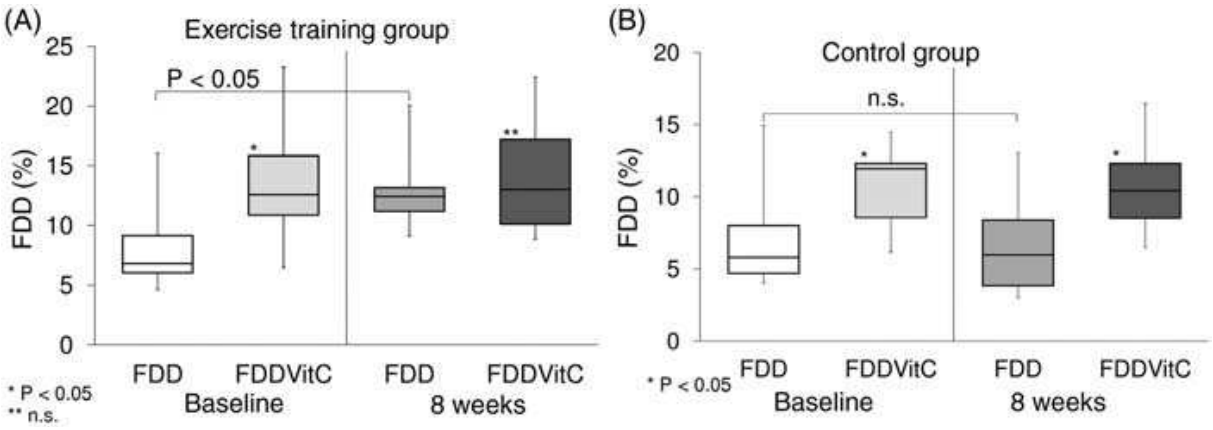

Figure 4. Flow-dependent, endothelium-mediated dilation (FDD) of the radial artery in response to reactive hyperaemic blood flow at baseline and after 8 weeks in the exercise-training group (**A**) or in the control group (**B**) before and after acute administration of the antioxidant vitamin C (3 g i.v.).

	Exercise-training Group ($n = 12$)		Control group ($n = 12$)		
Parameter	Baseline	8 weeks	Baseline	8 weeks	p
Age (years)	58 ± 3		64 ± 3		
Gender (m/f)	10/2		12/0		
BMI (kg/m^2)	33 ± 1	33 ± 1	31 ± 1	31 ± 1	NS
Waist circumference (cm)	115 ± 3	114 ± 3	108 ± 2	108 ± 2	NS
MAP (mmHg)	99 ± 4	98 ± 3	97 ± 4	95 ± 3	NS
HbA1c (%)	6.3 ± 0.2	6.03 ± 0.1[a]	6.8 ± 0.3	6.6 ± 0.3	<0.05 vs baseline
Fasting glucose (mg/dl)	113 ± 14	112 ± 8	149 ± 12	132 ± 10	NS
Cholesterol (mg/dl)	191 ± 7	187 ± 6	158 ± 11	162 ± 13	NS
LDL cholesterol (mg/dl)	129 ± 6	128 ± 5	111 ± 10	111 ± 11	NS
HDL cholesterol (mg/dl)	37 ± 1	39 ± 3	33 ± 2	35 ± 2	NS
Triglycerides (mg/dl)	236 ± 32	217 ± 35	189 ± 33	202 ± 44	NS
Medication:					
ASS	5/12	5/12	7/12	7/12	
ACE/AT1	7/12	7/12	11/12	11/12	
Diuretic	4/12	4/12	4/12	4/12	
Calcium antagonist	5/12	5/12	1/12	2/12	
Nitrate	0	0	1/12	1/12	
Beta-blocker	4/12	4/12	5/12	5/12	
Statin	3/12	3/12	7/12	7/12	
Oral antidiabetic drug	1/12	1/12	6/12	6/12	
Insulin	1/12	1/12	4/12	4/12	

BMI, body mass index; MAP, mean arterial pressure.
[a]$p < 0.05$ vs baseline.
Values expressed as mean ± SEM.

Table 1: Characteristics of subjects with metabolic syndrome