Endothelial progenitor cells are reduced in refractory hypertension

Circulating endothelial progenitor cells (EPCs) play a key role in the maintenance of endothelial homeostasis and promote vascular repair. They may also be of predictive value for cardiovascular events. Reduced EPC number and functional activity have been associated with several cardiovascular risk factors, but their relationship with hypertension remains unclear. The objective of this study was to investigate if number and function of circulating EPCs are reduced in patients with refractory hypertension (RHT). Circulating EPCs (CD34⁺CD133⁺/CD45⁻) were isolated from peripheral blood by flow cytometry in 39 RHT and 30 normotensive controls. EPC number was also determined in vitro after 7 days in culture. After age adjustment, EPC concentration was significantly reduced in RHT as compared with controls (mean (95% CI), 33.8 (18.1–49.6) vs 69.1 (50.7–87.5) EPCs per 10⁵ peripheral mononuclear cells (MNCs), respectively; \( P = 0.014 \)). After in vitro culture, EPCs were also reduced in patients as compared with controls (mean (95% CI), 142.3 (49.5–235.0) vs 611.0 (480.2–741.8) EPCs per field, respectively, \( P < 0.001 \)). In multiple linear regression analysis, circulating EPCs were significantly reduced by 56.3% in RHT as compared with control (\( P = 0.006 \)), independently of all other known risk factors. Moreover, RHT had a high independent predictive value for lower EPC proliferation. The number of EPCs per field was reduced by 76.7% in RHT with respect to controls (\( P < 0.001 \)). In summary, the number of circulating EPCs after culture is reduced in patients with RHT, which may be related to the increased rate of endothelial dysfunction, atherosclerotic disease and cardiovascular events observed in this population.

Keywords: endothelial progenitor cells; refractory hypertension; cardiovascular risk factors; cardiovascular diseases; endothelium

Introduction

Hypertension is one of the best-known cardiovascular risk factors for target organ damage and cardiovascular events. This relationship is even stronger for refractory hypertension (RHT).¹,² There is no doubt that the effect of hypertension on the vascular wall is one of the most important determinants of this enhanced risk. The dysfunctional endothelium, that is, the imbalance of homeostasis and function and the resulting structural changes, may be responsible for the adverse outcomes due to hypertension.³,⁴ Circulating endothelial progenitor cells (EPCs) represent a bone-marrow-derived cell population of distinct phenotypes, which share the ability to differentiate into mature and functionally competent endothelial cells. These progenitor cells co-express endothelial and immature cell surface molecules and are able to form colonies in vitro.⁵ The discovery of EPCs in 1997 suggested that postnatal neovascularization may be due to the synergistic action of angiogenesis, that is, the formation of new vessels by the migration and differentiation of the existing mature endothelial cells, and vasculogenesis, in which EPCs home, differentiate, proliferate and incorporate into resident endothelial cells to form new vessels.⁶,⁷ Several studies have confirmed that EPCs are derived from bone marrow, circulate in peripheral blood and may contribute to new vessel formation by homing to sites such as ischaemic tissues and tumour microenvironments in vitro.⁵,⁶–¹⁰ So, EPCs would appear to play a key role in the maintenance of endothelial homeostasis.

Recent studies have shown that EPC number and function can predict the occurrence of cardiovascular events¹¹,¹² and, most importantly, their
imbalance may lead to the development of atherosclerosis. Moreover, the number and functional capacity of circulating EPCs are lowered in the setting of some known traditional and newer cardiovascular risk factors. With regard to hypertension, it has been shown that patients with coronary artery disease have reduced levels and migratory capacity of EPCs, the latter mainly influenced by hypertension. However, at present, there is no evidence of a clear independent relationship between hypertension and the number of circulating EPCs. In fact, very recently Delva et al. reported no alteration in the number or functional activity of EPCs in 36 patients with essential hypertension. The purpose of our study was to investigate if the circulating EPC levels are altered in patients with RHT who are at high risk for cardiovascular disease. Moreover, we studied the influence of other cardiovascular risk factors on EPCs in RHT patients. We also assessed the increase in the number of these isolated EPCs after in vitro culture as a marker of functional impairment.

Materials and methods

The local Institutional Ethical Committee approved the study protocol. Written informed consent was obtained from all participants. Thirty-nine consecutive subjects with RHT were studied. According to current guidelines, RHT was considered as the failure to reach goal blood pressure in patients who are adhering to full doses of an appropriate three-drug regimen that includes a diuretic, besides attention to lifestyle measures and after excluding secondary hypertension. After 5 min of rest in the sitting position, blood pressure was measured and considered as the average of three measurements spaced by 1–2 min with validated oscillometric semiautomatic device and using the appropriate cuff sizes. Hypertension was defined as the office blood pressure values equal or higher than 140/90 mmHg. Ambulatory blood pressure monitoring (24 h) was performed in all RHT patients with validated Spacelabs-90207 device. Hypertension was confirmed if hypertensive individuals had mean daytime values of more than 135/85 mmHg. Left ventricular ejection fraction and left ventricular mass index were assessed by echocardiography according to standard guidelines. The healthy control group consisted of 30 subjects with normotension confirmed by office blood pressure measured as mentioned above.

Hyperlipidaemia was defined as serum cholesterol level greater than 220 mg dl⁻¹ (5.7 mmol⁻¹) and/or serum triglyceride level above 150 mg dl⁻¹ (1.5 g l⁻¹) or if treatment with lipid-lowering drugs had been implemented. Smokers were considered as those with an active smoking habit over the last year. Subjects with recent cardiovascular events, concomitant malignant diseases or active inflammation were excluded. Diabetic patients were also excluded in an attempt to avoid one of the well-known determinants of EPCs. Medications were maintained in this observational study.

In all participants, the total number of EPCs was assessed by using an in vitro assay, as described in detail previously. In brief, mononuclear cells (MNCs) were obtained from peripheral blood sample (20 ml) by Ficoll density-gradient centrifugation (Histopaque 1077; Sigma, St Louis, MO, USA). EPCs were identified by flow cytometry and their phenotype was determined by immunohistochemistry after staining with fluorescein isothiocyanate-conjugated CD45 (Becton Dickinson PharMingen, San Diego, CA, USA), phycoerythrin-Cy5-conjugated CD34 (Becton Dickinson PharMingen) and with phycoerythrin-conjugated CD133 antibodies (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Progenitor cells were identified by CD45 expression and separated by flow cytometry with right-angle light scatter properties. Cells expressing CD34 and CD133 were identified and quantified out of the progenitor population. To assess background, cells were stained with isotype-identical nonspecific antibodies as negative controls. We analysed a minimum of 2 × 10⁵ CD45⁺ cells. Circulating EPCs were enumerated as CD45⁺/CD34⁺/CD133⁺ via fluorescence-activated cell-sorting analysis and expressed in absolute number of cells per 1 × 10⁵ peripheral MNCs. After identification and quantification of circulating EPCs, we evaluated the functional activity of these EPCs by assessing their proliferation after in vitro culture. In this way, 0.5 × 10⁶ MNCs were seeded onto six-well plates coated with human fibronectin (10 μg ml⁻¹; Sigma) in endothelial basal medium-2 (Clonetics) supplemented with microvascular endothelial cell-medium-2 (EGM-2) MV single aliquots consisting of 5% fetal bovine serum, vascular endothelial growth factor, fibroblast growth factor-2, epidermal growth factor, insulin-like growth factor-1 and ascorbic acid in appropriate amounts. After 4 days in culture, nonadherent cells were removed and fresh media were added; cells were then cultured through day 7 with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-labelled acetylated low-density lipoprotein (acLDL-Dil) (Molecular Probes) 2.5 μg ml⁻¹ at 37 °C for 4 h. Cells were then fixed with 1% paraformaldehyde for 15 min and incubated with fluorescein isothiocyanate-labelled Ulex europaeus agglutinin-1 (10 μg ml⁻¹) (Sigma) for 2 h. The quantification of the number of EPCs after 7 days in culture was done using a computerized method. Briefly, 10 images of randomly selected high-power field were captured on a Leica inverted phase-contrast microscope. Double-stained cells for both Ulex europaeus agglutinin-1 and acLDL-Dil, which were considered as EPCs, were counted using digital software (Meta- morph Imaging System, Universal Imaging Corp., West Chester, PA, USA). The number of cultured EPCs was expressed as cell number per power field.
Statistical analysis was performed with statistical package SAS 9.0 (SAS Institute Inc., Cary, NC, USA). Variables following normal distribution are presented as mean ± s.d.; the non-normally distributed data are presented as median (quartile 1; 3). Categorical data are summarized as percentages. Univariate comparisons of continuous variables within groups were performed by unpaired Student’s t-test or the non-parametric Mann–Whitney U-test according to normality distribution assumptions. Categorical variables were compared by means of the χ² test and the Fisher’s exact test. Univariate correlations were made with Spearman correlation coefficient. Those variables that in the univariate analyses showed a marginally statistical significant association (that is \( P < 0.15 \)) with hypertension or with any of the dependent variables, that is, circulating EPC number or EPCs after culture, were selected to be included in linear multiple regression models. None of the variables in the final model showed collinearity among them. Relationship other than linear was tested in a Smoothed General Additive model, although none of the smoothing was significantly better than linear. Statistical significance was assumed if a null hypothesis could be rejected at \( P = 0.05 \).

### Results

**Characteristics of study patients and control subjects**

The clinical characteristics and laboratory parameters of patients with RHT (\( n = 39 \)) and a cohort of healthy subjects (\( n = 30 \)) are shown in Table 1. Patients with RHT were older and had significantly higher body mass index, hyperlipidaemia, LDL cholesterol and glycosylated haemoglobin levels, urinary albumin/creatinine ratio, fibrinogen and C-reactive protein serum levels as compared with normotensive subjects. In total, 65% of RHT patients had hyperlipidaemia and 16% were current smokers. Seven RHT patients had developed a cardiovascular event prior to the study (stroke in four patients, myocardial infarction in two other patients and one patient had experienced an episode of acute heart failure). Hypertensive patients also had worse renal function than the control group as measured by estimated glomerular filtration rate by the Modification of Diet in Renal Disease (eGFR-MDRD) study equation.

Forty-four percent of the patients received statins as part of their lipid-lowering treatment and 92% out of all RHT patients were treated with one or two renin–angiotensin system blockers. None of the controls were treated with either drug.

### Table 1 Baseline demographic data, clinical characteristics and laboratory tests of patients with refractory hypertension and control subjects

<table>
<thead>
<tr>
<th></th>
<th>Refractory hypertensive patients</th>
<th>Normotensive controls</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td><strong>Number</strong></td>
<td>39</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>57.7 ± 11.7</td>
<td>37.0 ± 9.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Gender, male (%)</strong></td>
<td>15 (38.5)</td>
<td>11 (36.7)</td>
<td>0.879</td>
</tr>
<tr>
<td><strong>Body mass index (kg m⁻²)</strong></td>
<td>31.2 ± 4.8</td>
<td>23.5 ± 3.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Office SBP (mm Hg)</strong></td>
<td>161.2 ± 19.4</td>
<td>111.8 ± 11.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Office DBP (mm Hg)</strong></td>
<td>93.4 ± 13.0</td>
<td>69.3 ± 9.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Medical history</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Hyperlipidaemia (%)</strong></td>
<td>25 (64.1)</td>
<td>10 (33.3)</td>
<td>0.011</td>
</tr>
<tr>
<td><strong>Current smoking (%)</strong></td>
<td>6 (15.4)</td>
<td>4 (13.3)</td>
<td>0.812</td>
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<tr>
<td><strong>Lipid profile</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total cholesterol</strong></td>
<td>208.7 ± 31.9</td>
<td>195.6 ± 32.4</td>
<td>0.098</td>
</tr>
<tr>
<td><strong>HDL cholesterol</strong></td>
<td>56.2 ± 16.2</td>
<td>61.2 ± 19.6</td>
<td>0.248</td>
</tr>
<tr>
<td><strong>LDL cholesterol</strong></td>
<td>134.0 ± 23.9</td>
<td>119.9 ± 30.0</td>
<td>0.034</td>
</tr>
<tr>
<td><strong>Triglycerides</strong></td>
<td>130.2 ± 111.8</td>
<td>93.0 ± 55.2</td>
<td>0.075</td>
</tr>
<tr>
<td><strong>Renal function</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SCreat (mg dl⁻¹)</strong></td>
<td>1.05 [0.94; 1.21]</td>
<td>1.08 [0.93; 1.16]</td>
<td>0.818</td>
</tr>
<tr>
<td><strong>eGFR-MDRD (ml min⁻¹ 1.73 m⁻²)</strong></td>
<td>64.7 ± 14.4</td>
<td>72.3 ± 9.5</td>
<td>0.014</td>
</tr>
<tr>
<td><strong>UACR (µg mg⁻¹)</strong></td>
<td>7.3 [4.6; 37.8]</td>
<td>2.5 [1.9; 4.0]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Other laboratory parameters</strong></td>
<td></td>
<td></td>
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<tr>
<td><strong>HbA1c (%)</strong></td>
<td>4.7 [4.5; 5.1]</td>
<td>4.3 [4.2; 4.4]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Homocysteine (µmol l⁻¹)</strong></td>
<td>9.5 ± 3.5</td>
<td>9.5 ± 2.9</td>
<td>0.995</td>
</tr>
<tr>
<td><strong>C-reactive protein</strong></td>
<td>0.50 [0.30; 0.80]</td>
<td>0.20 [0.20; 0.23]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Fibrinogen</strong></td>
<td>332.7 ± 71.7</td>
<td>276.8 ± 65.7</td>
<td>0.004</td>
</tr>
<tr>
<td><strong>EPO levels</strong></td>
<td>19.2 [14.6; 26.3]</td>
<td>17.0 [13.4; 22.2]</td>
<td>0.292</td>
</tr>
</tbody>
</table>

Abbreviations: DBP, diastolic blood pressure; eGFR-MDRD, estimated glomerular filtration rate by the Modification of Diet in Renal Disease study equation; EPO, erythropoietin; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SBP, systolic blood pressure; SCreat, serum creatinine; UACR, urinary albumin/creatinine ratio.

“Median [quartile 1; 3]. Remaining parameters: mean ± SD.”
Circulating number of EPCs

In the univariate analysis, after age adjustment, the concentration of circulating EPCs was significantly reduced in RHT patients in comparison with controls (mean (95% CI): 33.8 (18.1–49.6) vs 69.1 (50.7–87.5) EPCs per 10^5 MNC respectively; \( P = 0.014 \)) (Figure 1). We also examined whether the number of circulating EPCs correlated with any of the above-mentioned variables as well as with echocardiographic parameters (left ventricular ejection fraction and left ventricular mass index), family history of cardiovascular disease, physical inactivity, cardiovascular complications, obstructive sleep apnoea syndrome, statin therapy and renin–angiotensin system blockers treatment (data not shown). The only parameter which significantly correlated with EPC concentration was blood glucose level (\( r = -0.412; \ P = 0.01 \)); higher glycaemia correlated with a reduced number of circulating EPCs. None of the remaining factors were associated with the EPC concentration in a univariate analysis in RHT patients.

All the above-mentioned variables associated with RHT, that is, age, body mass index, hyperlipidaemia, serum levels of glucose, LDL cholesterol, glycosylated haemoglobin, C-reactive protein and fibrinogen, as well as urinary albumin/creatinine ratio and eGFR-MDRD were included in a multiple linear regression analysis. We found that RHT was associated with a significant reduction in circulating EPC number (Table 2). The absolute number of circulating EPCs was significantly reduced by 56.3% in hypertensive patients as compared with control subjects (\( P = 0.006 \)). In this multivariate analysis, none of the other analysed parameters except reduced eGFR-MDRD remained significantly associated with a lower circulating number of EPCs (Table 2).

EPCs after in vitro culture

In the univariate analysis, after age adjustment, the \textit{in vitro} studies showed that the number of EPCs after culture was significantly lower in hypertensive patients as compared with normotensive subjects (mean (95% CI): 142.3 (49.5–235.0) vs 611.0 (480.2–741.8) EPCs per field, respectively; \( P < 0.001 \)) (Figure 2). In these patients with RHT, of all the analysed parameters, the only parameter that significantly and positively correlated with number of EPCs after culture was left ventricular mass index (\( r = 0.417; \ P = 0.01 \)). In a multiple linear regression analysis model including all these variables, RHT had a high independent predictive value for lower EPC number after culture and it was the only analysed parameter that remained significantly associated with this result. The number of EPCs per field was reduced by 76.7% in RHT patients with respect to control subjects (\( P < 0.001 \)) (final model shown in Table 3).

We observed mean differences of 28.2 in EPC concentration (EPCs per 10^5 MNC) and 408.8 in EPCs per field after culture. We have undertaken a power calculation on our sample size and we have confirmed that it has a power of 80% to detect differences even smaller, 27 and 173, respectively.
Taking into account age differences between hypertensive patients and healthy subjects, we have done further statistical analyses with a sample of 13 age-matched pairs (RHT: 45 years, s.d. ± 7.6; controls: 45 years, s.d. ± 7.8). Results were very similar to those described above. The regression analysis showed that unstandardized coefficient (B) (95% CI) and P-value for circulating EPCs (EPCs per 10³ MNC) were −53.8 (−94.0 to −13.6), P = 0.011. Likewise, unstandardized coefficient (B) (95% CI) and P-value for EPCs after culture (EPCs per field) were −464.5 (−721.8 to −207.2), P = 0.001.

### Discussion

The results of the present study clearly point to RHT as an independent determinant of lower number of circulating and after culture EPCs. Our data show that the concentration of circulating EPCs is significantly reduced in RHT as compared to healthy subjects. Moreover, the increase in EPCs after in vitro culture was also lower in RHT patients, suggesting a functional impairment. In a multiple regression analysis, RHT was an independent predictor of peripheral blood EPC concentration and in vitro proliferation, indicating that RHT is a determinant of EPC downregulation.

To our best knowledge, this is the first time that such a clear relationship between EPCs and hypertension is documented in the scientific literature. Only Vasa et al. found that hypertension negatively correlated with the migration capacity of EPCs in patients with coronary artery disease. Other authors also found fewer circulating EPCs to be related to hypertension, but this relationship was not in any case independent of age or other factors. For instance, Hill et al. found a strong correlation between the number of circulating EPCs and the patient’s combined Framingham risk factor score, as well as between a reduced number of EPC colony-forming units and hypertension, but the latter correlation disappeared after age adjustment. Similarly, Werner et al. described a relationship between a lower number of circulating EPCs and higher systolic blood pressure, but this relationship disappeared in the multivariate analysis. The same not independent link between circulating EPC number and hypertension has been stated by other authors.

There is no doubt that the maintenance of the integrity and functional activity of the endothelial monolayer is pivotal to prevent atherogenesis. EPCs contribute to angiogenesis and vascular remodelling and may be found in the peripheral circulation, as has been documented by recent studies in animal models and humans. These EPCs play a key role in the maintenance of endothelial homeostasis. Several studies suggest that most of the major known cardiovascular (CV) risk factors negatively influence the number and function of EPCs. This is the case for ageing, type I and II diabetes mellitus, hyperlipidaemia, smoking, as well as physical inactivity, family history of premature coronary artery disease or renal insufficiency. This relationship with impaired renal function has also been found in our study. It could be speculated that this is the reason for differences in circulating EPC number between both groups. However, as shown in multivariate analysis, both factors independently predict decreased EPC number. Regarding the connection between EPCs and CV events, Schmidt-Lucke et al. recently demonstrated that patients with CV events had fewer circulating EPCs and that a low number of EPCs independently predicted a higher occurrence of CV events. Similar results were obtained by Werner et al. who found in patients with coronary artery disease (EPCAD study) a significant inverse relationship between EPC level and CV death, first major CV event, revascularization and hospitalization. Given these findings, one may speculate that the continuous detrimental effects of CV risk factors on circulating EPC number and function would result in an impairment of the endothelial monolayer and its regenerative capacity, leading to atherosclerotic diseases and its associated CV consequences. On the other hand, we found in the univariate analysis that left ventricular mass index positively correlated with EPCs after culture. We can hypothesize that this could be a response to the hypoxia-induced small vessel damage due to the hypertensive ventricular hypertrophy, that is, an attempt to induce neovascularization and improve angiogenesis at this level. Anyway, this is not investigated in our study and at last this relationship disappeared in the multivariate analysis. As for the factors influencing EPCs, consistent studies have reported that drugs such as HMG-CoA reductase inhibitors (statins) or renin–angiotensin system blockers increase the number and function of EPCs, strongly suggesting that at least part of their pleiotropic, vasculoprotective action is mediated via EPCs.

Our study clearly demonstrates that EPCs are downregulated in refractory hypertensive patients. The value of our results is even higher, given the fact that most of these patients were treated with a renin–angiotensin system blocker and/or a statin.
which are shown to increase EPCs. Thus, RHT becomes definitively added to the list of CV risk factors that are known to negatively influence EPCs. Delva et al.22 did not find a reduction in EPCs in patients with essential hypertension and mean systolic and diastolic blood pressure levels of 148 and 91 mm Hg, respectively, with most of them being on monotherapy or without antihypertensive treatment. Therefore, as compared to our own results, it seems that the severity of hypertension plays a key role in the downregulation of EPCs. Several underlying mechanisms could account for hypertension-induced downregulation of EPCs, such as an enhanced consumption of EPCs due to an increase in endothelial damage, an impairment in the mobilization process from bone marrow into peripheral blood or an increase in EPC apoptosis and/or impaired proliferation after culture, but we did not explore these mechanisms. In fact, it has been suggested that the number of EPCs after culture is reduced in patients with RHT. This decrease is independent of other risk factors and known determinants of EPCs. This reinforces a likely role of EPCs in atherosclerosis development. The knowledge of the specific pathways and modulators of EPCs related to hypertension could be helpful for the prevention of imbalanced endothelial homoeostasis and the consequent adverse CV outcomes.

In conclusion, this study demonstrates that EPC concentration in peripheral blood and after in vitro culture is reduced in patients with RHT. This decrease is independent of other risk factors and known determinants of EPCs. This reinforces a likely role of EPCs in atherosclerosis development. The knowledge of the specific pathways and modulators of EPCs related to hypertension could be helpful for the prevention of imbalanced endothelial homoeostasis and the consequent adverse CV outcomes.

What is known about this topic
- Endothelial progenitor cells play a key role in the maintenance of endothelial homoeostasis. They may also be of predictive value for cardiovascular events.11–43
- Number and functional activity of endothelial progenitor cells are reduced in the setting of several cardiovascular risk factors.
- Hypertension is a well-known cardiovascular risk factor and one of the initial steps in the development of atherosclerosis.1–5 No clear relationship has been found between hypertension and a lower number and deficient activity of endothelial progenitor cells until now.21

What this study adds
- This study shows for the first time an independent relationship between hypertension and a reduced number and functional activity of endothelial progenitor cells.
- We have demonstrated this relationship in patients with refractory hypertension. The severity of hypertension seems to play a role in the reduction of circulating number and functional activity of these cells.

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Conflict of interest

None.

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36 Laufs U, Werner N, Link A, Endres M, Wassmann S, Jürgens K et al. Physical training increases endothelial progenitor cells, inhibits neointima formation, and


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