Author's response to reviews

Title: Higher numbers of circulating endothelial progenitor cells in stroke patients with intracranial arterial stenosis

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Author's response to reviews: see over
Response to Reviewers’ comments

We thank the Editor and the Journal for their consideration to publish our work. We responded to all issues raised by the Reviewers, and provided a point-by-point response below.

Best regards,

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Reviewer 1

1- Patients included in the study must be better characterized. The authors provide only data on risk factors of patients. Conversely, more information on the index event and the therapeutic options must be provided. It is worth noting that most cardiovascular drugs affect the amount of cEPCs (i.e. statins).

Response: We added more patients’ characteristics in the new Table 1. We also included CRP levels in the multivariate analyses and replaced Table 2.

2- Apart from cEPCs, the authors have assessed only plasma fibrinogen levels. Can the authors provide information on inflammatory indexes?

Response: In the new Table 1, we now provide CRP levels, and these levels were different between the three groups.

3- In the results section, the authors compare cEPCs in patients grouped according to age (>60 years or <60 years). The authors should compare also patients stratified according to other parameter (i.e., gender – male vs females-, diabetes – present or not present-, etc.)

Response: There was no difference between male and female patients (0.063±0.039 vs. 0.055±0.032%, P=0.198). Diabetic patients had significantly less EPCs than non-diabetics (0.043±0.019 vs. 0.065±0.042%, P=0.024) (Figure 1C). This was added to the manuscript. (Paragraph 1, page 11)

4- The authors present only data on CD34+/CD133+/KDR+. In order to allow
comparison with previous works, the authors should also provide data on the levels of other subtypes of EPCs, such as CD34+/KDR+ and CD133+/KDR+

**Response:** In the present study, we assessed the proportions of CD34+, CD34+/CD133+ and CD34+/CD133+/KDR+ subpopulations in the HC, CP and IAS groups. The proportion of the CD34+/CD133+ subpopulation was significantly higher in IAS patients (0.246±0.142%) than in HC (0.098±0.032%) and CP (0.113±0.064%) (all P<0.001), but there was no difference between the HC and CP groups (P=0.382). A similar tendency was observed in the CD34+/CD133+/KDR+ subpopulation between the three groups. Proportion of CD34+ cells was significantly higher in the IAS group, followed by the CP group and the HC group (all P<0.05). Taken together, our results suggest that CD34+ cells could not help to differentiate the presence or absence of stenosis, while the CD34+/CD133+ and CD34+/CD133+/KDR+ subpopulations could. These were added in the Results (Section of Endothelial Progenitor Cells, page 10) and in Table 1.

In the present study, we assessed the relation of CD34+, CD34+/CD133+ and CD34+/CD133+/KDR+ with the presence of stenosis. In the study by Rafat et al. (Stroke 2009), only the CD34+/CD133+ and the CD34+/CD133+/KDR+ subpopulations were assessed, which was similar with our study. We did not assess other subpopulations since we did not believe that it could add further benefits.

5- The authors should add a section dedicated to the Limitations of the study. They should recognize that the methods used to assess EPCs still lack standardization and this might therefore have affected the results. Specifically, they can not rule out the possibility that the increase in CD34+ and CD133+ cells was caused by tissue ischemia, which can ‘per se’ contribute to raise VEGF levels and mobilize cells into peripheral blood. Furthermore, they should admit that pharmacologic agents (in particular statins) might have affected numbers of EPCs.

**Response:** We added these limitations in a separate paragraph. (Last paragraph, page 15)

**Reviewer 2**

1. At 'Introduction’ section, the authors could mention more recent similar articles regarding the association between EPC number and atherosclerosis disease.

**Response:** We added some references in the Introduction about the association between EPC numbers and atherosclerosis. (Second paragraph, page 5)

2. The 'Materials and Methods’ section would benefit of more data about patients such as: age, body weight, sex, plasmatic parameters.
Response: We provided a new Table 1 with more details about the study sample.

3. At ‘Results’ section, the authors should bring more explanations or data regarding EPC analysis by flow cytometry: it is necessary to distinguish between EPC (+CD34), EPC (+CD133) and EPC (+ KDR) and to give some different percent after flow cytometry analysis. The authors should explain how measured the EPC levels positive for the three specific markers (CD34/CD133/KDR). Anyway, the percent for EPC (+CD34/+CD133/ +KDR) is too low.

Response: In the present study, we assessed the proportions of CD34+, CD34+/CD133+ and CD34+/CD133+/KDR+ subpopulations in the HC, CP and IAS groups. The proportion of the CD34+/133+ subpopulation was significantly higher in IAS patients (0.246±0.142%) than in HC (0.098±0.032%) and CP (0.113±0.064%) (all P<0.001), but there was no difference between the HC and CP groups (P=0.382). A similar tendency was observed in the CD34+/CD133+/KDR+ subpopulation between the three groups. Proportion of CD34+ cells was significantly higher in the IAS group, followed by the CP group and the HC group (all P<0.05). Taken together, our results suggest that CD34+ cells could not help to differentiate the presence or absence of stenosis, while the CD34+/CD133+ and CD34+/CD133+/KDR+ subpopulations could. These were added in the Results. (Section of Endothelial Progenitor Cells, page 10)

EPCs were assayed as follows:

Peripheral blood (2 mL) was collected in tubes containing K₂-EDTA on the 7th day after acute onset. All blood samples were processed within 1 hour of collection. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Hypaque (Amersham, GE Healthcare, Waukesha, WI, USA), washed with PBS, and counted for recovery and viability using Trypan Blue. Since EPCs are characterized by the co-expression of CD34, CD133 and KDR (Urbich et al., Circ Res 2004; Peichev et al. Blood 2000), we determined the content of EPCs among PBMCs by flow cytometry using a triple staining with fluorescein-conjugated monoclonal antibodies against these markers. Briefly, PBMCs (1×10⁶) were incubated with CD34-PerCP-Cy5.5 (Beckton Dickinson, Franklin Lake, NJ, USA), CD133-PE (eBioscience, San Diego, CA, USA), and KDR-Alexa Flour647 (Beckton Dickinson, USA) for 25 min at 4°C in a dark room. Non-specific binding was determined by staining an aliquot of cells with fluorescein-conjugated IgG1 and IgG2a isotype controls (Becton Dickinson, Franklin Lake, NJ, USA). Following one wash with PBS, cells were fixed with 1% paraformaldehyde and analyzed using a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lake, NJ, USA). We gated CD34+ peripheral blood cells in the mononuclear cell fraction, followed by examining the resulting subpopulation for expression of KDR, CD133 or both. The frequency of peripheral blood cells positive for the aforementioned markers was
determined by a 2-dimensional side-scatter fluorescence dot-plot analysis, after appropriate gating. A minimum of 50,000 events was acquired for each sample. Data were analyzed using the Cellquest software provided by the manufacturer. EPC numbers were expressed as the proportion of total PBMCs.

We added more details in the Methods. (Second paragraph, page 8)

4. Along of the whole text, the authors should pay attention at ‘EPCs’: it is correct to write EPC levels not EPCs levels. When EPCs appear alone it is correct to write EPCs not EPC.

Response: This was corrected.