Author's response to reviews

Title: Smoking decreases the level of circulating CD34+ progenitor cells in young healthy women - a pilot study

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Author's response to reviews: see over
Dear Editors,

Herewith we submit a revised version of our manuscript entitled "Smoking decreases level of circulating CD 34+ progenitor cells in young healthy women-a pilot study" (MS: 2842876873147733). We were pleased by the evaluations and highly appreciate the constructive comments and suggestions made by the reviewers. As detailed below, in the revised manuscript we have addressed all points raised by the reviewers.

We hope that our article is now acceptable for publication in your journal, and we keenly look forward to your response.

Yours sincerely,

Verena Stangl
1. Reviewer 1 asked for a reference for the sentence: elevated estrogen plasma concentrations in women correlate with higher levels of circulating EPCs:


2. Reviewer 1 asked for an explanation why we have decided to exclude men from the study.

The reviewer raises a very interesting point. Nevertheless, the intention of our study was not the investigation of a possible sex difference regarding the effects of smoking on circulating progenitor cells. Our intention was to investigate the influence of smoking on circulating progenitor cells in young healthy women. For a better understanding of the influence of different levels of sexual steroids we investigated in a subgroup three time points of the menstrual cycle. The investigation of possible sex differences regarding smoking status and circulating progenitor cells could be an interesting question to answer in future studies.

3. Reviewer1: The authors have assessed only two subgroups of EPCs, that are CD34+ cells and CD34+/CD133+ cells. This is at variance with most recent works which have taken into account many other subtypes of cells, such as CD34+/KDR+/CD45- and CD133+/KDR+/CD45- cells. The authors should explain why they have not assessed these commonly evaluated subtypes.

Reviewer 2: In their introduction, the authors describe that to define EPC, VEGFR2 is used. Why then, was this not replicated in their study?

The identification, characterization, and exact role of EPCs in vascular biology are subject of much discussion – as recently excellently reviewed (Timmermans et al. 2009).

We shortly addressed this issue in the introduction section of the manuscript:

“In particular, a subset of circulating stem cells, designated endothelial progenitor cells (EPCs), is considered to contribute to endothelial cell regeneration and neovascularisation [1]”
“Since a clear and generally accepted definition of EPCs has until now not been established, most studies investigating the nature and function of EPCs have focused on flow-cytometric analysis of circulating cells that are positive for the haematopoietic stem cell markers CD34, CD133 and for the vascular endothelial growth factor receptor2 (VEGFR2), and/or have concentrated on analysis of in vitro formation of colony-forming units (EPC-CFU) [1,6]. There is some evidence that levels of circulating progenitor cells positive for CD34 (CD34+ cells) are more strongly correlated with cardiovascular risk factors than are progenitor cell populations with various combinations of CD34, CD133, and VEGFR2 [6]. “

For our study we decided to measure two different but possibly overlapping progenitor cell populations by different methods:

1) We measured putative endothelial progenitor cells as EPC-CFU as originally described by Asahara et al. 1997 [1]. We are aware that the combination of CD34, CD133, and VEGFR2 is often used to measure a very rare subgroup of bone marrow derived circulating hematopoietic progenitor cells which are often designated as endothelial progenitor cells. However, recent studies critically revised this point of view (reviewed in Timmermans et al. 2009). That is why we decided for the “classical Asahara assay” to estimate the number of putative EPCs.

2) We measured bone marrow derived circulating progenitor cells (PC) with the hematopoietic stem cell marker CD34 (which were previously shown to be strongly correlated with cardiovascular risk factors) and a subpopulation of these progenitor cells with additional expression of CD133. These cells are not defined to be EPCs. However, if true EPCs are really existing (until now EPCs are still putative!) they are probably a subpopulation of bone marrow derived circulating progenitor cells. There is a lack for a unique EPC marker. Also the question, if EPCs are negative for CD45 or not is still under debate. That is why we decided to not exclude CD45+ and CD45- cells for the estimation of bone marrow derived circulating progenitor cells. Actually, the PCs measured in our study were predominantly CD45dim, but not exclusively.

4. Following the suggestion of reviewer 1, we changed the title of the paragraph:

Effect of smoking on the number circulating progenitor cells in young healthy women (Page 8, line 182)

5. Reviewer 1: Conclusion is completely unrelated to the aim of study. The authors are not allowed to derive any conclusion based on their results on the significance of CD34+ cells evaluation in risk factor characterization of women.

Reviewer 3: The conclusion that the number of CD34+ progenitor cells may be a tool for risk stratification in young smoking women seems very speculative and should be rewritten.

We certainly agree that the small sample size of our study allows not the statement that CD34+ level is a marker for cardiovascular risk in young healthy women. Accordingly we reworded the conclusion:

“The number of CD34+ progenitor cells positively correlates with FMD in young healthy women and is decreased by smoking.” (Page 12, lines 281-282)

6. Reviewer 1: The authors report that smoking women were older. This is a major limitation of the study, as age has been previously told to influence itself the circulating levels of EPCs. The authors should discuss this point reporting references of recent researches that have shown no major impact of age on EPCs.

Reviewer 2: In relation to remark 3, the authors describe on the last page of their results, last line, that, after correction for age, no influence of “this parameter” was seen. What exactly do they mean?

All women were very young (25-35 years) and the difference between the mean age of both groups was only ~30 months. To our knowledge no study has investigated the impact of such minimal differences in age on progenitor cell levels in human. However, we cannot rule out, that minimal higher age in smoking women influences our study results. That is why we performed a multivariable regression analysis for CD34+ and CD34+/CD133+ and EPC-
CFU. Independent variables were adjusted age and smoking status. Smoking status was the only variable showing a significant difference in the model for CD34+ progenitor cells. (described in the extension in the method section of the manuscript page 7, lines 164-168)

7. Reviewer 2: How comparable were these groups in reality? Tot chol, LDL and triglycerides, as well as age were higher, HDL was lower in smoking women. While only age was significantly lower, total cardiovascular risk is a sum of different components.
I would like to see the Framingham risk score for both groups:

Both study groups were highly comparable regarding the cardiovascular risk. As written in the method section of the manuscript: “Subjects with chronic diseases or known cardiovascular risk factors other than smoking were excluded.” That means, for each individual woman every single relevant parameter was not afflicted with a higher cardiovascular risk (gender, age, total cholesterol, LDL, BMI, triglycerides, blood pressure etc. as described in table1). The only risk factor potentially counting for a higher Framingham risk score was smoking. The only significant difference between smoking and nonsmoking women was age. However, all women were very young (25-35 years) and the difference between both groups was only ~30 months.
The Framingham risk score would be in fact a useful parameter to document the low cardiovascular risk in both groups. However, the Framingham risk score, which was developed in the United states and is only evaluated for the US population, is not validated for individuals under 30 years [Wilson et al. 1998].


8. Reviewer 2: For table 3, we need repeated measures ANOVA, time x group effect with post-hoc comparison.

The statistical model used for evaluation of the variables CD34+, CD34+/CD133 and EPC-CFU over time was a linear mixed model with fixed and random effects. Fixed effects were the time with three time points of measurements during menstrual cycle and smoking status. The random effect was women as individual subjects. In the analysis for CD34+ only the smoking status showed a significant influence on the depending variables. (described in the extension in the method section of the manuscript page 7, lines 164-168)
9. Reviewer 3: It should be VEGF receptor 2 (VEGFR2) instead of VEGFR
PC should be mentioned

Following the reviewers suggestion, we changed VEGFR into VEGFR2 in the text.
(page 3, line 60 and 64; page 10, line 243)

“circulating bone marrow-derived progenitor cells (PCs)” (Page 3, line 51)