Author's response to reviews

Title: CD133+ CELLS ARE ASSOCIATED WITH ADIPOCYTOKINES AND ENDOTHELIAL DYSFUNCTION IN HEMODIALYSIS PATIENTS

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Prof. Faical Jarraya
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Dear Faical Jarraya,

We are pleased to learn that you might consider accepting our manuscript for publication provided with appropriate changes requested by the reviewers accordingly. We made changes in our manuscript in the light of your and the reviewer’s suggestions. We hope you will find the changes satisfactory.

We are looking forward to hearing from you soon,
Sincerely yours,

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Responses to Reviewer 1 (Wei Chen, MD, MS):

This is an interesting cross-sectional study of hemodialysis patients. The study examines the association of CD133+ cells with adipocytokines and cardiovascular parameters (using LMVI, FMD and CIMT). The main finding is that CD133+ cells were associated with inflammation and endothelial dysfunction in HD patients. The strength of the study is the findings of the novel relationships with CD133+ cells, as stated in the article. It is a hypothesis-generating study, and it suggests that CD133+ cells might play a role in endothelial dysfunction, which is early stage of atherosclerosis rather than in the advanced atherosclerosis.

We thank the reviewer for these nice comments on our manuscript.

1. For Table 1, please include participant characteristics by the CD133+ status (high vs. low), and the description of the participant characteristics should be discussed.

Table 1 is reconstructed to include the groups with high CD133+ and low CD133+ cell counts.

2. For Table 2, the methods for the linear regression model need to be stated in the article (under statistical analyses). Why were those covariates chosen? Were the assumptions of the model met? The units for the covariates need to be stated because without the units, the results cannot be interpreted. This is especially true for the covariate "gender".

Authors would like to thank to the reviewer for these constructive comments. Covariates that significantly correlated with the dependent variable (LogFMD) were included in the multiple linear regression analysis such as dialysis duration, LVMI and logCD133+ count. (R=0.57, adjusted R square=0.27, p=0.001). All the assumptions of the multiple linear regression were confirmed to be met including normal distribution of the dependent and independent variables, presence of linear relationship between the outcome variable and the independent variables, homoscedasticity, lack of multicollinearity. Age and gender has previously been customly included in the analysis as they are the most basic parameters even though they were not significantly correlated with FMD in the univariate analysis. Thus, we did not include these two variables in the new version of the regression model. As requested, the units for the covariates were stated. We added the following paragraph into the statistical analysis section:
“Independent variables that significantly correlated with the dependent variable in univariad analysis were included in the multiple linear regression analysis. Dialysis vintage, LVMI and LogCD133 counts were the independent variables entered into the multiple regression performed to predict LogFMD (model R=0.57, adjusted R square=0.27, p=0.001).”

3. Log-transformation of CD133+ should be stated in the statistical analysis section. In table 2, since the logCD133+ was used as a variable, the back transformation or geometric means of CD133+ should be discussed in the results.

The following statement was added to the statistical analysis section as requested:

“Since CD133+ cell count and FMD were not normally distributed, logarithmic transformation (Log10) was applied to these variables.”

Back-transformed unstandardized beta coefficient of LogCD133 was added to the results section as requested as follows:

“Back-transformed unstandardized beta coefficient of LogCD133 in multiple linear regression model was found to be -23.80 (CI: 95%; lower bound: -411.20 and upper bound: -2.26).”

4. The limitations of the study should be further discussed. Whether the study has sufficient power to detect a difference between CD133+ and CIMT should be discussed.

We agree with the reviewer that relatively small sample size might be responsible for the lack of association CD133+ cell count with CIMT. We added this subject into the limitation section. We expanded the limitations of the study as follows:

“Cross-sectional nature of the study, relatively small sample size, lack of control group are the limitations of the study. Possible significant relationships between CD133+ cell counts and atherosclerosis (namely CIMT) might not be found due to relatively small sample size.”

Responses to Reviewer 2 (A Jaroszynski)

This is a well written manuscript which reports on the relationships between CD133+ cell counts, some adipocytokines, parameters of endothelial dysfunction as well as atherosclerosis in a small group of HD patients. The findings are interesting with novel data presented regarding the link between CD133+ cells and endothelial dysfunction. Moreover, the findings may have potential clinical applications.

We would like to thank the reviewer for the favorable comments on our manuscript.

However, there are some issues that need to be addressed by the Authors:
Introduction: The introduction is generally sound. However, though the hypothesis is outlined the rationale for selecting prominin1 as well as analyzing the relation/relations between CD133+ cell counts and endothelial dysfunction is/are not convincingly detailed in the introduction.

In view of the comments of the reviewer, we reconstructed the background section detailing the reasons why we select CD133+ cells as a research topic in this manuscript. The new background section is as follows:

"Increased CV risk in CKD patients is not explained completely with the conventional CV risk factors (1,2) and new pathophysiological mechanisms such as impaired stem cell turnover might be responsible for dramatically increased CV mortality in this patient population. In general, circulating stem cells are known to have pro-angiogenic and repair properties in various tissues. CD133+ identifies a subset of undifferentiated stem cells which have been investigated for pathogenesis and prevention of CV diseases in several studies (3-5). In the study by Stamm et al (4), purified CD133+ progenitor cells were injected in the infarct border zone during the coronary artery bypass grafting operation in patients with chronic ischemic heart disease and ejection fraction was found to be higher in this cell therapy group. In another study, CD133+ cell administration was found to reduce cortical infarct volumes in mice following cerebral ischemia (5). However, in several other studies, CD133+ cells were found to have possible detrimental effects such as increased inflammation, atherosclerotic plaque instability and progression of atherosclerosis (6-10). In a study by Pizarro et al (10), CD45+/CD34+/CD133+ cells were found to be inversely associated with flow mediated dilatation (FMD) in patients with chronic obstructive pulmonary disease (COPD). All these studies were performed on non-CKD population. In the literature, there is only one study investigating the possible effect of CD133+ cells on a CV parameter in CKD patients. In this study performed on HD patients, CD133+ cell count were not found to be associated with left ventricular mass index (LVMI) (11).

Adipocytokines may also contribute to increased CV risk in CKD patients. Adipocytokines such as leptin, adiponectin, resistin, interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) are mediators produced by adipocytes which have important roles in the pathophysiology of insulin resistance, inflammation, hypertension, endothelial dysfunction and atherosclerosis (12-18). In this study, we aimed to investigate the relationships between CD133+ cell counts, adipocytokines and parameters of endothelial dysfunction and atherosclerosis in HD patients."

Methods: There are several limitations of the methods as I will describe further.

- A major flaw is that there is no control group.

Lack of control group was added as a limitation of the study as follows:

“Cross-sectional nature of the study, relatively small sample size, lack of control group are the limitations of the study. Possible significant relationships between CD133+ cell counts and atherosclerosis (namely CIMT) might not be found due to relatively small sample size.”
Diabetics were merely 10.3% of all HD patients. Are etiologies of chronic kidney disease presented in the paper similar to those of Turkish HD patients?

We agree with reviewer that the rate of diabetic patients in our dialysis clinic is lower than that of general Turkish HD patients which has been reported to be 41%.

- Were there any exclusion/inclusion criteria?

We included the chronic HD patients who had been on HD for more than 6 months between the ages 18-85. Exclusion criteria were as follows, having a recent acute CV event within 6 months of period, advanced heart failure, decline of the consent and unsuitable upper extremities for FMD measurements. These criteria were also included in the methods section of the manuscript.

"We included the chronic HD patients who had been on HD for more than 6 months between the ages 18-85. Exclusion criteria were as follows, having a recent acute CV event within 6 months of period, advanced heart failure, decline of the consent and unsuitable upper extremities for FMD measurements."

- What was the definition of a carotid plaque definition in your study?

We would like to thank to the reviewer for this helpful comments. We defined the carotid plaque as a thickness >1.5 mm as measured from the media–adventitia interface to the intima–lumen interface. We also added this statement to the methods section of the manuscript as follows:

"Carotid plaque was defined as a thickness more than 1.5 mm as measured from the media–adventitia interface to the intima–lumen interface (*)..


- The multivariate analysis has to be described in detail in the methods section.

As written for the reviewer 1 above, we added the detailed description of multivariate analysis in the statistical analysis section of the manuscript as follows:

“Independent variables that significantly correlated with the dependent variable in univaried analysis were included in the multiple linear regression analysis. Dialysis vintage, LVMI and LogCD133 counts were the independent variables entered into the multiple regression performed to predict LogFMD (model R=0.57, adjusted R square=0.27, p=0.001).”
Results: Reporting of the results follows a logical order. The Authors should, however, explain why were clinical factors which were not significant in univariate analysis or were not evaluated in univariate analysis included into the multivariable model (for instance age and gender)?

As pointed out above in the response to reviewer 1, we did not include these two variables in the new version of the regression model.

Similarly, why factors that were correlated with CD133+ (leptin, resistin and TNF-alpha) were not included into the final model/models? It would be advisable to check the results using other multivariable models.

Multivariable analysis (Table-2) was performed for the examining factors that were associated with FMD. Since leptin, resistin and TNF-alpha were not significantly associated with FMD in univariate analysis, they were not included in the linear regression.

Discussion: Discussion is sound and follows a logical sequence.

We would like to thank the reviewer for the positive comments on our manuscript.

Responses to Reviewer 3 (Xiong-Zhong Ruan)

The authors examined LVMI, CIMT and FMD of the brachial artery in 58 maintenance hemodialysis patients, counted CD133+ cells and measured adipocytokines (leptin, adiponectin, resistin, IL-6, TNF-α) levels in their blood samples. They found that CD133+ cell counts were associated with endothelial dysfunction in HD patients. CD133+ cell counts were positively related to serum leptin, resistin and TNF-α levels. We know that HD patients have increased risk of CVD and circulating progenitor cell are associated with CVD. But the role of circulating progenitor cells in HD patients' CVD is still not clear. This article proves that circulating progenitor cell (CD133+ cell) is important for CVD (endothelial dysfunction) in HD patients. In general, this study is innovative, practical and well designed.

We thank to the reviewer for these favorable comments.

Comments:

1) The author found that CD133+ cell counts were associated with endothelial dysfunction in HD patients with a correlation analysis. This article is mainly focused on the relationship between CD133+ cell, adipocytokines and CV paramaters. What is the cause or consequence among CD133+ cells, endothelial dysfunction and adipocytokines? This should be clarified or discussed clearly.

In the view of reviewer's suggestions, we reconstructed and improved the introduction section (please find the new version of “Background” section in the responses to the Reviewer-2). CD133+ cells have been rigorously investigated mainly in pathogenesis and prevention of CV
disease in non-uremic population. These studies were concisely described in this section. In this study performed in HD patients, we hypothesized that changes in circulating CD133+ cells might be associated with increased CV risk as measured by FMD, CIMT and LVMI. We also suggested that disordered adipocytokine levels in uremia might also affect the CD133+ cells. Possible relationships between CD133+ cell counts and adipocytokine metabolism has not been previously investigated in the literature. The cross-sectional nature of the study does not allow us to clarify more about the cause or consequence relationships among CD133+ cells, endothelial dysfunction and adipocytokines.

2) The figure 4 shows that serum leptin levels is higher in high CD133+ group than in lower CD133+ group. How to define the 'high' or 'lower' levels of CD133+ cells in this study? The authors should provide the correlation analysis between leptin and CD133+ cells using similar approach as the figure 2.

We divided the patients into two groups according to median CD133+ cell count and thus defined the high and low CD133+ levels accordingly. No significant relationship was found between CD133+ cell count (Log) and serum leptin levels (r=0.21, p=0.12). This was also added to the manuscript as requested.

"No significant relationship was found between LogCD133+ cell count and serum leptin levels (r=0.21, p=0.12)."

3) The central hypothesis of this study needs to be clear. The authors show the correlation between serum resistin/leptin and TNF-α levels in figure 5,6. However, all these factors are adipocytokines/cytokine and the relationships between these adipocytokines should not be the focus of this study. The author should provide analysis/correlation between these adipocytokines and endothelial dysfunction which should be the main target of this study and should be emphasized.

All the requested analyses had already been performed under the subheading of "Adipocytokines and CV parameters" in the results section. Accordingly, no adipocytokine except IL-6 was significantly associated with CV parameters. We agree with the reviewer that the main target of our study is not the relationships between different adipocytokines however we believe that significant correlations among adipocytokines might reflect the robustness of the data. Thus we preferred to keep these findings in the results section.

4) The references in this article are too old. There are only four articles cited after 2010.

We made a new search in the literature and updated references. In the light of the new references, we expanded the background and discussion sections as follows:

“In a recent study performed on HD patients by Lineen et al (*), the relationships between CD133+ cells and LVMI were investigated. CD133+ cell count were not found to be associated with LVMI in parallel to our findings. Hypervolemia and anemia are known to be the strong factors in the pathogenesis of LVH in HD patients, thus the role of stem cells in the remodelling of myocardium may be outweighed by these factors.”