Author's response to reviews

Title: Cystatin C as a potential predictor of osteoprotegerin levels in healthy men. A cross-sectional, observational study.

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Cystatin C as a potential predictor of osteoprotegerin levels in healthy men. A cross-sectional, observational study.
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Grazyna GS Sypniewska’s comments and our responses:

- The manuscript should be rewritten and presented in the easy readable form as it contains some quite interesting findings which are lost in the text.

Thank you very much. We have rewritten the manuscript as suggested and as such have tried our best to improve readability. We have made all efforts to highlight our findings in the manuscript as much as possible.

- Results should be presented in more clear form, instead this section is filled with numbers. Results presented in the tables 1 and 2 require only a short comment in the text. Page 7 line 187- should be p=0.049 as in the fig.4 not 0.49.

Results presented in tables 1 and 2 have been abbreviated in the text. The error as mentioned has been corrected to “0.049”.

- Results of multivariate regression analysis are not presented in the table, instead the text is full of numbers. This has to be changed. Among plenty of given numbers the most essential findings become less visible.

We have reduced mention of numerical data in the text as suggested. New tables have been included.

- Discussion is really difficult to read, plenty of names, discussion very often repeats the results. It should be written more clearly. In the discussion the most important finding on cystatin C should be emphasized at the beginning, instead it is lost somewhere between numbers and names.

We have made efforts to clarify the text and make it as straight forward as possible, hopefully meeting the standards of the Journal.
• Introduction
The Introduction does not contain appropriate information for its purpose. There are extensive data already published on OPG and RANKL levels, and the authors don’t provide the rationale for why it is important to examine the selected confounders. I believe the authors use the expression ‘confounders’ and ‘predictors’ interchangeably, but their meaning is very different. Moreover, why is the association between cystatin C and OPG and RANKL expected and relevant? And why it is relevant to examine these associations in healthy men? What are the study hypotheses? These are some of the issues that must be improved.

Thank you very much for emphasizing this huge lapse in the manuscript. Indeed the rationale for the study was not enumerated extensively in the introduction section. Indeed “confounder” and “predictor” are not the same. In the revised version we have taken care to use the expression “predictor” rather than “confounder”.

Kindly note that we have made the following additions to the introduction:
“Furthermore, the association of cystatin C with OPG and RANKL has only been limitedly investigated in the healthy, where no significant correlation was found [14]. Nonetheless, in-vitro studies have implied that cystatin C is a cysteine proteinase inhibitor that decreases osteoclastogenesis by interfering at a late stage of pre-osteoclast differentiation [15,16]. Additionally, cystatin C possesses the advantage of being independent of gender, muscle mass and age; and consequently may qualify better than creatinine as a surrogate marker of renal function in investigating the role of decreased protein clearance as a potential cause of age-related OPG elevation [17].”

• Methods
This section is difficult to follow because of the lack of subheadings (research design, subjects, measurements, statistical analysis). How was creatinine measured? Data was not normally distributed but the authors used linear regression, thus violating one of the linear regression assumptions. Although we may assume that linear regression is fairly robust for validity against nonnormality, using a nonparametric regression method, or employing a transformation of the variables may result in a more powerful test. The authors should describe the statistical methods with more detail. For instance they should explain how were the variable selected for the multiple regression. Were all variables included or only the variables that correlated significantly (spearman correlation) with OPG and RANKL?
As suggested, subheadings have been added. Creatinine was measured using the Creatinine Jaffé 2nd generation (compensated) test on the cobas c 111 system (Roche Diagnostics GmbH, Mannheim, Germany). This information has been added to the methods section. The reviewer is completely right, and it was a lapse on our part in not detailing our statistical steps. Our not normally distributed data was in fact log transformed prior to executing the linear regression. This has been added to the statistical analysis section. Only variables that showed significant correlation were included in the regression analysis. For example, to determine the statistically significant confounders of serum OPG levels, age, cystatin C, FTI and E2 were included in the analysis.

- Results
Considering that the aim of the study was to explore the associations of OPG and RANKL with the other factors (age, sex hormones, vitamin D, BMD and bone turnover markers), why was the correlation of cystatin C presented in this section? The authors performed an additional data analysis dividing the sample in two age groups (using the median), but again this was not previously mentioned as a secondary aim of the study. Moreover, dividing only in two groups may not be the best approach, probably dividing into tertiles or quartiles would be a more suitable method; classifying the older age group as ‘elderly’ is not accurate, as the minimum age is 59 years old (too young to be labeled as elderly). The figures are not clear enough; thus tables with the numeric data for correlation and multiple regression should be added and figures should be removed. Using tables will help reducing the text when presenting the most important results.

Hopefully the revised (clarified) version shall help address the issues correctly pointed out by the reviewer. We have placed maximal effort to better emphasize the aim of our present study, i.e., to explore the association between cystatin C and OPG, in addition to the other known predictors.

As pointed out by the reviewer, it was not our primary aim to divide our cohort into two age-groups. This was an extension of our attempt to reduce the confounding effect of age on OPG. Nonetheless, we have included this concept into the statistical analysis section. Indeed dividing the study population into tertiles or quartiles may be more suitable, but given our limited sample size, further fractionation did not reveal meaningful statistical data.

We take your point; it would probably be wiser to discard the use of the term “elderly”, instead the term “older” may better characterize those over “> 59 years of age” since they truly are older than those “≤ 59 years of age”. We hope that the reviewer agrees with our point of view.

As suggested we have removed all figures and additional tables are used.
to summarize the correlation and regression data. Consequently the text has been abbreviated.

- Discussion
The authors focus the discussion or at least the main findings of the study on the comparison of both age groups, instead of addressing the results related with the aim of the study.
I believe that table 2 is dispensable.
The authors speculate that the present results provide evidence of a triad E2 - cystatin C - OPG. I believe, based on the present study results that there is no sufficient and robust evidence to support this statement.
A section describing the major strengths and limitations of the study should be provided.
The conclusion does not reflect what was found. Why is cystatin C considered a confounder? Between which relationship (predictor and outcome) is cystatin C involved? The authors may be assuming that the predictor is age and the outcome OPG, but this is confusing because cystatin c was not the only variables associated with OPG (FTI, E2). Thus, I believe that the results of this study demonstrated that in addition to age (which was the stronger predictor), other modifiable factors such as cystatin C, FTI and E2 were also significant predictors of OPG , and that the association between cystatin C and OPG was more evident with increased age (older age group).
The authors must remember that when they included age, FTI, and E2 the association between cystatin C and OPG remained significant even when considered the other variables. In other words, cystatin c is a significant predictor of OPG independently of age, FTI and E2.

As suggested we have thoroughly restructured the discussion section. We have opted to keep table 2 (now table 4) since quite a few points in the discussion refer to the table, additionally it summarizes the studies published on this topic.
We have reformulated our conclusion and the limitations of the present study have been further expanded upon,

Thank you for all your constructive and thought provoking suggestions.
Summary of the changes in the revised version:

All 4 figures have been deleted.
Two additional tables have been added (table 2 and 3), as such the original table 2 is now table 4.
The references have been renumbered.
Numerical data is primarily presented in the tables.
Maximum emphasis has been made to highlight the main aim and findings of the study.
All remarks and comments have been addressed, hopefully adequately.
Text confirms to editorial requirements.