Author’s response to reviews

Title: Effect of a rare genetic variant of TM7SF4 gene on osteoclasts of patients with Paget's disease of bone

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Version: 1 Date: 30 Sep 2017

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RESPONSES TO REVIEWER’S COMMENTS

We wish to thank reviewers for their constructive comments, which have helped us to improve this manuscript. Modifications in the manuscript have been made in the enclosed revised version and can be seen in track changes.

Reviewer #1:

1. Results - In abstract it is noted that DC-STAMP expression was elevated in patients with carrier of the variant than in non-carrier patients. Please clarify the sentence in Results section (p.8) that nuclei/multinucleated cell was higher in patients carrier of the variant than in healthy controls but not different from non-carrier patients.

Response: As suggested, the sentence has been reformulated (page 8): The mean number of nuclei per multinucleated cell was significantly higher in patients carrier of the variant (7.2 ± 4.3) than in healthy controls (4.9 ± 2.0), p=0.04. But this mean number of nuclei in patients carrier of the variant was not significantly different from patients non carrier of the variant (6.1 ± 2.9).
2. Figure 2 show immunofluorescence of DC-STAMP expression in osteoclast cultures with similarity in healthy and carriers with PDB. I suggest removing the words (p.9; line 5) "but not at the plasma membrane" as DC-STAMP is known as a transmembrane protein.

Response: This change has been made (page 9): The immunofluorescence of the DC-STAMP protein in osteoclast cultures after 21 days of differentiation showed a similar distribution between the patient with PDB carrier of the rare variant and a healthy control, mainly intracellular internalized (Figure 2).

3. I suggest to include a citation for OS-9 (p.9; line 45).

Response: The sentence has been clarified (page 9): SQSTM1 and OS-9 expressions were lower in patients than in healthy controls, in particular in patients non carrier of the rare variant for isoforms 1 and 2 of OS-9 which were more than twice lower than in healthy controls (see the left part of the bottom line of Figure 4).

4. Figs.1&3-the authors may consider noting index (health controls; paget patients non mutated; paget patients mutated) once either in the figure or in the figure legend as they are same for all the graphs. Also, shorten (write in two lines than all in single line) the axis labeling in graphs.

Response: As suggested by reviewer 1, improvement in the presentation has been made in Figures 1 and 3 (see revised Figures 1 and 3).

Reviewer #2:

1. The authors need to reflect in the text if there is any significant difference in age, gender or extent of disease between the different groups analysed in the study.

Response: There was no significant difference in terms of age and gender between the three studied groups and participants, nor difference in extent of the disease between the two groups of participants with PDB. This information was added in page 5 of the manuscript.

2. Although the authors claim to follow Bustin et al (Clinical Chemistry 2009 and BMC Mol Biol 2010) for qPCR publication, these papers strongly advise to disclose the probe sequence, which is missing in the present manuscript. The authors also need to clarify the
Response: In this study, the qPCR were performed by the use of the SYBR green reagent but not a Taqman assay, reason why we did not need to add a probe sequence in addition to forward and reverse primers which are shown in table 2.

We performed absolute quantification. This information has been clarified (page 7): Absolute quantification of the number of copies of each mRNA was performed according to Luu-The et al. [20] using second derivative method and a standard curve of Cp versus logarithm of the quantity.

The PCR amplification efficiency has been added (page 7): PCR amplification efficiency varied between 1.93 and 2.20.

3. Following the above, on figure 3, does the ratio represented in the figures correspond to fold change? Besides, all figures are normalised by HKG, which is not mentioned in the manuscript. The authors used 3 endogenous controls, are those 3 showing similar results? The results from each of the endogenous control need to be discussed in the paper.

Response: The ratio corresponds to the ratio of the studied gene expression by the geometric mean of the gene expression of the three housekeeping genes (which has been added as a footnote to figure 3), but not to fold change. We added the information on the normalization by the three housekeeping genes in the title of figure 3, as follows: Gene expression analyses of candidate genes quantified by PCR from cell lysates of in vitro differentiated osteoclasts and normalized by the geometric mean of the three housekeeping genes.

In addition, we made the following changes in the results section for gene expression analyses in page 9: At the end of the differentiation period, gene expression analyses performed in cell lysates from mature osteoclasts and normalized by the geometric mean of the three housekeeping genes did not provide any significant differences between patients with PDB carrier of the rare variant, patients non carrier of the variant and healthy controls (Figure 3). The gene expression analyses normalized by each housekeeping gene separately shown similar results (see Additional Files 1 to 3). In Figure 3, gene expressions of LAMP1 and NFATc1 trended to be lower in patients, carrier of the variant or not, than in healthy controls, whereas gene expressions of CREB, TM7SF4 and OC-STAMP trended to be higher in patients than in healthy controls.

The results of gene expression analyses normalized by each housekeeping gene separately has been added as additional files 1 to 3.

4. The definition of PAL in Table 1 needs a better elaboration.
Response: The definition has been clarified at the bottom of the table 1 (page 20): *PAL= total alkaline phosphatase, expressed as the number of time the midpoint of the normal range of total alkaline phosphatase levels.

Reviewer #3:

Although in the method section is included the number of patients, it should also be included in the abstract.

Response: The number of participants has been added in the abstract (page 2): 50 ml of peripheral blood were collected in pagetic patients carrier of this variant (n= 4) or not (n= 4) and healthy controls (n= 4).