Author’s response to reviews

Title: Structure/function relationship and retinal ganglion cells counts to discriminate glaucomatous damages

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Version: 9  Date: 22 September 2015

Author's response to reviews: see over
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Version: 3  Data: 21/09/2015
Object: MS: 1467303059161015 - Structure/function relationship and retinal ganglion cells counts to discriminate glaucomatous damages

Dear Dr Martini,

Dear Dr Goni,

First of all thank you so much for your review to our study and for your suggestions. Your observations have been really helpful for us. We will answer point by point.

Reviewer #1 (Dr E. Martini)

MAJOR COMPULSORY REVISIONS

1. [...]The declared aim of the study is to assess the correlation between the estimated RGC count and structural and functional parameters in different stages of the disease. In the paper there is only a general correlation between many parameters but always regarding the entire study group and not the different stages. [...] You should add the correlation in the different groups or change the declared aim of the study.

   We changed the aim of the study in order to be more coherent to our results, to our tables and to data we decided to show. (page 2, lines 34-37; page 4, lines 89-92; page 14, lines 324-327. “The aim of the current study is to analyse the correlation between RGC count, estimated by Medeiros’ formula, and the structural and functional parameters in patients examined for glaucoma and to evaluate SAP, OCT and RGC counts capability to discriminate the weight of the disease itself.”)

2. In the methods section you diffusely explain Global loss volume and Focal loss volume (OCT parameters), in the table 1 there are data about correlation of these 2 parameters and the other OCT, visulf field and RGC count parameters but in the text there is no comment on this. [...]I feel that you should comment on it.

   Comments about GLV and FLV have been added (page 13, lines 290-292: “There is a good inverse correlation between FLV and the two RCG counts (r=-0.79); likewise, GLV is in a very good inverse relation with RGC count (r=-0.81) and RGC count GCC (r=-0.82).”; page 17, lines 387-389: “FLV and GLV have a good or very good correlation with ganglion cells counts: in particular, GLV correlates with circumpapillary and...
macular RGC counts better than FLV and could be used during clinical practice instead of Medeiros' formula")

3. Some statements are contradictory: I think that if you use and accept GSS that is based entirely on visual field as your reference standard you cannot assume that perimetry is not able to discriminate early damage from normal (lines 232-234).

   We fully agree with your observation and we tried to better explain it the text (page 11, lines 255-261: “MD and PSD derived from SAP always showed a statistically significant difference (p<0.01) in the comparison between groups. Nevertheless, VFI was not statistically significant (p=0.563) comparing groups 0 and 1: this could demonstrate that not all SAP parameters, considered separately, are always so sensitive in discriminating healthy subjects from those with early/moderate perimetric defects. As demonstrated by Brusini's GSS2, these parameters should be considered in couple at least (and not individually) to discriminate various stage of glaucoma.”).

4. in table 1 you should add the significance of difference between groups for all parameters. This would be very useful in the judgment about the performance of different tests and different parameters in discriminating between the different stages.

   Considering the suggestion of the other reviewer, we add Table 2 as a part of the text (pages 8-9, lines 189-192; page 9, lines 201-204). In this way, we were able to insert a new "Table 2" containing the p values of all considered parameters comparing the three groups.

MINOR ESSENTIAL REVISIONS

1. In the DISCUSSION section the last sentence (lines 328-330) is quite obscure how "glaucoma characteristics of each individual patient " could affect the estimate of RGC count. You should explain better or else eliminate this sentence.

   We tried to better explain that sentence (page 15, lines 341-345: “Moreover, each patient has different characteristics as age, stage of disease, neuronal and non-neuronal structures, differential light sensitivities. All these features are directly or indirectly involved in RGCs number determination, because some of them are considered within Medeiros formulas, while others (as non-neuronal structures) influence thicknesses measured by OCT.”).

2. In table 2 in the SAP derived formula there is in the final formula a "gl" that is not cited or explained in the legend. The "m" and "b" values how can be obtained? The linear function that is cited how is calculated? I think that some explanation is due in addition to the citation of the original work.

   We tried to better explain those formulas (page 8, line 192: “gl” was an error instead of “gc”; page 8, lines 194-199: “The structural part of the model consisted of estimating the number of RGC axons from RNFL and GCC thickness measurement obtained by OCT (OCTrgc). Below, formulas used are showed: d corresponds to the axonal density (axons/μm²) and c is a correction factor for the severity of the disease to take into
account remodeling of the RNFL axonal and non-axonal composition. The average RNFL and GCC thicknesses correspond to the 360-degree measures automatically calculated by OCT software.”). We also think that the way to calculate RGC count is quite clear for our article, also considering that the aim of our study is not to scrupulously describe how to estimate the RGCs number (this kind of work has been already made by Medeiros et al. as we indicated in our References at point 12. Medeiros FA, Weinreb RN et al. Estimating the rate of retinal ganglion cell loss in glaucoma. Am J Ophthalmol 2012; 154:814-824).

3. In the line 104 is cited BCVA > 0,5: is it an inclusion criterium? If it is, it should be clearly stated.

You’re right. We controlled our data and there was an error in the text: the BCVA was >0.7 and in the text we have now specified that it was an inclusion criterium (page 5, line 105: “(BCVA) >0.7 (if less than 0.7 the patient was excluded from the study)”).

4. In statistical analysis description at line 206 is written "three treatment groups". I think it refers to 3 groups of severity and it should be corrected.

Page 10 Lines 221: it has been corrected (“...comparisons among three groups...”)

5. It would be advisable to better explain which are the parameters whose correlations are explored, otherwise the table 3 is the only point where you can find every data and most of them are highly significant, so it is difficult to extract any sense.

We add at the end of the “Statistical analysis” all the parameters we decided to evaluate and to correlate (page 10, lines 232-238: “To summarize, parameters we chose to correlate are: age, Mean Deviation (MD), Pattern Standard Deviation (PSD), Visual Field Index (VFI), Global Loss Volume (GLV), Focal Loss Volume (FLV), average RNFL thickness (RNFL av.), RNFL superior thickness (RNFL av. sup.), RNFL inferior thickness (RNFL av. inf.), RNFL temporal thickness (RNFL av. temp.), GCC average thickness (GCC av.Total), GCC superior thickness (GCC av. sup.) and GCC inferior thickness (GCC av. inf.). To these, we also added RGC count and RGC count GCC obtained using Medeiros’ algorithm.”)

6. I think that it is obvious that any global index will be poorly sensitive in differentiating between normal and early disease and will be more sensitive in established and advanced disease. Perhaps it would be interesting to investigate whether focal damage index is more sensitive in these cases.

We took into account in our analysis all parameters provided by SAP and OCT and indicating focal and global damages.

**DISCRETIONARY REVISIONS:**

Discretionary revisions: quality of written English has been reviewed by an English native speaker.
Kind regards

Dr Pietro Distante (on behalf of all other authors)
1. Is the question posed original, important and well defined? The research question posed by the authors should be easily identifiable and understood. Lines 87-90 of the paper describe the aim of the study. It remains unclear for me what is the main objective of the study. [...] According to your observations, we changed the aim of the study and tried to better explain it (page 4, lines 89-92: “The aim of the current study is to analyse the correlation between RGC count, estimated by Medeiros’ formula, and the structural and functional parameters in patients examined for glaucoma and to evaluate SAP, OCT and RGC counts capability to discriminate the weight of the disease itself.”).

2. Are the data sound and well controlled? In methods, it is not specified how many examinations (SAP and OCT) were performed to obtain the data. If only one exam was performed, there is no chance to control the variability of measurements, an important parameter to correctly interpret results. RGC count may vary significantly mainly due to SAP variability. This will have an impact in the results of RGC count’s discriminant capacity to reproducibly separate levels of damage.

We explained why we performed just one visual field test and one OCT for each patient (page 6, lines 118-121: “All patients were well trained to SAP with more than one reliable visual field test in the past. All the visual field considered were reliable with fixation losses <20% and false positive and negative errors <15% and were performed only once on the examination day.”; page 8, line 171: “The first OCT test with a “Good” Scan Quality Index (≥40) was taken into account.”; page 16, lines 376-379: “Nevertheless, in our study we tried to simulate a daily clinical practice, in which just one reliable test (both SAP and OCT) is performed and evaluated by the ophthalmologist: higher is the number of the tests, smaller will be the compliance of the patient and the reliability of the test itself.”).

- In line 212 it is said that differences were considered significant when the two-sided p value was < 0.05. In lines 231, 237,240 and 251, p is related to the level 0.01 (either bigger or lower).

There was an error (difference significant with p value<0.05). We corrected it (page 10, line 229: “...significant when the two-sided p value was <0.01”).

- Also, Pearson’s coefficients are showed in the text as (r >...) instead of r=...). Please clarify.

About Pearson’s coefficient, “r >...” (page 12, line 282 and page 13, line 287) means that for all those parameters “r” is always better than the specified value (specific values can be read in Table 3). We also added a description of the strength of correlation (page 10, lines 226-228: “The strength of correlation was defined as “very poor” (r<20), “poor” (0.21<r<0.40), “moderate” (0.21<r<0.60), “good” (0.61<r<0.80) or “very good” (0.81<r<1). All reported p-values were two-sided.”)
3. Is the interpretation (discussion and conclusion) well balanced and supported by the data?
   - The discussion properly addresses the two main questions of the study. Some argumentations are questionable nevertheless. In lines 302 to 309 it is said that results can be considered a proof of GSS2 efficacy, but they simply confirm that GSS2 separates levels of damage according only to SAP and MD-PSD values. Any other variable considered (VFI or pure structural measurements) will probably show lower discriminant performances as they are not included in the method defining the scoring system. The argument exposed in lines 271-273 is also questionable, as reasons to explain the “delay” of SAP to detect glaucoma damage are more related to the semilogarithmic conversion of differential light sensitivity, stimulus size, grid density, etc, according to studies.
   
   Sentences in lines 302-309 and in lines 271-273 have been crossed out.
   
   - The conclusions look like an extension of the discussion and can’t be easily found. They should be summarized in a short and clear way.

   According to your suggestion, a part of conclusion has been added to the discussion (pages 15-16, line 346-370). Moreover, we tried to make shorter and clearer conclusions (page 16-17, lines 381-396: “A very good correlation exists between MD, PSD and VFI and an analogous result can be noticed between RNFL and GCC, both total and sectorial, above all in early glaucoma. This confirm the anatomic relation existing between macular RGCs and their axons and dendrites measured around the optic nerve. Therefore in clinical practice both GCC and RNFL could be used to detect structural changes usually occurring in early glaucoma. FLV and GLV have a good or very good correlation with ganglion cells counts: in particular, GLV correlates with circumpapillary and macular RGC counts better than FLV and could be used during clinical practice instead of Medeiros’ formula. RGC counts estimated with Medeiros’ formula is not just an interesting combination of functional and structural parameters, but also a method to summarize in an objective way a wide and partially personal clinical reasoning. Moreover, RGC counts discriminate various stages of disease better than any other parameter singularly considered. Although further studies with a larger number of patients are necessary.”)

4. Are the methods appropriate and well described, and are sufficient details provided to allow others to evaluate and/or replicate the work?
   - Methods are well described, but some aspects, like examination quality control (in both SAP and OCT measurements) or number of examinations are lacking.
   
   As told, we tried to better explain quality control and number of examinations in the text (page 6, lines 118-121; page 8, line 171; page 16, lines 376-379).
   
   - In line 122-124 it is described that a central MD was calculated (16 central points). In lines 180-183, sensitivities are considered instead. I guess sensitivities were used for RGC count algorithm estimation, but don’t find in results what central MD was used for.
We specify the use of central MD in page 8, lines 186-187 “…using, into Medeiros’ formula, the MD calculated in the 16 central point of the Total Deviation map.” So, central MD was simply used to calculate RGC count GCC.

5. What are the strengths and weaknesses of the methods? Add in Discussion strengths and weaknesses of the study.

Done. Strengths and weaknesses of the study have been added (page 16, lines 371-379: “The main strength of our study is represented to the potential use of RGC counts for their classification and diagnostic capabilities. Furthermore, other interesting indices, as GLV, could be used instead of RGC counts, whose estimation requires time and data elaboration. On the other hand the not so great number of patients could be surely considered a limit of our study, together with the impossibility to evaluate the measurement variability as explained in “Methods”. Nevertheless, in our study we tried to simulate a daily clinical practice, in which just one reliable test (both SAP and OCT) is performed and evaluated by the ophthalmologist: higher is the number of the tests, smaller will be the compliance of the patient and the reliability of the test itself.).

6. Can the writing, organization, tables and figures be improved?
   - Writing is poor in my opinion. Text must be reduced, and sentences should be shorter. Some words are incorrect (ie: hypotonic, line 106; unity, line 313; actually, line 333)

As suggested some words have been modified (page 5, line 108: “hypotonic therapy” substituted with “eye drop therapy”; page 14, line 331: “unity” substituted with “scale”; page 15, line 346: “actually” substituted with “nowadays”).

- Describe properly the conclusions
  We tried to better describe our conclusions (page 16-17, lines 381-396).

- Table 2 is not a table in fact. Suggest to add as part of text

Table 2 has been crossed out: according to your suggestion, we add its contents as part of the text (pages 8-9, lines 189-192; page 9, lines 201-204). The new Table 2 shows p values of all considered parameters comparing the three groups.

- Figure 1: Add (cursive) -16 central threshold sensitivity points of the visual field corresponding to GCC area.
  As suggested, the comment to Figure 1 has been modified.

- Maybe one more figure (VFI vs groups) is lacking, as it measures function in a slightly different way than MD and adds significant information.
Please take into account that the maximum number of figures allowed is six (6) and we decided to not to emphasize VFI vs groups in a figure because that parameter is not included into Medeiros’ algorithm.

Kind regards

Dr Pietro Distante (on behalf of all other authors)
Note for the Editor

We would like to thank you for your observation. We better explained in “Methods” the procedure we used in recruiting patients (page 5, lines 99-101).