Reviewer 1

The manuscript describes a method for the detection of IgG and IgA isotypes of anti-tissue transglutaminase (anti-tTG) antibodies in human serum using electrochemical sensors based on gold membrane templated gold nanoelectrode ensembles (NEEs). As both the sensor and the separate detection of analytes has been reported previously, the main novelty of this manuscript is the possibility to detect both those new markers of celiac disease in a single run implementing two different enzyme labels and obtain the signal for each one of them at a different potentials. Although the way the detection of the two analytes is presented in the introduction creates the impression that their detection is performed in one run, the experimental part shows that they are detected separately. If the latter is the case, then the novelty of the work is very weak. The authors should clearly state which case applies in the current manuscript.

We thank the reviewer for the comments that allowed us to improve the manuscript, making some important points clearer. Indeed, with the enzyme labels used here, the possibility to detect the two antibodies in a single run, is not realistic. As explained now on lines 126-130 this is because "mM concentrations of H_2O_2 used for IgG detection can inactivate the GOx enzyme used in the IgA-NEE [48], while H_2O_2 eventually produced by GOx can interfere with the HRP response, therefore, at the present stage we preferred to keep separated the two voltammetric detection steps." Anyhow, even if the final detection of the two isotypes is performed in two separated steps, the possibility to distinguish each isotype by its own voltammetric signature is an important step forward in isotypes analysis. For instance, this distinction can avoid procedure errors in routine analyses, where a mismatching in the addition of the secondary antibodies can reflect in mismatching one isotype for the other (note that this mistake possibility applies also to FEIA). It is worth stressing that such a development can be extended also to the detection of different isotypes of other antibodies of clinical relevance, not only to anti-tTG. This work represents a first step in this direction, which can finally evolve to the possibility of distinguishing the two isotypes just in one voltammetric run however it requires individuating (see new lines 426-428) "alternative enzyme labels, suitable to provide separated voltammetric signals without interfering each other when working in the same solution.". An additional progress point of this work with respect to previous literature is the very low detection limit achieved in serum samples, which is 3-4 times lower than previously reported immunosensors approaches (see line 414). We believe that the above outlined progress points represent a step forward not only for anti-tTG detection, but also for the reliable isotyping of antibodies in general, by developping suitably advanced electrochemical immunosensors.

Our point-to-point answers to the other specific comments by reviewer 1, are reported below.

In addition, the following points should be addressed prior to manuscript acceptance for publication.

1) A schematic of the electrodes structure would be helpful for the reader.

We added a SEM image showing the structure of the electrode, that is Figure 1 A in the new version of Figure 1.

2) The cyclic voltammograms presented in Figure 2 and 3 have been taken using PBS buffers of different molarity. Is there any reason for that?

Indeed, we didn't observe any change in the CV by varying the PBS concentration from 10 to 50 mM. Anyhow, for homogeneity, in the revised paper, all data reported are in 10 mM PBS.

3) The authors used serum samples of know concentration at different dilutions in order to establish calibration curves for IgG and IgA anti-tTG antibodies. This means that the different solutions contain different percentages of serum. What is the range of serum content in these solutions? How the serum presence affects the sensor signal? Some CVs of serum dilutions in PBS are required to determine the serum effect.

We thank the reviewer for this observation. Indeed we forgot to specify that standardized serum samples were "eventually diluted with human serum (Sigma-Aldrich) to 100 µL volume and finally diluted 1:10 with 10 mM PBS (pH 7.4)" (see lines 264-265 and 328-329 of the revised manuscript).

4) Based on the 3SD/m value, the authors claim a LOD of 0.7 U/mL for IgG. However, from the values presented in Figure 5b the signals obtained for solutions with concentration 0.7, 1 and 1.5 U/mL are undistinguishable. So the LOD value claimed should be reviewed.

Indeed, the first three points of the calibration plot, now Figure 4B (see previous Figure 6B) are signficantly different each other, the net current increasing (even if not remarkably on the scale of the figure) with the target concentration. On suggestion fo reveiwer 3, we deleted previous Figure 5 , referring to the more detailed description and discussion of calibration plot for IgG reported by us in ref. [29]. Anyhow, we wish to point out that the calibration points here commented by reviewer 1 are set at concentration values between detection (0.72 U/mL) and quantification limit (2.4 U/mL). By definition, the detection limit is the minimal concentration which provides a signal statistically different from the background noise. As recommended by specilaized literature, the detection limit is calculated as LOD = (3 x standard deviation)/sensitivity. This means that the standard deviation of the signal at the LOD is 33 % of the signal. Therefore, a relative scattering from linearity (up to ± 30 %) of data near the LOD is obviously acceptable. This scattering should decrease to about ± 10 % by approaching the limit of quantification (LOQ).

5) The curve in Figure 5b is sigmoidal and not linear. The authors should exclude the value corresponding to 0.7 U/mL from the calculation of linear regression curve. It would be also advisable to try other ways to linearized the curves in Figures 5b and 6b, e.g., in linear-log scale.

Indeed, a sigmoidal shape characterizes the plot A and not B (now Figure 4A, previously, Fig. 6 A). The plot in the new Figure 4B, that is the first part (zoom in) of 4 A, is clearly linear (see also previous Fig. 6B). The linear 4B plot is indeed the one used by us for the quantification of the analyte concentration. Note that proper dilution of the samples is eventually applied, to stay approximately within the linearity range, as described on lines 286-288, 351-353, 358-365.

6) Have the authors check the cross-reactivity of the secondary antibodies used? They should include in Figures 5 and 6, respectively, the signal obtained when the secondary antibody against IgG (along with the respective enzyme substrate) is used with IgA solutions, and vice versa.

From a general viewpoint, the selectivity and lack of cross reactivity of IgA anti-tTG and IgG anti-tTG has been proven previosuly by the studies reported in references 1 and 19. Information on this has been added on lines 162-163 of the revised manuscript. This selectivity was confirmed also in our experiments and on line 319-321, we specify now that (for IgA) "No electrocatalytic current was detected in the absence of any anti-tissue transglutaminase nor when incubation was performed with IgG anti-tTG instead of IgA anti-tTG".

7) The four serum samples analysed are two few to provide a reliable correlation. It is better to add in the table the % deviation of the values determined with the sensor to those determined by FEIA. This deviation is more than 50% in some cases. Have the authors any explanation for this?

We thank the reviewer for this observation. Consequently, we have revised all the discussion of the data in Table 2; the new discussioni is now reported on lines 377-399 of the revised manuscript. Note also that the results for samples 10641 and 10645, from healthy individuals, have been added in Table 2 and discussed accordingly. Taking into account the small number of samples analyzed, the correlation equations have been deleted.

8) A short discussion regarding comparison of the method developed with the literature methods for IgG and IgA anti-tTG antibodies determination accompanied by a table to summarize the methods analytical characteristics would be very helpful for the reader.

We added in the main text comparison and discussion with previous studies aimed to determine IgG and IgA antiTG with EC immunosensors (mainly ref.15,17,19). Doing this in a table does not seem feasible because of the differences in the way used to express concentrations (some as U/mL, other as ug/L) and also because, among previous works, only ref. 19 reports LOD/LOQ data. A comparative discussion ref. 17 is reported on lines 390-392 and with ref. 19 on lines 414-415.

9) Some minnor corrections:

• Replace "sec-antibody" by "secondary antibody" throughout the text

Done, thanks

• H2Q is written in two different ways. Select one and use it throughout the manuscript.

H2Q is for hydroquinone, BQ is for benzoquinone, as it appears in the revised manuscript.