PEER REVIEW HISTORY

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ARTICLE DETAILS

| TITLE (PROVISIONAL) | Assessment of blood clot formation and platelet receptor function ex vivo in patients with primary Sjögren’s syndrome |
|---------------------|----------------------------------------------------------------------------------------------------------|
| AUTHORS             | Ng, Wan-Fai; Collins, Katherine; Balasubramaniam, Karthik; Viswanathan, Girish; Natasari, Andini; Tarn, Jessica; Lendrem, Dennis; Mitchell, Sheryl; Zaman, Azfar |

VERSION 1 - REVIEW

| REVIEWER | Christian Lood |
|----------|----------------|
|          | Post doctoral associate |
|          | Lund University |
|          | Department of Clinical Sciences Lund |
|          | Section of Rheumatology |
|          | Lund |
|          | Sweden |
|          | I have no competing interests. |

| REVIEW RETURNED | 03-Mar-2013 |

| RESULTS & CONCLUSIONS | In my opinion, the authors should be more careful in their interpretation of the data since there are few individuals included. Perhaps the authors also could discuss the cytokine data more. Now, it sounds as if a lot of things were measured and by luck some laboratory findings were associated to platelet function. I have found one reference from 1986 by Peter Oxholm discussing platelet function in patients with pSS. I did not have full access to the paper but the authors should consider including this key reference. |

| GENERAL COMMENTS | The paper by Katherine Collins and colleagues was interesting to read and it discusses the important question of whether patients with primary Sjögren’s syndrome have an aberrant platelet function increasing the susceptibility to thromboembolic disease. In all, the research question is relevant, limitations of the study highlighted and the results are nicely presented. I have some questions and comments, as stated below, which might be relevant to consider in order to improve the paper further. |
|                 | 1. In the strengths and limitations you mention this to be the first study to examine platelet aggregation in patients with pSS. I have to admit I did not have full access to the paper, but Peter Oxholm and colleagues have published a paper in Acta Med Scand in 1986 entitled “Platelet function in patients with primary Sjögren’s syndrome”. I don’t know the methods used in the study by Oxholm but in the abstract, the authors claim to observe increased platelet aggregation upon stimulation with epinephrine, ADP and collagen in pSS patients as compared to healthy individuals. You should look at this paper and also discuss the potential differences observed in your study. |
2. With regard to the methods I have never used TEG previously but it seems adequate and is nicely combined with MEA. Even though the manufacturers seem to allow a long time period between blood sampling and analysis, in my experience, platelets become activated quite fast, at least when analysed by flow cytometry for platelet-monocyte complexes. Do you have information about how long time each and every sample stood before being analysed? Where there any differences in time between healthy individuals and pSS patients? Or between time before analysis and TEG/MEA parameters?

3. In the discussion you mention that there were no correlation between age and TEG/MEA and that is correct, but in healthy volunteers the correlation is almost statistically significant (p=0.053) and the correlation is inverse. Since the healthy individuals are younger than pSS patients, adjusting for age might reveal that platelets from pSS patients indeed are more prone to aggregate upon certain stimuli. You might consider doing this statistical analysis. You might also consider including more healthy individuals to strengthen your findings.

4. You mention that platelet number is one important determinant of TEG parameters. Were there any differences in platelet count between the groups, and should it be taken into account in the statistical analyses?

5. The discussion is nice and discusses several important things, among others the patient selection criteria. However, perhaps the platelets should be discussed in more detail. As stated in your paper, patients with pSS have increased platelet activation and generation of platelet microparticles.

6. In the discussion you tend to focus on the cytokine analysis which, even though interesting, is highly speculative and the results rendered by random rather than hypothesis as far as I understand. In light of the paper by Ng et al, it is interesting that you also observe inverse correlation to TEG parameters. It is tempting to speculate of potential receptor interactions as you do, but why not try them? Why not add purified cytokines to the system and see whether or not the cytokines per se affect TEG/MEA parameters or if it is only correlations to inflammation in general?

7. One other possibility for the inverse correlation between inflammation and TEG parameters could be prior platelet activation. We have previously described associations between certain cytokines (IFN-alpha) and platelet activation in lupus. Previously activated platelets might not be as prone to participate in coagulation and platelet aggregation as non-activated platelets. It would be interesting to study markers of platelet activation such as soluble P-selectin and soluble CD40L in your patient cohort and see whether or not platelet activation could affect the TEG/MAE parameters. Both this experiment and the addition of cytokines, described above, are quite easily performed and should add some useful information to the paper.

8. You state MIP-1a and IL-1a to be independent predictors for clot strength and overall coagulability. Are the cytokines also predictors independently of each other, or are they highly correlated?

9. In Figure 1, why are only 17-18 patients included/visible in the graphs?

10. Have you investigated the effect of treatment on any of the TEG/MAE parameters? Hydroxychloroquine is associated to reduced cardiovascular events and may be able to reduce anti-phospholipid antibody-mediated platelet activation in lupus (Espinola RG et al 2002). Do you see any effects of this treatment in your group? Are pSS patients without hydroxychloroquine different in
TEG/MAE parameters as compared to healthy individuals?

11. In Table 2, why are only 12 healthy individuals included and not all 13?

12. In supplementary table S2, why do you use the non-parametric test (Mann-Whitney) when assessing influence of gender and the parametric test (Paerson’s correlation) when assessing influence of age on the TEG/MEA parameters? Why not use non-parametric tests in both analyses?

REVIEWER
Manuel Ramos-Casals, MD, PhD
Sjögren Syndrome Research Group (AGAUR), Laboratory of Autoimmune Diseases Josep Font, IDIBAPS, Department of Autoimmune Diseases, ICMiD, Hospital Clinic, Barcelona, Spain

REVIEW RETURNED
10-Mar-2013

GENERAL COMMENTS
Collins et al present the results of a study that analyses blood clot formation and platelet receptor function in primary SS.

I have the following comments:

- Please increase the clinical characterization of patients, including cardiovascular risk factors, previous thrombotic events or history of pregnancy losses.
- Some features may suggest that the two populations at study (primary SS and SLE) are biased. First, the high % of SLE patients with positive (Ro/La) (even higher than the % found in primary SS). Second, the high % of primary SS patients treated with hydroxychloroquine (similar to the % found in SLE).
- Page 4: some studies have suggested a higher frequency of antiphospholipid antibodies in primary SS. This should be add as an additional reason to investigate a potential thrombotic disorder in primary SS.
- Page 6: more information about patient selection is needed. Were the patients selected consecutively? From a statistic point of view, the predominance of female gender in SS and SLE is not a valid reason to exclude males.

REVIEWER
Pasoto, Sandra
Universidade de São Paulo

REVIEW RETURNED
19-Mar-2013

THE STUDY
This is an interesting and original study. However, some concerns regarding the selection of patients with primary Sjögren's syndrome (pSS) and systemic lupus erythematosus (SLE) may have influenced the results.

1) Hydroxychloroquine may inhibit platelet activation, causing an antithrombotic effect. However, the current use of this drug was not an exclusion criterion, and a percentage of pSS and SLE patients was under treatment with this medication. Espinola RG, Pierangeli SS, Gharavi AE, Harris EN. Hydroxychloroquine reverses platelet activation induced by human IgG antiphospholipid antibodies. Thromb Haemost. 2002 Mar;87(3):518-22. Erratum in: Thromb Haemost. Thromb Haemost. 2002 May;87(5):IX. Ghara AE [corrected to Gharavi AE].

2) Antiphospholipid antibodies may be present in the sera from pSS
and SLE patients, and they are a known cause of thromboembolic events in these patients. However, these antibodies were not considered in the present study.

Fauchais AL, Lambert M, Launay D, Michon-Pasturel U, Queyrel V, Nguyen N, Hebbar M, Hachulla E, Devulder B, Hatron PY. Antiphospholipid antibodies in primary Sjögren’s syndrome: prevalence and clinical significance in a series of 74 patients. Lupus. 2004;13(4):245-8.

Al-Homood IA. Thrombosis in systemic lupus erythematosus: a review article. ISRN Rheumatol. 2012;2012:428269.

RESULTS & CONCLUSIONS

Thromboembolic phenomena are uncommon in pSS, so the sample of patients evaluated is small to obtain relevant conclusions.

Haga HJ, Jacobsen EM, Peen E. Incidence of thromboembolic events in patients with primary Sjögren's syndrome. Scand J Rheumatol. 2008 Mar-Apr;37(2):127-9.

Fauchais AL, Lambert M, Launay D, Michon-Pasturel U, Queyrel V, Nguyen N, Hebbar M, Hachulla E, Devulder B, Hatron PY. Antiphospholipid antibodies in primary Sjögren's syndrome: prevalence and clinical significance in a series of 74 patients. Lupus. 2004;13(4):245-8.

VERSION 1 – AUTHOR RESPONSE

Reviewer 1: Christian Lood

I have no competing interests.

In my opinion, the authors should be more careful in their interpretation of the data since there are few individuals included. Perhaps the authors also could discuss the cytokine data more. Now, it sounds as if a lot of things were measured and by luck some laboratory findings were associated to platelet function.

I have found one reference from 1986 by Peter Oxholm discussing platelet function in patients with pSS. I did not have full access to the paper but the authors should consider including this key reference.

The paper by Katherine Collins and colleagues was interesting to read and it discusses the important question of whether patients with primary Sjögren’s syndrome have an aberrant platelet function increasing the susceptibility to thromboembolic disease. In all, the research question is relevant, limitations of the study highlighted and the results are nicely presented. I have some questions and comments, as stated below, which might be relevant to consider in order to improve the paper further.

1. In the strengths and limitations you mention this to be the first study to examine platelet aggregation in patients with pSS. I have to admit I did not have full access to the paper, but Peter Oxholm and colleagues have published a paper in Acta Med Scand in 1986 entitled “Platelet function in patients with primary Sjögren’s syndrome”. I don’t know the methods used in the study by Oxholm but in the abstract, the authors claim to observe increased platelet aggregation upon stimulation with epinephrine, ADP and collagen in pSS patients as compared to healthy individuals. You should look at this paper and also discuss the potential differences observed in your study.
Response:
We thank the reviewer for informing us of an earlier publication in 1986 examining platelet aggregation which we were unaware of. We have obtained the full-text article and read it with interest. The study by Oxholm et al examined 15 patients with PSS and 15 normal controls. PSS patients were defined by the presence of keratoconjunctivitis sicca and xerostomia without other chronic inflammatory connective tissue disease. They found that 13 out of 15 PSS patients had platelet counts within “normal” ranges whilst the remaining 2 patients had mild thrombocytopenia (120, 127 respectively). All 15 controls had platelet counts within the normal ranges. There was no statistically significant difference in the levels of P-β-thromboglobulin. To study platelet aggregation, Oxholm and colleagues separated platelet-rich plasma and platelet-depleted plasma and then stimulated these plasma fractions with various agonists. Platelet aggregation was measured using Dual Channel Payton Aggregometer, which as far as we can work out, relied on measuring light transmission, and the data were presented according to the “lowest concentrations of various agonists that causes an irreversible aggregation and a difference of >= 80% in light transmission between platelet-rich and platelet-depleted plasma during 5 minute incubation.”

Thus, there are important differences between the study by Oxholm et al and our study. Firstly, the classification criteria used for PSS patients were different. Before the AECG consensus criteria 2002 were developed, studies of PSS used different criteria for the disease, which has been a source of many discrepant data in PSS. AECG criteria are arguably the most widely accepted classification criteria for PSS to date. Secondly, Oxholm et al studied platelet aggregation in “isolation”, whereas in our study, platelet aggregation was measured in “whole blood”, which we believe are more physiologically relevant (see page 5 of original manuscript). In addition, the process of platelet enrichment can activate platelet and may introduce variability and inconsistency of the data obtained. In contrast, the method we used in this study involved minimal handling of the samples. Thirdly, the methods used for measuring platelet aggregation and how the results are presented differ between the two studies.

In light of the knowledge of the study by Oxholm et al, we have modified our statement on our study being the first to examine “ex vivo blood clot formation and platelet aggregation” to “ex vivo clot kinetics and platelet aggregation in whole blood”.

2. With regard to the methods I have never used TEG previously but it seems adequate and is nicely combined with MEA. Even though the manufacturers seem to allow a long time period between blood sampling and analysis, in my experience, platelets become activated quite fast, at least when analysed by flow cytometry for platelet-monocyte complexes. Do you have information about how long time each and every sample stood before being analysed? Where there any differences in time between healthy individuals and pSS patients? Or between time before analysis and TEG/MEA parameters?

Response:
We thank the reviewer for the helpful comment. Although we did not record the exact time intervals, we had aimed to analyse all samples between 30 and 120 minutes after collection. All blood samples were collected with specimen tubes containing either citrate (to minimise initiation of clotting cascade for TEG measurement) or hirudin (to minimise initiation of platelet aggregation) and the minimum time of 30 minutes allows sufficient time for the citrate and hirudin to have their inhibitory effects. Furthermore, TEG measurement is routinely performed in our intensive care unit and theatre settings as a clinical test to guide treatment, so we have made the assumption that quality control data would have been obtained by the manufacturer in order to make the specific recommendations. Furthermore, Zambruni and colleagues have showed that all TEG parameters are stable between 30 minutes and 2 hours following blood sampling with added citrate as an anti-coagulant (Zambruni A et al. Blood Coagul Fibrinolysis. 2004;15:103-7). However, in light of the interesting flow cytometric data that the reviewer has shared, it would be interesting to take this into consideration in future studies using TEG and MEA.
3. In the discussion you mention that there were no correlation between age and TEG/MEA and that is correct, but in healthy volunteers the correlation is almost statistically significant (p=0.053) and the correlation is inverse. Since the healthy individuals are younger than pSS patients, adjusting for age might reveal that platelets from pSS patients indeed are more prone to aggregate upon certain stimuli. You might consider doing this statistical analysis. You might also consider including more healthy individuals to strengthen your findings.

Response:
We again thank the reviewer for this very helpful comment. Although the p-value between age and one of the MEA parameters (ADP) was close to statistical significance (p=0.053), the p values for all the remaining 10 correlations tests results were over 0.1 (with the exception of α-angle (0.07)). Therefore, we believe that overall, there is no evidence to support a link between age and the TEG/MEA parameters.
We agree that our sample size is relatively small and a larger sample size may reveal smaller differences between groups. However, as we have mentioned in our original manuscript (page 9), the mean/median values for many TEG parameters were remarkably similar between all 3 subject groups and standard deviations were small. Therefore, a substantially larger sample size will be needed to demonstrate any statistically significant differences between groups. Furthermore, whether such small differences in TEG/MEA parameters are of physiological significance is uncertain.

4. You mention that platelet number is one important determinant of TEG parameters. Were there any differences in platelet count between the groups, and should it be taken into account in the statistical analyses?

Response:
Platelet count was measured only for patient participants. The platelet counts for all PSS patients were within the normal range and there was no significant difference in platelet count between the PSS and SLE groups. (see revised Table 1). Upon bivariate correlation analysis, platelet count inversely correlated with Ly30 (r= -0.359, p=0.040 (uncorrected)) and Ly60 (r= -0.355, p=0.042 (uncorrected)), which were not statistically significant upon correction for multiple comparison. Thus, there was no significant correlation between platelet count and other TEG/MEA parameters. These figures have been incorporated in supplementary Table S3.

5. The discussion is nice and discusses several important things, among others the patient selection criteria. However, perhaps the platelets should be discussed in more detail. As stated in your paper, patients with pSS have increased platelet activation and generation of platelet microparticles.

Response:
We thank you the reviewer for the compliments and helpful suggestion. We agree that extending our discussion of the role of platelet in more details will improve the manuscript and have done so accordingly in the revised manuscript (page 12).

6. In the discussion you tend to focus on the cytokine analysis which, even though interesting, is highly speculative and the results rendered by random rather than hypothesis as far as I understand. In light of the paper by Ng et al, it is interesting that you also observe inverse correlation to TEG parameters. It is tempting to speculate of potential receptor interactions as you do, but why not try them? Why not add purified cytokines to the system and see whether or not the cytokines per se
Response:
We too share the enthusiasm of the reviewer to test our hypothesis and are certainly keen to do so in the future. There are several reasons why we did not conduct these experiments in this current study. Firstly, although conceptually it would be relatively simple to test our postulation that MIP-1α may provide a negative feedback mechanism on platelet function, at a minimum, we will need to establish the optimal in vitro condition (e.g. dose and period of pre-exposure of platelet to MIP-1α) to test the hypothesis, we believe that obtaining such data may require considerable time and additional resource. Secondly, we have limited financial resource to carry out additional experiments. With regard to the possible relationship between our observation and inflammation in general, the only significant correlation we have identified between any of the TEG/MEA parameters and C-reactive protein (CRP) was Ly30, which was not statistically significant after corrections for multiple comparison. Although there were a few more significant correlations between erythrocyte sedimentation rate (ESR) and TEG parameters, none were statistically significant after corrections for multiple comparisons. Furthermore, although ESR is used as a marker of more "sustained" inflammatory clinically, the levels of ESR are influenced by many factors, and cannot be used as a reliable marker of inflammation. Therefore, we believe that our data do not support that a simple relationship between TEG/MEA and inflammation in general.

7. One other possibility for the inverse correlation between inflammation and TEG parameters could be prior platelet activation. We have previously described associations between certain cytokines (IFN-alpha) and platelet activation in lupus. Previously activated platelets might not be as prone to participate in coagulation and platelet aggregation as non-activated platelets. It would be interesting to study markers of platelet activation such as soluble P-selectin and soluble CD40L in your patient cohort and see whether or not platelet activation could affect the TEG/MAE parameters. Both this experiment and the addition of cytokines, described above, are quite easily performed and should add some useful information to the paper.

Response:
We thank again for the insightful comment. We have indeed examined the relationship between soluble CD40L and TEG/MEA parameters but found no statistically significant correlations (See supplementary Table S3 in the original manuscript). We have not, however, studied the relationship between P-selectin and TEG/MEA parameters but agree that it would be of interest in future studies. At the suggestion of the reviewer, we were able to measure serum P-selectin levels on 13 stored serum samples but found no significant correlation between serum P-selectin levels and any of the clot kinetic parameters (Spearman’s R between 0.04 and 0.3 with p value between 0.3 and 0.9). We have also included the potential link between prior platelet activation and TEG/MEA parameters in our discussion as well as appropriate references in the revised manuscript (page 12).

8. You state MIP-1a and IL-1a to be independent predictors for clot strength and overall coagulability. Are the cytokines also predictors independently of each other, or are they highly correlated?

Response:
We thank the reviewer for this helpful suggestion. There were significant correlations (Table 3) between many cytokines which were not entirely unexpected given the known physiological links between some of these cytokines. Some of the correlations were particularly strong e.g between IL-1a, MIP-1α, IL-17a and IL-21 (r>0.8), which may suggest potential multi-collinearity and in turn may lead to over-fitting of the regression model. In order to mitigate these potential issues, we have performed the following additional analyses (using SPSS version 19):
(i) We first identified independent variables that may give rise to potential problem with collinearity (based on Eigenvalue <0.1). We then removed each independent variable in turn in the regression analysis. We found that for Clot Strength (MA), MIP-1α remained the key predictor when each of the cytokines (IL-1α, IL-17α, TNF-α, IL-21) that were strongly correlated to MIP-1α were removed in the regression analysis. When MIP-1α was removed, IL-1α and IL-8 were found to be the key predictors (with a slightly improved r value (0.785) compared to MIP-1α as a lone predictor (r=0.725)). For Clotting Index (CI), when MIP-1α, IL-17α, TNF-α or IL-21 was removed, in addition to IL-1α, IFN-γ was also found to be an independent predictor (improving the r value from 0.647 to 0.805). When IL-1α was removed, MIP-1α was identified as the only independent predictor (r=0.637). It should be noted that however, neither IL-8 nor IFN-γ correlated with any of the TEG or MEA parameters on bivariate correlation analysis.

(ii) We performed factor analysis (Supplementary Table S4), followed by stepwise regression (Table 4) using the factors generated. For Clot strength (MA), we identified factor 1 (which was predominantly represented by IL-1α, MIP-1α and IL-17α) being the key predictor (r=0.69, p=0.002). For Clotting index (CI), the key independent predictors were factors 1 and 7 (r=0.807, p=0.021). The latter was predominantly represented by IFN-γ.

In light of the results of these additional analyses, we have revised our manuscript accordingly and included the findings of the additional analyses using factor analysis in the result section. In the discussion, we acknowledge “the potential problems with collinearity and that our findings suggest several cytokines may correlate with clot strength and clotting index. Furthermore, based on our factor analysis, cytokines such as MIP-1α, IL-17α, IL-1α and IFN-γ may associate with clot kinetics such as clot strength and clotting index.”

9. In Figure 1, why are only 17-18 patients included/visible in the graphs?

Response:
Cytokine analyses were only carried out in 18 patients as we did not collect serum at the beginning. We have clarified this in the method section of our revised manuscript.

10. Have you investigated the effect of treatment on any of the TEG/MAE parameters? Hydroxychloroquine is associated to reduced cardiovascular events and may be able to reduce anti-phospholipid antibody-mediated platelet activation in lupus (Espinola RG et al 2002). Do you see any effects of this treatment in your group? Are pSS patients without hydroxychloroquine different in TEG/MAE parameters as compared to healthy individuals?

Response:
We again thank the reviewer for the helpful suggestion. We have performed the suggested additional analyses: There was no statistical significance in all TEG/MEA parameters between those who were on HCQ treatment and those who were not. Same results were obtained when we combined the PSS and SLE groups – i.e. no significant difference was detected between those receiving HCQ treatment and those who did not (Supplementary Table S5). We have included this in the discussion of our revised manuscript as well as the appropriate references accordingly.

11. In Table 2, why are only 12 healthy individuals included and not all 13?

Response:
We apologise for the typos, which have been corrected in the revised manuscript.
12. In supplementary table S2, why do you use the non-parametric test (Mann-Whitney) when assessing influence of gender and the parametric test (Paerson’s correlation) when assessing influence of age on the TEG/MEA parameters? Why not use non-parametric tests in both analyses?

Response:
Although the data were normally distributed as a whole, upon division into subgroups of males and females – the subgroups no longer followed normal distribution and therefore non-parametric (Mann Whitney) test was used.

Reviewer: Manuel Ramos-Casals, MD, PhD
Collins et al present the results of a study that analyses blood clot formation and platelet receptor function in primary SS. I have the following comments:

1. Please increase the clinical characterization of patients, including cardiovascular risk factors, previous thrombotic events or history of pregnancy losses.

Response:
We thank the reviewer for the suggestion. These have now been included (See revised Table 1).

2. Some features may suggest that the two populations at study (primary SS and SLE) are biased. First, the high % of SLE patients with positive (Ro/La) (even higher than the % found in primary SS). Second, the high % of primary SS patients treated with hydroxychloroquine (similar to the % found in SLE).

Response:
We thank the reviewer for pointing this out. The % of Ro/La positivity among the SLE group was incorrect. Only 4 out of 11 SLE patients were positive for either anti-Ro or anti-La antibodies. We have revised Table 1 accordingly. (The original figure of 9 was extracted from the incorrect data column of “ENA positivity”).
With regard to relatively high percentage of HCQ use among PSS patients in this study, this may in part reflects local practice and in part the relative short disease duration of the PSS patients in this study. As the other reviewers have pointed out, HCQ may be associated with reduced cardiovascular events and reduced platelet activation, we have included this in the discussion in our revised manuscript.

3. Page 4: some studies have suggested a higher frequency of antiphospholipid antibodies in primary SS. This should be add as an additional reason to investigate a potential thrombotic disorder in primary SS.

Response:
We thank the reviewer for the helpful comment, we have revised the manuscript accordingly.

4. Page 6: more information about patient selection is needed. Were the patients selected consecutively?

Response:
PSS patients for this study were recruited via two separate routes: First, patients with a confirmed diagnosis of PSS according to the AECG criteria attending the outpatient clinics run by investigator
WFN between 1 March and 1 May 2012 were invited. The sample collection and processing were performed predominantly by a single researcher (KSC). Because the equipment for measuring TEG and MEA is located on a different site from where the outpatient clinic was being held and the requirement to process the samples within 3 hours of collection, therefore not all PSS patients who had attended the clinic during this period had been invited and there was also a possible bias for selecting those with an early morning or early afternoon appointments.

Second, PSS patients who were participants of the UK primary Sjogren’s syndrome registry (UKPSSR) and attending the outpatient clinics of the Freeman Hospital (but were not expected to attend the clinics during the above mentioned period) were sent an invitation letter to participate in this study (n=63). In total after the first round of invitation, 28 responded “Yes”, 5 responded “No” and no response received from the remaining patients. Overall, 18 female PSS patients were recruited from the outpatient clinics, and 16 female patients via invitation. Not all who have accepted the invitations were studied due to limitation of financial and personnel resources.

We have not included these additional details in the revised manuscript for reasons of readability of the manuscript. Instead, we have included the recruitment strategy as a supplementary document.

5. From a statistic point of view, the predominance of female gender in SS and SLE is not a valid reason to exclude males.

Response:
With regard to the exclusion of male subjects in our analysis, our rationale was not because of the female predominance in PSS, but because we found gender differences in TEG data in our healthy volunteers (see page 6 of the original manuscript). However, we believe that the wording may have led to confusion with the readers, we have therefore restructured the sentence to clarify this.

Reviewer: Sandra Pasoto
This is an interesting and original study. However, some concerns regarding the selection of patients with primary Sjögren’s syndrome (pSS) and systemic lupus erythematosus (SLE) may have influenced the results.

1) Hydroxychloroquine may inhibit platelet activation, causing an antithrombotic effect. However, the current use of this drug was not an exclusion criterion, and a percentage of pSS and SLE patients was under treatment with this medication.

Espinola RG, Pierangeli SS, Gharavi AE, Harris EN. Hydroxychloroquine reverses platelet activation induced by human IgG antiphospholipid antibodies. Thromb Haemost. 2002 Mar;87(3):518-22. Erratum in: Thromb Haemost. Thromb Haemost. 2002 May;87(5):IX. Gharavi AE [corrected to Gharavi AE].

Response:
We thank the reviewer for this helpful comment. In this study, there was no significant difference in all TEG/MEA parameters between patients taking HCQ and those who did not (see also response to a very similar comment 10 by reviewer Lood).

2) Antiphospholipid antibodies may be present in the sera from pSS and SLE patients, and they are a known cause of thromboembolic events in these patients. However, these antibodies were not considered in the present study.

Fauxchais AL, Lambert M, Launay D, Michon-Pasturel U, Queyrel V, Nguyen N, Hebar M, Hachulla E, Devulder B, Hatron PY. Antiphospholipid antibodies in primary Sjögren's syndrome: prevalence and clinical significance in a series of 74 patients. Lupus. 2004;13(4):245-8.

Al-Homood IA. Thrombosis in systemic lupus erythematosus: a review article. ISRN Rheumatol.
Response:
We thank the reviewer for this helpful comment and agreed that this is a weakness of the study. In this study, at least 3 PSS were positive for anti-phospholipid antibodies (anti-phospholipid antibodies however were not systematically tested for all patients) although the titre was low in one patient. Since the presence of anti-phospholipid antibody is expected to increase thrombogenicity, our findings that PSS patients as a group did not have altered thrombogenicity compared to healthy controls further strengthen our conclusion that PSS is not associated with altered clot kinetics and platelet aggregation in whole blood ex vivo.

3) Thromboembolic phenomena are uncommon in pSS, so the sample of patients evaluated is small to obtain relevant conclusions.
Haga HJ, Jacobsen EM, Peen E. Incidence of thromboembolic events in patients with primary Sjögren's syndrome. Scand J Rheumatol. 2008 Mar-Apr;37(2):127-9.
Fauchais AL, Lambert M, Launay D, Michon-Pasturel U, Queyrel V, Nguyen N, Hebbar M, Hachulla E, Devulder B, Hatron PY. Antiphospholipid antibodies in primary Sjögren's syndrome: prevalence and clinical significance in a series of 74 patients. Lupus. 2004;13(4):245-8.

Response:
In this study, the primary outcome was not thromboembolic phenomena. Since thromboembolic phenomena is an “end-product” of multiple factors, therefore, while a much larger sample size might be needed to demonstrate differences in thromboembolic phenomena between PSS patients and controls, it does not necessarily imply that the same sample size is needed to demonstrate abnormality in platelet aggregation or clot kinetics (because platelet aggregation or clot kinetic abnormality alone may not be sufficient to lead to thromboembolic events (even though they may increase the susceptibility of thromboembolic phenomena)). We, however, agree that the relatively small sample size of this study is a weakness which we have acknowledged in our original manuscript (in Article Summary and in Discussion).

| REVIEWER          | Christian Lood, PhD       |
|-------------------|---------------------------|
|                   | Lund University, Sweden   |
| REVIEW RETURNED   | 16-Apr-2013               |

GENERAL COMMENTS
The authors have nicely revised their manuscript and adequately answered all of my comments. Even though the study still is somewhat preliminary due to the small number of included patients and controls the authors report several interesting findings which are well discussed.

I only have two minor comments you might consider.

1. It’s good that you discussed your findings in relation to the paper by Oxholm et al. Please also add the reference to the reference list.

2. In the discussion you mention that the inability to find differences in clotting between SLE patients and healthy controls suggest alternative mechanisms operating in this disease. I agree that there are several mechanisms which contribute to thromboembolic risk in SLE patients; however I don’t think that you should rule of
differences in clotting based on 11 SLE patients and 13 healthy volunteers.

**REVIEWER**
Ramos-Casals, Manuel
Hospital Clinic, Department of Autoimmune Diseases.

**REVIEW RETURNED**
19-Apr-2013

- The reviewer completed the checklist but made no further comments.