Author's response to reviews

Title: Thromboelastography on plasma reveals delayed clot formation and accelerated clot lyses in HIV-1 infected persons compared with healthy controls

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Author's response to reviews: see over
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Dear editor and reviewers,

Thank you for having reviewed our paper and for giving us the opportunity to revise it. We have read the comments carefully and hope that we have addressed the reviewers’ concerns satisfactorily. Below we have inserted our answers to the questions raised by the reviewers.

We believe the manuscript has benefited from the review process and we hope you will find it suitable for publication in the revised form. Changes in the manuscript are highlighted with yellow.

We are of course willing to make further changes if necessary. Should you have questions or concerns regarding the manuscript, please do not hesitate to contact me.

On behalf of the authors,

Frederikke Rönsholt

Reviewer 1:

1-1 I would suggest to report a thorough analysis of coagulation factor concentrations, D-dimer and FSP. Furthermore an analysis of thrombingeneration would be worthwhile.

- Unfortunately sufficient plasma for further analysis does not remain, however we agree on the relevance of the suggestions. This also applies to comment 2-2. We have added a comment about these limitations in the manuscript (line 274).

1-2 As there seems no values of the lipid-analysis I would suggest to leave this completely out.

- We have left out the lipid analysis

1-3 Please comment on the impact of EDTA on the TEG-analysis. Although it is been applied in all analysis (and might be a structural problem) this might have an impact.

- We have previously conducted a quality control study comparing TEG values in citrate and EDTA plasma and found that these were comparable with the only exception being slightly longer r-time values in EDTA plasma compared to citrate plasma. It is well described that platelets contribute to r-time so extreme thrombocytopenia or platelet inhibition/dysfunction will result in prolonged r-times. Based on our TEG data on EDTA plasma we chose to use this matrix in the present study as the citrate plasma we had access to was incomplete for the cohort and had been thawed several times. As the reviewer mentions, we analyzed the same plasma type from HIV infected patients and controls so we don’t expect this to yield any problems with regard to results. For your information reference values for the EDTA plasma TEG parameters were determined in 8 healthy individuals (blood donors) by running TEG ±tPA in duplicate. The mean (95% CI) and
intra-assay CV% of the plasma TEG parameters R, α angle and MA (-tPA) and Ly30 were as follows: R mean 12.5 min (95%CI 7.2-17.9) and CV%=11; α angle 37.6 º (95%CI 9.9-65.4) and CV%=7; MA 28.9 mm (95%CI 16.1-41.7) and CV%=4 and Ly30 51.0 % (95%CI 17.4-84.6) and CV%=5.

1-4 What is the purpose of stratification for smoking history? Please introduce this in the background section and mention the data-sampling in the methods section.

- Smoking is known to influence coagulation and the purpose of the stratification was to see if smoking alone was a major contributing factor. A sentence with references has been added in the background section (line 38) and it has been clarified in the methods section that the information came from the patients’ charts (line 72).

1-5 Please avoid the last sentence (given the thromboembolic events...) as it is not shown in the current paper.

- This sentence has been removed

1-6 Page 3, line 4, please describe the life style factors which are specific for HIV, or are this general factors?

- This has been clarified in line 4-5

1-7 Page 3 lines 10-11 please re-word this sentence as it is very vague. What about the hypofibrinolytic state? ...Similarly, the flowing blood comprised of plasma and blood cells may change from its normal state to hypercoagulant, hypocoagulant and hyperfibrinolytic depending on the disease/condition of the patient.

- This sentence has been rephrased to: Similarly, in disease conditions, the flowing blood representing the sum of function in plasma proteins and blood cells may change from “normal” to hyper- or hypocoagulant or to hyper- or hypofibrinolytic like e.g. the hypocoagulability and hyperfibrinolysis observed in severe trauma or the fibrinolytic shut down observed in sepsis with DIC (line 11)

1-8 Page 3 lines 12-14 ... Importantly, there appears to be a balance between the endothelium and the flowing blood indicating that a pro-coagulant state of one part will be counterbalanced. Please add „ under stable conditions”, because you describe a steady state.

- Line 14 now reads “. Importantly, under stable conditions there appears to be a balance between the endothelium and the flowing blood indicating that a pro-coagulant state of one part will be counterbalanced by an anti-coagulant/hypocoagulable state of the opposite part [5]”

1-9 Page 3 lines 21-22. Please re-word this sentence as HIV-infection itself does NOT cause this alterations (it is at least not proven that this is a direct action of the virus).
This sentence has been rephrased to: HIV infection, treated as well as untreated, has been associated with altered levels of circulating markers of endothelial activation/damage, coagulation, anti-coagulation, fibrinolysis, and platelet function (line 23)

1-10 Page 4 lines 43-45. ..When evaluated in plasma, TEG/ROTEM provides a unique opportunity to examine functional coagulation and especially fibrinolysis and/or fibrinolytic resistance of the fibrin clot in stored samples. Please avoid the word „unique“ and mention the disadvantage of plasma analysis in contrast to whole blood analysis which might be of importance during inflammation.

• This sentence has been rephrased to: When evaluated in plasma, TEG/ROTEM provides a distinctive opportunity to examine functional coagulation and especially fibrinolysis and/or fibrinolytic resistance of the fibrin clot in stored samples albeit the analysis does not necessarily reflect the functional properties of fresh, whole blood. (line 47)

1-11 Page 5 line 63-64 ... Several articles... Please leave this sentence out.

• This sentence has been left out.

1-12 Page 6 line 95. Please give this sentence a place more in the beginning of this section as it seems a little late here.

• The sentence has been moved to line 88.

1-13 Page 7 line 111. Please describe more precise the platelet analysis. I guess you mean here platelet count? Please display the count in the result section.

• Corrected to “platelet count” in line 116 and results added in line 188 (HIV+ and controls had similar platelet counts (240 x 10^9/L (197-273) vs. 210 x 10^9/L (187.5-249.25), p=0.115).

1-14 Page 9 line 153. .....indicative of a slower thrombin generation and/or higher levels of anticoagulant factors.. This is interpretation, please leave this out here.

• This has been left out

1-15 Page 9 line 164 pp. ...The findings were not reproduced when the analyses were performed on HIV+ and controls separately suggesting that these differences were driven by the differences in lymphocyte subsets between the two groups. Again the latter is an interpretation, which might be discussed later in the manuscript.

• This has been left out

1-16 Page 10 line 188: ..suggesting enhanced in vivo activation of platelets. See above.

• This has been left out
Page 11 lines 194-198 please leave the whole lipid analysis out as it does not contribute to the rest of the paper.

- We have left out the lipid analysis

Page 12 lines 222-223. ... anticoagulant factors (antithrombin, protein c, protein s, FII, FVII, FIX, FX) in untreated HIV+ suggesting that impaired hepatocyte function plays a role for the IMBALANCE OF coagulation.

- This has been corrected inline 219

Page 12 lines 231-233. ... Thus, some studies have found elevated levels of d-dimer in treated HIV infected persons [18,42], in accordance with our finding of a pro fibrinolytic state of plasma. Please be more precise: ...which might be a result of a pro fibrinolytic state as we could demonstrate...

- This sentence has been rephrased to: Thus, some studies have found elevated levels of d-dimer in treated HIV infected persons [18,44], which might be a result of a pro- or enhanced fibrinolytic state as demonstrated in plasma in the present study. (Line 228)

Page 12 lines 238-end of page ... In addition to factors circulating in plasma, whole blood contains red blood cells, leukocytes, platelets and microparticles that all contribute to hemostasis [7,44-46]. Platelets are key players in coagulation and as the results of this study are based on plasma samples, soluble factors derived from platelets may influence the results.... This is a very complicated description of the fact that the analyses were done in plasma which lacks the cellular contribution to coagulation. Please re-word.

- This sentence has been rephrased to: Whole blood contains red blood cells, leukocytes, platelets and microparticles [7,46-48] and their contribution to hemostasis is not assessed in this study, as it is based on plasma samples. However, circulating levels of factors derived from cells may influence coagulation and fibrinolysis. (Line 235)

Page 13 line 243. Please comment on the fact that the level of sCD40L is inversely correlated with the R-time but only the relation sCD40L/platelet count are different between the groups. What about the induced thrombopenia due to cART therapy? Maybe the impact of this finding is not due to platelet activation? Please comment on this.

- It is well described that platelet function influences TEG r-time when assessed in whole blood and sCD40L is closely correlated with platelet count and activation. In the present study platelet count and sCD40L correlated in both HIV+ and CON though more strongly in CON than in HIV+ (HIV+ (Rho=0.30, p=0.017) and controls (Rho=0.755, p=0.002)). We infer that the correlation between TEG r-time (and MA) and sCD40L reflects that platelet activation in vivo (and sCD40L shedding, may result in reactions / changes in the plasma phase that makes this more procoagulant which is hence reflected in vitro in plasma). In the present study, HIV+ tended to have lower platelet-count (p<0.15) and tended to have...
higher sCD40L (p<0.15) and thus had significantly higher sCD40L/platelet. Given that sCD40L release / level per platelet was different in HIV+ and CON, this may reflect different (patho)physiologic reactions in vivo and therefore we are not surprised that this is not simply correlated to TEG r-time. Also, the per platelet sCD40L release does not reflect the bulk activation but activation per platelet and thus cannot be expected to correlate to a global analyses (TEG r-time or TEG MA).

1-22 Page 14 line 270. Please add some comments on the fact that TEG was done in plasma and not in whole blood. What about EDTA? Please add comment on this here.

- This is mentioned in line 273. With regards to EDTA, please refer to point 1-3.

1-23 Page 14, lines 280-281.... by TEG and also we found evidence for enhanced plasma activation in vivo. Please be more precise. What do you mean by „plasma activation“? As the study was performed in vitro this conclusion is supported by the data.

- This sentence has been removed.

1-24 Page 14 lines 281-283. ... Together these findings suggest that hypocoagulability in HIV infected persons may reflect a universally adaptive response, comparable to that observed in acute critically ill patients, ensuring blood flow through an activated, procoagulant microvasculature. Please avoid this in the conclusion. It was not topic of the investigation. Please re-word the conclusion section as it is highly suggestive and not completely based on the results of the paper.

- The conclusion section has been restricted to: In conclusion, we found that plasma from long term well treated HIV infected persons is hypocoagulable with reduced fibrinolytic resistance as assessed by TEG. The clinical implications of the findings are unknown, but the observed coagulation anomalies could play a role in the well described excess of thromboembolic events in HIV infected persons. The study introduces the potential use of plasma TEG to further investigate coagulation anomalies in conditions with low grade inflammation. (Line 280)

Reviewer 2:

The authors present an interesting snapshot of plasmatic clot formation in treated HIV patients vs. normal controls. The results are interesting, showing an overall hypocoagulable state with increased lytic susceptibility. While, very interesting, the manuscript is also quite complex and confusing given the wide range of coagulation, platelet, and endothelial markers presented. There are also several key omissions from the data which require acknowledgment. A more concise reporting of the results is needed in this case.
2-1 The goal of "comparing the vascular system" is too broad, please refine and present a specific hypothesis to be tested.

- This sentence has been rephrased to: To assess whether coagulation and fibrinolysis differ between long-term treated HIV infected individuals (HIV+) and healthy controls (CON), we investigated functional plasma coagulation by thrombelastography (TEG) and plasma markers of endothelial and platelet activation.

2-2 Limiting the investigations to direct measurements of fibrin clot formation, which are the strongest results, would provide more focus and clarity.

- We have removed the lipid analysis for clarity.

2-3 The key finding of a functional hypocoagulable state with increased lytic susceptibility would benefit from further investigation. If plasma samples remain, please consider adding measurements of thrombin generation (PF 1-2), fibrin activation (fibrinopeptide-a), fibrinogen concentration, plasminogen concentration, and D-Dimer. If these are unavailable, please acknowledge the limitation.

- We agree with the reviewer on this; please refer to comment 1-1.

2-4 Please report all TEG parameters for each group, including the runs with added tPA.

- R (+tpa), Angle (+tpa), and TMA (+tpa) have been added in line 154. Lysis parameters without tpa did not yield meaningful results.

2-5 In addition, the Angles are very low in the HIV group, yet the MA are very similar. This is an inherent limitation of using TEG, in that MA is not measured at a standardized time during clot formation.

- We agree with the reviewer that MA is not a kinetic variable (how far is the clot formation after a given time) but other parameters reflect kinetics (r-time, k-time, α angle and A30 when available). In the TEG analysis the MA parameter is per definition the maximum clot strength, in this study reflecting mainly fibrinogen function. If MA had been a dynamic variable it would have been reduced in HIV+. Now, MA is interpreted as a functional fibrinogen level that is overall comparable between groups when the clot initiation dynamics are not taken into account.

2-6 Please also report the time to MA (TMA), which may shed more light on the different results between groups. Also consider reporting the 1/2 lysis time. (the total time from start of the run to achieve 1/2 of the MA during lysis ). This is a more standard way to report fibrinolysis.

- TMA have been added but we did not have access to ½ lysis time as it is not estimated by the used software/methods.
The TEG results may be mostly explained if the fibrinogen concentration is known for each sample. This is a major limitation.

- Please refer to comment 1-1.

The results do not necessarily support that the vasculature is activated similar to results found in critical illness, as stated in the conclusion. Please revise.

- The conclusion has been reduced, please refer to comment 1-24.

Line 280 "with reduced fibrinolytic resistance as assessed by TEG and also we found evidence for enhanced plasma activation in vivo." ....should this read “platelet activation” instead?

- Please refer to comment 1-23.

**Editorial requirements**

**E-1** By way of a section ?Acknowledgements?, please acknowledge anyone who contributed towards the article by making substantial contributions to conception, design, acquisition of data, or analysis and interpretation of data, or who was involved in drafting the manuscript or revising it critically for important intellectual content, but who does not meet the criteria for authorship. Please also include the source(s) of funding for each author, and for the manuscript preparation. Authors must describe the role of the funding body, if any, in design, in the collection, analysis, and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication. Please also acknowledge anyone who contributed materials essential for the study. If a language editor has made significant revision of the manuscript, we recommend that you acknowledge the editor by name, where possible. The role of a scientific (medical) writer must be included in the acknowledgements section, including their source(s) of funding. We suggest wording such as ‘We thank Jane Doe who provided medical writing services on behalf of XYZ Pharmaceuticals Ltd.’ Authors should obtain permission to acknowledge from all those mentioned in the Acknowledgements section.

- An acknowledgement section has been added on page 15 containing information on funding; we have no further acknowledgements and no writing assistance was used in the preparation of this manuscript.

**E-2** Please also ensure that your revised manuscript conforms to the journal style ([http://www.biomedcentral.com/info/ifora/medicine_journals](http://www.biomedcentral.com/info/ifora/medicine_journals)). It is important that your files are correctly formatted.

- The title page has been edited to fit your format; we believe the manuscript conforms to the journal style.