PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	The effect of empagliflozin on oxidative nucleic acid modifications in patients with type 2 diabetes: protocol for a randomised, double-blinded, placebo-controlled trial
AUTHORS	Larsen, Emil List; Cejvanovic, Vanja; Kjær, Laura; Vilsbøll, Tina; Knop, Filip; Rungby, Jørgen; Poulsen, Henrik

VERSION 1 - REVIEW

REVIEWER	Anna Solini, Associate Professor in Internal Medicine University of Pisa, Italy
REVIEW RETURNED	19-Jan-2017

GENERAL COMMENTS	The Authors propose a protocol aimed at exploring potential effects of SGLT2 inhibition on urinary excretion of 8-oxo-7,8-dihydriguanosine (8-oxoGuo) and 8-oxo-7,8-dihydro-2'- 50 deoxyguanosine (8-oxodG), two well recognized markers of DNA and RNA damage and oxidative stress related with CV risk. These two biomarkers have so far never measured after administration of a SGLT2 inhibitor, and in this light the protocol, even simple and merely descriptive, can be considered novel. However, I suggest to consider some points 1. I suggest a more narrow range of HbA1c of the recruited patients, trying to exactly match this parameter (major determinant of the level of oxidative stress, and affecting the level of 8-oxoGuo itself. The ideal would be to pick relatively well-controlled patients (HbA1c below 8%). The proposed number of individuals to study is small, and it would not be difficult to reduce variability of clinical parameters to the minimum possible level 2. Please explore also the effect on lipid peroxidation by measuring creatinine-normalized urinary thiobarbituric acid reactive substances (TBARS) and 8-isoprostane 3. I am afraid 2 weeks will be a too short study period to observe any effect on body iron storage 4. Coffee and tea consumption need to be recorded and monitored, especially in the presence of such short-term study. The ideal would be to refrain from assuming these substances, able to influence body iron storage 5. 8-guanosine compounds have been recently described as potent and efficacious potassium-sparing diuretics; therefore, please combine the measurement of 24-h diuresis with Na/K urinary excretion, to eventually correlate these parameters with urinary levels of 8-oxoGuo

	6. Given that urinary 8-oxodG and 8-oxoGuo are higher in patients with micro and macrovascular complications vs those without (Liu X et al., Oxid Med Cell Longev 2016), I suggest more strict exclusion criteria (recruiting just people without complications)

REVIEWER	Andreas Daiber University Medical Center Mainz Germany I am involved in a similar clinical Trial at our Institution (EmDiaOx) but without relation to the authors of the present study. Therefore, I feel able to provide a Review for this paper.
REVIEW RETURNED	28-Jan-2017

GENERAL COMMENTS	Major Points 1) The exclusion criteria should be Extended to the intake of antioxidants (drug or excessive intake of fruits etc.) since this will interfere with the Primary and secondary objectives in the present study, which is measuring oxidative stress. 2) The power calculation should be described in full Detail. On which previous data are the calculations based that lead to the conclusion that 17 participants in each Group will be enough? 3) The methods for the measurement of Primary and secondary endpoints (8-oxo-Guo and MDA) should be described in more Detail. 4) Comparison of the Gold Standard methods for 8-oxo-Guo and MDA) used by the authors should be compared with commonly used ELISA techniques - this would greatly improve the importance of the study. Minor Points 1) Abstract: Correct "8-oxo-7,8-dihydriguanosine"

VERSION 1 – AUTHOR RESPONSE

Reviewer: 1
Reviewer Name: Anna Solini, Associate Professor in Internal Medicine Institution and Country: University of Pisa, Italy Competing Interests: None declared

The Authors propose a protocol aimed at exploring potential effects of SGLT2 inhibition on urinary excretion of 8-oxo-7,8-dihydriguanosine (8-oxoGuo) and 8-oxo-7,8-dihydro-2'- 50 deoxyguanosine (8-oxodG), two well recognized markers of DNA and RNA damage and oxidative stress related with CV risk. These two biomarkers have so far never measured after administration of a SGLT2 inhibitor, and in this light the protocol, even simple and merely descriptive, can be considered novel. However, I suggest to consider some points

1.I suggest a more narrow range of HbA1c of the recruited patients, trying to exactly match this parameter (major determinant of the level of oxidative stress, and affecting the level of 8-oxoGuo itself. The ideal would be to pick relatively well-controlled patients (HbA1c below 8%). The proposed number of individuals to study is small, and it would not be difficult to reduce variability of clinical parameters to the minimum possible level.

First of all, thank you very much for reviewing our protocol manuscript. We think that some background information to our responses is appropriate.

After the contracts for this trial were signed in the beginning of October 2016, we submitted this manuscript. We received trial medicine in November and started the trial. Therefore, some participants have already completed the trial, and we cannot change the in- and exclusion criteria. With that in mind, we truly appreciate your great advices about the exclusion criteria, since we are planning other clinical trials in the field. And we will explain the rationale for our choices.

We chose the upper limit of HbA1c in order to include patients who are not in the middle of adjusting their medicine. Previously, we have not found a correlation between HbA1c and 8-oxoGuo/dG. Hereby, we did not find a problem to the bit more wide range than you suggested. We will have your comment in mind, when setting the criteria for the participants in our next trial.

2.Please explore also the effect on lipid peroxidation by measuring creatinine-normalized urinary thiobarbituric acid reactive substances (TBARS) and 8-isoprostane.

We do explore the effect of empagliflozin on lipid peroxidation. We chose to determine the effect on lipid peroxidation by measuring malondiadehyde (MDA) by high performance liquid chromatography (HPLC) in one of our collaborational laboratories due to the high specificity of the method. Thiobarbituric acid reactive substances (TBARS) is still frequently used, but criticized for the lack of specificity and artificial formation. Our laboratory is not able to measure 8-isoprostane. Isoprostanes are often determined by ELISA – due to the low cost and simplicity. However, the ELISA method is unspecific. We have added a line in the manuscript to describe why we prefer MDA measured by HPLC [line 291 (in manuscript without track changes)].

3.I am afraid 2 weeks will be a too short study period to observe any effect on body iron storage

Thank you for pointing this out. You are probably right, and perhaps the analysis at the end-of-study is needless. The iron measurements were at first included in order to make a correlation to 8-oxoGuo/8-oxodG at baseline, not as an effect parameter. We have in previous and ongoing studies found a correlation between iron and the oxo measurements that we would like to confirm.
We chose to measure body iron storage at end-of-study to be able to correlate changes in 8-oxoGuo/8-oxodG/MDA to the iron storage.
We have clarified our rationale to measure body iron storage in the manuscript [line 124-126 + 145-147].

4.Coffee and tea consumption need to be recorded and monitored, especially in the presence of such short-term study. The ideal would be to refrain from assuming these substances, able to influence body iron storage

Thank you for pointing out this issue. Many lifestyles habits could potentially influence our endpoints. To avoid influence of changes in these lifestyle habits, all participants are asked to continue their current lifestyle (i.e. diet, exercise, and smoking). We have clarified this in the manuscript [line 192-193]. And since our endpoint is the delta values, not the absolute values, we do think that lifestyles habits will influence our result.

5.8-guanosine compounds have been recently described as potent and efficacious potassium-sparing diuretics; therefore, please combine the measurement of 24-h diuresis with Na/K urinary excretion, to eventually correlate these parameters with urinary levels of 8-oxoGuo.

We think that this is a very interesting point. However, the recent paper [Jackson E. et al, Journal of Pharmacology and Experimental Therapeutics, 2016], uses micromole doses of 8-guanosine

compounds. The endogenous production of 8-oxoGuo/8-oxodG is in the nanomol range. The endogenous production is stable in most individuals, and the changes we have observed is in the range less than 50%, and several orders of magnitude from the pharmacological doses used. Furthermore, the diuretic effect in [Jackson E. et al, Journal of Pharmacology and Experimental Therapeutics, 2016] is particularly related to the nitro substitution. Therefore, we do not believe that such small changes present a confounder in our study. We thank you for this comment and we will consider including it in the discussion when the trial is over.

6. Given that urinary 8-oxodG and 8-oxoGuo are higher in patients with micro and macrovascular complications vs those without (Liu X et al., Oxid Med Cell Longev 2016), I suggest more strict exclusion criteria (recruiting just people without complications).

Thank you for pointing out this issue. We are expecting to include this in the discussion of our main paper. The association of 8-oxoGuo and increased mortality is based on data of both patients with and without complications. Since our primary endpoint is the delta values of 8-oxoGuo/8-oxodG we do not expect this to influence our result.

Reviewer: 2
Reviewer Name: Andreas Daiber
Institution and Country: University Medical Center Mainz, Germany Competing Interests: I am involved in a similar clinical Trial at our Institution (EmDiaOx) but without relation to the authors of the present study. Therefore, I feel able to provide a Review for this paper.

Major Points
1) The exclusion criteria should be Extended to the intake of antioxidants (drug or excessive intake of fruits etc.) since this will interfere with the Primary and secondary objectives in the present study, which is measuring oxidative stress.

First of all, thank you very much for reviewing our protocol manuscript. We think that some background information to our responses is appropriate.
After the contracts for this trial were signed in the beginning of October 2016, we submitted this manuscript. We received trial medicine in November and started the trial. Therefore, some participants have already completed the trial, and we cannot change the exclusion criteria.

It is a very valid point, that lifestyle habits e.g. diet could influence the biomarkers. However, all the participants are asked not to change their lifestyle during the trial (i.e. diet, exercise, smoking). This is clarified in the manuscript [line 192-193 (in manuscript without track changes)]. And since we use delta values as our primary as secondary objective, we do not expect lifestyle habits to influence our result.

2) The power calculation should be described in full Detail. On which previous data are the calculations based that lead to the conclusion that 17 participants in each Group will be enough?

We agree that this should be pointed out and we have revised the section in the manuscript[line 229-236]. The mean and sd for the power calculation are based on the baseline values from the PENTRIOX trial(Larsen et al. 2017). Smoking increases urinary excretion of 8-oxodG by 50% (95% CI 31-69%) (Loft et al. 1992). We wish to be able to detect an effect size of 20%, since we do not believe smaller changes are clinical relevant.

3) The methods for the measurement of Primary and secondary endpoints (8-oxo-Guo and MDA) should be described in more Detail.

We agree that a more detailed description of the measurements is appropriate, and we have included this in the manuscript [line 270-279 and line 283-290].

4) Comparison of the Gold Standard methods for 8-oxo-Guo and MDA) used by the authors should be compared with commonly used ELISA techniques - this would greatly improve the importance of the study.

We appreciate and agree with your concern regarding the methodology. Another of our recently accepted papers has a section in the discussion about the comparison of the methodology(Larsen et al. 2017) and we have done a large multi-laboratory comparison that include the ELISA teqnique that clearly pin-points the deficiencies and low specificity of the ELISA technique(Barregard et al. 2013). We think a discussion is out of scope for this protocol manuscript, but we have added some comments to why we prefer the UPLC-MS/MS and HPLC methods[line 280-281 and line 291].

Minor Points
1) Abstract: Correct "8-oxo-7,8-dihydriguanosine"

Thank you for pointing this out. We have corrected the typo.

References:
Barregard, Lars et al. 2013. “Human and Methodological Sources of Variability in the Measurement of Urinary 8-Oxo-7,8-Dihydro-2’-deoxyguanosine.” Antioxidants & redox signaling 18(18): 2377–91. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3671631&tool=pmcentrez&rendertype=abstract.

Larsen, E. L. et al. 2017. “Clarithromycin, Trimethoprim, and Penicillin and Oxidative Nucleic Acid Modifications in Humans: Randomised, Controlled Trials.” Br J Clin Pharmacol.

Loft, Steffen et al. 1992. “Oxidative DNA Damage Estimated by 8-Hydroxydeoxyguanosine Excretion in Humans: Influence of Smoking, Gender and Body Mass Index.” Carcinogenesis 13(12): 2241–47.

VERSION 2 – REVIEW

REVIEWER	Andreas Daiber University Medical Center Mainz, Germany I am involved in a similar clinical Trial at our Institution (EmDiaOx) but without relation to the authors of the present study. Therefore, I feel able to provide a Review for this paper.
REVIEW RETURNED	10-Mar-2017

GENERAL COMMENTS	My comments were addressed or in case they were not addressed this was explained in the Response. However, I would like to mention that it is not optimal to Hand in a study protocol for publication that undergoes Evaluation by reviewers and, when they come up with suggestions, state that the study was already started and nothing can be changed anymore. Nevertheless, I respect that contract signature and Need to start a clinical Trial sometimes goes fast. For me the study protocol is fine and can be published.