Point-by-point reply to reviewers

We thank the Reviewer and the Scientific Editor for their helpful comments. We address the comments from the Reviewer and the Scientific Editor below. All answers to the below questions are given in red colour font. In addition, changes are tracked throughout our text document.

Reply to the Reviewer

1) **Provide in the methods section how many mice of each type and for each SGLT2 inhibitor were used in each experiment.**

This information has now been included in the revised manuscript under the topic 'Materials and Methods' (sub topic 'Animals') on page 8.

2) **Supplementary fig 1 shows reduction of succinate in empagliflozin treated Akimba mice, but we don't know if this is statistically significant. Why you did not include suppl fig 1 in fig 3A, like you did in fig 3b?**

Unfortunately, the comparison displayed in the original Supplementary figure 1 did not reach statistical significance. However, Empagliflozin resulted in an interesting trend for reduced levels of succinate. As suggested by the reviewer, we have now included Supplementary figure 1 as Figure 3B.

3) **I would suggest also, to mention in the figures 3A and 3B that you refer to Akimba and Akita mice respectively (not only in the legend). (The same for figures 4, 5).**

The strain names have now been added above each bar graph for Figures 3, 4 and 5.

4) **In addition, include suppl. Fig 2 in Figure 5B.**

We have now added Supplementary figure 2 as the new Figure 5B. We are required to make it a separate figure as each SGLT2 inhibitor had its own control group.

5) **You need to comment that empagliflozin and dapagliflozin had not, both of them, the same effects in the different parameters studied and in the different mice species.**

We have now added the text below to the discussion on page 16 of the revised manuscript:

"It appears that Dapagliflozin was more bioactive than Empagliflozin when it comes to the effects on succinate levels (Figure 3A and 3B). It has been shown in mouse models that Dapagliflozin is superior to Empagliflozin as it has an increased half-life, longer duration of action, higher distribution and long retention period in the kidney (Tahara et al. 2016). Therefore, this could be the likely reasoning for the Dapagliflozin being more bioactive in relations to succinate levels.

Interestingly, Empagliflozin was able to reduce norepinephrine levels to a greater degree compared to Dapagliflozin in diabetic Akita mice (Figure 4A). This is interesting in the context of the EMPA-REG study (Wanner et al. 2016). In this study, factors driven by an increased SNS such as mortality from cardiovascular disease were reduced to a markedly greater degree compared to the Dapagliflozin DECLARE–TIMI 58 clinical trial (Wiviott et al. 2018). Hence, regulation of the SNS may be one factor where Empagliflozin may be specifically more effective than Dapagliflozin."

Reply to the Science Editor

1) **Reviewer#00503199 suggests the authors to provide in the methods section how many mice of each type and for each SGLT2 inhibitor were used in each experiment.**

Please refer to the reply to Reviewer 1 (Question 1) above.

2) **Supplementary figure 1 shows reduction of succinate in empagliflozin treated Akimba mice, but we don't know if this is statistically significant.**

Please refer to the reply to Reviewer 1 (Question 2) above.

3) **The authors need to discuss the empagliflozin and dapagliflozin had not, both of them, the same effects in the different parameters studied and in the different mice species.**

Please refer to the reply to Reviewer 1 (Question 5) above.

4) **I found the authors did not provide the approved grant application form(s). Please upload the approved grant application form(s) or funding agency copy of any approval document(s).**

This is now uploaded with the revised manuscript.

5) **I found the authors did not provide the original figures. Please provide the original figure documents. Please prepare and arrange the figures using PowerPoint to ensure that all graphs or arrows or text portions can be reprocessed by the editor;**

The original figure document in the format of PowerPoint is now included. As requested we have now changed the colours red and green in figures to alternate colours.

6) **I found the authors did not add the PMID and DOI in the reference list. Please provide the PubMed numbers and DOI citation numbers to the reference list and list all authors of the references.**

The PMID and the DOI is not added to the main manuscript.

7) **I found the authors did not write the "article highlight" section. Please write the "article highlights" section at the end of the main text.**

This is now added to the main manuscript, as indicated below:

Article Highlight

Research background

Type 1 diabetes (T1D) dramatically increases chronic microvascular complications which is a leading cause of diabetes associated morbidity. Both human and murine studies highlight the role of the gut microbiome and gut dysbiosis in the pathogenesis of numerous diseases. It is well-established that diabetes and its complications are of

multifactorial aetiology. Recent studies have highlighted the importance of perturbations in the gut microbiota as a contributing factor in the development and progression of diabetes and related complications. Therefore, many studies are now focusing on the gut microbiome as a potential source of biomarkers of diabetes and its complications.

Research motivation

The sodium glucose cotransporter 2 (SGLT2) inhibitors are a novel class of oral antidiabetic medications specifically used in the treatment of type 2 diabetes. It is well established that SGLT2 inhibitors block glucose reabsorption in the renal proximal tubules, thereby resulting in excretion of glucose in the urine and leads to improvements in metabolic and glycaemic parameters. However, preclinical and human studies investigating the beneficial mechanisms of SGLT2 inhibition in T1D and its complications are currently limited. Further pre-clinical investigations are essential to elucidate the underlying mechanisms by which SGLT2 inhibitors may impact the progression of T1D and its related complications. Therefore, we hypothesised that SGLT2 inhibition may exert its protective effects via alterations of the gut microbiome and tested this in a mouse model of T1D and diabetic retinopathy.

Research objectives

To investigate whether the treatment of type 1 diabetic mice with two independent SGLT2 inhibitors (Empagliflozin and Dapagliflozin) will affect gut health.

Research methods

To address the specific aims of our study, we used two of the most widely investigated SGLT2 inhibitors, Empagliflozin or Dapagliflozin and administered it to 10 week old C57BL/6J, Akita, Kimba and Akimba mice for 8 weeks via drinking water. At the end of the experiment, all mice were scarified and sera was collected. The concentration of succinate and the short-chain fatty acid (SCFA) butyric acid was measured using gas

chromatography-mass spectrometry and enzyme immunoassays were conducted to determine insulin, leptin and norepinephrine concentrations. Pancreatic tissue was also wax embedded, sectioned and stained with haematoxylin and eosin and analysed using brightfield microscopy.

Research results

In comparison to C57BL/6J and Kimba mice, both Akita and Akimba mice showed reduced levels of insulin production due to the presence of the Akita allele. In line with this, Akita mice also showed the presence of apoptotic bodies within the pancreatic islets and the acinar cells of both the Akita and Akimba mice displayed swelling which is suggestive of acute injury or pancreatitis. In Akimba mice, SGLT2 inhibition with Dapagliflozin for 8 weeks significantly reduced succinate levels when compared to vehicle treated mice. Furthermore, succinate levels in Akimba mice treated with the SGLT2 inhibitor Empagliflozin showed a similar trend. In diabetic Akita mice, the beneficial short-chain fatty acid butyric acid was significantly increased after Dapagliflozin treatment when compared to vehicle. There was a significant reduction in the kidney norepinephrine content in both Dapagliflozin and Empagliflozin treated Akita mice. Furthermore, the diabetic Akimba mice also showed a significant reduction in kidney norepinephrine content when treated with Empagliflozin. Lastly, both non-diabetic C57BL/6J and Kimba mice showed significantly reduced serum leptin levels after Dapagliflozin therapy.

Research conclusion

Our novel study compares and contrasts the effects of SGLT2 inhibition on the main products and intermediate metabolites of gut metabolism particularly in Akita and Akimba mice. We conducted studies using two independent SGLT2 inhibitors and showed that both inhibitors reduced the pathogenic biomarker succinate in our novel T1D Akimba mouse model of retinopathy. However, in relation to succinate levels in Akimba mice, Dapagliflozin was more bioactive than Empagliflozin, potentially due to factors such as increased half-life, longer duration of action, higher distribution and

long retention period in the kidney. Furthermore, we demonstrate for the first time that SGLT2 inhibition is sympathoinhibitory in a T1D mouse model.

Research Perspective

In line with our findings, it would be mechanistically insightful in the future to assess the expression of the succinate specific receptor GPR91 in ocular tissue before and after SGLT2 inhibition as SGLT2 is expressed in the eye. Furthermore, it is important to determine whether our findings can be reproduced in patients with T1D and its complications who are treated with SGLT2 inhibitors.