nature biomedical engineering | PEER-REVIEW INFORMATION

Stimulation of the hepatoportal nerve plexus with focused ultrasound restores glucose homeostasis in diabetic mice, rats, and swine

Corresponding author: Chris Puleo

Editorial note

This document includes the relevant written communications between the manuscript's corresponding author and the editor and reviewers of the manuscript during peer review. It includes decision letters relaying any editorial points and peer-review reports, and the authors' replies to these (under 'Rebuttal' headings). The editorial decisions are signed by the manuscript's handling editor, yet the editorial team and ultimately the journal's Chief Editor share responsibility for all decisions.

Any relevant documents attached to the decision letters are referred to as **Appendix #**, and can be found appended to this document. Any information deemed confidential has been redacted or removed. Earlier versions of the manuscript are not published, yet the originally submitted version may be available as a preprint. Because of editorial edits and changes during peer review, the published title of the paper and the title mentioned in below correspondence may differ.

Correspondence

Wed 29 Sep 2021
Decision on Presubmission Enquiry nBME-21-2167-PE

Dear Dr Puleo,

Thank you for submitting to *Nature Biomedical Engineering* your Presubmission Enquiry, "Focused Ultrasound Modulation of Hepatic Neural Plexus Restores Glucose Homeostasis in Diabetes".

As you may know, we screen Presubmission Enquiries against our editorial criteria. These editorial judgements are based on considerations of fit to the journal's scope and, when enough information is provided, of the degree of advance, broad implications, and breadth and depth of the work.

In this case, the topic of the Presubmission Enquiry is within the remit of the journal, and we appreciate that the stimulation of the hepatic neural plexus via pulses of focused ultrasound for therapeutic outcomes regarding glucose homeostasis is unprecedented and broadly relevant. The evidence in multiple animal models is welcome and a necessary requirement for this journal and for this type of work. We would therefore be glad to send the manuscript out for peer review. However, I recommend that you consider the following:

* The p values in Fig. 3b,c for the rat and swine models are not encouraging, despite the consistent trend across species. Could you please discuss this in the manuscript.

* Please provide all the precise p-values (ideally in the plots themselves) rather than as ranges.

* Figs. 4 and 6 would be easier to lay out and visualize if each is split into two. Our Articles can have up to 8 main figures

I should also ask you to please fill in our reporting summary and policy checklist. (Please note that these

forms are dynamic PDF files that can only be properly visualized and filled in by using Acrobat Reader.)

Both documents are aimed at ensuring good reporting standards and at easing the interpretation of results, and will be available to any reviewers. Should the manuscript be eventually published, the reporting summary will be attached to the published PDF of the paper and will also be available as supplementary information. More information is available on the editorial policies page.

Moreover, we highly recommend that you use our manuscript template. This will help you ensure that the manuscript complies with our data-presentation recommendations, that it includes all the necessary sections, and that it is structured to facilitate the assessment of peer reviewers and editors. In particular, please make sure that the manuscript provides thorough information on statistics and methods, and that the images comply with our guidelines for image integrity.

When you are ready to resubmit the manuscript, please upload the manuscript files as well as the reporting summary and policy checklist.

Best wishes,

Pep

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Pep Pàmies
Chief Editor, *Nature Biomedical Engineering*

Thu 18 Nov 2021
Decision on Article nBME-21-2167A

Dear Dr Puleo,

Thank you again for submitting to *Nature Biomedical Engineering* your manuscript, "Focused Ultrasound Modulation of Hepatic Neural Plexus Restores Glucose Homeostasis in Diabetes". The manuscript has been seen by three experts, whose reports you will find at the end of this message. You will see that the reviewers find the work compelling, and that they raise a number of technical criticisms and questions that I am sure you and your team will be able to address. In particular, we would expect that a revised version of the manuscript provides:

* Discussion of the main translational bottlenecks for this therapeutic strategy to restore glucose homeostasis.

* Thorough methodological detail, as per the questions and suggestions of the reviewers (and, in particular, points #1 and #5 from Reviewer #2 and points #5 and #6 from Reviewer #3).

Also, please do enlarge and better lay out the main figures (you can use the full-page width), so that the data can be more easily interpreted. In particular, most of the axes displaying glucose concentrations could be elongated so as to allow for easier visualization of concentration differences. Moreover, we allow for up to 10 Extended Data figures, and I suggest that the current Supplementary Figs. 9–13 would be most useful as such.

When you are ready to resubmit your manuscript, please upload the revised files, a point-by-point rebuttal to the comments from all reviewers, the reporting summary, and a cover letter that explains the main improvements included in the revision and responds to any points highlighted in this decision.

Please follow the following recommendations:

* Clearly highlight any amendments to the text and figures to help the reviewers and editors find and understand the changes (yet keep in mind that excessive marking can hinder readability).

* If you and your co-authors disagree with a criticism, provide the arguments to the reviewer (optionally, indicate the relevant points in the cover letter).

* If a criticism or suggestion is not addressed, please indicate so in the rebuttal to the reviewer comments and explain the reason(s).

* Consider including responses to any criticisms raised by more than one reviewer at the beginning of the rebuttal, in a section addressed to all reviewers.

* The rebuttal should include the reviewer comments in point-by-point format (please note that we provide all reviewers will the reports as they appear at the end of this message).

* Provide the rebuttal to the reviewer comments and the cover letter as separate files.

We hope that you will be able to resubmit the manuscript within 15 weeks from the receipt of this message. If this is the case, you will be protected against potential scooping. Otherwise, we will be happy to consider a revised manuscript as long as the significance of the work is not compromised by work published elsewhere or accepted for publication at *Nature Biomedical Engineering*.

We hope that you will find the referee reports helpful when revising the work, which we look forward to receive. Please do not hesitate to contact me should you have any questions.

Best wishes,

Pep

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Pep Pàmies
Chief Editor, *Nature Biomedical Engineering*

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Reviewer #1 (Report for the authors (Required)):

This manuscript describes a highly comprehensive set of experiments that demonstrate the effectiveness of peripheral focused ultrasound stimulation (pFUS) of the hepatic neural plexus in improving glucose homeostasis in diabetic animal models. Specifically, these experiments demonstrate:

1. pFUS prevents and reverses the onset of hyperglycemia in the Zucker Diabetic Fatty rat and in the diet-induced obesity rat models of type 2 diabetes.
2. pFUS improves glucose tolerance as indicated by glucose tolerance tests in diabetic rats.
3. pFUS modulated glucose uptake and utilization as demonstrated by hyperinsulinemic-euglycemic clamp techniques in diabetic rats and swine.
4. The effects of pFUS on glucose metabolism is medicated through neuronal pathways projecting from the liver through the IML and NTS, to the hypothalamus.
5. The effect of pFUS depends on activation of mechanically-activated ion channels.
6. pFUS induces specific transcriptomic and metabolomic changes consistent with improved glucose metabolism in type 2 diabetes.

General Comments
This manuscript describes fascinating experiments that suggest that pFUS may potentially be useful as a non-invasive, non-pharmacologic treatment for type 2 diabetes. The experiments appear to be properly conducted with inclusion of appropriate control experiments. The manuscript is very well written but contains some redundancies.

Major Comment
1. Daily pFUS applications were required to achieve chronic therapeutic effects in the diabetic animal models. Obviously, an experienced sonographer and specialized equipment would be needed to perform the stimulation every day in diabetic patients. Maybe the authors can speculate on how they envision to translate these fascination animal data to a chronic clinical application in diabetic patients.

Minor Comments
1. Due to the comprehensive set of experiments the manuscript is quite long. Maybe consider shortening the manuscript by removing some of the redundancies (e.g., some of the Fig. legends are identical to the text in the manuscript, some techniques are described multiple times if used in different experiments).
2. Figure legend to Fig. 1, line 3 from bottom: “sham” not “shame”.
3. Section “Outlook”, 2nd paragraph, last 2 sentences: Please avoid references to “future reports”.
4. Section “Image Target Identification Methods”, line 2: “… and position [of] the focused ultrasound transducer …”.
5. Methods Section, “Mechanical vs. Ultrasound Neuromodulation”. This section describes experiments related to the cholinergic anti-inflammatory pathway where the spleen was stimulated. While these experiments supposedly demonstrate the importance of the mechanical stimulus to initiate a physiologic response to pFUS, these experiments seem to be out of place, given that this manuscript is about glucose homeostasis and not inflammation.

Harald M. Stauss

Reviewer #2 (Report for the authors (Required)):

Cotero et l present a highly in depth study in which they demonstrate how focused ultrasound directed at the hepatic neural plexus can reverse hyperglycemia in models of type2 diabetes. There are many strengths to this study, including i) demonstrating reversal of hyperglycemia in multiple models of diabetes, including in different species; ii)in depth characterization of the changes in metabolism including glycemia, food intake,

glucose clamps for insulin sensitivity, circulating hormones. The investigators also perform a detailed characterization of relevant nucelli in the hypothalamus, including electrophysiology measurements, neurotransmitter/neuropeptide release and gene expression changes. The investigators also characterize the pathways involved (sympathetic afferents and vagal nerve) and the mechanoreceptive channel involved. Overall this represents an extremely thorough study that demonstrates a readily translatable approach that can robustly ameliorate diabetes, and
detailed characterization for how this reversal occurs in all peripheral and central tissues. I have a few minor comments that I believe could provide some improvements to this manuscript. However I would not characterize these as current weaknesses in the manuscript, rather ways to make the manuscript more accessible and to answer lingering questions.

1. Central to this whole study is targeting the hepatic neuro plexus with focused ultrasound. While this is described fairly well in figure S2, I think a bit more elaboration is needed. How exactly do they locate the portal vein consistently (e.g. position of probe). Where is the portal vein is the focused ultrasound applied, or is it applied over the whole vein? Were any differences in glucose lowering observed between the two methods employed?

2. I was wondering if some of the observations could result from changes in glucagon and insulin release. A). recent studies have discovered alpha cell and liver cross-talk (ie changes in amino acid utilization by the liver affects alpha cell glucagon release). Figure S10 D does reveal progressive decline in glucagon levels, albeit with substantial variability reducing any significance of the change. Nevertheless I think this is worth acknowledging. B) Decreases in insulin levels appear to occur prior to glycemia decreasing. This may be contributing to improved insulin sensitivity. This should be briefly discussed.

3. Data in 4B is unclear. Why was blockade in the NTS missing an equivalent sham NTS control group? While glucose elevates in the IML: group, this occurs under both sham and FUS cases so it is unclear if FUS has an effect. This is better illustrated in 4C with vagal manipulation.

4. Data in figure 7 presents transcriptional changes in different tissues following 7 weeks pFUS. However at this point glyemcia, plus insulin, glucagon, leptin, ghrelin etc are substantially different between treatment and sham, controls groups. Thus it is unclear how one can interpret these data -would these gene transcriotn changes be observed comparing sham untreated with a lean animal?. Data in figure 8 are a little more revealing given they including a 3 day time point where glycemia isn't substantially changed (although it is nice that many changes are conserved between 3 days and 7 weeks). Nevertheless do the authors have tissue from earlier time points hat can eb analyzed?

5. I think it would be helpful for the authors to provide a schematic, similar to that summarizing known data in figure S1B, that summarizes the authors data regarding the pathways activated by pFUS and how they may be responsible for reducing glycemia. For example it is unclear if the authors are proposing that activation of the hepatic neuroplexus activates afferent fibres of the vagal nerve or efferent fibres of the vagal nerve? What is the role of circulating factors vs altered sympathetic activation? This would be very useful for the reader who is not intricately knowledgeable about the different nuclei involved in metabolism and their corresponding pathways.

6. Figure S4 – numbers and statistics should be included here (even if numbers are the same as in figure 1)

Reviewer #3 (Report for the authors (Required)):

The article describes that noninvasive pulsed focused ultrasound (pFUS) stimulated the hepatoportal neuronal plexus and significantly improved glucose homeostasis via the brain-liver neuromodulation. The type 2 diabetes model in transgenic Zucker rats and high-fat diet plus low-dose STZ induced Sprague-Dawley (SD) rats and C57BL/6J mice, the type 1 diabetic model in high-dose STZ-induced SD rats, and normal swines were used for experimental pFUS treatments. The experimentally in vivo results demonstrated significant improvement in measures of hypothalamic insulin signalling and glucose utilization, associated with down-regulation of markers of the hypothalamic NPY pathway. The ex vivo study also showed the importance of the TRPA1-specific ion channel in the transduction of the ultrasound stimuli. The strong evidence supports the conclusion. The novelty and significance of the present study are high. Here are some comments for better readability.

1. In Fig 1. B.i, the glucose level increased once stopping pFUS stimulation (orange line). Does daily pFUS treatment for 20 days has no any effects on improvement of hyperglycemia?

2. In Fig 2, why were different models of Zucker rats and black C57BL/6J mice used for comparison of glucose tolerance?

3. In Fig 3, the data of glucose infusion rate (GIR) was significantly different only for DIO rats (p value < 0.05). Is the experimental number of T1D rats and swines too few to have statistic significance?

4. It's still unclear why and how pFUS stimulation improved hyperglycemia of T1D rats with severely STZ-damaged pancreas.

5. Please write the dimensions of hepatoportal neuronal plexus and discuss if the ultrasonic focal zone covers the nerve or acts other cells outside the nerve.

6. For the swine study, Is the ultrasound applicator a convex or concave type? Please specify it and discuss potential ultrasonic interaction at off-target tissue.

Thu 01 Feb 2022
Decision on Article nBME-21-2167B

Dear Chris,

Thank you for your patience in waiting for the feedback on your revised manuscript, "Focused Ultrasound Modulation of Hepatic Neural Plexus Restores Glucose Homeostasis in Diabetes". Having consulted with the original reviewers (whose comments you will find at the end of this message), I am pleased to write that we shall be happy to publish the manuscript in *Nature Biomedical Engineering*, provided that the points specified in the attached instructions file are addressed.

When you are ready to submit the final version of your manuscript, please upload the files specified in the instructions file.

Also, please address the minor points of Reviewer #2, and provide a point-by-point reply.

For primary research originally submitted after December 1, 2019, we encourage authors to take up transparent peer review. If you are eligible and opt in to transparent peer review, we will publish, as a single supplementary file, all the reviewer comments for all the versions of the manuscript, your rebuttal letters, and the editorial decision letters. **If you opt in to transparent peer review, in the attached file please tick the box 'I wish to participate in transparent peer review'; if you prefer not to, please tick 'I do NOT wish to participate in transparent peer review'.** In the interest of confidentiality, we allow redactions to the rebuttal letters and to the reviewer comments. If you are concerned about the release of confidential data, please indicate what specific information you would like to have removed; we cannot incorporate redactions for any other reasons.
More information on transparent peer review is available.

Please do not hesitate to contact me should you have any questions.

Best wishes,

Pep

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Pep Pàmies
Chief Editor, *Nature Biomedical Engineering*

P.S. Nature Research journals encourage authors to share their step-by-step experimental protocols on a protocol-sharing platform of their choice. Nature Research's Protocol Exchange is a free-to-use and open resource for protocols; protocols deposited in Protocol Exchange are citable and can be linked from the published article. More details can be found at www.nature.com/protocolexchange/about.

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Reviewer #1 (Report for the authors (Required)):

My only major comment in response to the original submission was related to translational hurdles that may be encountered during testing of a pFUS-based therapy in the clinic. The authors have added a paragraph to the Discussion addressing these translational hurdles and, importantly, outlined some potential ways and technologies to overcome these hurdles. I just wished they had used the word "may" instead of "will" in the last sentence of this paragraph that reads: "These advances will enable the development of novel wearable ultrasound systems that can be applied by unskilled users, and further enable use across clinical applications and settings." The authors also addressed all minor comments listed in my original review.

Reviewer #2 (Report for the authors (Required)):

The authors have nicely addressed most of my prior comments related to their already impressive study. However i do have a lingering concern related to my previous point 3 that focused on data presented in figure 4B where there is an absence of a sham NTC lidocaine injection.

If one focuses on the IML treatment, comparing Sham-control and Sham-IML it appears that lidocaine injection into IML elevated glucose levels during OGTT. When comparing pFUS IML with pFUS no block groups, glucose is elevated by a similar amount. Thus one may argue the Lidocaine injection into the IML elevates glucose whether pFUS is present or not; and thus could be independent. Is this a fair comment?

When examining the lidocaine injection into NTS one cannot make such an assessment as there is no Sham-NTS group. If lidocaine injection into the NTS substantially elevated glucose itself, irrespective of pFUS then this may suggest lack of interaction between the lidocaine administration and pFUS. However if there was not such an elevation this would be more supportive of the authors conclusions. So it is unclear.

The authors indicate that their animal care and use committee would not approve a sham-NTS group and i do not suggest they try and repeat these experiments. However some acknowledgement may be needed.

Further, the authors state in the caption for figure 4 "Lidocaine at the IML in the absence of pFUS (black circles) did not yield any significant change in circulating glucose as compared to standard ZDF sham treated animals (dark blue circles)." but this is not consistent with what is presented where a difference is apparent. What statistical analyses were used to make this assessment? (and also those in panel C)? These should be indicated.

Reviewer #3 (Report for the authors (Required)):

No further comments.

Rebuttal 1

The authors thank the editor and reviewers for comments meant to improve the manuscript; we've included a point-by-point response to each comment below. In addition, please also find yellow highlights (on main text or caption figures) in the revised manuscript to help reference where the manuscript was changed to address each point.

Editor Comments:

1) Revised manuscript to include "Discussion of the main translational bottlenecks for this therapeutic strategy to restore glucose homeostasis."

The revised manuscript includes two additions in the discussion section, which describe translational hurdles that may be encountered during testing of a pFUS based therapy in the clinic (highlighted in the revision text and included below).

"Despite the non-invasive nature of pFUS stimuli and potential benefits compared to traditional implant-based approaches to neuromodulation, some translational challenges will need to be addressed for clinical use. Currently, ultrasound imaging is practiced by technicians and physicians, trained on manual handling and use of the ultrasound probe and system software to achieve quality images and image-based diagnostic measurements. Unlike pharmaceutical treatments or implant-based therapies (both of which enable therapy to take place at home and without supervision), ultrasound neuromodulation tools based on current clinical systems would hinder at home use, daily application of the therapy, or cost-effective clinical testing across large cohorts of patients. However, low-cost wireless ultrasound systems are now available commercially,[112] wearable ultrasound probes have minimized the need for manual handling of the probe during use,[113] and automated anatomical target detection software is now available to enable target tracking in real-time using convolution neural-network models.[114] These advances will enable the development of novel wearable ultrasound systems that can be applied by unskilled users, and further enable use across clinical applications and settings. "

"Ultimately, the efficacy, safety, and duration of glycemic reduction found using a pFUS treatment in clinical trials, compared to current pharmaceutical treatments for T2D, will dictate utility in clinical practice. "

2) Revised manuscript to include "Thorough methodological detail, as per the questions and suggestions of the reviewers (and, in particular, points #1 and #5 from Reviewer #2 and points #5 and #6 from Reviewer #3)."

Please find responses/edits based on each methodological query raised by the reviewer below; each point has been corrected in the revised manuscript and highlighted in yellow.

3) Altered formatting to figures to include "Also, please do enlarge and better lay out the main figures (you can use the full-page width), so that the data can be more easily interpreted. In particular, most of the axes displaying glucose concentrations could be elongated so as to allow for easier visualization of concentration differences. Moreover, we allow for up to 10 Extended Data figures, and I suggest that the current Supplementary Figs. 9–13 would be most useful as such."

All figures have been changed to full-page width to enable better reading and interpretation (which also enabled elongation of axes displaying glucose concentrations from the widths shown in the original manuscript). Previous supplemental figures 9 – 13 have been separated into an Extended Data figure section, and the remaining supplementary figures remain in the supplement file.

Reviewer #1
We thank the reviewer for the supportive comments and enthusiastic summary comments. Please find the following point-by-point edits made to address/adopt each suggestion to improve the manuscript further.

1) Daily pFUS applications were required to achieve chronic therapeutic effects in the diabetic animal models. Obviously, an experienced sonographer and specialized equipment would be needed to perform the stimulation every day in diabetic patients. Maybe the authors can speculate on how they envision to translate these fascination animal data to a chronic clinical application in diabetic patients.

We added a paragraph in the discussion section, which outlines potential challenges to translation (including eliminating the need for skilled users/sonographer). It is included above in the answer to editor comment #1 and highlighted in yellow in the text. In short, wireless and wearable probes already exist that enable low-cost devices to be used without the need to manually handle the ultrasound probe. Furthermore, our group and others have demonstrated the use of convolutional neural networks to automatically detect and track (in real time) anatomical targets, which further eliminates the need of a trained user to identify and target for ultrasound stimuli. Our group is already implementing these tools in clinical pFUS and demonstration trials.

2) Due to the comprehensive set of experiments the manuscript is quite long. Maybe consider shortening the manuscript by removing some of the redundancies (e.g., some of the Fig. legends are identical to the text in the manuscript, some techniques are described multiple times if used in different experiments).

A significant amount of redundant information has been removed from several figure captions; those caption edits are highlighted in yellow in the revised manuscript.

3) Figure legend to Fig. 1, line 3 from bottom: “sham” not “shame”.

Thank you for catching this; the error has been corrected and highlighted in the revised manuscript.

4) Section “Outlook”, 2nd paragraph, last 2 sentences: Please avoid references to “future reports”.

This reference to “future reports” has been eliminated from the text.

5) Section “Image Target Identification Methods”, line 2: “… and position [of] the focused ultrasound transducer …”.

Thank you; this addition has been made in the revised text.

6) Methods Section, “Mechanical vs. Ultrasound Neuromodulation”. This section describes experiments related to the cholinergic anti-inflammatory pathway where the spleen was stimulated. While these experiments supposedly demonstrate the importance of the mechanical

stimulus to initiate a physiologic response to pFUS, these experiments seem to be out of place, given that this manuscript is about glucose homeostasis and not inflammation.

We respectfully would like to keep this figure in the supplement. We believe that it further supports the mechanical mechanism of action of the pFUS stimulus, and the motivation for the TRPA1 blocking data/experiments within the manuscript (a mechanically sensitive ion channel). However, we have added further description to the caption in supplemental figure S10:

"We have previously demonstrated use of pFUS to activate the splenic cholinergic anti-inflammatory pathway. In this figure, we demonstrate that pulsed mechanical ultrasound and direct mechanical pulsed stimuli (at similar pulse repetition frequencies) result in nerve activation and neuromodulation of CAP. This data enabled focused investigation of ion channel blockers that have previously been shown to inhibit channels affected by mechanical stimuli (such as TRPA1)."

This additional description in the caption outlines how the data is relevant to this manuscript, as it enabled focused investigation of mechanically sensitive ion channels in figure 6 D and E.

Reviewer #2:
We thank the reviewer for the summary and enthusiasm around the data sets included in the original manuscript. Please find the following point-by-point edits made to address/adopt each suggestion to improve the manuscript further.

1. Central to this whole study is targeting the hepatic neuro plexus with focused ultrasound. While this is described fairly well in figure S2, I think a bit more elaboration is needed. How exactly do they locate the portal vein consistently (e.g. position of probe). Where is the portal vein is the focused ultrasound applied, or is it applied over the whole vein? Were any differences in glucose lowering observed between the two methods employed?

We have further detailed our ultrasound targeting procedures, including the image-based anatomical landmark used to identify the position of the porta hepatis, and the stand utilized to maintain fixed height of the ultrasound transducer (and thus depth of focus in the animals). These additions are found in the methods section "Peripheral focused ultrasound stimulation in ZDF and DIO models", highlighted in yellow, and included below:

"Passage of the hepatic and portal artery through the target region and localization in the central intraperitoneal fissure of the liver allowed for ready identification of the porta hepatis under ultrasound imaging. This target was visualized as the entrance of the portal vein (i.e., closest imaging plane in which the portal vein was observed prior to entrance into the liver). The target location was then marked with a permanent marker and a pFUS stimulation probe was placed on the target area (represented in Figure 1A) for application of either 3 minutes of ultrasonic stimulus (ZDF-pFUS; 1.1 MHz, 200mV per pulse, 150 burst cycles, 500 µs burst period) or Sham stimulus where no energy was applied through the transducer up to once a day. Although not statistically compared, there was no observed differences in glucose reduction in pilot animals treated using image-guidance for every stimulus, and animals in which the ultrasound transducer stand-off was simply placed above the previously marked target on the skin. The depth of the probe with reference to the animal was held constant using a metal stand (holding the focused ultrasound transducer at a fixed height above the laboratory table)."

2. I was wondering if some of the observations could result from changes in glucagon and insulin release. A). recent studies have discovered alpha cell and liver cross-talk (ie changes in amino acid utilization by the liver affects alpha cell glucagon release). Figure S10 D does reveal progressive decline

in glucagon levels, albeit with substantial variability reducing any significance of the change. Nevertheless, I think this is worth acknowledging. B) Decreases in insulin levels appear to occur prior to glycemia decreasing. This may be contributing to improved insulin sensitivity. This should be briefly discussed.

We thank the reviewer for suggesting that we make it clear that although our data shows that liver-hypothalamic sensory pathways are important for the pFUS effect, it does not rule out additional contributing mechanisms and effects. We've added the following sentence to the discussion, highlighted the addition in yellow in the revised text, and added the reference/citation suggested by the reviewer.

"In addition, while the nerve resection and lidocaine blocking data (figure 4) clearly demonstrate the importance of hypothalamic sensory pathways on the effect of hepatoportal pFUS, our work does not yet rule out other contributing effects, including activation of neural reflex or neuroendocrine mediated effects between the liver and other organs (such as the pancreas[117])."

3. Data in 4B is unclear. Why was blockade in the NTS missing an equivalent sham NTS control group? While glucose elevates in the IML: group, this occurs under both sham and FUS cases so it is unclear if FUS has an effect. This is better illustrated in 4C with vagal manipulation.

Figure 4 B and C show data from two different methods of "blocking" the sensory nerve pathway between the liver and hypothalamus (i.e., nerve resection and lidocaine blocking). For lidocaine, blocking at the NTS is known to block both sympathetic and vagal pathways, while blocking at the IML more specifically blocks sympathetic or spinal pathways (as referenced in our original text). For nerve resection, cutting at the hepatic vagus blocks vagal pathways, while cutting at the dorsal root ganglia block sympathetic or spinal pathways.

The intent of figure 4B and C was to test whether spinal, vagal, or both pathways contributed to the pFUS effect. In figure 4B, pFUS IML reduced glucose levels in the late stage of the OGTT compared to sham IML, but did not achieve the glucose lowering shown using pFUS with no block. In figure 4C, pFUS VgX reduced glucose levels in the late stage of the OGTT compared to sham VgK, but did not achieve the glucose lowering shown using pFUS with no block. Additional data in each plot showed that the pFUS effect is further reduced when performing block of both the vagus and spinal pathways (i.e., either NTS or VgX + DrGX cohorts). This combined data set supports that multiple (i.e., vagal and spinal) pathways contribute to the hepatoportal plexus pFUS effect.

[redacted]

4. Data in figure 7 presents transcriptional changes in different tissues following 7 weeks pFUS. However, at this point glycemia, plus insulin, glucagon, leptin, ghrelin etc are substantially different between treatment and sham, controls groups. Thus it is unclear how one can interpret these data - would these gene transcript changes be observed comparing sham untreated with a lean animal? Data in figure 8 are a little more revealing given they include a 3 day time point where glycemia isn't substantially changed (although it is nice that many changes are conserved between 3 days and 7 weeks). Nevertheless, do the authors have tissue from earlier time points hat can eb analyzed?

The team did take all samples that were allowed by project budget and time restrictions, and unfortunately no more samples/data are available for this set. However, as previously described in the text, the data clearly shows improvement across multiple organ systems, and metabolic markers (i.e., glycemia, hormone, leptin, ghrelin, etc; as described by the reviewer). As both the ZDF and DIO animals are common models, several previous studies exist that clearly show that improvement in these markers would not be expected in sham/untreated animals. These references have now been added to the main text in the discussion section:

"Our transcriptomic and metabolomic data sets (Fig. 7 and 8, and Suppl. Fig. 12-20) clearly demonstrate pFUS-induced shifts in overall metabolism that would not be expected in the T2D models without treatment,[92] including improvements in fatty acid, cholesterol, vitamin, and bile acid metabolism. Whether each of these improvements is a direct result of pFUS neuromodulation of nerve pathways that directly affect fat, cholesterol, vitamin, and bile acid metabolism, or these observed effects are simply an indirect result of improved glycemic control and hormone levels remains an important question for further investigation."

5. I think it would be helpful for the authors to provide a schematic, similar to that summarizing known data in figure S1B, that summarizes the authors data regarding the pathways activated by pFUS and how they may be responsible for reducing glycemia. For example, it is unclear if the authors are proposing that activation of the hepatic neuroplexus activates afferent fibres of the vagal nerve or efferent fibres of the vagal nerve? What is the role of circulating factors vs altered sympathetic activation? This would be very useful for the reader who is not intricately knowledgeable about the different nuclei involved in metabolism and their corresponding pathways.

Respectfully, the authors believe that since there were no direct experiments contained in the manuscript that tested for "efferent versus afferent" contribution to different metabolic effects of hepatoportal pFUS, a schematic describing the potential contributions of each would be speculative. The hepatoportal plexus pathways have been shown to be "connected" to nearly every metabolic organ, and the experiments required to test for every potential efferent effect (i.e., all those originating from the hypothalamic nuclei and activated by liver pfUS) are beyond the scope of this manuscript. However, the authors agree with the reviewer that additional guidance on the pathways that are currently known to exist (that is, sensory versus efferent pathways that are affected by the hepatoportal plexus glucose sensors) would be informative for the readership. We have therefore added supplemental table 1, which outlines the liver-brain (or hepatoportal plexus) pathways that are known to exist (right most column) and effect each metabolic organ system (each row). In addition, we include information on the overlap between each of those nerve pathways and the known T2D disease defects and current pharmaceutical treatments. The addition of the overlap between the known hepatoportal/liver-brain pathways and organ systems/mechanisms of action enables the reader to understand the potential interplay between purely nerve-mediated versus neuroendocrine effects of pFUS.

6. Figure S4 – numbers and statistics should be included here (even if numbers are the same as in figure 1)

We have added animal numbers used, p-values, and statistical test information to the caption in supplemental figure S4.

Reviewer #3:
We thank the reviewer for the supportive summary description and suggestions to improve readability, which we have addressed/adopted in the following responses.

1. In Fig 1. B.i, the glucose level increased once stopping pFUS stimulation (orange line). Does daily pFUS treatment for 20 days have no effects on improvement of hyperglycemia?

The authors respectfully guide the reviewer to the paragraph in the original manuscript which clearly describe the effect of stimulation for 20 days, and then the washout period of ~ 3 days.

"Daily pFUS (figure 1B) prevented the onset of hyperglycemia, attenuated hyperinsulinemia compared to sham treated controls, and maintained blood glucose at levels comparable to non-diabetic animals in the ZDF rats (Fig. 1 B.i., n=12; Sprague Dawley (SD) naïve). Following 20 days of treatment, a sub-set of treated and sham cohorts were interchanged (Fig. 1 B.i.). pFUS decreased circulating glucose in the severely hyperglycemic animals (previous sham controls), while cessation of treatment resulted in onset of hyperglycemia within three days."

The authors add that wash-out periods of even the most potent anti-diabetic drugs (i.e., liraglutide) are typically 3-5 days in clinical cross-over studies. Thus, while wash-out of the effect clearly demonstrates that pFUS is responsible for the glucose reduction demonstrated, it in no way suggests a future limitation of a pFUS therapy.

2. In Fig 2, why were different models of Zucker rats and black C57BL/6J mice used for comparison of glucose tolerance?

There is no perfect rodent model of human T2D, and therefore it is typical (and even encouraged) to use multiple models to test novel therapies. The Zucker and DIO models represent two of the most common models used to test new drug candidates for T2D and are therefore among the best models to demonstrate a completely new therapeutic modality (as ample data is available in literature to compare drug effects to pFUS effects in these models). Furthermore, we used a third model (western diet mouse model; figure 2 B) to demonstrate the effect in multiple species and provide evidence that the pFUS effect shown were not specific to one species.

3. In Fig 3, the data of glucose infusion rate (GIR) was significantly different only for DIO rats (p value < 0.05). Is the experimental number of T1D rats and swines too few to have statistical significance?

Additional swine experiments have been completed and added to the manuscript. We have replaced the previous data (which did not contain matched shams for each stimulated animal, and only included two sham animals) with a final data set performed in 5 animals that each had a sham and pFUS procedure performed (i.e., 10 matched experiments). Please find the new data in figure 3c, including the new statistical analysis and achievement of p=0.04 (at n=5), in the revised manuscript.

4. It's still unclear why and how pFUS stimulation improved hyperglycemia of T1D rats with severely STZ-damaged pancreas.

Please find the description of the insulin-independent pathway previously shown to enable increased glucose uptake into skeletal muscle and the significant evidence in figure 1 that hepatoportal pfUS activates this pathway.

"Previous studies using invasive portal glucose infusions to activate the hepatoportal plexus have demonstrated transient enhancement of muscle glucose uptake.[34,41,42] In these previous studies, enhanced glucose uptake in skeletal muscle was mediated by an insulin-independent/AMPK-dependent pathway, resulting in activation of GLUT4.[41] Consistent with these previous observations, skeletal muscle samples from pFUS treated cohorts in the ZDF model demonstrated an increase in AMPK and GLUT 4 activity of 35.8±9.6% and 65.7±20.2% respectively, compared to sham controls (Fig. 1E; n=5, P<0.05). Glycogen content of the terminal muscle samples was also altered after 4 weeks of daily pFUS, resulting in an increase of 91±22.3% (Fig. 1.E.) in the pFUS treated ZDF animals compared to sham controls. Unlike skeletal muscle, autonomic regulation of glucose uptake in the liver has been previously shown to affect the temporal variability of glycogen synthesis and gluconeogenesis, but not long-term glycogen content.[43–46] Subsequent reports have shown that this is due to the liver's inherent autoregulatory mechanisms for glucose uptake and the interaction of nerve-mediated regulatory mechanisms with insulin and glucagon signaling.[43] In this initial experiment, we could not rule out transient effects of pFUS on glucose uptake, storage, or production in liver. However, the terminal samples collected did not show significant change in hepatic GLUT2 or AMPK activity, or glycogen content (Fig. 1.E)."

Thus, a clear mechanism for insulin-independent glucose clearance was previously provided and described. Note that this is further described in the discussion section:

"Other pharmaceutical-based hypothalamic neuromodulation therapies have also recently demonstrated dependence on an intact insulin pathway to achieve long-lasting versus transient effects on glucose utilization,[112,113] suggesting that the hypothalamic nerve pathways modulate and refine, but do not replace tissue response to insulin. These results in the T1D model are informative, but do not take away from the potential impact that pFUS may have in T2D models and applications."

5. Please write the dimensions of hepatoportal neuronal plexus and discuss if the ultrasonic focal zone covers the nerve or acts other cells outside the nerve.

The following text has been added to the methods section to adequately describe the width and depth of the ultrasound focal zone vs. the porta hepatis anatomical target: (located in methods section: 1.1-MHz Single-element Focused Ultrasound System (Rodent Models))

"The simulated pressure profile has a full width half maximum amplitude of 1.8 mm laterally and 6 mm in the depth direction using Field II[114,115] (Fig S3A). The -6dB diameters at the peak were measured to be 1.45 (+/- 0.02) mm in the X scan axis and 1.46 (+/- 0.02) mm in the Y scan axis at the accredited laboratory. The width and depth of the ultrasound focal zone adequately covered the width and depth of the porta hepatis (i.e., size of the portal vein and hepatic artery at the entrance to the liver).[120]"

6. For the swine study, Is the ultrasound applicator a convex or concave type? Please specify it and discuss potential ultrasonic interaction at off-target tissue.

The word convex has been added to the title of the GE LOGIQ E10 description section in the methods section, to ensure the reader knows the configuration of the cited and commercially available probe used in the swine studies.

GE LOGIQ E10 and C1-6 (Convex) Probe (Swine Model)

Rebuttal 2

Response to Referees for: "Focused Ultrasound Modulation of Hepatic Neural Plexus Restores Glucose Homeostasis in Diabetes".

We thank the editor for the invitation to publish, pending response to the final points raised by the referees. Below we have listed the final edits (below in bold; with edits in yellow highlight) made in response to those referee comments.

Reviewer #1:
"My only major comment in response to the original submission was related to translational hurdles that may be encountered during testing of a pFUS-based therapy in the clinic. The authors have added a paragraph to the Discussion addressing these translational hurdles and, importantly, outlined some potential ways and technologies to overcome these hurdles. I just wished they had used the word "may" instead of "will" in the last sentence of this paragraph that reads: "These advances will enable the development of novel wearable ultrasound systems that can be applied by unskilled users, and further enable use across clinical applications and settings." The authors also addressed all minor comments listed in my original review."

In the conclusion:

"These advances may enable the development of novel wearable ultrasound systems that can be applied by unskilled users, and further enable use across clinical applications and settings."

The word "may" was added per comment by reviewer #1.

Reviewer #2:
"The authors have nicely addressed most of my prior comments related to their already impressive study. However, i do have a lingering concern related to my previous point 3 that focused on data presented in figure 4B where there is an absence of a sham NTC lidocaine injection.

If one focuses on the IML treatment, comparing Sham-control and Sham-IML it appears that lidocaine injection into IML elevated glucose levels during OGTT. When comparing pFUS IML with pFUS no block groups, glucose is elevated by a similar amount. Thus one may argue the Lidocaine injection into the IML elevates glucose whether pFUS is present or not; and thus could be independent. Is this a fair comment?

When examining the lidocaine injection into NTS one cannot make such an assessment as there is no Sham-NTS group. If lidocaine injection into the NTS substantially elevated glucose itself, irrespective of pFUS then this may suggest lack of interaction between the lidocaine administration and pFUS. However if there was not such an elevation this would be more supportive of the authors conclusions. So it is unclear.

The authors indicate that their animal care and use committee would not approve a sham-NTS group and i do not suggest they try and repeat these experiments. However some acknowledgement may be needed.

Further, the authors state in the caption for figure 4 "Lidocaine at the IML in the absence of pFUS (black circles) did not yield any significant change in circulating glucose as compared to standard ZDF sham treated animals (dark blue circles)." but this is not consistent with what is presented where a difference is apparent. What statistical analyses were used to make this assessment? (and also those in panel C)? These should be indicated."

In the caption to Figure 4:

B. Microinjection of lidocaine into the nucleus of the solitary tract (NTS) abolished the effects of pFUS stimulus on blood glucose during GTT (ZDFs; light blue squares), as compared to pFUS alone (red triangles). In contrast, lidocaine injection into the intermediolateral (IML) nucleus attenuated, but did not completely block, the effects of pFUS (pink inverted triangles). Lidocaine injection at the IML (black diamonds) resulted in increased glucose excursion (AUC: 31,443) compared to ZDF sham treated animals without block (AUC: 26,087; p<0.057). No sham NTS experiments were performed. C. Hepatic vagotomy and dorsal root ganglion resection at the T1 vertebrae (DRGX/VGX) abolished the effects of pFUS on blood glucose during GTT in ZDFs (brown boxes), as compared pFUS alone (red dotted line; replotted from 4.C.). In contrast, vagotomy alone (VGX) attenuated, but did not completely block the effects of pFUS (yellow inverted triangles (AUC: 37,336) vs. gray circles (AUC: 42,074); p<0.19). Hepatic vagotomy in the absence of pFUS (gray circles) led to an increase in circulating glucose as compared to standard ZDF sham treated animals (dark blue dotted line; replotted from 4.C. for comparison).

Details on statistical comparisons were added, as well as acknowledgement that no sham NTS experiments were performed, per comment by reviewer #2.

Reviewer #3:

"No further comments."

Reviewer 3 required no further edits.