

Non-damaging retinal laser treatment with ERG-based thermal dosimetry activates hormetic heat response in pig RPE in vivo

Corresponding Author: Professor Ari Koskelainen

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

A version of this paper was originally rejected for publication by Nature Communications, however that decision was reconsidered after appeal by the authors.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In their article, the authors describe fERG-controlled SRT laser treatment and its effect in vivo in pigs eyes. The study is of high interest, however, some aspects have to be addressed before publication can be considered.

Major

The authors have to clearly explain what distinguishes this paper from the one they have already published, establishing the method presented here (IEEE Trans Biomed Eng. 2022 Feb;69(2):991-1002. doi: 10.1109/TBME.2021.3111533.)

The authors seem to be oblivious to studies of other researchers on the field of SRT and their results, as seen both in the introduction and in the discussion.

Introduction

Line 65, "thermal dosimetry for retinal laser treatment is currently unavailable", there has been quite some work on optoacoustics for controlling SRT in the retina (e.g. Photoacoustics. 2021 Nov 10;25:100316. doi: 10.1016/j.pacs.2021.100316.). It should be mentioned here and compared to the method presented here in the discussion. What is better and why?

Line 59 ff, the temperature-dependent expression of Hsp70 has been shown for the porcine retina before with different temperature dependencies as found here (Exp Eye Res. 2018 May;170:117-126. doi: 10.1016/j.exer.2018.02.013). This should be mentioned and again, discussed in the discussion why the temperatures differ between these studies.

Methods

Line 219 "A retinal area was selected", was the area the same for all treated eyes? This might be of importance as pigs have an area centralis with a high cone percentage, where RPE cells may react differently from areas with a higher rod density.

Lines 264-265, "4-6 non-treated samples were collected from each eye", how far away were these samples from the treatment area? Were also not-treated eyes used as controls to rule out any off-target effect of the treatment?

Lines 278-279, was the signal quantified? How? Did the authors quantify the area of expression? Was it consistent with the area of treatment or did they see off-target effects?

Line 280, please state in the methods the positive control used.

Line 303, ACTB was used as control, however, a recent publication showed for inflammatory activation that ACTB was changed by the stimulus (doi: 10.1016/j.exer.2023.109469), did the authors check that their controls were not changed by

temperature?

Line 334 following, please include sample size (flatmounts, molecular testing) in paragraph on statistics.

Results

Line 378, “number of treatments per eyes ranging from 2-6”, are the authors sure that the treatments do not have any additive effects on the RPE?

Line 396 “heated areas”, I would strongly suggest to quantify the areas affected.

Line 435, if there is discussion in the result section, the work of other authors investigating the effect of temperature on the hsp expression in the RPE needs to be included.

Line 473, 489, why are significances not shown in the graphs of the respective figures?

Several figures, the different lanes on the Western Blot images are not defined. What are we seeing here?

Discussion

As pointed out above, a discussion concerning previous studies by other authors is missing, e.g. the comparison of this method with opto-acoustics or the differences between this study and other studies investigating RPE temperature and hsp70 expression.

In addition to what was already pointed out above:

Line 582, there is no mentioning of other studies which have investigated VEGF secretion in porcine RPE/choroid cultures after SRT (e.g. doi: 10.1167/iov.17-23163) or that have investigated the effect of temperature on the release of VEGF in porcine RPE cells (doi: 10.1007/s00417-014-2750-z.). Please compare your results with what has already been published and discuss the differences.

Line 589, “we demonstrate the feasibility of fERT-based thermal dosimetry for STLT in vivo”, didn’t the authors already prove this in a previous paper (doi: 10.1109/TBME.2021.3111533.)?

Minor

Line 129, there is a dot missing at the end of the sentence.

Reviewer #2

(Remarks to the Author)

Overview:

The results of this study are interesting and useful, as they corroborate and add to prior studies on the thermodynamics and proteomics of retinal laser treatment, but also demonstrate the utility of ERG to monitor laser effects. A major weakness is that concepts that the authors use to provide context and justification for their approach are dated. In short, the authors present ERG monitoring as useful because of the narrow therapeutic range of CW laser and the difficulty otherwise of performing “subthreshold” laser treatment and avoiding retinal damage with CW laser. The issue is that, for this reason, retina-sparing treatments have abandoned CW and gone to micropulsed laser modes that are extremely safe and predictable due to extremely wide therapeutic ranges (TR), rendering the clinical utility of ERG monitoring as presented here, much like opto-acoustic real-time monitoring, clinically unnecessary.

More specific comments:

The authors do not discriminate between the various modes of “subthreshold” retinal laser treatment, so diverse as to make the term meaningless. “Subthreshold” needs to be clearly qualified with the laser mode being employed (conventional CW, short pulse CW, nanosecond CW, microsecond pulsed, etc), and the level of scrutiny. In other words, “subthreshold” to what? Detection by the naked eye? FFA? FAF? OCT? RF photos? Histology? Electron microscopy? Biomicroscopy? Absent such defining terms, “subthreshold” has no meaning.

The authors describe the therapeutic range (TR) of “subthreshold” laser as dangerously narrow (true), thus indicating the need for the author’s technology (not true). They should note that the TR of CW laser, the laser mode they used in this study, is indeed extremely narrow (0.010 W in breadth). But they importantly fail to note that the TR for microsecond pulsed lasers can be vast – 15 watts or more – precluding the need for thermal (or any other) real-time temperature monitoring, including functional monitoring with ERG. The fact that laser treatment within the very wide microsecond pulsed TR using fixed (non titrated) laser parameters in all patients that is both effective and safe is supported by a vast amount of clinical and experimental research and publications. Along these lines, the authors state that titration of laser exposures is state of the art. It is not. Titration is an anachronism associated with use of CW lasers for retinal photocoagulation. Keunen JEE, Battaglia-Parodi M, Vujosevic S, Luttrull JK. International Retinal Laser Society Guidelines for Subthreshold Laser Treatment. Transl Vis Sci Technol. 2020 Aug 7;9(9):15. doi: 10.1167/tvst.9.9.15. eCollection 2020 Aug. PMID: 32879771

Following on the above, the individual variations in eyes in such as pigment density, choroidal perfusion, etc, while possibly important in CW retinal laser treatment, are inconsequential with very large TRs such as can be obtained with pulsed laser

modes. Thus, eye specific variations are also no longer a consideration requiring treatment intensity adjustment such as that which might be aided by ERG. Again, this is because of the extremely wide TRs achievable with microsecond pulsed lasers. Chang DB, Luttrull JK. Comparison of subthreshold 577nm and 810nm micropulse laser effects on heat-shock protein activation kinetics: Implications for treatment efficacy and safety. *Transl Vis Sci Technol*. 2020 Apr 28;9(5):23.doi: 10.1167/tvst.9.5.23. eCollection 2020 Apr.

The authors state that their tech helps achieve “personalized” treatment, as if personalized treatment is a good and desirable thing. It is quite the opposite. The ideal is one simple treatment that works for all people and all problems all the time, not turning each individual into a different disease. The concept of personalized medicine is, while popular, absurd.

Finally, it is not reasonable to suggest that real-time ERG monitoring to accomplish retina-sparing laser treatment may have clinical application. First, as discussed above, it is wholly unnecessary. Further, the author’s themselves note that laser has been shown to benefit many common problems that affect millions of patients, often requiring regularly repeated treatments for years on end. How would they imagine this technology being used routinely in clinical practice for these hundreds of millions of potential retinal laser treatments around the world? One cannot. The technology the authors report is an interesting research tool, but has no indication or application clinically.

I recommend the authors re-cast this otherwise interesting study as a lab study of laser thermal proteomics and eliminate their discussion of clinical laser treatment and references to any clinical need or application for this technology. An interesting follow up study would be to repeat this study with low-duty cycle microsecond pulsed laser parameters known to be safe and effective in all eyes, to show why ERG monitoring is unnecessary.

Additional important references:

Caballero S, Kent DL, Sengupta N, et al. Bone marrow derived cell recruitment to the neurosensory retina and retinal pigment epithelial cell layer following subthreshold retinal phototherapy. *Invest Ophthalmol Vis Sci*. 2017;58:5164e76

Frizziero L, Calciati A, Midena G, et al. Subthreshold Micropulse Laser Modulates Retinal Neuroinflammatory Biomarkers in Diabetic Macular Edema. *J Clin Med*. 2021;10(14):3134. Published 2021 Jul 15. doi:10.3390/jcm10143134

Midena E, Bini S, Frizziero L, Pilotto E, Esposito G, Micera A. Aqueous humour concentrations of PEDF and Erythropoietin are not influenced by subthreshold micropulse laser treatment of diabetic macular edema. *Biosci Rep*. 2019;39(6):BSR20190328. Published 2019 Jun 18. doi:10.1042/BSR20190328

Midena E, Micera A, Frizziero L, Pilotto E, Esposito G, Bini S. Sub-threshold micropulse laser treatment reduces inflammatory biomarkers in aqueous humour of diabetic patients with macular edema. *Sci Rep*. 2019;9(1):10034. Published 2019 Jul 11. doi:10.1038/s41598-019-46515-y

Respectfully,
Jeffrey K Luttrull, MD

Reviewer #3

(Remarks to the Author)

The manuscript well described biological reactions after controlled temporal (60s) laser induced retinal temperature increase in pigs. The temperature was determined with focal electroretinography (fERG)-based temperature monitoring during laser exposure. This is a very acute and relevant topic, since retinal laser treatments, especially hyperthermic sub-damaging irradiations so far have no dosing endpoints. The manuscript focusses on the investigation of RPE and retina tissue and investigated in detail markers for oxidative stress, structural features and apoptosis, as well as heat shock protein and gene expression. Very interesting results were achieved and discussed. The manuscript should be published after some revisions addressed below.

The manuscript is thoroughly written with respect to all biological reactions including the investigation towards the damage threshold. What is less good described are the physical and fERG aspects with respect to the temperature measurement. Even though I acknowledge that this is not the topic of the manuscript and references for more details are presented, nevertheless the reader needs to know some more details, since all aspects investigated here rely on the temperature rise. Thus a proper description how and in which layer the temperature is obtained and how it was calibrated is necessary, and especially the fERG temperature response value of 3.7%/C. These aspects need to be better described for the reader, however, its doesn’t mean to go too deep into the mathematics, but a verbal clear description would be very helpful.

Some points to be considered in this context:

Title:

1. “subthreshold”; which threshold is meant? I suggest to write non-damaging or sub-damaging or hyperthermal or another unambiguously word. Further also super-threshold experiments are performed, so this word can also be omitted.

Introduction:

2. How could the method be beneficial for the treatment of AMD as mentioned in line 89? Which stage of AMD? And is the fERG signal not altered by a non-healthy neural retina?
3. There are also other temperature controlled retinal laser treatments under investigation, in open and closed control settings and approved in clinical trials already. This should at least be partly mentioned in this context.

Material and methods:

4. Why is a wavelength of 810 nm been chosen, even though this is only very rarely used in retinal laser treatments due to its low absorption of melanin leading to a brought and deep heating into the choroid rather than just the RPE and its surroundings? In my opinion a green wavelength would be much better suited and would require much less power by avoiding choroidal heating.
5. Did you carefully check, whether coagulation in the choroid was avoided?
6. How homogeneous was the beam profile with respect to top hat or Gaussian, and spatial modulation? This is essential if such a large spot is illuminated.
7. Which temperature all over the manuscript is given with respect to time? The average temperature after equilibrium is obtained? Or the final temperature after 60s, somehow averaged?
8. What are the temperature equilibrium times for both the spot sizes?
9. Laser induced heating results in a complex 3D-temperature distribution, also changing over time. E.g. at the layer of the photoreceptors outer segments high temperatures in the center and falling temperatures at and beyond the irradiated spot will be found. Is the temperature given here the average temperature across all layers excited by the flicker or the peak temperature at the central spot (in with layer)?
10. In order to value the temperature stability, a time plot of the temperature over the exposure time of 60 s would be helpful, best exemplary plots for all three addressed temperatures, respectively.
11. I assume the temperature is also measured with the flicker rate of 16 or 17 Hz, right? Thus about 1000 data points are achieved per spot, for which a standard deviation or error should be given, at least after equilibrium is reached.
12. Which wave of the fERG is used, referring to which neuronal cells?
13. The value of 3.7%/C is used as a fERG tissue response value. Is it present in all waves? How is this value determined and what is the temperature reference for it. Please shortly describe. This constant has a very strong influence on the temperature accuracy
14. fERG reflects cellular potentials in the neural retina. The temperature given in the value above refers somehow to neuronal cells? However, the highest temperatures are found in the RPE and choroid. How does this fits together?

Discussion:

15. At least some of the points addressed above with respect to the temperature should be also discussed.
16. In the discussion (line 519) it is mentioned that the power required for the same temperature varies widely by more than a factor of four. What is the reason for this? Just absorption differences? Please report this in the results section and provide a distribution graph for all spots with the corresponding fluence. This is, as mentioned, the main argument for a temperature controlled therapy.
17. The discussion somehow lacks the comparison to other works using different temperatures and exposure times. Can the temperature ranges found here be transferred to higher temperatures likely needed for shorter irradiation times, typically few hundred milliseconds in other retinal laser treatments?
18. Could this method presented here also work with smaller spot sizes and much shorter irradiation times? What do you think are the limitations with this method?
19. In the end, a roadmap towards a clinical study would be great. Which factors therefore need to be improved? What is otherwise also required? What would be a retinal disease this treatment options could be used for first?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have submitted a well revised article. However, there are some aspects that still should be addressed.

The authors did explain the difference between this paper and a previously published paper by their group (IEEE Trans Biomed Eng. 2022 Feb;69(2):991-1002.) in the rebuttal letter. However, this should also be included in the introduction of the paper.

The authors did explain in the rebuttal letter that the control area was separated from the treatment area by appr. 5 mm. This should be included in the method section.

The authors pointed out that in addition to actin, protein loading was controlled additionally by UV or Ponceau staining. This needs to be included in the method section.

Reviewer #2

(Remarks to the Author)

I appreciate the opportunity to review the revised paper and author responses. I address some specific responses to my prior comments below. However, my over all view of the current paper is unchanged; that (1) the arguments and understanding of retinal laser treatment widely described throughout the paper are out-of-date, and that (2) the out-of-date viewpoints expressed are used to justify use of the reported ERG technology, which in current times, has nothing to offer clinically. CW lasers for sublethal but therapeutic treatment of the retina have been abandoned for good reason. They are unsafe. There is no need to find a way to make them safe because by simply using a more appropriate laser mode – a low frequency micropulsed laser – safety and efficacy can be assured very simply. Despite the author's many suggestions, no evidence is presented that their approach offers any actual clinical benefits or improved clinical outcomes compared to the far simpler, safer, and more sensible approach of simply using MPL, as most currently do. As a research tool the tech may be of interest. But this study asserts both clinical utility and a need for such tech. This is not the case at all.

"We appreciate the view of Dr. Luttrull, who we know to be an experienced physician and a pioneer in the field of micropulsed lasers. However, we disagree with Dr. Luttrull about the therapeutic range of micropulsed or CW lasers, see detailed reasoning below. Additionally, we are talking about two different treatment modalities, micropulsed laser treatment aims to heat primarily the RPE while longpulse treatments aim to extend the therapeutic benefits also to the retina and choroid in addition to RPE, which are all affected in diseases like AMD, cCSC, and DME. According to our view, the micropulsed approaches aim to limit the heating maximally to the RPE, because without thermal dosimetry, this would be the only known way to guarantee the treatment safety, but it also limits the achievable therapeutic effects. With controlled long pulse, large spot treatment, we can activate the cells' natural restoration mechanisms in all tissues affected by the disease, not just in RPE."

Therapeutic range is defined as "distance" between no effect and the ED50, or 50/50 retinal burn risk. It is not defined by any particular laser modality (CW vs MPL, etc), but is influenced by it. I am not aware of evidence of any unique clinical benefits arising from sublethally heating the choroid or neurosensory retina in the absence RPE hyperthermia. If there is any evidence for this, the authors' need to present it. Transpupillary thermotherapy heated the choroid nicely. That's why it was abandoned.

"We agree with the reviewer that the term "subthreshold", although widely used, is ambiguous. We decided the change the term for our treatment to non-damaging retinal laser therapy and define the term. "

The only term more corrupt than "subthreshold" is "non-damaging". It also is used to create the false impression that treatment is safe. Note that "non damaging" is used in marketing to describe both the PASCAL, and 2RT, both of which are designed to be reliably destructive. The only useful term is "sublethal". Treatment is either sublethal to the RPE, or it isn't.

"Since micropulse treatments use extremely short pulses, the power during the pulse is substantially higher than with CW exposure to reach a corresponding effect. As the laser power required to reach any effect grows with shorter pulses, so does the difference in laser powers required to cause damage. This, however, is not a meaningful way to describe the width of the therapeutic window. We define the therapeutic window as the ratio of the temperature elevation required to cause apoptotic changes and the temperature elevation required to trigger therapeutic effects. This ratio may differ slightly with the duration of laser exposure, but the difference is small (Wang et al., 2017). As the temperature elevation depends on the physiological parameters of the treated eye and the physiological parameters vary between patients and even between different retinal areas, the temperature elevation varies as well. As temperature feedback has not been available, the development of laser therapy modalities has focused on designing the laser exposure in a way that a) minimizes the effect of variability in the relevant physiological parameters on temperature elevation, and b) minimizes the extent of the damage in case of overtreatment. "

The therapeutic range (from no effect to 50/50 thermal burn risk) is dependent on a specific combination of laser parameters. It is not clear what the authors' think laser power has to do with the TR, other than it is just one parameter that contributes. There is no TR for a particular laser power, or wavelength, etc. Only for specific combinations of all parameters (power, wavelength, spot size, duration, pulse frequency). One is not guided by the parameters (power, wavelength, etc) but by the degree of safety one wishes to achieve. One then tailors the laser parameters to those that best achieve that goal (see the LIGHT treatment recommendations). The only importance of the TR is the likelihood that any particular eye will fall well within that range and expect safe and effective treatment. Thus, narrow TR bad, broad TR good, and it doesn't matter how you get the. But some modes, like MPL get to broad TRs better than others (like CW). With appropriately selected MPL parameters one can create a very broad TR (> 15 watts), using a set of parameters to accommodate safe and effective treatment every eye of every patient for every indication. This has been clearly demonstrated in clinical application in many studies over 25 years in thousands of human patients. Note that there has never been a single case of retinal damage reported from an 810nm laser at a 5% DC at any spot size or any power using current laser systems. And this without real-time retinal temperature monitoring! This is a fact, not a point of view. This creates a problem for the authors' technology, and others like optoacoustic temperature measurements, because there is simply no clinical need for the technology the authors describe to directly or indirectly measure temperatures during treatment, if one instead uses the appropriate mode of treatment (long-wavelength, low-frequency MPL) to preclude any risk of thermal retinal damage (ie, treatment lethal to the RPE).

"It is important to note that laser exposure paradigms with pulse durations in the microsecond and minute ranges create very different temperature elevations in the fundus. With very short laser exposures, the thermal elevation in the retina occurs primarily where the laser energy is absorbed, creating a very short and high transient peak. In long-pulse treatments, the

heat has time to spread to surrounding tissues. Since no harmful effects of non-damaging heating have been found, we consider temperature-guided long-pulse treatments as an excellent opportunity to increase the therapeutic effect of non-damaging laser treatments, which already show significant potential in treating multiple retinal diseases.”

There is no evidence to support the above assertion, which, as the sentence continues, already show a high level effectiveness in many different types of disorders without need for temp monitoring or choroidal heating. See also prior comments on “non-damaging” and lacking evidence for therapeutic benefits from heat spread from the RPE (below)

“According to our view, the short-pulse methods aim to limit the heating only to the RPE, because without thermal dosimetry, this is the only way to guarantee the treatment safety, but it also limits the achievable therapeutic effects. With controlled long pulse, large spot treatment, we can activate the cells' natural restoration mechanisms in also other tissues affected by the disease (like AMD, cCSC, and DME), not just in RPE.”

This is an interesting point of view, but there is no evidence that I am aware of for any notable independent therapeutic benefit from sublethal hyperthermia of either the choroid or neurosensory retina independent of the RPE, which to my knowledge have no paracrine function and operate under the control of the RPE, and not the other way around. If the authors have evidence to support this interesting hypothesis, they should present it.

“2. How could the method be beneficial for the treatment of AMD as mentioned in line 89? Which stage of AMD? And is the fERG signal not altered by a non-healthy neural retina?” “There is evidence that non-damaging laser treatments can be used to reduce the number of needed anti-VEGF injections (Söderberg et al., 2012), abolish drusen (Eng et al., 2019; Virgili et al., 2015), aggregations of cellular waste materials...”

The above comment was taken from the notes from reviewer #3, as the authors' answer illustrates a fundamental misunderstanding of common retinal laser effects. Acute drusen and debris reduction is dependent on the inflammatory and debridement response to laser-induced retinal damage. It is thus not observed in “non damaging” laser treatment sublethal to the RPE. This is why the failure of laser for dry AMD parallels laser damage: more laser damage, more drusen reduction, faster the progression, and more wet AMD. See the “laser for drusen” and LEAD studies.

Reviewer #3

(Remarks to the Author)

The manuscript has been improved and most of my questions have been answered.

However, I am still concerned about the accuracy of the given temperatures determined by the method. How accurate are the two explicitly addressed temperatures of 44.2 and 46.5 C for all biological effects investigated? The decimal place indicated reflects an accuracy to a tenth of a degree. When thinking about future reference of this paper with respect to temperature depended biological effects, the uncertainty range and the limitations must clearly be defined and should also be mentioned in the abstract and critically discussed also with respect to k and not just alpha(opt). The reader should understand, which uncertainty in the temperature values are present for future discussions in the whole field of hyperthermia.

With respect to this:

Eq. 2 has been changed (why?), k is now in the denominator, and thus the units don't fit any more. So there is obviously something wrong. Further the value k is referred to the paper of Kaikkonen IEEE 2021 being $k=0.037\text{ C}$. I read this paper and learned that k strongly depends on the flash strength. The flash used here was $46\text{ lx}\cdot\text{s}$, while Kaikkonen found this k value for just $10\text{ cd}\cdot\text{s}/\text{m}^2$, for which k was not determined at all. The temperature slope value strongly depends on this parameter. Thus it remains erratic while $k=0.037\text{ C}$ was chosen. This needs to be clearly clarified.

Further alpha(opt) given in eq. 2 is not explained and unclear, how it was determined, and what the error of this value is with respect to the measured data. I recommend to use the same nomenclature as Kaikkonen, if this paper should be the reference standard for temperature determination.

The discussion section should be extended with respect to the error. I cannot comprehend, how the error of 0.6 C was determined for the 5mm spot, and which of the mentioned sources of error mentioned have which influence. And how is it for the error for the smaller 3.4 mm spot? The influence of k to the error is not mentioned, and obviously the chosen k does not fit to the used flash strength (see above). The Kaikkonen paper did not investigate this flash strength.

Reviewer #4

(Remarks to the Author)

This is an interesting manuscript on retinal laser treatment with ERG- based thermal dosimetry. The manuscript has undergone a previous round of review and the authors have responded to the comments. The revised version reads well and the revision process has improved the manuscript substantially. I only have two comments that should be addressed by the reviewers.

1. Using fERG in humans is associated with significant variability in the amplitudes. Whereas this may be less of a problem in animal experiments where the eye can be stabilised over prolonged periods of time it may not be easy to translate this to the clinical setting. This is also the reason why fERG has lost importance in the recent years. Why dont the authors try to guide the treatment via Optoretinography? I understand that this technique is still in its infancy, but on the long run this may be the better choice.

2. The authors hypothesise that all molecular changes are caused by thermal effects. Is there any proof for this concept. Could the selected wavelength play a role as well? What is the rationale for using a laser diode with 810 nm? Would the same results been achieved with a 670 nm diode?

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have answered all of the remarks of this reviewer to this reviewer's satisfaction.

Reviewer #3

(Remarks to the Author)

My concerns and questions were almost all answered well. Overall, this is a very good paper, especially with regard to the temperature-relevant expression of the different factors studied.

The only remaining point is the accuracy of the temperature determination and the determination of the value of the constant α (previously k). I am not convinced by the reasoning and argumentative justification of the authors for $\alpha=3.7\text{ \%}/\text{K}$, as too many assumptions were made to be able to state the accuracy postulated here. Since this value is directly and inversely proportional to the temperature calculation, α is very decisive for the absolute, specified temperatures.

Since I don't see an easy way of obtaining better substantiated values here, I suggest that the remarks made in the reply to my comments be included directly and in detail in the manuscript (and not in the supplements) so that every reader can understand exactly under which assumptions the temperature determination and the α value were derived.

Beyond that, I have no objections for publication.

Reviewer #4

(Remarks to the Author)

All comments have been addressed

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Response to reviewers

Reviewer #1 (Remarks to the Author):

In their article, the authors describe fERG-controlled SRT laser treatment and its effect in vivo in pigs eyes. The study is of high interest, however, some aspects have to be addressed before publication can be considered.

Major

The authors have to clearly explain what distinguishes this paper from the one they have already published, establishing the method presented here (IEEE Trans Biomed Eng. 2022 Feb;69(2):991-1002. doi: 10.1109/TBME.2021.3111533.)

The referred publication is a study conducted by us. It describes the mathematical and physiological basis for photopic ERG-based thermal dosimetry. The research is conducted by heating the whole isolated retina or whole pig and collecting ERG signals from the whole retina. The current submission utilizes the method for the first time in practice to control actual local laser treatments in anesthetized pigs and investigates the local biological effects of the heat treatment to different temperatures.

The authors seem to be oblivious to studies of other researchers on the field of SRT and their results, as seen both in the introduction and in the discussion.

The selective retina therapy (SRT) is one modality of photothermal therapies where intense laser pulses are given to a small local area to induce microbubble formation inside RPE cells which leads to destruction of those cells. While our technique and SRT share some similarities, we consider the techniques mostly different, since our treatment pursues to a gentle heating of a large local area in retina with no harmful effects to cells. We extended our introduction and discussion to describe different techniques and different investigated and existing methods to control laser treatments.

Introduction

Line 65, “thermal dosimetry for retinal laser treatment is currently unavailable”, there has been quite some work on optoacoustics for controlling SRT in the retina (e.g. Photoacoustics. 2021 Nov 10;25:100316. doi: 10.1016/j.pacs.2021.100316.). It should be mentioned here and compared to the method presented here in the discussion. What is better and why? The reviewer is right in that optoacoustics is used even commercially to detect the formation of microbubbles in RPE, a phenomenon that is temperature-dependent but requires high local temperatures (presumably higher than 100 °C). This is not directly applicable as a thermal dosimetry for non-damaging treatments. Yet, there has been extensive research of using optoacoustic methodology also as molecular thermometer and as far as we know, there is a clinical trial currently on a way where the method is studied in controlling non-damaging laser treatment. Another method uses OCT measurement to detect thermal expansion caused by heating. Both thermal dosimetry methods are still investigational and not currently commercially available or in clinical use. We added discussion of these methods and comparison of the techniques to ours. However, we are not experts on those techniques and can only talk about them on a general level. What we can say is that our method is reliable, accurate, easy to use, and it is based on methodology (ERG) familiar to ophthalmologists.

Line 59 ff, the temperature-dependent expression of Hsp70 has been shown for the porcine retina before with different temperature dependencies as found here (Exp Eye Res. 2018 May;170:117-126.

doi: 10.1016/j.exer.2018.02.013). This should be mentioned and again, discussed in the discussion why the temperatures differ between these studies.

We thank the reviewer for this comment. In the referred article, the authors studied HSP70 expression in cultured porcine RPE cells under laser heating. In their measurement configuration, the temperature distribution in the cell culture dish was nicely gaussian. However, the HSP70 expression pattern (a stripe of HSP70 expressing cells, their figure 3) did not follow the gaussian temperature distribution, especially in fig. 3C with the peak temperature of 47 °C, making quantitative HSP70 expression temperature evaluation somewhat uncertain. Further, the temperature-dependence of HSP70 expression may differ in *in vivo* and in cell cultures. However, taking into account the minor temperature uncertainties, we rather interpret that the findings of the referred paper are consistent with our data than remarkably different. More specifically, they were not able to find a statistically significant increase in HSP70 production in 44 °C with their 10 second heating. However, they found a tendency for HSP70 to increase after 24h and 48h in response to a 10 second heating to 44 °C although the finding was not statistically significant. In immunofluorescent images, they also saw an increase of HSP70 production in the center of the area heated to 44 °C. Signs of apoptosis and cell death were found after heating to above 47 °C. To us it seems that these results are well in line with our findings that 60 s treatment to 44 °C induced significant increase in HSP70 production and heating to 48 °C was damaging. This comparison was already mentioned in the discussion related to damaging temperatures, but now we added also the comparison of temperatures for inducing HSP70 production.

Methods

Line 219 “A retinal area was selected”, was the area the same for all treated eyes? This might be of importance as pigs have an area centralis with a high cone percentage, where RPE cells may react differently from areas with a higher rod density.

This is a good comment and we have now made it clearer how the areas were selected. We did not make separation between areas with higher cone percentage but selected the areas randomly throughout the retina. However, we did not conduct treatments on top of large vessels and avoided the optic nerve.

Lines 264-265, “4-6 non-treated samples were collected from each eye”, how far away were these samples from the treatment area? Were also not-treated eyes used as controls to rule out any off-target effect of the treatment?

Only controls from the treated eyes were used. The control areas were selected randomly throughout the retina, and they were always separated from the treated areas by approximately 5 mm. This can be expected to be enough to prevent the heating to directly affect that area. However, if the tissue's reaction to the heating spreads (through a mechanism other than temperature) to a much larger area (e.g. up to the control sample areas) than anticipated, any off-target effects would manifest as a reduction of the measured effect.

Lines 278-279, was the signal quantified? How? Did the authors quantify the area of expression? Was it consistent with the area of treatment or did they see off-target effects?

The method for signal quantification is described under the title “Data and Statistical Analysis”.

“The treatment was considered to increase HSP70 production if the relative fluorescent intensity increases in the treated area compared to the surrounding areas was 2-times larger than the relative standard deviation of the fluorescent intensity in the surrounding areas in the flat mounted immunofluorescent images”.

We did not quantify the area of expression for individual spots but always measured the mean intensity under the 3.4 mm diameter treated area and compared this to the mean intensity in the near surroundings. Visually, the area of HSP70 production varied inside the treated area with mild temperatures and filled the whole heated area in treatments to higher temperatures. We were not able to see any off-target effects.

Line 280, please state in the methods the positive control used.

Non-treated areas were used as a negative control and treatment causing a visible lesion was used as a positive control.

Line 303, ACTB was used as control, however, a recent publication showed for inflammatory activation that ACTB was changed by the stimulus (doi: 10.1016/j.exer.2023.109469), did the authors check that their controls were not changed by temperature?

We would like to clarify that for all of the experiments, we used equative loading of 15 µg protein per lane, which serves as a secondary loading control alongside actin. Additionally, the transfer of the proteins on the gel was assessed by UV or Ponceau staining.

Line 334 following, please include sample size (flatmounts, molecular testing) in paragraph on statistics.

We added sample sizes for the different analysis techniques in the Data and Statistical Analysis chapter.

Results

Line 378, “number of treatments per eyes ranging from 2-6”, are the authors sure that the treatments do not have any additive effects on the RPE?

In this study, we only studied the local effects in the heated area compared to non-heated areas from the same eye. Indirect additive effects were not investigated.

Line 396 “heated areas”, I would strongly suggest to quantify the areas affected.

We added a scale bar to the figures to quantify the affected areas.

Line 435, if there is discussion in the result section, the work of other authors investigating the effect of temperature on the hsp expression in the RPE needs to be included.

We removed this discussion from the results section.

Line 473, 489, why are significances not shown in the graphs of the respective figures?

Only transcripts with a p-value of <0.05 were selected for these figures.

Several figures, the different lanes on the Western Blot images are not defined. What are we seeing here?

The meaning of different rows are defined with the legend text next to the row in question. The different columns represent individual samples.

Discussion

As pointed out above, a discussion concerning previous studies by other authors is missing, e.g. the comparison of this method with opto-acoustics or the differences between this study and other studies investigating RPE temperature and hsp70 expression.

We extended our discussion to cover the work of other authors on temperature dependence of HSP expression and other investigational methods for thermal dosimetry.

In addition to what was already pointed out above:

Line 582, there is no mentioning of other studies which have investigated VEGF secretion in porcine RPE/choroid cultures after SRT (e.g. doi: 10.1167/iovs.17-23163) or that have investigated the effect of temperature on the release of VEGF in porcine RPE cells (doi: 10.1007/s00417-014-2750-z.). Please compare your results with what has already been published and discuss the differences.

We added comparison of our results to literature findings to the discussion

Line 589, “we demonstrate the feasibility of fERT-based thermal dosimetry for STLT in vivo”, didn’t the authors already prove this in a previous paper (doi: 10.1109/TBME.2021.3111533.)?

We thank the reviewer for pointing this out. We corrected the manuscript to clarify the point that here we demonstrate the feasibility by using fERG-based thermal dosimetry for the first time to successfully control local heat treatments.

Minor

Line 129, there is a dot missing at the end of the sentence.

Typo corrected, thanks.

Reviewer #2 (Remarks to the Author):

Overview:

The results of this study are interesting and useful, as they corroborate and add to prior studies on the thermodynamics and proteomics of retinal laser treatment, but also demonstrate the utility of ERG to monitor laser effects. A major weakness is that concepts that the authors use to provide context and justification for their approach are dated. In short, the authors present ERG monitoring as useful because of the narrow therapeutic range of CW laser and the difficulty otherwise of performing “subthreshold” laser treatment and avoiding retinal damage with CW laser. The issue is that, for this reason, retina-sparing treatments have abandoned CW and gone to micropulsed laser modes that are extremely safe and predictable due to extremely wide therapeutic ranges (TR), rendering the clinical utility of ERG monitoring as presented here, much like opto-acoustic real-time monitoring, clinically unnecessary.

We appreciate the view of Dr. Luttrull, who we know to be an experienced physician and a pioneer in the field of micropulsed lasers. However, we disagree with Dr. Luttrull about the therapeutic range of micropulsed or CW lasers, see detailed reasoning below. Additionally, we are talking about two different treatment modalities, micropulsed laser treatment aims to heat primarily the RPE while long-

pulse treatments aim to extend the therapeutic benefits also to the retina and choroid in addition to RPE, which are all affected in diseases like AMD, cCSC, and DME.

According to our view, the micropulsed approaches aim to limit the heating maximally to the RPE, because without thermal dosimetry, this would be the only known way to guarantee the treatment safety, but it also limits the achievable therapeutic effects. With controlled long pulse, large spot treatment, we can activate the cells' natural restoration mechanisms in all tissues affected by the disease, not just in RPE.

More specific comments:

The authors do not discriminate between the various modes of “subthreshold” retinal laser treatment, so diverse as to make the term meaningless. “Subthreshold” needs to be clearly qualified with the laser mode being employed (conventional CW, short pulse CW, nanosecond CW, microsecond pulsed, etc), and the level of scrutiny. In other words, “subthreshold” to what? Detection by the naked eye? FFA? FAF? OCT? RF photos? Histology? Electron microscopy? Biomicroscopy? Absent such defining terms, “subthreshold” has no meaning.

We agree with the reviewer that the term “subthreshold”, although widely used, is ambiguous. We decided to change the term for our treatment to non-damaging retinal laser therapy and define the term.

The authors describe the therapeutic range (TR) of “subthreshold” laser as dangerously narrow (true), thus indicating the need for the author’s technology (not true). They should note that the TR of CW laser, the laser mode they used in this study, is indeed extremely narrow (0.010 W in breadth). But they importantly fail to note that the TR for microsecond pulsed lasers can be vast – 15 watts or more – precluding the need for thermal (or any other) real-time temperature monitoring, including functional monitoring with ERG. The fact that laser treatment within the very wide microsecond pulsed TR using fixed (non titrated) laser parameters in all patients that is both effective and safe is supported by a vast amount of clinical and experimental research and publications. Along these lines, the authors state that titration of laser exposures is state of the art. It is not. Titration is an anachronism associated with use of CW lasers for retinal photocoagulation. Keunen JEE, Battaglia-Parodi M, Vujosevic S, Luttrull JK. International Retinal Laser Society Guidelines for Subthreshold Laser Treatment. Transl Vis Sci Technol. 2020 Aug 7;9(9):15. doi: 10.1167/tvst.9.9.15. eCollection 2020 Aug. PMID: 32879771

Since micropulse treatments use extremely short pulses, the power during the pulse is substantially higher than with CW exposure to reach a corresponding effect. As the laser power required to reach any effect grows with shorter pulses, so does the difference in laser powers required to cause damage. This, however, is not a meaningful way to describe the width of the therapeutic window. We define the therapeutic window as the ratio of the temperature elevation required to cause apoptotic changes and the temperature elevation required to trigger therapeutic effects. This ratio may differ slightly with the duration of laser exposure, but the difference is small (Wang et al., 2017). As the temperature elevation depends on the physiological parameters of the treated eye and the physiological parameters vary between patients and even between different retinal areas, the temperature elevation varies as well. As temperature feedback has not been available, the development of laser therapy modalities has focused on designing the laser exposure in a way that a) minimizes the effect of variability in the relevant physiological parameters on temperature elevation, and b) minimizes the extent of the damage in case of overtreatment.

The key aspects to consider when comparing CW or micropulse treatments with or without thermal dosimetry are:

- What is the therapeutic benefit with optimal dosage?
- How much does the treatment temperature fluctuate between patients or laser exposures?
- What is the effect of the temperature variation on beneficial and harmful effects of the treatment?

It is important to note that laser exposure paradigms with pulse durations in the microsecond and minute ranges create very different temperature elevations in the fundus. With very short laser exposures, the thermal elevation in the retina occurs primarily where the laser energy is absorbed, creating a very short and high transient peak. In long-pulse treatments, the heat has time to spread to surrounding tissues. Since no harmful effects of non-damaging heating have been found, we consider temperature-guided long-pulse treatments as an excellent opportunity to increase the therapeutic effect of non-damaging laser treatments, which already show significant potential in treating multiple retinal diseases.

It is true that the LIGHT (International Retinal Laser Society, chaired by Dr. Luttrull) recommends the use of constant power for all patients when conducting micropulse laser treatment. On the other hand, SOLS (Subthreshold Laser Ophthalmic Society) recommends conducting the titration before every micropulse treatment. Chhablani J; SOLS (Subthreshold Laser Ophthalmic Society) writing committee. Subthreshold laser therapy guidelines for retinal diseases. Eye (Lond). 2022 Dec;36(12):2234-2235. doi: 10.1038/s41433-022-02136-w. Additionally, the treatment schemes with subthreshold lasers vary profoundly between clinical trials and clinicians. This is partly because the endpoint of the treatment is not clearly visible (subthreshold) and clinicians do not have a clear idea what should the optimal treatment be like. The beneficial effects of these treatments are thought to be induced by heat and thus the knowledge of the laser-induced temperature elevation in each patient is crucial for standardization of the treatments between clinicians.

Following on the above, the individual variations in eyes in such as pigment density, choroidal perfusion, etc, while possibly important in CW retinal laser treatment, are inconsequential with very large TRs such as can be obtained with pulsed laser modes. Thus, eye specific variations are also no longer a consideration requiring treatment intensity adjustment such as that which might be aided by ERG. Again, this is because of the extremely wide TRs achievable with microsecond pulsed lasers. Chang DB, Luttrull JK. Comparison of subthreshold 577nm and 810nm micropulse laser effects on heat-shock protein activation kinetics: Implications for treatment efficacy and safety. Transl Vis Sci Technol. 2020 Apr 28;9(5):23.doi: 10.1167/tvst.9.5.23. eCollection 2020 Apr.

According to our view, the short-pulse methods aim to limit the heating only to the RPE, because without thermal dosimetry, this is the only way to guarantee the treatment safety, but it also limits the achievable therapeutic effects. With controlled long pulse, large spot treatment, we can activate the cells' natural restoration mechanisms in also other tissues affected by the disease (like AMD, cCSC, and DME), not just in RPE.

The paper, Chang DB, Luttrull JK., Transl Vis Sci Technol. 2020, referred by the reviewer is hard to evaluate since the model is quite complex and the supplement is missing the list of references completely. The modeling seems to use ten equations and sixteen rate constants but shows no experimental data to support the model outcomes.

The authors state that their tech helps achieve “personalized” treatment, as if personalized treatment is a good and desirable thing. It is quite the opposite. The ideal is one simple treatment that works for all people and all problems all the time, not turning each individual into a different disease. The concept of personalized medicine is, while popular, absurd.

We understand this point when viewed with the eyes of the clinician since all additional time spent performing the treatments reduces the number of treated patients. Thus, whatever method used for treatment personalization, it should not complicate the treatment excessively or otherwise it will not be taken into common practice if there are no substantial benefits. Here, we demonstrate that the ERG-based thermal dosimetry can be used to control non-damaging laser treatments and found a temperature consistently inducing beneficial biological effects without damaging tissue. Yet, for clinical use, the methodology will be refined taking into account the needs of the ophthalmologists.

Finally, it is not reasonable to suggest that real-time ERG monitoring to accomplish retina-sparing laser treatment may have clinical application. First, as discussed above, it is wholly unnecessary. Further, the author's themselves note that laser has been shown to benefit many common problems that affect millions of patients, often requiring regularly repeated treatments for years on end. How would they imagine this technology being used routinely in clinical practice for these hundreds of millions of potential retinal laser treatments around the world? One cannot. The technology the authors report is an interesting research tool, but has no indication or application clinically.

As described earlier, we understand that new clinical tools should not complicate the existing standards of care unnecessarily. However, with refined methodology, our treatment can be made as simple for the clinician to administer as current laser treatments. Assuming improved clinical outcomes with same effort for the ophthalmologist, the new treatment method would be clinically appealing. However, the clinical benefits must still be shown in clinical trials.

I recommend the authors re-cast this otherwise interesting study as a lab study of laser thermal proteomics and eliminate their discussion of clinical laser treatment and references to any clinical need or application for this technology. An interesting follow up study would be to repeat this study with low-duty cycle microsecond pulsed laser parameters known to be safe and effective in all eyes, to show why ERG monitoring is unnecessary.

Additional important references:

Caballero S, Kent DL, Sengupta N, et al. Bone marrow derived cell recruitment to the neurosensory retina and retinal pigment epithelial cell layer following subthreshold retinal phototherapy. Invest Ophthalmol Vis Sci. 2017;58:5164e76

Frizziero L, Calciati A, Midena G, et al. Subthreshold Micropulse Laser Modulates Retinal Neuroinflammatory Biomarkers in Diabetic Macular Edema. J Clin Med. 2021;10(14):3134. Published 2021 Jul 15. doi:10.3390/jcm10143134

Midena E, Bini S, Frizziero L, Pilotto E, Esposito G, Micera A. Aqueous humour concentrations of PEDF and Erythropoietin are not influenced by subthreshold micropulse laser treatment of diabetic macular edema. Biosci Rep. 2019;39(6):BSR20190328. Published 2019 Jun 18. doi:10.1042/BSR20190328

Midena E, Micera A, Frizziero L, Pilotto E, Esposito G, Bini S. Sub-threshold micropulse laser treatment reduces inflammatory biomarkers in aqueous humour of diabetic patients with macular edema. Sci Rep. 2019;9(1):10034. Published 2019 Jul 11. doi:10.1038/s41598-019-46515-y

Respectfully,

Jeffrey K Luttrull, MD

Reviewer #3 (Remarks to the Author):

The manuscript well described biological reactions after controlled temporal (60s) laser induced retinal temperature increase in pigs. The temperature was determined with focal electroretinography (fERG)-based temperature monitoring during laser exposure. This is a very acute and relevant topic, since retinal laser treatments, especially hyperthermic sub-damaging irradiations so far have no dosing endpoints. The manuscript focusses on the investigation of RPE and retina tissue and investigated in detail markers for oxidative stress, structural features and apoptosis, as well as heat shock protein and gene expression. Very interesting results were achieved and discussed. The manuscript should be published after some revisions addressed below.

The manuscript is thoroughly written with respect to all biological reactions including the investigation towards the damage threshold. What is less good described are the physical and fERG aspects with respect to the temperature measurement. Even though I acknowledge that this is not the topic of the manuscript and references for more details are presented, nevertheless the reader needs to know some more details, since all aspects investigated here rely on the temperature rise. Thus a proper description how and in which layer the temperature is obtained and how it was calibrated is necessary, and especially the fERG temperature response value of 3.7%/C. These aspects need to be better described for the reader, however, its doesn't mean to go too deep into the mathematics, but a verbal clear description would be very helpful.

Some points to be considered in this context:

Title:

1. "subthreshold"; which threshold is meant? I suggest to write non-damaging or sub-damaging or hyperthermal or another unambiguously word. Further also super-threshold experiments are performed, so this word can also be omitted.

We agree with the reviewer and chose to use non-damaging to replace subthreshold.

Introduction:

2. How could the method be beneficial for the treatment of AMD as mentioned in line 89? Which stage of AMD? And is the fERG signal not altered by a non-healthy neural retina?

There is evidence that non-damaging laser treatments can be used to reduce the number of needed anti-VEGF injections (Söderberg et al., 2012), abolish drusens (Eng et al., 2019; Virgili et al., 2015), aggregations of cellular waste materials, which are typical signs of AMD development, increase the thickness of the photoreceptor layer (Querques et al., 2021), delay the progression of geographic atrophy (Luttrull et al., 2020), and prevent AMD conversion from dry to wet (Huang et al., 2022; Luttrull et al., 2018, 2023; Luttrull & Gray, 2022). The main hypothesis behind all these treatments is the activation of heat shock reaction and production of heat shock proteins. In addition to these, we demonstrate heat-induced activation of autophagy that can provide cellular "waste" clearance and thus reduce harmful accumulation of aggregates. The above-mentioned studies have been conducted without thermal dosimetry and thus we consider the temperature optimized non-damaging laser therapy to be a highly interesting option for treatment of both wet and dry AMD.

According to literature (Forshaw et al., 2022) and our own usability trials (unpublished results), the photopic ERG signal amplitude in AMD is only slightly reduced and this should not hamper our temperature determination.

3. There are also other temperature controlled retinal laser treatments under investigation, in open and closed control settings and approved in clinical trials already. This should at least be partly mentioned in this context.

There are indeed some investigational techniques for improving thermal dosing for different types of laser exposures. Since the laser exposure paradigms that these techniques are designed for differ substantially from each other and the method presented here, direct comparison is not straight forward. We added a paragraph in the discussion about this, which concentrates on most widely studied methods for thermal dosimetry, optoacoustic method and OCT.

Material and methods:

4. Why is a wavelength of 810 nm been chosen, even though this is only very rarely used in retinal laser treatments due to its low absorption of melanin leading to a brought and deep heating into the choroid rather than just the RPE and its surroundings? In my opinion a green wavelength would be much better suited and would require much less power by avoiding choroidal heating.

Using a green laser with this exposure time and spot size would be unfeasible for two main reasons:

1. The brightness experienced by the patient would be intolerable.
2. Recording fERG inside the laser spot would be practically impossible due to the excessive photoreceptor excitation from the green laser beam.

It is indeed a feature of this laser exposure paradigm that the temperature of the choroid and neural retina is also elevated. However, the absorbance of the RPE is still substantially stronger at 810 nm compared to the choroid, and thermal simulations show that the highest temperature is experienced by the RPE (Narasimhan & Sundarraj, 2013). It is not known whether the best therapeutic response in any retinal disease is reached by limiting the heat shock to the RPE versus giving a mild exposure also to the choroid and neural retina. Our approach enables these two laser paradigms to be compared in a clinical setting.

Additionally, it is noteworthy that almost all retinal laser treatments with long pulses and large spot areas were performed with an 810 nm laser, which encouraged us to choose the same wavelength instead of some other usable wavelength in the near-infrared regime.

5. Did you carefully check, whether coagulation in the choroid was avoided?

A treatment was judged to cause a lesion if retinal whitening was visible in fundus imaging, fluorescein angiography, or color photography from opened pig eyecup in the treated area 24 hours after treatment. We did not study coagulation in the choroid specifically.

6. How homogeneous was the beam profile with respect to top hat or Gaussian, and spatial modulation? This is essential if such a large spot is illuminated.

The laser beam has a tophat beam profile at the retina. We added a mention of this to methods.

7. Which temperature all over the manuscript is given with respect to time? The average temperature after equilibrium is obtained? Or the final temperature after 60s, somehow averaged?

The temperature is determined by averaging the fERG responses recorded during the laser exposure, excluding the first 15 seconds when the temperature changes rapidly. The result is effectively the average temperature during the laser exposure, excluding the first 15 seconds.

8. What are the temperature equilibrium times for both the spot sizes?

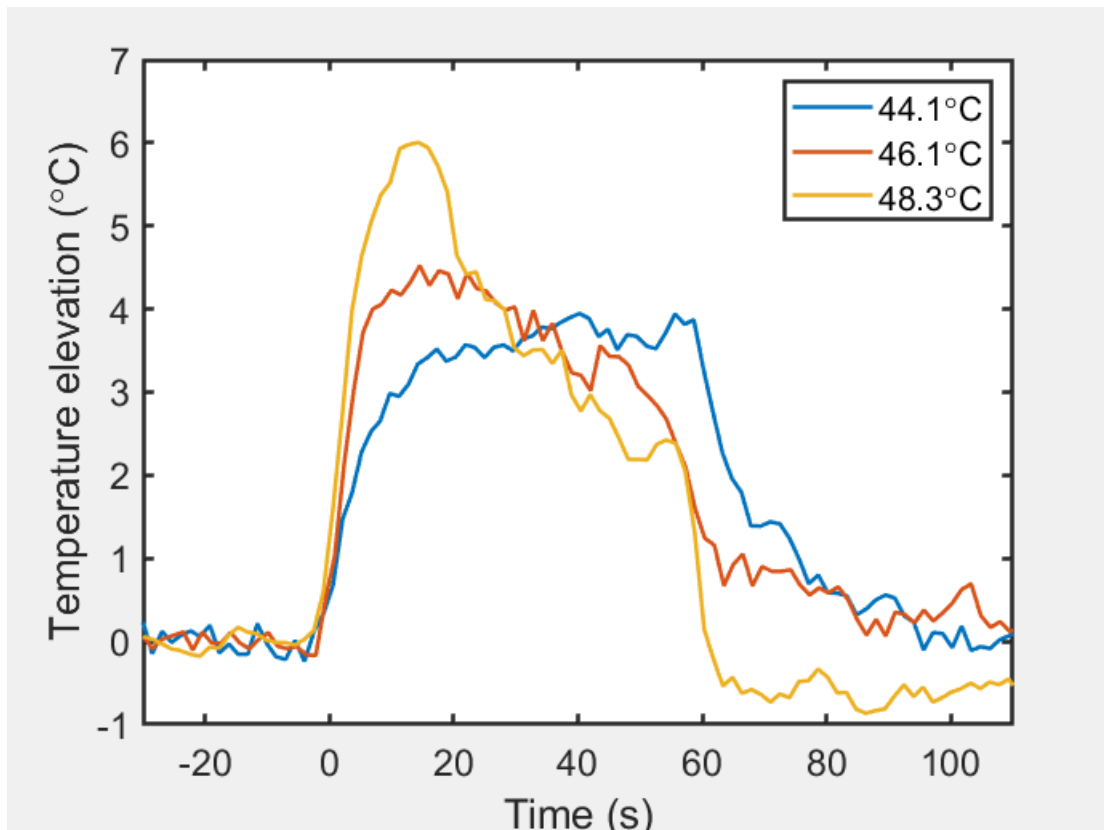
As shown in Fig. 2C, the temperature rises rapidly during the first 15 seconds, followed by a very slow drift. After 15 seconds, approximately 90% of the temperature rise happening in a 60 second heating has already taken place.

9. Laser induced heating results in a complex 3D-temperature distribution, also changing over time. E.g. at the layer of the photoreceptors outer segments high temperatures in the center and falling temperatures at and beyond the irradiated spot will be found. Is the temperature given here the average temperature across all layers excited by the flicker or the peak temperature at the central spot (in with layer)?

Our fERG signal is averaged over the whole stimulated area. Additionally, the recorded signal arises mainly from photoreceptor cells and bipolar cells, and the signal therefore reflects the temperature of the photoreceptor layer and the synaptic layer. The used fERG stimulation spot diameter was always 80% from the diameter of the laser spot. According to our modeling, the temperature at the edge of the fERG stimulation spot is around 80% from the peak temperature and the temperature estimate is around 90% from the peak temperature assuming even pigmentation and best guesses for the needed parameters. However, the model is rough and cannot be assumed to give accurate absolute temperature values. In our study, we determined the lesion threshold temperature and our “optimal” temperature for non-damaging heat treatment with the same method and both describe the fERG-based average temperatures. Thus, even though our temperature estimate does not describe the peak temperature, it can be used to accurately determine the laser power to reach the “optimal” temperature and the margin to damage.

10. In order to value the temperature stability, a time plot of the temperature over the exposure time of 60 s would be helpful, best exemplary plots for all three addressed temperatures, respectively.

As shown in Fig. 2 D, the kinetics of the fERG signal accelerates linearly with temperature up to around 44°C, while in higher temperatures the kinetics starts to slow down. When the temperature determination method is used to generate a time series of temperature estimates during treatments that elevate retinal temperature substantially above 44°C, the temperature estimate is not reliable anymore, as explained in the manuscript. In the figure below, we show the temperature extracted from the fERG signal with a 6 second moving average during treatments to 44.1°C, 46.1°C and 48.3°C. The lowest of the three temperatures allows for a reliable temperature estimate during the treatment while the two higher temperatures are above the temperature range where the temperature determination functions well. To overcome this, we determine the thermal responsivity of the treated area using calibration exposures with mild temperature increases as shown in the manuscript.



11. I assume the temperature is also measured with the flicker rate of 16 or 17 Hz, right? Thus about 1000 data points are achieved per spot, for which a standard deviation or error should be given, at least after equilibrium is reached.

It is true that the number of individual fERG responses used for temperature determination is measured in the hundreds. However, enhancing the signal-to-noise ratio to a point where temperature determination is precise requires tens or hundreds of responses to be averaged. Individual fERG responses are dominated by noise, and performing such analyses would not give meaningful results.

12. Which wave of the fERG is used, referring to which neuronal cells?

The time interval of the signal used in the computation of the temperature dependent feature was selected to include the a- and b-waves. Hence the signal is generated primarily by cone photoreceptors and their bipolar cells. We added further explanation about the origin of the used fERG components to the section “ERG-based thermal dosimetry” of Methods.

13. The value of 3.7%/C is used as a fERG tissue response value. Is it present in all waves? How is this value determined and what is the temperature reference for it. Please shortly describe. This constant has a very strong influence on the temperature accuracy

The value for thermal responsivity of ERG kinetics was determined for pig cones by heating an anesthetized pig and recording ERG at different body temperatures (Kaikkonen *et al.*, 2021). In that study, we did not see any differences in thermal responsivity of different ERG-wave components, the a- and b-waves, which suggests that the thermal responsivity for both waves are similar. The threshold temperature for visible lesions in our study, determined using the 3.7%/°C temperature dependence

for ERG kinetics acceleration, was near 48 °C, which is close to values determined by Kern and others for 10-second laser exposures in cultured pig RPE cells for apoptotic changes, and necrosis, 47 °C, and 50 °C, respectively (Kern et al., 2018) and close to the threshold for visible lesions in humans in TTT treatments, determined by modeling at approximately 10°C of temperature elevation (M A Mainster et al., 1970; M A Mainster & Reichel, 2000). The similarity between the lesion threshold temperature found in this study and by others implies that the temperature dependence for ERG kinetics acceleration is essentially correct.

We extended our description of how thermal responsivity was determined in the section “ERG-based thermal dosimetry” of Methods.

14. fERG reflects cellular potentials in the neural retina. The temperature given in the value above refers somehow to neuronal cells? However, the highest temperatures are found in the RPE and choroid. How does this fits together?

With long laser exposures, the photoreceptor outer segments partly surrounded by the apical RPE protrusions as well as the bipolar cells are expected to be at almost the same temperature as the RPE cells. We added a mention about this to the section “ERG-based thermal dosimetry” of Methods.

Discussion:

15. At least some of the points addressed above with respect to the temperature should be also discussed.

Thanks, most of the points addressed above are now discussed further in the manuscript as described in the individual responses.

16. In the discussion (line 519) it is mentioned that the power required for the same temperature varies widely by more than a factor of four. What is the reason for this? Just absorption differences? Please report this in the results section and provide a distribution graph for all spots with the corresponding fluence. This is, as mentioned, the main argument for a temperature controlled therapy.

We delivered six treatments to depigmented areas of the fundus and all of these areas showed a lower thermal responsivity than any of the pigmented areas. Hence, we expect pigmentation to play the largest role in explaining the difference between the highest and lowest thermal responsivity found in our study. Aside from pigmentation, differences in ocular opacity and choroidal pigmentation may also play a role in explaining the variability in thermal responsivity, but the relative extent of these factors in explaining the variability was not studied. We added a histogram showing the thermal responsivities found in the study as part of Fig. 2.

17. The discussion somehow lacks the comparison to other works using different temperatures and exposure times. Can the temperature ranges found here be transferred to higher temperatures likely needed for shorter irradiation times, typically few hundred milliseconds in other retinal laser treatments?

Unfortunately, the temperature ranges found here are not directly translatable to treatments with shorter irradiation times. The physiological phenomena affecting the temperature elevation in long and short timescales are different. With sub-second laser pulses and smaller spot sizes, which are typically employed with short pulses, pigmentation of the treated area and amount of ocular scattering are the key parameters affecting the temperature rise in the retina. With long pulses and large spot sizes, the effect of ocular scattering is reduced due to a larger proportion of scattered light falling within laser spot boundaries (Ginis et al., 2012), but heat dissipation, primarily from differences in choroidal perfusion, plays a larger role (Herrmann et al., 2007).

It is not known how well the thermal responsivities determined with our laser treatment approach correlate with the temperature elevations in short-pulse laser exposures of the same retinal area. However, there are studies showing that the laser power needed to induce heat shock protein production is proportional to the laser power causing damage, as 50 – 80 % of a damaging power has been shown to increase HSP70 production in RPE and choroid (Amirkavei et al., 2020; Desmettre et al., 2001; Sramek et al., 2011). This is briefly mentioned in the introduction of our manuscript.

18. Could this method presented here also work with smaller spot sizes and much shorter irradiation times? What do you think are the limitations with this method?

The amplitude of the fERG signal is roughly proportional to the stimulated area. The signal-to-noise ratio of the fERG signal, which is the main limiting factor in reducing the spot size or exposure duration, is directly proportional to signal amplitude and inversely proportional to the square of recording time. Hence, if the spot diameter is halved, the noise-induced error in the signal is doubled, or alternatively, the recording time is increased 4-fold to counteract this. In addition to decreasing the amplitude of the fERG signal, small spot sizes will additionally lead to a larger proportion of the fERG signal to be stray light -induced, reducing the accuracy of the method. We do not expect our method to be applicable to sub-second or sub-millimeter spot size treatments without fundamental changes. A more detailed analysis of possible spot sizes and exposure times is found in our earlier publication (Kaikkonen et al., 2021), but we added a brief mention about the applicability of the method to the discussion.

19. In the end, a roadmap towards a clinical study would be great. Which factors therefore need to be improved? What is otherwise also required? What would be a retinal disease this treatment options could be used for first?

We added a chapter considering the translation from animals to humans at the end of the discussion before the summary chapter. We have already run some initial usability tests without laser in humans and recognized that a device that does not use a contact fundus lens is advisable due to irritation from the contact fundus lens and unwanted movement of the treatment spot in the fundus. As for the first indication where this could be tested, the main options are neovascular AMD, intermediate AMD, GA, diabetic macular edema, retinal vein occlusion and central serous chorioretinopathy. However, we feel that detailed clinical roadmap is not in the focus of the manuscript.

Point-by-point response to reviewers' comments

Reviewer #1 (Remarks to the Author)

The authors have submitted a well revised article. However, there are some aspects that still should be addressed.

We thank the reviewer for the positive assessment of our revisions and for highlighting the remaining aspects that required further attention. We appreciate your thoughtful feedback, which has helped us refine the manuscript further.

The authors did explain the difference between this paper and a previously published paper by their group (IEEE Trans Biomed Eng. 2022 Feb;69(2):991-1002.) in the rebuttal letter. However, this should also be included in the introduction of the paper.

The following text is now included in the introduction of the manuscript:

“The mathematical and physiological basis of the thermal dosimetry is described in (Kaikkonen et al., 2021), and this study demonstrates its feasibility in anesthetized pigs by treating the retina with 60-second near-infrared laser exposures and 3.4- and 5-mm diameter heating spots.”

The authors did explain in the rebuttal letter that the control area was separated from the treatment area by appr. 5 mm. This should be included in the method section.

The following text is now included in the method section:

“The control samples were collected from all over the retina and were separated by at least approximately 5 mm from the treated areas.”

The authors pointed out that in addition to actin, protein loading was controlled additionally by UV or Ponceau staining. This needs to be included in the method section.

The following text is now included in the method section under ‘Immunoblotting’:

“The transfer of proteins on the gel was assessed by UV or Ponceau staining.”

Reviewer #2 (Remarks to the Author):

I appreciate the opportunity to review the revised paper and author responses. I address some specific responses to my prior comments below. However, my over all view of the current paper is unchanged; that (1) the arguments and understanding of retinal laser treatment widely described throughout the paper are out-of-date, and that (2) the out-of-date viewpoints expressed are used to justify use of the reported ERG technology, which in current times, has nothing to offer clinically. CW lasers for sublethal but therapeutic treatment of the retina have been abandoned for good reason. They are unsafe. There is no need to find a way to make them safe because by simply using a more appropriate laser mode – a low frequency micropulsed laser – safety and efficacy can be assured very simply. Despite the author’s many suggestions,

no evidence is presented that their approach offers any actual clinical benefits or improved clinical outcomes compared to the far simpler, safer, and more sensible approach of simply using MPL, as most currently do. As a research tool the tech may be of interest. But this study asserts both clinical utility and a need for such tech. This is not the case at all.

We thank the reviewer for the critical feedback. While we acknowledge that there is plenty of clinical evidence on benefits of both micropulse (MP) and continuous wave (CW) subthreshold retinal lasers, we maintain our view that there is a persistent need for more accurate thermal dosimetry—regardless of the laser mode used. The notion that MP lasers are inherently safer and more efficacious solely by virtue of their mode of operation is not well established. Two recent studies, (Wang et al., 2017) and (Miura et al., 2022), have directly compared MP and CW delivery modes and reported no definitive advantage of one mode over the other in terms of overall safety and tissue response. Specifically, Wang et al. (2017) concluded that while MP reduces heat spread due to a shorter duty cycle, this only decreases the treated volume and diminishes the therapeutic window. Miura et al. (2022) further showed that for the same average power, MP and CW modes led to similar average temperature elevation and similar levels of RPE cell death, but MP had higher temperature peaks and larger temperature fluctuation than CW. Thus, underlying physical principles do not substantiate the claim that one modality is inherently superior to the other.

We believe the key clinical challenge lies not merely in different laser modes, but in ensuring that the chosen laser settings yield a reproducible, sublethal thermal dose that elicits beneficial tissue responses—particularly the activation of heat shock proteins (HSPs)—without causing damage. Our own work and that of others (Lappin & Coogan, 1970; Theisen-Kunde et al., 2023; von der Burchard et al., 2024) demonstrate that the same laser power can result in significantly different temperature elevations between retinal treatments, varying by a factor of two to four. Moreover, it is well documented that achieving a therapeutic response requires reaching a narrow thermal window: approximately 50–80% of the damaging laser power is needed to upregulate production HSP70 (Amirkavei et al., 2020; Desmettre et al., 2001; Sramek et al., 2011; and the current study). In practice, consistently achieving this narrow therapeutic window across a diverse patient population is highly unlikely without personalized laser dosing, regardless of the laser mode used.

In other words, the fundamental issue is not solely whether CW or MP lasers are used, but how to ensure that subthreshold therapy is actually “subthreshold” for each patient while inside the narrow therapeutic window. Thermal dosimetry offers a path to tailor the treatment to individual tissue responses, thereby improving the likelihood of achieving the intended therapeutic effect safely and consistently. We thus maintain that our approach addresses a critical, ongoing need: the ability to deliver a precisely controlled, patient-specific thermal stimulus that genuinely distinguishes “non-damaging” therapy from potential over- or undertreatment.

“We appreciate the view of Dr. Luttrull, who we know to be an experienced physician and a pioneer in the field of micropulsed lasers. However, we disagree with Dr. Luttrull about the therapeutic range of micropulsed or CW lasers, see detailed reasoning below. Additionally, we are talking about two different treatment modalities, micropulsed laser treatment aims to heat primarily the RPE while longpulse treatments aim to extend the therapeutic benefits also to the retina and choroid in addition to RPE, which are all affected in diseases like AMD, cCSC, and DME. According to our view, the micropulsed approaches aim to limit the heating maximally to the RPE, because without thermal dosimetry, this would be the only known way to guarantee the treatment safety, but it also limits the achievable therapeutic effects. With controlled long

pulse, large spot treatment, we can activate the cells' natural restoration mechanisms in all tissues affected by the disease, not just in RPE.”

Therapeutic range is defined as “distance” between no effect and the ED50, or 50/50 retinal burn risk. It is not defined by any particular laser modality (CW vs MPL, etc), but is influenced by it. I am not aware of evidence of any unique clinical benefits arising from sublethally heating the choroid or neurosensory retina in the absence RPE hyperthermia. If there is any evidence for this, the authors’ need to present it. Transpupillary thermotherapy heated the choroid nicely. That’s why it was abandoned.

Transpupillary thermotherapy uses 60-second laser exposure with large spot size (0.8 to 6 mm in diameter) to heat the fundus for around 10 °C (Mainster & Reichel, 2000). With these treatments, the heat dissipated also to retina and choroid in addition to RPE. When the therapy was investigated for wet AMD, a common method to determine sufficient laser power was to use mild retinal graying or whitening in slitlamp-based fundus imaging as a treatment endpoint – they did not have thermal dosimetry. Current knowledge is that already before visible damage, detrimental effects can be seen in more accurate imaging modalities (Deák et al., 2012). Hence, earlier studies with TTT have most probably caused unnecessary damage by aiming to deliver too high thermal dose without having means to monitor the temperature elevation.

Some studies investigated using TTT with milder power. Söderberg and others (Söderberg et al., 2012) used around half the power commonly used for same population in TTT. They investigated whether low-dose TTT treatment can affect the need for anti-VEGF injections for patients with wet AMD. They showed in a 24-month, double-masked, randomized, active-controlled clinical trial that the need for anti-VEGF injections could be reduced almost to half when combining the injection routine with quarterly TTT sessions. Additionally, we found 8 studies testing TTT for treatment of central serous chorioretinopathy (CSC), which recruited from 7 to 25 eyes to TTT (one study used 689 nm laser, others used 810 nm). TTT led to complete resolution of subretinal fluid in 71 to 100% of patients in different studies (Giudice et al., 2011; Hussain et al., 2006; Kawamura et al., 2012; Manayath et al., 2012, 2017; Mathur et al., 2009; Russo et al., 2017; Shukla et al., 2008). The average, weighted by the number of treated eyes, was 87.8% (total 131 eyes).

While these studies do not provide conclusive evidence, they hint that long-pulse treatment, which heats also the choroid and retina, may have a clear clinical benefit, even when conducted without personalized dosing. We acknowledge that many different laser settings have been used in the mentioned studies, which increases the variance in the clinical effects found between trials. However, this again highlights the need for a proper way to monitor the temperature increase to find suitable treatment settings for all.

“We agree with the reviewer that the term “subthreshold”, although widely used, is ambiguous. We decided to change the term for our treatment to non-damaging retinal laser therapy and define the term. “

The only term more corrupt than “subthreshold” is “non-damaging”. It also is used to create the false impression that treatment is safe. Note that “non damaging” is used in marketing to describe both the PASCAL, and 2RT, both of which are designed to be reliably destructive. The only useful term is “sublethal”. Treatment is either sublethal to the RPE, or it isn’t.

We consider that “non-damaging” is a clear term and highlights the aim not to cause damage to the heated tissue.

“Since micropulse treatments use extremely short pulses, the power during the pulse is substantially higher than with CW exposure to reach a corresponding effect. As the laser power required to reach any effect grows with shorter pulses, so does the difference in laser powers required to cause damage. This, however, is not a meaningful way to describe the width of the therapeutic window. We define the therapeutic window as the ratio of the temperature elevation required to cause apoptotic changes and the temperature elevation required to trigger therapeutic effects. This ratio may differ slightly with the duration of laser exposure, but the difference is small (Wang et al., 2017). As the temperature elevation depends on the physiological parameters of the treated eye and the physiological parameters vary between patients and even between different retinal areas, the temperature elevation varies as well. As temperature feedback has not been available, the development of laser therapy modalities has focused on designing the laser exposure in a way that a) minimizes the effect of variability in the relevant physiological parameters on temperature elevation, and b) minimizes the extent of the damage in case of overtreatment. “

The therapeutic range (from no effect to 50/50 thermal burn risk) is dependent on a specific combination of laser parameters. It is not clear what the authors’ think laser power has to do with the TR, other than it is just one parameter that contributes. There is no TR for a particular laser power, or wavelength, etc. Only for specific combinations of all parameters (power, wavelength, spot size, duration, pulse frequency). One is not guided by the parameters (power, wavelength, etc) but by the degree of safety one wishes to achieve. One then tailors the laser parameters to those that best achieve that goal (see the LIGHT treatment recommendations). The only importance of the TR is the likelihood that any particular eye will fall well within that range and expect safe and effective treatment. Thus, narrow TR bad, broad TR good, and it doesn’t matter how you get the. But some modes, like MPL get to broad TRs better than others (like CW). With appropriately selected MPL parameters one can create a very broad TR (> 15 watts), using a set of parameters to accommodate safe and effective treatment every eye of every patient for every indication. This has been clearly demonstrated in clinical application in many studies over 25 years in thousands of human patients. Note that there has never been a single case of retinal damage reported from an 810nm laser at a 5% DC at any spot size or any power using current laser systems. And this without real-time retinal temperature monitoring! This is a fact, not a point of view. This creates a problem for the authors’ technology, and others like optoacoustic temperature measurements, because there is simply no clinical need for the technology the authors describe to directly or indirectly measure temperatures during treatment, if one instead uses the appropriate mode of treatment (long-wavelength, low-frequency MPL) to preclude any risk of thermal retinal damage (ie, treatment lethal to the RPE).

We do not find it meaningful to describe the width of the therapeutic range in terms of laser power. The tissue targeted by the therapy responds to a transient increase in temperature, which depends also on other laser parameters besides laser power, most importantly pulse duration and spot size, as well as physical properties of the eye. If the spot size is increased or the pulse duration is decreased, the required laser power to achieve any temperature dependent effect will increase, also increasing the therapeutic range determined in this way. However, this has nothing to do with the safety or efficacy of the treatment as with this logic a higher therapeutic range would be achieved by maximizing spot size and minimizing pulse duration. We find it meaningful to determine the therapeutic range as the temperature interval

where therapeutic benefits occur, but deleterious effects do not. The next question is how reliably a laser treatment modality can produce an ideal temperature elevation without individualized control of laser parameters and what is the extent of improvement with individualized dosing. The answer to this depends on the width of the therapeutic temperature range relative to the magnitude of the temperature elevation and the variability of temperature elevation in response to the laser exposure due to differences in eye physiology and anatomy. We discussed these aspects in the first answer to reviewer #2.

“It is important to note that laser exposure paradigms with pulse durations in the microsecond and minute ranges create very different temperature elevations in the fundus. With very short laser exposures, the thermal elevation in the retina occurs primarily where the laser energy is absorbed, creating a very short and high transient peak. In long-pulse treatments, the heat has time to spread to surrounding tissues. Since no harmful effects of non-damaging heating have been found, we consider temperature-guided long-pulse treatments as an excellent opportunity to increase the therapeutic effect of non-damaging laser treatments, which already show significant potential in treating multiple retinal diseases.”

There is no evidence to support the above assertion, which, as the sentence continues, already show a high level effectiveness in many different types of disorders without need for temp monitoring or choroidal heating. See also prior comments on “non-damaging” and lacking evidence for therapeutic benefits from heat spread from the RPE (below)

See our responses above.

“According to our view, the short-pulse methods aim to limit the heating only to the RPE, because without thermal dosimetry, this is the only way to guarantee the treatment safety, but it also limits the achievable therapeutic effects. With controlled long pulse, large spot treatment, we can activate the cells' natural restoration mechanisms in also other tissues affected by the disease (like AMD, cCSC, and DME), not just in RPE.”

This is an interesting point of view, but there is no evidence that I am aware of for any notable independent therapeutic benefit from sublethal hyperthermia of either the choroid or neurosensory retina independent of the RPE, which to my knowledge have no paracrine function and operate under the control of the RPE, and not the other way around. If the authors have evidence to support this interesting hypothesis, they should present it.

Various cell types in the fundus of the eye—including all retinal neurons, Müller cells, astrocytes, microglia, and vascular endothelial cells—use paracrine signaling to influence neighboring neurons and tissues. Among these, the stimulation of Müller cells is one key hypothesis for how micropulse laser treatment (MP) achieves its therapeutic effects in diabetic macular edema (DME), though it remains unclear whether this occurs via the RPE or through alternate pathways. Further discussion of this hypothesis can be found in (Mei & Lin, 2024).

Until now, investigating the beneficial effects of controlled sublethal heating on the neural retina or choroid in a clinical setting has been limited by the absence of reliable thermal dosimetry. Earlier transpupillary thermotherapy (TTT) studies offered both cautionary examples of overheating and preliminary indications of potential benefit. However, these studies often aimed to deliver high thermal doses without precise temperature monitoring, despite evidence—including our current findings—that retinal temperature elevation in response to a standard laser exposure may vary by a factor of four.

Our recent work demonstrates that even mild heating of approximately 5 °C above baseline can activate the heat shock response and engage other potential therapeutic targets. By employing accurate thermal dosimetry, we can now explore and refine non-damaging retinal laser treatments. This approach opens the door to optimizing therapeutic efficacy while maintaining safety, potentially improving treatment outcomes in many retinal diseases.

“2. How could the method be beneficial for the treatment of AMD as mentioned in line 89? Which stage of AMD? And is the fERG signal not altered by a non-healthy neural retina?” “There is evidence that non-damaging laser treatments can be used to reduce the number of needed anti-VEGF injections (Söderberg et al., 2012), abolish drusens (Eng et al., 2019; Virgili et al., 2015), aggregations of cellular waste materials,...”

The above comment was taken from the notes from reviewer #3, as the authors’ answer illustrates a fundamental misunderstanding of common retinal laser effects. Acute drusen and debris reduction is dependent on the inflammatory and debridement response to laser-induced retinal damage. It is thus not observed in “non damaging” laser treatment sublethal to the RPE. This is why the failure of laser for dry AMD parallels laser damage: more laser damage, more drusen reduction, faster the progression, and more wet AMD. See the “laser for drusen” and LEAD studies.

It is well established that the amount of drusens or pseudodrusens can be acutely reduced by physically destroying them with laser energy, but this approach has not been shown to be beneficial in treating AMD (Guymer et al., 2019; Virgili et al., 2015). Notably, the LEAD study utilized a nanosecond-pulse laser specifically designed to induce microbubble formation and create small-scale RPE ruptures (Guymer et al., 2019), which is a different mechanism of action than non-damaging treatments.

It is also noteworthy that pseudodrusen can also disappear spontaneously after macular neovascularization develops (Menean et al., 2024). These spontaneous changes highlight that drusen or pseudodrusen reduction alone is not a reliable endpoint for assessing therapeutic benefit in AMD.

In contrast, a systematic review (Eng et al., 2019) found that, among subthreshold laser studies for nonexudative AMD, those reporting significant improvements in visual acuity were also associated with notable drusen reduction. Similarly, in subthreshold laser treatment for reticular pseudodrusen secondary to AMD, reduction in pseudodrusen correlated with increased outer nuclear layer thickness (Querques et al., 2021). While we agree that acute drusen reduction achieved through damaging laser interventions is likely not beneficial, these findings suggest that drusen/pseudodrusen reduction through non-damaging methods could still be associated with both anatomical and functional benefits.

Reviewer #3 (Remarks to the Author):

The manuscript has been improved and most of my questions have been answered. However, I am still concerned about the accuracy of the given temperatures determined by the method. How accurate are the two explicitly addressed temperatures of 44.2 and 46.5 C for all biological effects investigated? The decimal place indicated reflects an accuracy to a tenth of a degree. When thinking about future reference of this paper with respected to temperature

depended biological effects, the uncertainty range and the limitations must clearly be defined and should also be mentioned in the abstract and critically discussed also with respect to k and not just $\alpha(\text{opt})$. The reader should understand, which uncertainty in the temperature values are present for future discussions in the whole field of hyperthermia.

Thank you for this comment. The mentioned temperatures represent group mean temperatures, with a standard deviation of 0.3 °C inside each of the groups. While we chose not to round to whole degrees (e.g., 44°C or 47°C) to avoid unnecessary loss of resolution, we agree it is important to clearly communicate the inherent uncertainty.

Whenever we refer to these temperatures, we now explicitly state that they are “group mean values.” In addition, figure captions and the text highlight that these values have a standard deviation of 0.3 °C.

With respect to this:

Eq. 2 has been changed (why?), k is now in the denominator, and thus the units don't fit any more. So there is obviously something wrong.

We thank the reviewer for noticing an error in the unit. The correct unit is $1/^{\circ}\text{C}$.

Further the value k is referred to the paper of Kaikkonen IEEE 2021 being $k=0.037\text{ C}$. I read this paper and learned that k strongly depends on the flash strength. The flash used here was 46 lx*s, while Kaikkonen found this k value for just 10 cd*s/m², for which k was not determined at all. The temperature slope value strongly depends on this parameter. Thus it remains erratic while $k=0.037\text{ C}$ was chosen. This needs to be clearly clarified.

In our previous paper we investigated the temperature dependence of the ERG flash response kinetics at various flash strengths with constant background illumination. In this paper we needed to use a higher overall brightness primarily to provide sufficiently bright background illumination to enable retinal imaging of the pig fundus using a slit lamp biomicroscope.

The strong background light fully saturates the rods, and the ERG signal is generated primarily by the M-cone pathway. In our previous study (Kaikkonen et al., 2021), we estimate the combined retinal illuminance from the background and stimulation be 150 lx, while in the present work, it is 1800 lux. These levels cause approximately 70% and 96% bleaches, respectively, assuming that the half-bleaching constant of 70 lx at the retina, found for human M-cones (Stockman et al., 2006), is approximately valid for pig M-cones as well.

Under these backgrounds, the M-cones are in the Weber region, where M-cone sensitivity is determined by pigment bleaching (Stockman et al., 2006). In the Weber region, the primary difference in the state of the phototransduction machinery can be attributed to the amount of chromophore-free cone opsin that can weakly activate phototransduction (Kefalov et al., 2005). The difference between the amounts of free pigment at 70% and 96% bleaches is small, and we expect it to have negligible effects on photoreceptor signaling and its temperature dependence.

Furthermore, the luminance contrast between the stimulus and the background used in the current study is the same as that leading to a temperature dependence of 3.7%/°C in our previous work (Kaikkonen et al., 2021). With stronger contrasts, the temperature dependence stabilized near the minimum value of 3.6%/°C within the investigated dynamic range. In current work, we verified that we were at a comparable location on the stimulus-response curve by

recording responses with varying flash strengths in selected experiments. Thus, we consider the value of 3.7%/ °C reliable.

Additional support for the accuracy of our temperature estimates comes from their close alignment with previously published values. Kern et al. (2018) observed an increase in HSP70 production in cultured porcine RPE cells following a 10-second exposure to 44 °C, while signs of apoptosis and cell death were detected above 47 and 50 °C, respectively (Kern et al., 2018). Similarly, Miura et al. (2019) reported that cell damage occurred at temperatures exceeding 45–50 °C in experimental conditions comparable with Kern et al. (Miura et al., 2019). Denton et al. (2009) further demonstrated that, when human-derived RPE cells were heated with a laser for 0.1 to 1 second, the threshold for cell death fell within the range of 46–50 °C (Denton et al., 2009).

Further $\alpha(\text{opt})$ given in eq. 2 is not explained and unclear, how it was determined, and what the error of this value is with respect to the measured data. I recommend to use the same nomenclature as Kaikkonen, if this paper should be the reference standard for temperature determination.

We have changed the symbols of Equation 2 to match the nomenclature of our previous paper.

The discussion section should be extended with respect to the error. I cannot comprehend, how the error of 0.6 C was determined for the 5mm spot, and which of the mentioned sources of error mentioned have which influence. And how is it for the error for the smaller 3.4 mm spot? The influence of k to the error is not mentioned, and obviously the chosen k does not fit to the used flash strength (see above). The Kaikkonen paper did not investigate this flash strength.

The calculation of the temperature error is shown in the supplementary material and comprises all random errors, not systematic ones. We clarified this in the discussion and added a reference to the Supplementary materials to the results section where error values are presented. Systematic errors in temperature determination would influence the lesion threshold temperature without significantly affecting the error value measured as a percentage of the temperature elevation. We added a mention of this to the discussion and improved the nomenclature when using the terms accuracy and precision. The error values were calculated identically for both spot sizes, fitting a probit model between the fERG-based temperature estimates and binary lesion data for corresponding treatments.

Reviewer #4 (Remarks to the Author):

This is an interesting manuscript on retinal laser treatment with ERG- based thermal dosimetry. The manuscript has undergone a previous round of review and the authors have responded to the comments. The revised version reads well and the revision process has improved the manuscript substantially. I only have two comments that should be addressed by the reviewers.

1. Using fERG in humans is associated with significant variability in the amplitudes. Whereas this may be less of a problem in animal experiments where the eye can be stabilised over prolonged periods of time it may not be easy to translate this to the clinical setting. This is also the reason why fERG has lost importance in the recent years. Why dont the authors try to guide

the treatment via Optoretinography? I understand that this technique is still in its infancy, but on the long run this may be the better choice.

Our temperature determination method only looks at the changes in the kinetics of the fERG waveform and the resulting temperature estimate does not depend on signal amplitude. Additionally, temperature elevation happens gradually, and hence small fluctuations in the fixation should not be a concern. If needed, unstable fixation can be compensated using modern retinal tracking -based feedback that would keep the beams in targeted area – a feature found in many diagnostic devices and e.g., in OD-OS Navilas retina laser.

Using optoretinography is a very interesting approach and its suitability for temperature monitoring could be investigated. However, ERG is a well-established technology, and its signal origin is known at detailed level. Additionally, integrating fERG capabilities to a retinal laser therapy system is straightforward when taking into account ERG specific details described in this study and (Kaikkonen et al., 2021).

2. The authors hypothesise that all molecular changes are caused by thermal effects. Is there any proof for this concept. Could the selected wavelength play a role as well? What is the rationale for using a laser diode with 810 nm? Would the same results been achieved with a 670 nm diode?

The thermal effect is expected to be similar with both wavelengths. The rationale for using 810 nm laser is that it does not light adapt cone photoreceptors as much as 670 nm laser. High power 670 nm laser beam could be visible for the pig M-cone and cause substantial light adaptation affecting the ERG signal while the sensitivity for 810 nm light is lower by a factor of >10 000 compared to than for 670 nm light (Govardovskii et al., 2000). The light adapting effect of 810 nm laser can be considered insignificant with the used background illumination but achieving the same for 670 nm light would require an excessively bright background.

Our strong hypothesis is that the changes investigated are caused by temperature elevation and many effects were noted to be temperature dependent. The effect on heat shock proteins and autophagy are similar to those detected when heating ARPE-19 cells in incubator (Amirkavei et al., 2022) and thus we believe that the thermal effect is the dominant factor. Yet we cannot completely rule out additional effects of photobiomodulation caused by the laser or LED beams that could be masked by the thermal effects. Photobiomodulation has recently been shown to have some benefits for treating intermediate AMD (Boyer et al., 2024; Burton et al., 2023) and possible additional photobiomodulation might enhance the beneficial effects of non-damaging heating.

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Point-by-point response to reviewers' comments

Reviewer #1 (Remarks to the Author)

The authors have answered all of the remarks of this reviewer to this reviewer's satisfaction.

We appreciate the reviewer's time and thoughtful feedback throughout the review process. We are glad that our revisions have addressed all concerns to the reviewer's satisfaction.

Reviewer #3 (Remarks to the Author):

My concerns and questions were almost all answered well. Overall, this is a very good paper, especially with regard to the temperature-relevant expression of the different factors studied.

The only remaining point is the accuracy of the temperature determination and the determination of the value of the constant α (previously k). I am not convinced by the reasoning and argumentative justification of the authors for $\alpha = 3.7\%/\text{K}$, as too many assumptions were made to be able to state the accuracy postulated here. Since this value is directly and inversely proportional to the temperature calculation, α is very decisive for the absolute, specified temperatures.

Since I don't see an easy way of obtaining better substantiated values here, I suggest that the remarks made in the reply to my comments be included directly and in detail in the manuscript (and not in the supplements) so that every reader can understand exactly under which assumptions the temperature determination and the α value were derived.

Beyond that, I have no objections for publication.

We thank the reviewer for the positive evaluation and valuable final comment. Following the suggestion, we have moved a condensed version of the previously provided detailed justification and explanations regarding the choice and accuracy of $\alpha = 3.7\%/\text{K}$ directly into the discussion of the main manuscript.

Reviewer #4 (Remarks to the Author):

All comments have been addressed

We appreciate the reviewer's careful evaluation and are pleased that all comments have been addressed satisfactorily. Thank you for your constructive feedback.