Selective Retina Therapy and Thermal Stimulation of the Retina: Different Regenerative Properties - Implications for AMD Therapy

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Abstract

Background

Selective Retina Therapy (SRT) and Thermal Stimulation of the Retina (TSR) have shown therapeutic effects on Age-related Macular Degeneration (AMD) in mice. We investigate the differences between both laser modalities concerning RPE regeneration.

Methods

For PCR array, 6 eyes of apolipoprotein E and nuclear factor erythroid-derived 2-like 2 knock out mice respectively were treated by neuroretina-sparing TSR or SRT. Untreated litter mates were controls. Eyes were enucleated either 1 or 7 days after laser treatment. For morphological analysis, porcine RPE/choroid organ cultures underwent the same laser treatment and were examined by calcein vitality staining 1 h and 1, 3 or 5 days after irradiation.

Results

TSR did not induce the expression of cell-mediators connected to cell death. SRT induced necrosis associated cytokines as well as inflammation 1 but not 7 days after treatment. Morphologically, 1 hour after TSR, there was no cell damage. One and 3 days after TSR, dense chromatin and cell destruction of single cells was seen. Five days after TSR, there were signs of migration and proliferation. In contrast, one hour after SRT a defined necrotic area within the laser spot was seen. This lesion was closed over days by migration and proliferation of adjacent cells.

Conclusions

SRT induces RPE cell death, followed by regeneration within a few days, accompanied by necrosis induced inflammation, RPE proliferation and migration. TSR does not induce immediate RPE cell death; however, migration and mitosis can be seen a few days after laser irradiation, not accompanied by necrosis-associated inflammation.

Background

Age related macular degeneration (AMD) is the most common cause for legal blindness in the industrialized world (1,2). The pathogenesis of AMD is multifactorial. Altered lipid metabolism (3–5), disturbed extracellular matrix homeostasis (6–8), inflammatory processes (9–11), and altered angiogenesis (12–14) are the four major pathways of AMD pathogenesis. Metabolites and cell-waste accumulate within and adjacent to Bruch's membrane (BrM) (3), increasing BrM thickness and thereby diffusion barrier (15), inhibiting gas and nutrient exchange. Under the influence of external factors like smoking (16), Western diet (17) and oxidative stress (18,19) as well as genetic predisposition (20), inflammatory processes appear, RPE cells and, consequently, photoreceptors degenerate leading to late stage dry AMD with patchy RPE/photoreceptor atrophy, called geographic atrophy. Pro-angiogenic factors may lead to choroidal neovascularization (CNV), forming fast progressive late stage neovascular (n)AMD. Both late-stage types of AMD are followed by vision deterioration.

Currently there is no treatment for early AMD, intermediate AMD, or geographic atrophy. Only nAMD can be treated by anti-vascular endothelial growth factor (VEGF) injections, mostly on a monthly schedule (21). The need for treatment options for early and intermediate AMD is unmet.

We could show that novel laser therapies, thermal stimulation of the retina (TSR) and selective retina therapy (SRT), reduce pathologically thickened BrM and partially restore RPEs physiological morphology (22,23). Therefore, both laser therapies might be therapeutic options for early and intermediate AMD. TSR is a continuous wave laser irradiation therapy that leads to a photothermal increase of temperature to about 45°C (22). It induces no anatomical or functional damage to neuroretina. SRT is a micro pulsed laser irradiation therapy that creates a photodisruptive selective damage to RPE, leaving the neuroretina intact (23,24).

Recently, we evaluated the influence of both TSR and SRT on inflammatory mediators (25). We could show that TSR initially acts anti-inflammatory and is followed by chemotactic processes. SRT, on the other hand, initially leads to an inflammatory response, most likely linked to the necrosis of RPE, followed by mild suppression of inflammatory mediators, like complement components, after a week. This led to the hypothesis that in SRT, RPE regeneration is the consequence of selective RPE necrosis. In TSR, RPE regeneration is the consequence of delayed RPE cell death. This hypothesis is addressed here by the evaluation of cell-death linked cell-mediator expression in murine AMD models and by the evaluation of cell morphology in calcein stained porcine organ cultures.

Materials And Methods
AMD Mouse Models

Both knock out AMD mouse models, Apolipoprotein (Apo)E knock out (−/−) and Nuclear factor erythroid 2-related factor 2 (NRF2) −/− have been described in detail (22,26,27). ApoE −/−, NRF2 −/− and C57BL6/J control mice were purchased from the Jackson Laboratories (Bar Harbour, ME, USA). The homozygous genotype and screening for Crumbs homologue 1 (CRB1) retinal degeneration (rd)8 mutation, known to interfere with the AMD phenotype of NRF2−/− mice (28), was confirmed by PCR from tail clips. Mice were kept on a regular 12 hours night and day cycle and fed standard murine diet and water ad libitum. All animal experiments were conducted in accordance with the EU directive 2010/63/EU for animal experiments. They were approved by the animal ethics and welfare committee (approval number: V 242-7224.121-12 (61-5/14)) located at the ministry of energy transition, agriculture, environment and rural areas in Schleswig-Holstein according to German federal and European law. Animal experiments adhere to the NIH Guide for Care and Use of Laboratory Animals.

Animal Maintenance and Anesthesia During Experiments

All examinations and laser treatments were conducted under general anesthesia, like described before (22).

Anesthetized animals were placed on a rigid examination platform and body temperature was maintained within normal limits using a heating mat. Pupils were dilated and eyes were covered with a protective moisturizing gel. After examinations, the anesthesia was antagonized, like described before (22). Anesthesia was uneventful in all mice. Animal wellbeing was evaluated by a standard score sheet and was uneventful in all included mice. After the final examination animals were euthanized by cervical dislocation at the day of enucleation under deep anesthesia.

Examinations

All examinations were conducted under general anesthesia. All mice were examined by funduscopy (MICRON III, phoenix research labs, Pleasanton, CA, USA), to assess integrity of retina, hallmarks of AMD (drusen-like retinal spots (DRS)), RPE atrophy and CNV.

Optical coherence tomography (OCT) (small animal OCT, thorlabs, Lübeck, Germany) was applied to evaluate retinal structure, confirm retinal integrity after laser treatment and to confirm CNV.

All examinations were repeated at the day of enucleation, thus 1 day or 1 week after laser treatment. Untreated controls also were examined twice, at inclusion and at enucleation day.

Laser Treatment

For both SRT and TSR a frequency doubled Neodym-Vanadate (Nd:VO₄) experimental laser (Carl Zeiss Meditec AG, Jena, Germany) with a wavelength of 532 nm was used. The light was coupled to an optical multimode fiber with a 70 x 70 µm² core profile. The laser light was applied via contact laser-injector (Phoenix) attached to the Micron III camera. The pilot laser was controlled visually via live fundus imaging. Spot size was fixed to 50 µm².

For TSR duration of irradiation was fixed to 10 ms continuous wave mode. For SRT duration of irradiation was fixed to 300 ms, pulse-duration was ~1.4 µs at 100 Hz, creating 30 pulses per spot. Comparable to our previous work (22,23), the intended effect was titrated visually by decreasing energy at the peripheral retina from a clearly visible white burn at higher energy to a barely visible spot at lower energy. The barely but instantly visible spot was classified as threshold of definite retinal burn/RPE destruction with visible neuroretinal involvement. Power was reduced by 70 % to ensure neuroretina-sparing temperature increase for TSR, or an RPE-selective laser damage for SRT respectively. The invisible 50 µm TSR/SRT laser spots were distributed uniformly across the retina at 1 spot interspot spacing to an optic disc centered approx. 50° field of view. No laser spot was applied to vasculature or the optic disc.

PCR Array

RT² profiler PCR array (Qiagen®, Frederick, Maryland, USA; Mouse Inflammatory Response & Autoimmunity; PAMM-077Z) was used to determine regulation of inflammation and cell-death related mediators of TSR or SRT treated eyes in comparison with untreated littermates in AMD mouse models. The procedure was described in detail before (25). Briefly, for RNA isolation, posterior cups were homogenized and total RNA was isolated using TRI Reagent® according to the manufacturer's instructions.
Isolated RNA was converted to cDNA using the RT² First Strand Kit (Qiagen). The mixture was aliquoted (25 μl) to each well of the same RT² Profiler PCR Array plate (96-well plate) containing the pre-dispensed gene-specific primer sets. PCR was performed using a 7500 Fast Real Time cycler (Applied Biosystems).

Qiagens online Web analysis tool (Gene globe) was used to calculate the fold change by determining the ratio of mRNA levels to control values using the Δ threshold cycle (Ct) method (2^(-ΔΔCt)). All data were normalized to the housekeeping genes of PAMM007Z panel (Quiagen). PCR conditions used: hold for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C.

### Porcine Organ Cultures Preparation

Fresh porcine eyes were acquired from a local abattoir. The preparation has been described in detail elsewhere (29). Briefly, the eye bulbs were cut at the limbus removing the anterior segment, including lens and vitreous body. Eyes were opened by longitudinal incisions and neuroretina was removed. The complex of RPE, BrM and choroid was removed carefully from sclera. A plastic ring-system was inserted, and the RPE/BrM/choroid complex fixed to it. Rings were placed into 12-well-culture plate and kept warm at 37°C in 1.5 ml organ culture medium (see Richert et al. (29)).

### TSR and SRT in Organ Cultures

Organ cultures were placed under a slit-lamp adapted laser system in organ culture medium in 12-well plates. Organ cultures were irradiated by either TSR (100 ms duration, 200 μm spot size, power titrated to no instant cell-death and a cell death rate of ~ 2 % one day after TSR), or by SRT (300 ms duration, 100 Hz, 1.4 μs pulse duration, 200 μm spot size, energy titrated to an initial cell-death rate of 80 %). Calcein-assays were performed afterwards to confirm cell-death rates. This method was also applied to check the quality of organ cultures (30).

### Calcein Assay

Calcein assays were conducted to examine integrity and vitality of RPE organ cultures. Calcein fluoresces if cleaved by active enzymes integrated into vital RPE cell membranes. Dead cells do not fluoresce, since enzymatic cleavage does not function in dead cells (31). Organ culture explants were incubated in 2 ml culture medium with 4 μg/ml Calcein at 37 °C for 45 minutes. Afterwards they were rinsed twice in phosphate buffered saline. Cell vitality was measured by fluorescence microscopy (Axiovert 100, Zeiss, Jena, Germany) at λex/λem = 497/517 nm and documented photographically.

### Statistics

#### Gene Expression by PCR Arrays

Fold changes in gene expression for pairwise comparison using the ΔΔCT method was calculated through Qiagen® Web analysis tool and p-values were provided, at a confidence interval of 95 % and a type-1 error of 5 %.

For comparison of TSR or SRT treated to untreated eyes, one randomized eye of ApoE-/- or NRF2-/- was treated by TSR or SRT. One day or one week after treatment these eyes were compared to entirely untreated age-matched randomized control eyes of the same genotypes in groups of 6 eyes each.

#### Determination of Cell Size and Number by Calcein Assays

For determination of cell death and regeneration, 1 hour, 1, 3 and 5 days after TSR or SRT laser treatment, calcein assay photographs were analyzed semiautomatically by AxioVision (Zeiss, Jena, Germany). Cell size and number of vital cells within the defined 200 μm laser spot were measured and noted for statistical analysis. The median percentage of non-fluorescent area within the defined laser spot was calculated from 12 spots each.

### Results

#### In-Vivo Imaging
All mice showed certain signs of AMD, such as drusen-like retinal spots (DRS), RPE pigmentation irregularities and mottling. CNV or geographic atrophy, as markers for late AMD, were not seen in any mouse. AMD disease grading, like explained before (28) (1 = physiological retina, 2= 1-14 DRS, 3= 15-100 DRS, 4= >100 DRS, 5= any number of DRS plus signs of late AMD), revealed a mean of grade 2.6 +/- 0.6 in NRF2-/- and 1.7 +/- 0.6 in ApoE-/- mice. Laser treatment did not significantly alter the AMD grade of either NRF2 -/- or ApoE -/- mice. There were no signs of neuroretinal damage in fundus examination or in OCT after either laser treatment.

**PCR-Array for the Expression Level of Inflammatory Cell Mediators Linked to Apoptosis and Necrosis**

Table 1 shows the results of PCR array-based analysis of cell mediator expression. Cell-death-linked apoptotic factors, like Fas ligand (FasL), Interferon gamma (IFNg), Interleukin (IL)1 beta and IL18, as well as Nuclear factor kappa light-chain enhancer of activated B-cells (NFkb) were examined. FasL was not altered neither by TSR nor by SRT, 1 or 7 days after laser treatment. IFNg was downregulated by TSR, 1 day after treatment in ApoE-/- and upregulated 1 day after SRT in NRF2-/- mice. IL1b was downregulated 7 days after SRT in NRF2-/- mice. IL18 was not altered in either treatment. Expression of NFkb1 was increased 1 day after SRT.

Necrosis-linked factors were also examined. Complement system centered complement factor 3 (C3) expression, was increased 4-fold, 1 day after SRT in ApoE-/. Toll-like receptors (Tlr) were increased 1 day after SRT in both models. In NRF2-/- mice Tlr expression was decreased 1 day after TSR. Tumor necrosis factor superfamily (Tnfsf) was downregulated 1 day after TSR in ApoE-/- mice.

### Table 1

The results of PCR array-based analysis of cell mediator expression.

| Prot | ApoE 1d | TSR 1d | TSR 7d | SRT 1d | SRT 7d | NRF2 1d | TSR 1d | TSR 7d | SRT 1d | SRT 7d |
|------|---------|--------|--------|--------|--------|---------|--------|--------|--------|--------|
| FasL | 1.2     | 1.8    | 1.7    | -2.5   | -1.5   | 1.1     | -1.3   | 1.3    |
| IFNg | -5.1    | <0.01  | 1.6    | -1.9   | -1.3   | 1.1     | 2.3    | <0.01  | 1.9    |
| IL1b | -1.4    | 1.1    | 3.3    | -1.7   | -1.8   | -1.8    | -5.2   | -2.0   | 0.02   |
| IL18 | 1.4     | 1.1    | 1.6    | -1.5   | -1.6   | 1.1     | -1.1   | -1.3   |
| NFkb1| -1.3    | -1.6   | 5.0    | <0.01  | 1.3    | -1.2    | 1.1    | 1.2    | -1.7   |
| C3   | -1.9    | 1.1    | 4.0    | 0.01   | -1.4   | -1.2    | -1.8   | 1.2    | -3.1   |
| Tlr1 | -1.2    | 1.6    | 2.1    | -1.7   | -1.7   | 1.1     | 2.5    | 0.04   | -1.4   |
| Tlr2 | -1.8    | -1.1   | 4.1    | <0.01  | -1.3   | -1.9    | 1.2    | -1.1   | -1.1   |
| Tlr4 | -1.5    | 1.1    | 2.8    | 0.01   | -1.1   | -2.0    | <0.01  | -1.1   | 1.5    | -1.5   |
| Tlr7 | -1.3    | 1.6    | 1.9    | -2.3   | -2.3   | 0.05    | 1.7    | 1.2    | -1.4   |
| Tlr9 | -1.7    | 1.1    | 4.0    | <0.01  | -1.5   | -3.0    | <0.01  | -1.6   | -1.6   | -1.4   |
| Tnfsf14| -3.5   | <0.01  | 2.6    | 1.1    | -3.0   | 1.9     | 2.5    | -2.5   | 1.3    |

**Single Values of Examined Apoptosis and Necrosis linked Inflammatory Cell Mediators**

*Column 1 shows the name of the protein examined. For each genotype, x-fold expression in the treated eyes (TSR or SRT respectively) compared with untreated eyes and their p-values are given. FasL, Ifng, Il1b, Il18, Nfkbl may be linked to apoptosis (bold frame). C3, Tlr1,2,4,7,9 and Tnfsf14 may be linked to necrosis.*
Calcein Assay

In porcine organ cultures RPE cell vitality was examined by calcein assay. In addition, regeneration processes could be examined. Figure 1 displays calcein assays after TSR and after SRT at 200 µm spot-size 1 hour, 1, 3 or 5 days after laser irradiation. There was no instantly visible cell damage 1 hour after TSR. TSR-treated organ cultures showed cell mottling, condensed nuclei from day 1 (2 % cell death ± 4.3 % within the lasered area, n= 12 spots). From day 3 (mean cell damage 4.2 % ± 3.6 within the lasered area, n= 12 spots), regenerative signs, like cell migration and cell proliferation were seen. Cell replacement and lesion closure were seen in small patches across the spot. Lesion closure was achieved at day 5. SRT induced instant cell necrosis covering the whole spot, followed by proliferation and migration from day 1 after laser irradiation (mean area of cell death 60 % ± 36.5 n= 12 spots). At day three 6.7 % (± 6.5 %, n= 12 spots) of the initial spot area were not filled with new cells. Lesion closure was complete after 5 days.

Discussion

AMD is a multifactorial disease composed of an altered lipid metabolism, changed extracellular matrix, inflammatory processes and mislead angiogenesis. There is no pathogenesis-driven therapy that targets all the above-named aspects of AMD. Current therapeutic strategies aim at certain parts of AMD pathogenesis. To date, only the treatment of pathologic angiogenesis by anti-vascular endothelial growth factor (VEGF) antagonists(21) in neovascular AMD has shown great therapeutic benefit. A therapy for early or intermediate AMD has yet to be developed. We know that TSR, as well as SRT have a therapeutic effects on AMD-like alterations in AMD mouse models. Thickened BrM becomes thinner and pathologically altered RPE becomes a more physiological phenotype (22,23). BrM restructuring aims at extracellular matrix and is mediated by an increase in matrix metallo-protease (MMP) expression, especially active MMP-2 (29,32). However, RPE regeneration may have a positive influence not only on extracellular matrix.

As for inflammation, it is more likely that inflammatory processes are altered in short term due to the laser impact on the treated RPE cells. RPE cells are reduced in viability if put under constant pro-inflammatory stress (33). This condition can be found in AMD mouse models, like ApoE -/- mice (25). We could show earlier that TSR suppresses inflammatory processes one day after treatment and is followed by chemotaxis one week after laser irradiation. SRT induces inflammation instantly due to the intended necrosis. Inflammatory processes are unaltered or even suppressed one week after laser irradiation (25). A lasting therapeutic effect that derives from suppression of pro-inflammatory processes over a long time, useful for the treatment of AMD, has not been shown so far.

The effect on lipid metabolism has not been evaluated yet. It should be part of future studies to increase the understanding of the way of action of both TSR and SRT.

The effect of TSR and SRT on neovascular AMD has also not been evaluated in a translational model. We know from organ culture experiments that both TSR and SRT lead to a reduction of VEGF expression and increase of PEDF expression (29,34). However, both laser modi have shown to influence more than only one aspect of AMD pathogenesis in AMD mouse models. Both may be therapeutic options. The unsuccessful LEAD study (35), as far as the inhibition of a conversion of intermediate to late AMD is concerned, has shown that patient selection and a good understanding of the molecular mechanisms of a new treatment are crucial.

So how exactly do TSR and SRT act?

Based on findings from this paper, as well as findings from former publications (22,23,25,29,34,36), we propose the following model, as depicted in figure 2.

SRT, as expected, leads to defined necrosis of RPE (24) (see Fig. 1, 2 and Table 1). The photo-disruptive effect induces RPE cell death that does not harm neuroretina (23,37). Necrosis is accompanied by inflammation and chemotaxis to remove cell-debris (25). RPE regenerates (29) and active MMP expression is increased, leading to thinning of the pathologically thickened BrM in AMD (23,38). Restoring and rejuvenating RPE could be a useful therapeutic approach to treat intermediate AMD.

TSR (see Fig. 2), on the other hand, leads to a delayed small scale RPE cell-death (see Fig.1), followed by immediate replacement of RPE cells (34), without inflammation (25) and, like SRT, without neuroretinal damage (22). The regenerative process is accompanied by an increase in active MMP expression (34), also leading to thinning of BrM. A regenerative process of RPE cells can also be seen (22). This less invasive, less immunogenic approach might be a therapeutic means for mild AMD or even for AMD prevention.

From the presented data and from what we have known so far, one cannot decide if either TSR or SRT are the better therapeutic option for the treatment of a certain type of AMD. More needs to be known about the way of action to better attribute either laser modus to a certain
type of AMD.

The above shown data is limited by small numbers of eyes and methods to differentiate between the different regenerative properties. However, the differences between both laser modi become clearly evident. SRT leads to necrosis followed by regeneration. TSR leads to delayed RPE cell death in absence of inflammation also followed by regeneration. A presumed apoptotic process needs to be looked at closer in future studies.

Despite uncertainties concerning laser therapy for early and intermediate AMD it is reasonable to consider both SRT and TSR potential therapeutic means for AMD. Both need to be evaluated in humans. Study populations need to be selected carefully, considering the different grades and forms of AMD. TSR might be an option for early AMD with small drusen, SRT for a more advanced type that might need more therapeutic effect. Future studies, especially human studies, will have to determine the benefit of TSR and SRT for the treatment of early and intermediate AMD.

**Abbreviations**

AMD age-related macular degeneration
ApoE -/- apolipoprotein E knock out
BrM Bruch's Membrane
C3 complement factor 3
CNV choroidal neovascularization
CRB1 crumbs homologue gene 1
DRS drusen like retinal spots
FasL Fas ligand
IFNg interferon gamma
IL interleukin
MMP matrix metallo proteinase
nAMD neovascular age-related macular degeneration
NFkb Nuclear factor kappa light-chain enhancer of activated B-cells
NRF2 -/- nuclear factor erythroid 2-related factor 2
OCT optical coherence tomography
RPE retinal pigment epithelium
SRT Selective Retina Therapy
Tlr toll-like receptor
Tnfsf Tumor necrosis factor superfamily
TSR Thermal Stimulation of the Retina
VEGF vascular endothelial growth factor

**Declarations And Acknowledgments**

**Ethics Approval**
All animal experiments were conducted in accordance with the EU directive 2010/63/EU for animal experiments. They were approved by the animal ethics and welfare committee (approval number: V 242-7224.121-12 (61-5/14)) located at the ministry of energy transition, agriculture, environment and rural areas in Schleswig-Holstein according to German federal and European law. Animal experiments adhere to the NIH Guide for Care and Use of Laboratory Animals.

Data Availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare that they have no competing interests

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Author’s Contributions

ER planning experiments, conducting experiments, data evaluation, manuscript revision
JP conducting experiments, data evaluation, manuscript revision
CvB data evaluation, manuscript revision, statistics
AK manuscript revision, laboratory support, experimental support
PA manuscript revision, laboratory support, experimental support
RL manuscript revision, laboratory support, experimental support
RB manuscript revision, laser device, experimental and technical support
CF manuscript revision, data revision
JR manuscript revision, data revision
JT planning experiments, conducting experiments, data evaluation, manuscript preparation, manuscript revision, funding, correspondence

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References

1. Schrader WF. [Age-related macular degeneration: a socioeconomic time bomb in our aging society]. Ophthalmol Z Dtsch Ophthalmol Ges. 2006 Sep;103(9):742–8.
2. Klein R, Klein BE, Linton KL. Prevalence of age-related maculopathy. The Beaver Dam Eye Study. Ophthalmology. 1992 Jun;99(6):933–43.
3. Curcio CA, Johnson M, Rudolf M, Huang J-D. The oil spill in ageing Bruch membrane. Br J Ophthalmol. 2011 Dec;95(12):1638–45.
4. Xu Q, Cao S, Rajapakse S, Matsubara JA. Understanding AMD by analogy: systematic review of lipid-related common pathogenic mechanisms in AMD, AD, AS and GN. Lipids Health Dis. 2018 Jan 4;17(1):3.
5. Wang L, Clark ME, Crossman DK, Kojima K, Messinger JD, Mobley JA, et al. Abundant lipid and protein components of drusen. PloS One. 2010;5(4):e10329.
6. Okubo A, Rosa RH, Bunce CV, Alexander RA, Fan JT, Bird AC, et al. The relationships of age changes in retinal pigment epithelium and Bruch's membrane. Invest Ophthalmol Vis Sci. 1999 Feb;40(2):443–9.

7. Beattie JR, Pawlak AM, Boulton ME, Zhang J, Monnier VM, McGarvey JJ, et al. Multiplex analysis of age-related protein and lipid modifications in human Bruch's membrane. FASEB J Off Publ Fed Am Soc Exp Biol. 2010 Dec;24(12):4816–24.

8. Ahir A, Guo L, Hussain AA, Marshall J. Expression of metalloproteinases from human retinal pigment epithelial cells and their effects on the hydraulic conductivity of Bruch's membrane. Invest Ophthalmol Vis Sci. 2002 Feb;43(2):458–65.

9. Jun S, Datta S, Wang L, Pegany R, Cano M, Handa JT. The impact of lipids, lipid oxidation, and inflammation on AMD, and the potential role of miRNAs on lipid metabolism in the RPE. Exp Eye Res. 2018 Oct 5;

10. Kauppinen A, Niskanen H, Suuronen T, Kinnunen K, Salminen A, Kaamiranta K. Oxidative stress activates NLRP3 inflammasomes in ARPE-19 cells—implications for age-related macular degeneration (AMD). Immunol Lett. 2012 Sep;147(1–2):29–33.

11. Anderson DH, Mullins RF, Hageman GS, Johnson LV. A role for local inflammation in the formation of drusen in the aging eye. Am J Ophthalmol. 2002 Sep;134(3):411–31.

12. Dvorak HF, Brown LF, Detmar M, Dvorak AM. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. Am J Pathol. 1995 May;146(5):1029–39.

13. Ohno-Matsui K, Morita I, Tombran-Tink J, Mrzak D, Onodera M, Uetama T, et al. Novel mechanism for age-related macular degeneration: an equilibrium shift between the angiogenesis factors VEGF and PEDF. J Cell Physiol. 2001 Dec;189(3):323–33.

14. Tong J-P, Yao Y-F. Contribution of VEGF and PEDF to choroidal angiogenesis: A need for balanced expressions. Clin Biochem. 2006 Mar;39(3):267–76.

15. Hussain AA, Starita C, Hodggets A, Marshall J. Macromolecular diffusion characteristics of ageing human Bruch's membrane: implications for age-related macular degeneration (AMD). Exp Eye Res. 2010 Jun;90(6):703–10.

16. Pons M, Marin-Castaño ME. Nicotine increases the VEGF/PEDF ratio in retinal pigment epithelium: a possible mechanism for CNV in passive smokers with AMD. Invest Ophthalmol Vis Sci. 2011 May;52(6):3842–53.

17. Boehme MWJ, Buechle G, Frankenhauser-Mannuss J, Mueller J, Lump D, Boehm BO, et al. Prevalence, incidence and concomitant comorbidities of type 2 diabetes mellitus in South Western Germany—a retrospective cohort and case control study in claims data of a large statutory health insurance. BMC Public Health. 2015 Sep 3;15:855.

18. Hollyeld JG, Bonilha VL, Raybom ME, Yang X, Shadrach KG, Lu L, et al. Oxidative damage-induced inflammation initiates age-related macular degeneration. Nat Med. 2008 Feb;14(2):194–8.

19. Klettner A, Roeder J. Constitutive and oxidative-stress-induced expression of VEGF in the RPE are differently regulated by different Mitogen-activated protein kinases. Graefes Arch Clin Exp Ophthalmol Albrecht Von Graefes Arch Klin Exp Ophthalmol. 2009 Nov;247(11):1487–92.

20. Fritsche LG, Igl W, Bailey JNC, Grassmann F, Sengupta S, Bragg-Gresham JL, et al. A large genome-wide association study of age-related macular degeneration highlights contributions of rare and common variants. Nat Genet. 2016 Feb;48(2):134–43.

21. CATT Research Group, Martin DF, Maguire MG, Ying G, Grunwald JE, Fine SL, et al. Ranibizumab and bevacizumab for neovascular age-related macular degeneration. N Engl J Med. 2011 May 19;364(20):1897–908.

22. Tode J, Richert E, Koinzer S, Klettner A, von der Burchard C, Brinkmann R, et al. Thermal Stimulation of the Retina Reduces Bruch's Membrane Thickness in Age Related Macular Degeneration Mouse Models. Transl Vis Sci Technol. 2018 May;7(3):2.

23. Tode J, Richert E, Koinzer S, Klettner A, von der Burchard C, Brinkmann R, et al. Selective Retina Therapy Reduces Bruch's Membrane Thickness and Retinal Pigment Epithelium Pathology in Age-Related Macular Degeneration Mouse Models. Transl Vis Sci Technol. 2019 Nov;8(6):11.

24. Roeder J, Michaud NA, Flotte TJ, Bimgruber R. Response of the retinal pigment epithelium to selective photocoagulation. Arch Ophthalmol Chic Ill 1960. 1992 Dec;110(12):1786–92.

25. Richert E, Tode J. Modulation of inflammatory processes by thermal stimulating and RPE regenerative laser therapies in age related macular degeneration mouse models | Elsevier Enhanced Reader. Cytokine:X [Internet]. 2020 [cited 2020 Aug 10]; Available from: https://reader.elsevier.com/reader/sd/pii/S2590153220300112?token=9066081D8D8C491D6E7BACAF79C73842446AB01FA82C68097301B2DDBA7747954ED22DAE046C83DD86FC6B456BDD4A4

26. Dithmar S, Curcio CA, Le NA, Brown S, Grossniklaus HE. Ultrastructural changes in Bruch's membrane of apolipoprotein E-deficient mice. Invest Ophthalmol Vis Sci. 2000 Jul;41(8):2035–42.

27. Zhao Z, Chen Y, Wang J, Stemberg P, Freeman ML, Grossniklaus HE, et al. Age-related retinopathy in NRF2-deficient mice. PloS One. 2011;6(4):e19456.
28. Richert E, Klettner A, von der Burchard C, Roiger J, Tode J. CRB1rd8 mutation influences the age-related macular degeneration phenotype of NRF2 knockout mice and favors choroidal neovascularization. Adv Med Sci. 2020 Jan 6;65(1):71–7.

29. Richert E, Koinzer S, Tode J, Schlott K, Brinkmann R, Hillenkamp J, et al. Release of Different Cell Mediators During Retinal Pigment Epithelium Regeneration Following Selective Retina Therapy. Invest Ophthalmol Vis Sci. 2018 Mar 1;59(3):1323–31.

30. Klettner A, Miura Y. Porcine RPE/Choroidal Explant Cultures. Methods Mol Biol Clifton NJ. 2019;1834:109–18.

31. Neri S, Mariani E, Meneghetti A, Cattini L, Facchini A. Calcein-Acetyoxymethyl Cytotoxicity Assay: Standardization of a Method Allowing Additional Analyses on Recovered Effector Cells and Supernatants. Clin Diagn Lab Immunol. 2001 Nov;8(6):1131–5.

32. Richert E, Koinzer S, Klettner A, Brinkmann R, Hillenkamp J, Roider J. Response of RPE-Choroid Explants to Thermal Stimulation Therapy of the Retinal Pigment Epithelium (TS-R). Invest Ophthalmol Vis Sci. 2016;57(12):4442.

33. Klettner A, Brinkmann A, Winkelmann K, Käckenmeister T, Hildebrandt J, Roider J. Effect of long-term inflammation on viability and function of RPE cells. Exp Eye Res. 2020 Sep 6;200:108214.

34. Richert E, Papenkort J, Klettner A, Tode J, Koinzer S, Brinkmann R, et al. Response of Retinal Pigment Epithelium (RPE)-Choroid Explants to Thermal Stimulation Therapy of the RPE (TS-R). Lasers Surg Med. 2020 Jun 21;

35. Guymer RH, Wu Z, Hodgson LAB, Caruso E, Brassington KH, Tindill N, et al. Subthreshold Nanosecond Laser Intervention in Age-Related Macular Degeneration: The LEAD Randomized Controlled Clinical Trial. Ophthalmology. 2018 Sep 20;

36. Treumer F, Klettner A, Baltz J, Hussain AA, Miura Y, Brinkmann R, et al. Vectorial release of matrix metalloproteinases (MMPs) from porcine RPE-choroid explants following selective retina therapy (SRT): Towards slowing the macular ageing process. Exp Eye Res. 2012 Apr;97(1):63–72.

37. Roiger J, Brinkmann R, Wirbelauer C, Laqua H, Birngruber R. Retinal sparing by selective retinal pigment epithelial photocoagulation. Arch Ophthalmol Chic Ill 1960. 1999 Aug;117(8):1028–34.

38. Guymer RH, Brassington KH, Dimitrov P, Makeyeva G, Plunkett M, Xia W, et al. Nanosecond-laser application in intermediate AMD: 12-month results of fundus appearance and macular function. Clin Experiment Ophthalmol. 2014 Jul;42(5):466–79.