PRO RETINA FOUNDATION

# 11th PRO RETINA
## RESEARCH-COLLOQUIUM POTSDAM

CONFERENCE REPORT

# Retinal Degeneration

## In Times of Transition and Translation

An Interdisciplinary Dialogue

April 08 / 09, 2016

Seminaris SeeHotel Potsdam

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# PRO RETINA DEUTSCHLAND E. V. & THE PRO RETINA-FOUNDATION FOR PREVENTION OF BLINDNESS

## WHO WE ARE

The patient-organisation, "PRO RETINA Deutschland e. V.", was founded in 1977 as „Deutsche Retinitis Pigmentosa-Vereinigung" by patients and their relatives intended to organize help for themselves. The three objectives mentioned in the constitution are to actively support research, to give psychological and social advice for its members and to strengthen public information.
Every member can join one of the 60 regional groups, which are spread throughout Germany. At present (2015), PRO RETINA Deutschland e. V. counts more than 5,800 members. The Board, the counsellors, the leaders of the regional groups and all active members are working on a non-profit basis, but they are supported by a fulltime working staff at our office which is located in Aachen (www.pro-retina.de).

## WHAT WE DO IN RESEARCH

The jewel of all this work is the PRO RETINA-Foundation for Prevention of Blindness, which was founded in 1996.
From the early beginning we have created a stable network with researchers and ophthalmologists for joined information and advice. We support research projects with direct financial funding – since the "Foundation for Prevention of Blindness" was established in 1996, more than two million Euro have been donated. We actively initiate research projects and therapy tests and contribute to their implementation.

Every year, we award two research prices and organize and support national and international seminars and conferences on relevant topics. We are financing PhD grants in order to foster research activities and networking between researchers.

We are consulted by a Scientific and Medical Advisory Board ("Wissenschaftlicher und Medizinischer Beirat", WMB) and a Working Group on Clinical Questions ("Arbeitskreis Klinische Fragen", AKF). In this Working Group scientists of different medical and other relevant disciplines are taking part.

The main objective is to secure a long-term support for research activities, e. g. by granting financial means for the development of new research projects or by financing the initial phase of relevant projects.

It is envisaged to increase the capital of the foundation to a minimum of Euro 5,000,000, which are to result in a steady source of funding for the support of research, independent from changing income of donations.
We guarantee that the benefits of the Foundation will only be dedicated to the research of retinal diseases, with the wider objective to develop applicable therapies for the patients.

# PROGRAMME

## Friday, April 08, 2016

13:00–13:05 **Welcome remarks**
Franz Badura, PRO RETINA-Foundation

**13:05–14:30 Session 1 Selected poster presentations**
*Eight abstracts to be selected from submissions*

14:30–15:00 **Keynote lecture**
Christian Grimm, Zürich
*Road to Therapy*

15:00–15:45 **Coffee break**

**15:45–17:25 Session 2 Biomarker – New developments**
Chairman: Prof. Dr. Klaus Rüther

15:45–16:10 M. Valeria Canto-Soler, Baltimore
*3D mini-retinas and its use in pre-clinical studies*

16:10–16:35 Seba Almedawar, Dresden
*Stem cells derived retinal pigment epithelium: From transwell to high-throughput screening format*

16:35–17:00 Felix Grassmann, Regensburg
*Genetic and epigenetic markers in AMD*

17:00–17:25 Sascha Fauser, Köln
*Cytokines in age-related macular degeneration: Pathogenesis and treatment*

17:30 **Dinner**

**19:00–20:00 Session 3 Back to the future – Recalling a scientist's working life**

19:00–19:30 Andreas Gal, Hamburg
*From congenital stationary night blindness to inherited retinal dystrophy: A journey of 30 years discovering the secrets of the emerging human genome*

19:30–20:00 Andreas Reichenbach, Leipzig
*Me and the Müller Cell – A quarter of a century-lovestory*

20:00–open **Swingin' poster session**

# PROGRAMME

## Saturday, April 09, 2016

| | | |
|---|---|---|
| **09:00–10:40** | **Session 4** | **Outcome monitoring in clinical trials**<br>Chairman: Prof. Dr. Olaf Strauß |
| | 09:00–09:25 | Thomas Ach, Würzburg<br>*RPE autofluorescence – An old biomarker revisited* |
| | 09:25–09:50 | Michael Bach, Freiburg<br>*Visual acuity – Conceptualisation, quantification and clinical validity* |
| | 09:50–10:15 | Michel Paques, Paris<br>*Short-term monitoring of AMD by flood-illumination adaptive optics and OCT* |
| | 10:15–10:40 | Ursula Schmidt-Erfurth, Wien<br>*Computational image analyses for outcome prediction in AMD trials* |
| 10:40–11:15 | **Coffee break** | |
| **11:15–12:55** | **Session 5** | **Preclinical and clinical trials**<br>Chairman: Prof. Dr. Bernhard Weber |
| | 11:15–11:40 | Don Zack, Baltimore<br>*Patient-derived cells and genome-editing as innovative tools for large-scale compound screenings* |
| | 11:40–12:05 | Stylianos Michalakis, München<br>*Gene therapy for achromatopsia: From mouse models to human clinical trials* |
| | 12:05–12:30 | Rupert Strauss, London<br>*ProgStar study – Update* |
| | 12:30–12:55 | Peter Charbel-Issa, Bonn<br>*Will there be tablets against Stargardt disease? The effect of deuterated vitamin A* |
| 12:55–13:00 | **Concluding remarks** | |
| 13:00 | **Lunch and end of meeting** | |

# SPEAKER ABSTRACTS

# Stem cells derived retinal pigment epithelium: from transwell to high-throughput screening format

*Seba Almedawar[1], Sven Schreiter[1], Elly Tanaka[1]*

*[1]Center for Regenerative Therapies TU Dresden (CRTD)*

**Purpose:** The retinal pigment epithelium (RPE) is a monolayer of pigmented epithelial cells that are required for the maintenance of photoreceptors, and their functionality is largely compromised in retinal degenerative diseases, which have so far no effective cure, such as age related macular degeneration and retinitis pigmentosa. Since RPE cells are post-mitotic, there is a limited source of primary RPE that can be used to study such diseases on the molecular and cell biological level and develop large scale RPE cell based compound screening assays required to reveal new therapies. In the last few years many protocols have been developed to generate RPE cells from human embryonic or induced pluripotent stem cells (hESC/iPSC). However, the efficiency and the length of these protocols do not meet industry standards which are required to carry high throughput compound screens.

**Methods:** In our lab we have established a protocol that generates RPE cells from hESC and iPSC in large quantities within 30 days. Three dimensional epithelial cyst cultures of hESCs in Matrigel combined with neural induction results in a quantitative conversion into neuroepithelial cysts containing a single lumen within 5 days. These cysts naturally generate RPE, in part due to IGF-1/insulin signaling after seeding on transwell filters. In addition, we have expanded our RPE preparation to generate enough cells and optimized a phagocytosis assay to carry a high throughput compound screen for stimulators of photoreceptor outer segment phagocytosis in the RPE.

**Results and conclusions:** RPE cells derived through this protocol are highly functional as direct transplantation into a rat model of retinal degeneration, without any selection or expansion of the cells, results in the formation of a donor-derived RPE monolayer that rescues photoreceptor cells. Our compound screen shows that our assay is quite robust and reproducible and can reveal not only stimulators of phagocytosis, but also modulators. In parallel, the availability of these cells allowed us to develop secondary assays that can be used to validate the hits and study the molecular and cell biology of phagocytosis in the RPE.

# Genetic and epigenetic markers in age-related macular degeneration (AMD) and their implications for therapy

*Felix Grassmann*

*Institute of Human Genetics, University of Regensburg, Germany*

Recently, the International AMD Genomics consortium (IAMDGC) has reported the latest findings on genetic factors conferring risk to develop AMD. In total, the consortium reported genome-wide significant associations of 34 loci with AMD, further implicating genes of the complement system in disease pathology.

Building on these results, the consortium is currently conducting additional analyses on the available data which will further elucidate the genetic architecture of the disease. Although such efforts continue to shed light on disease risk of AMD, still, little is known about factors governing disease progression, severity and treatment response. Those factors are, however, crucial to design targeted treatment options and to monitor and evaluate treatment success. Therefore, the presentation will address new approaches to investigate such factors and will discuss their advantages and shortcomings. This will highlight potential study settings that are greatly underrepresented in current study designs.

The presentation will also focus on potential genetic and epigenetic markers that can be queried in those studies and advocate their application in other retinal dystrophies. Examples of such studies already in progress will be outlined and their preliminary results will be given.

# Cytokines in age-related macular degeneration: Pathogenesis and treatment

*Sascha Fauser*

*University Hospital Cologne, Germany*

**Purpose:** In age-related macular degeneration (AMD) hypotheses on the pathomechanism and on treatments have to be evaluated in the human eye. The anterior chamber is an easily accessible compartment for such measurements.

**Methods:** Aqueous humor samples were collected in patients with AMD and in controls prior to intravitreal injections or cataract surgery. Cytokines were measured by ELISA and correlated with clinical and genetic data.

**Results:** Inflammatory cytokines and complement proteins are elevated in AMD patients compared to controls. Anti-VEGF drugs such as ranibizumab or aflibercept completely inhibit VEGF in the anterior chamber for a very stable, intra-individual period of time which is independent of choroidal neovascularization.

**Conlusion:** AMD is a local disease with the involvement of inflammation and the complement system. However, treatment targets only VEGF so far.

# RPE autofluorescence – An old biomarker revisited

*Thomas Ach*

*Department of Ophthalmology, University Hospital Würzburg*

The accumulation of autofluorescent granules (lipofuscin, melanolipofuscin) within retinal pigment epithelium (RPE) cells is a well-known phenomenon of the aging RPE cell. Macroscopically, granule accumulation within RPE cells is easily detectable by fundus cameras and has been intensely studied in clinical studies. RPE autofluorescence also became a biomarker for healthy and diseased RPE. Based mostly on clinical imaging, it was believed that lipofuscin accumulation in age-related macular degeneration (AMD) causes RPE cell death.

This paper presentation will summarize and discuss new histological data on RPE autofluorescence in normal aging and in AMD: recent systematic autofluorescence analysis of RPE flatmounts and histological retina sections questions lipofuscin's role in the development of AMD. Furthermore, with the advent of new imaging modalities (e.g., high-resolution structured illumination, mass spectrometry, spectral analysis) extensive new data on RPE autofluorescence is available. Careful data interpretation opens the way for a re-evaluation of RPE autofluorescence regarding its significance in health and disease.

# Visual acuity – Conceptualisation, quantification and clinical validity

*Michael Bach*

*Eye Center, University Medical Center, Freiburg, Germany*

Visual acuity is frequently seen as trivial in concept and methodology. This presentation aims to first addle such opinions and then proffer solid understanding.

I will cover the history of visual acuity and the influence of optics and neuronal processing. When defining "acuity", we will encounter international norms and multiple formats (decimal acuity, Snellen fraction, logMAR, lines and letters). The psychometric function is the basic rationale of subjective assessment, suggesting specific procedural approaches to obtain reliable quantitative values. This leads to a discussion of the range of normal values (is 20/20 = 1.0decimal = 0.0 logMAR normal?), reproducibility, influence of age and clinical validity.

I will introduce the Freiburg Visual Acuity (and Contrast) Test which allows us to quantify categories like CF (counting fingers) and HM (hand motion) in low vision. Current restorative approaches require quantitative such low vision assessment. Finally, one objective approach based on visual evoked potentials (VEP) will be mentioned which can solve clinical problems that occur when relying on subjective reports.

# Short-term monitoring of AMD by flood-illumination adaptive optics and OCT

*Michel Paques*

**Purpose:** During dry age-related macular degeneration (ARMD), photoreceptor loss is temporally and spatially correlated with the disruption of the retinal pigment epithelium monolayer and is accompanied by extensive redistribution of melanin. Recent histology data (Zanzottera et al. IOVS 2015) suggests that most melanin clumps seen by FIAO are intracellular. We have previously shown that flood illumination adaptive optics (FIAO) allows high resolution documentation of atrophy and of melanin redistribution (Gocho et al, IOVS 2013). Here, assuming that melanin clumps are cellular tags, we detail the cell kinetics that can be observed by time-lapse FIAO imaging in dry ARMD patients.

**Methods:** The cohort comprised 8 eyes of 6 patients (age, 68 to 74 years; VA, counting fingers to 20/20). Five eyes had foveal sparing, 2 had small parafoveal atrophic spots, and one had extensive atrophy involving the fovea. Margins of atrophic areas closest to the fovea were iteratively imaged by FIAO imaging (rtx1™; Imagine Eyes, Orsay, France; average image field, 1.2 mm x 1.2 mm; Intervals between imaging sessions 1.2 to 10.2 days; follow-up 10 to 205 days). Progression of atrophy and motion of melanin clumps were analyzed on time-lapse videosequences. Fusion and differential images were constructed using ImageJ.

**Results:** During follow-up, measurable progression was observed in 6 eyes, ranging from 8 to 12 µm/month, with significant local variations. Subretinal circulation of melanin clumps was observed in the subretinal space (n = 3) and in atrophic areas (n = 6). Their size ranged from 5 to 25 µm and their velocity peaked at ≈ 30 µm/month. Melanin clumps were also seen to agregate and deagregate. While melanin clumps distant from margins showed erratic, spatially restricted motion, some marginal melanin clumps were apparently moving ahead of and in synchrony with atrophy progression.

**Conclusions:** Time-lapse FIAO imaging allows short-term documentation of ARMD progression and reveals a complex kinetics of melanin clumps suggesting cellular migration. Therefore, our working hypothesis concerning the coordinated displacement of melanin clumps ahead of atrophy progression is that they correspond to marginal melanin-loaded cells involved in ARMD progression. Determining the epithelial or macrophagic nature of pigmented cells in lesional margins may therefore be important for the understanding of ARMD.

# Computational image analysis for outcome prediction in AMD trials

*Ursula Schmidt-Erfurth, Sebastian M. Waldstein, Amir Sadeghipour, Thomas Schlegl, Georg Langs, Bianca S. Gerendas, Hrvoje Bogunovic*

**Purpose:** To evaluate the potential of computational machine learning methods to predict visual acuity outcomes from structural and functional assessments during the treatment in patients receiving standardized anti-vascular endothelial growth factor (VEGF) therapy for neovascular age-related macular degeneration (AMD).

**Setting:** Analysis of randomized clinical trials at the Vienna Reading Center.

**Methods:** Prospective clinical trial data including 1,815 patients randomized to four-weekly (Q4wks) 0.5 mg ranibizumab, Q4wks 2 mg aflibercept or eight-weekly (Q8wks) 2 mg aflibercept (VIEW studies) and 563 evaluable patients receiving intravitreal ranibizumab monthly or PRN according to protocol-specified criteria (HARBOR trial). Monthly spectral-domain optical coherence tomography (SD-OCT) volume scans were processed using a validated, fully automated computational image analysis pipeline available at the Vienna Reading Center. This system performs three-dimensional segmentation of retinal layers, intraretinal cystoid fluid, subretinal fluid and pigment-epithelial detachments and vitreomacular adhesion, as well as detection of the foveal center, optic nerve head and retinal vasculature. All scans are longitudinally registered to a common reference frame using fully automated software algorithms. The extracted quantitative OCT biomarkers and visual acuity measurements at baseline and month 1 – month 3 were used for prediction of visual acuity scores at 12 months using random forest machine learning.

**Results:** In neovascular AMD, only IRC at baseline and persistent through week 12 had a negative impact on BCVA. With therapeutic intervention, exudative features such as IRC and SRF resolved rapidly in 74 % of eyes, whereas PED responded only slowly with 38 %. Independent of the type of regimen, fixed or flexible, retinal morphology correlated tightly with visual function. Intraretinal cysts consistently showed the lowest BCVA gains with either regimen or substance. With the switch from a fixed to a flexible pro re nata (PRN) regimen in the VIEW studies, progressive visual loss occurred exclusively in the group with primary PED presenting as the hallmark of neovascular activity and was induced by secondary formation of IRC in the neurosensory retina. The computational image analysis pipeline enabled a fully automated quantitative characterization of neovascular lesions in the large-scale SD-OCT data sets. In VIEW, the best correlation with BCVA at baseline was achieved using a coverage-based, foveal area-weighted IRC parameter ($R2 = 0.59$; $P < .001$). The same baseline parameter also predicted BCVA at 12 months ($R2 = 0.21$; $P = .003$). The BCVA gain correlated with IRC decrease in the exponential model ($R2 = 0.40$; $P < .001$) and linear model ($R2 = 0.25$; $P = .002$). No robust associations were found between SRF and baseline BCVA ($R2 = 0.06$; $P = .14$) or BCVA change ($R2 = 0.14$; $P = .02$). In this proof-of-principle study, IRC-derived morphometric variables correlated well with treatment-naive BCVA and BCVA outcomes in antiangiogenic therapy. While IRC reduction was associated with BCVA gains, some IRC-mediated neurosensory damage remained permanent. In HARBOR, at month 3, the machine learning algorithm predicted final visu-

al acuity outcomes with an accuracy of $R^2 = 0.64$. If only the baseline visit was considered for prediction, the performance was poorer with $R^2 = 0.34$. The most relevant SD-OCT biomarker for visual function was the horizontal extension of intraretinal cystoid fluid in the central foveal region.

**Conclusions:** Imaging biomarkers obtained by fully automated analysis of SD-OCT scans in conjunction with functional assessments during the loading phase allowed the prediction of final visual acuity outcomes in anti-VEGF therapy of neovascular AMD.

# Gene therapy for achromatopsia: From mouse models to human clinical trials

*Stylianos Michalakis*

*Center for Integrated Protein Science Munich CiPSM at the Department of Pharmacy – Center for Drug Research, Ludwig-Maximilians-Universität München, Munich, Germany*

Achromatopsia (ACHM) is a genetically and clinically well-defined inherited retinal disorder. The majority of ACHM patients carry mutations in one of the two genes (*CNGA3* or *CNGB3*) encoding for the cone photoreceptor cyclic nucleotide-gated (CNG) channel. Currently, there is no treatment available for ACHM. However, promising gene therapy treatments based on adeno-associated virus (AAV) vector-mediated gene supplementation are currently evaluated. Moreover, other than originally assumed cone photoreceptors do degenerate over time. Our knowledge about the mechanisms underlying the progressive degeneration of cones in ACHM is still limited. Here, I will provide an overview on our preclinical studies on pathomechanisms in *CNGA3*-linked ACHM. I will then summarize our preclinical data on the evaluation of *CNGA3*-gene supplementation in the *Cnga3*-deficient mouse model of ACHM.

The promising preclinical data have led to the development of an AAV.CNGA3 vector, which is currently evaluated in a phase I/II safety clinical study (NCT02610582) initiated by the RD-CURE consortium (www.rd-cure.de) and sponsored by the Tistou and Charlotte Kerstan Foundation. I will provide an overview on the approach and give an outlook on future projects.

# Progression of atrophic lesions prospectively determined by Fundus Autofluorescence: The natural history of the progression of atrophy secondary to Stargardt Disease (ProgStar) study

*Rupert Strauss[1,2], Xiangrong Kong[1], Alex Ho[3], Michel Michaelides[2], SriniVas Sadda[3], Sheila West[1], Hendrik Scholl[1]*

*[1] Wilmer Eye Institute, Johns Hopkins University Baltimore, USA*
*[2] Moorfields Eye Hospital, London, UK*
*[3] Doheny Imaging Reading Center, UCLA, USA*

**Purpose:** The multicenter ProgStar study aims to characterize the natural history of Stargardt disease (STGD1) and to develop new outcome measures for clinical trials. The yearly rate of progression of STGD1 using the growth of atrophic lesions as measured by fundus autofluorescence (AF) imaging is the primary endpoint.

**Methods:** FAF images from genetically confirmed STGD1 patients were sent from the nine participating sites to a central reading center (Doheny Image Reading Center, CA) and areas of definitely decreased AF (DDAF), well-demarcated questionably decreased AF (WD-QDAF), and poorly demarcated questionably decreased AF (PD-QDAF) were outlined and quantified. Based on background uniformity, homogeneous versus heterogeneous background was defined. Linear models with generalized estimating equations were used to estimate the mean changes of lesion areas while accounting for between-eye correlations.

**Results:** 489 study eyes of 259 study patients (54.4 % female) were enrolled in the prospective study and images from 444 eyes of 234 participants were graded for visits at baseline and six months follow-up. Mean age at baseline was 33.3 (sd 15.1) years. At baseline, DDAF was present in 306 (64 %) eyes, mean lesion size 3.96 (sd 4.38) $mm^2$; WD-QDAF in 71 (15 %) eyes, mean lesion size 1.54 (sd 1.38) $mm^2$, and PD-QDAF in 299 (62 %) eyes, mean lesion size 2.17 (sd 1.92) $mm^2$. Over six months, 14 eyes out of the 138 eyes (10.0 %) without any DDAF at baseline, have developed a lesion of DDAF by six months. Progression of DDAF in eyes with DDAF involvement at baseline was 0.33 (0.19 – 0.47) $mm^2$ ($p < 0.001$). There was a statistically significant difference in progression in lesion size over 6 months between eyes with a homogeneous (estimated progression rate: 0.18 (CI 0.01 – 0.35) $mm^2$ and a heterogeneous (estimated progression rate: 0.49 (CI 0.30 – 0.70) $mm^2$ ) background. Combining all lesion types, progression of areas of decreased AF was 0.33 (CI 0.27 – 0.40).

**Conclusions:** Mean increase of DDAF lesions in STGD1 was 0.24 $mm^2$ over six months. FAF may serve as a monitoring tool for interventional clinical trials in STGD1 that aim to slow down disease progression.

PRO RETINA
POTSDAM-MEETING

# POSTER ABSTRACTS

# The plasma protein and drusen component Apolipoprotein E is an inhibitor of the Classical Complement Pathway

*Susanne Ackermann[1], Changjun Yin[2], Andreas Habenicht[2], Martin Westermann[3], Peter Zipfel[1], Christine Skerka[1]*

*[1]Department of Infection Biology, Leibniz Institute for Natural Product Research and Infection Biology, Jena, Germany*
*[2]Institute for Cardiovascular Prevention, Ludwig-Maximilians University, Munich, Germany*
*[3]Electron Microscopy Center, Friedrich-Schiller University, Jena, Germany*

**Purpose:** Age-related macular degeneration (AMD) is a frequent, complement associated neurodegenerative disease in elderly people. AMD is characterized by the appearance of extracellular cell debris, called drusen in the retina which induces chronic inflammation. Drusen are composed of proteins and lipids, including apolipoprotein E (ApoE) and proteins of the complement system. ApoE, a lipid transporter protein, is genetically linked to many diseases such as Atherosclerosis, Alzheimer and AMD. Individuals carrying ApoE isoforms E2/E2 have a significantly increased risk to develop late AMD. Also ApoE deficient mice show a reduced cell layer thickness, indicating that ApoE deficiency influences degeneration of retinal cells. The occurrence of ApoE in drusen is explained by its role in clearance of extracellular lipids. As AMD is a complement associated disease, we studied whether ApoE in addition plays a role in the complement system.

**Methods:** The effect of ApoE on the three different complement pathways was analyzed by hemolysis-, complement activation- and bacterial survival assays. Binding of ApoE isoforms to divers complement proteins especially C1q was evaluated by ELISA, competition analysis, biolayer interferometry and MicroScale thermophoresis. Interaction between ApoE and C1q was visualized by electron microscopy. Complement inhibition by ApoE was confirmed in 'knock-in ApoE' mice by immunohistochemistry of brain sections.

**Results:** All three ApoE isoforms inhibit exclusively the classical pathway of complement at the level of initiation. Inhibition is mediated by binding of ApoE via the LDL-receptor binding site to the inner stalk of the tulip shaped C1q protein with high affinity (0.5 nM). ApoE knock-out mice show lipid accumulation in the choroid plexus of the brain combined with classical complement activation and inflammation. In contrast, human ApoE4 knock-in mice, which still have lipid deposition, showed significantly less complement activation, thus confirming the role of ApoE as regulator.

**Conclusion:** In addition to lipid transporter functions ApoE plays a regulatory role in innate immunity by inhibiting the classical pathway and regulating inflammation on lipid particles in the blood. Similar complement regulation by all three ApoE isoforms suggests that the differences are restricted to their lipid transporter function.

# HtrA1 expression in a murine model of dry AMD and *in vitro* effect of TGFβ on murine microglia cells

*Isha Akhtar, Patricia Klemm, Lebriz Altay, Sascha Fauser, Thomas Langmann*

*Experimental Immunology of the Eye, Department of Ophthalmology, University of Cologne, Cologne, Germany*

**Purpose:** HTRA1 polymorphisms are major risk factors for developing age-related macular degeneration (AMD). These HTRA1 variants impair transforming growth factor β (TGFβ) leading to profound dysregulation of both microglia homeostasis and inflammatory signaling which is detectible during AMD pathogenesis. Here we sought to investigate the expression of HtrA1 in a murine AMD model and examined the capacity of TGFβ to regulate microglia reactivity *in vitro.*

**Methods:** BALB/C mice were exposed to 15000 lux of bright light for 1 h and hyperreflective dots (HDs) were monitored via optical coherence tomography (OCT) at different time points. Expression level of HtrA1 was determined by qRT-PCR and in situ hybridization in light damaged and control retinas. Separately, the effect of TGFβ on classical and alternative activated BV-2 microglial cells was investigated *in vitro* by probing for pro-inflammatory as well as alternative markers using qRT-PCR. Furthermore, the effect of TGFβ on microglia morphology was analyzed in interferone γ (IFNγ)- and interleukin 4 (IL4)- activated primary mouse microglia using immunocytochemistry.

**Results:** OCT analysis revealed increased number of HDs in the plexiform layers. Furthermore, HtrA1 expression was elevated two days after light damage and decreased on the fourth day. This was also confirmed by in situ hybridization of retinal sections which showed an increased HtrA1 mRNA expression to the inner nuclear layer three days post light damage. TGFβ significantly increased transcription of the early inflammatory response gene plasminogen activator inhibitor type 1 (PAI-1) and HtrA1 and reduced the expression of iNOS, but not of TNFα in IFNγ- activated BV-2 microglia. Moreover, treatment with TGFβ enhanced the IL4- induced alternative activation through significant elevation of Arginase1 (Arg1) and Chitinase 3-like 3 (Ym1) transcription. Furthermore, TGFβ treatment of primary microglia cells promoted a more ramified phenotype after challenge with IFNγ.

**Conclusion:** Findings of this study provide further compelling evidence of anti-inflammatory properties of TGFβ on murine microglia cells and the induced expression of HtrA1 in a murine AMD model. Given that aberrant HtrA1 expression may contribute to dysregulated TGF-β signaling with concomitant initiation and perpetuation of neuroinflammation, strategies to target TGF-β signaling could be protective in our AMD mouse model.

# Molecular study of the MFRP gene in patients with posterior microphthalmia (MCOP) supports its role in autosomal recessive MCOP pathogenesis

*Basamat Almoallem[1, 2], Gavin Arno[3, 4], Julie De Zaeytijd[2], Sarah Hull[3, 4], Martina Suzani[3, 4], Thomy J. L. de Ravel[5], Andrew Webster[3, 4], Bart P. Leroy[2, 1], Tony Moore[3, 4], Elfride De Baere[1]*

[1]*Center for Medical Genetics, Ghent University and Ghent University Hospital, Ghent, Belgium*
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[3]*Institute of Ophthalmology, University College London, London, United Kingdom*
[4]*Department of Ophthalmology, Moorfields Eye Hospital, London, United Kingdom*
[5]*Center for Medical Genetics, University Hospitals of Leuven, Leuven, Belgium*

**Purpose:** Posterior microphthalmia (MCOP) is a rare developmental disease restricted to the posterior segment of the eye. To date, mutations in the MFRP gene, encoding a frizzled-related protein, have been reported in autosomal recessive MCOP (arMCOP). Here, we aimed to identify the underlying genetic cause of arMCOP in seven unrelated patients from different ethnicity.

**Methods:** All patients underwent detailed ophthalmological evaluations and Sanger sequencing of the coding region of MFRP (NM_031433.2). Two patients originating from a consanguineous marriage underwent homozygosity mapping using SNP arrays.

**Results:** MFRP was found in a homozygous region of 10.2 and 6.2 Mb in two patients respectively. Overall, eight distinct MFRP mutations were found in the patients studied. Five patients were homozygous for: two missense variants with predicted pathogenic effect (c.1231T>C p.Y411H, novel; c.1549C>T p.R517W, known) and three frameshift mutations (c.1090_1094del p.T364*, novel; c.498del p.N167T*25 and c.498dup p.N167fs*, known). Moreover, a sixth patient was compound heterozygous for a nonsense mutation (c.955C>T p.Q319*, novel) and novel deletion of 6,2 kb (c.16088_54+40delinsA), predicted to abolish the transcription initiation site. The seventh patient was heterozygous for a known frameshift mutation (c.491_492insT p.N167Qfs*34), no second mutation was found so far. All patients had short axial length (13-16.5 mm), reduced visual acuity (0.15-0.8 logMAR) and hyperopia (+13D to +17.25D). Crowded optic discs were noticed in 7/7 and macular folds in 3/7 patients. Optical coherence tomography showed intraretinal cysts in 5/7 patients. Peripheral pigmentary changes were observed in 5/7 patients.

**Conclusions:** Eight distinct MFRP mutations were identified, four of which were novel, including the first report of a genomic rearrangement. No clear genotype-phenotype correlations could be observed. The identification of new families with MFRP mutations might offer opportunities for potential gene-based therapies suggested by Dinculescu et al (2012).

# Genetic variants in *ARMS2/HTRA1* and *APOE* loci are associated with hyperreflective foci present in early forms of age-related macular degeneration

*Lebriz Altay[1,2], Paula Scholz[1], Tina Schick[1], Carel B. Hoyng[3], Anneke I. den Hollander[3,4], Thomas Langmann[2]*, Sascha Fauser[1]**

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**Purpose:** To evaluate the association of hyperreflective foci (HF) observed in early and intermediate age-related macular degeneration (AMD) with known AMD risk alleles.

**Methods:** In this case-control study, HF were defined as lesions with reflectivity equal or higher than the retinal pigment epithelium band in spectral domain optical coherence tomography. HF in the outer nuclear layer and photoreceptor complex were evaluated in 518 individuals with early and intermediate AMD. Genotyping was performed for 22 single nucleotide polymorphisms (SNPs). Associations between AMD severity stages, HF and SNPs were determined by univariate logistic regression analyses.

**Results:** HF (n≥10) were significantly associated with AMD severity and the association was strongest in bilateral intermediate AMD (odds ratio (OR): 41.78; p=3.57x10-7). HF were independently associated with *ARMS2/HTRA1* rs10490924/rs11200638 (p=0.018, OR: 1.65) and *APOE* rs2075650 variants (p=0.009, OR: 2.17).

**Conclusions:** The presence of HF is related to AMD severity and associated with known risk polymorphisms in *ARMS2/HTRA1* and *APOE* genes. Our findings suggest that modification of the extracellular matrix or altered lipid metabolism triggered by genetic components may play a role in the formation of HF.

# Isolated and syndromic retinal dystrophy caused by biallelic mutations in *RCBTB1*, implicated in ubiquitination

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**Purpose:** The aim of this study was to identify and functionally study a novel disease gene mutated in a Turkish consanguineous family with a syndromic inherited retinal dystrophy (iRD).

**Methods:** Two affected individuals underwent whole exome sequencing (WES) (HiSeq2000, Illumina) after homozygosity mapping. WES data of over 1000 iRD patients were inspected for *RCBTB1* mutations, and targeted next-generation sequencing of *RCBTB1* was performed in 281 iRD patients. Flanking markers were genotyped for haplotyping. *RCBTB1* qPCR expression analysis was performed in cDNA from human retina and retinal pigment epithelium (RPE). RCBTB1 immunostaining was performed on human and murine sections. As RCBTB1 has previously been identified as a Cullin3 substrate adaptor, different components of the Cullin3 and Nrf2 pathway were quantified using qPCR.

**Results:** WES identified a novel missense variant, c.973C>T p.(His325Tyr) (rs200826424) in RCBTB1 (NM_018191.3). Additional homozygous missense mutations were identified in 5 families with isolated and syndromic iRD. All changes segregate with disease, affect highly conserved amino acids and *in silico* predictions suggest a deleterious effect. Three mutations are located in the 6$^{th}$ repeat of the RCC1 domain, while two variants localize in the first BTB domain. In two Mediterranean families, a founder haplotype was identified for c.919G>A, p.(Val307Met). *RCBTB1* mRNA expression was demonstrated in human retina and RPE and protein immunostaining was observed mainly in the inner retina. Different components of the Cullin3 and Nrf2 pathway displayed decreased mRNA expression in patients' lymphocytes.

**Conclusions:** Hypomorphic *RCBTB1* missense mutations clustering in two protein domains were identified in families with non-syndromic and syndromic IRD respectively, putting forward *RCBTB1* as a new IRD disease gene. A founder effect was demonstrated for one *RCBTB1* mutation in two Mediterranean families. Finally, our data suggest a potential role of the ubiquitination pathway in the pathogenetic mechanism underlying *RCBTB1*-associated IRD.

# Polysialic acid prevents microglia/macrophage reactivity and reduces oxidative and complement-triggered retinal damage

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**Purpose:** Age-related macular degeneration (AMD) is a leading cause of visual impairment and associated with chronic reactivity of microglia/macrophages in the retina and subretinal space. Sialic acid-binding immunoglobulin-like lectin-11 (SIGLEC11) is a human-specific microglia receptor that recognizes polysialic acid (polySia) on the glycocalyx of neurons to suppress microglial activation in the brain. Here, we hypothesized that purified polysialic acid may reduce microglia/macrophage reactivity in the retina by induction of inhibitory SIGLEC11 receptor signaling and attenuate retinal damage in a humanized mouse model of AMD-like laser-induced retinal injury.

**Methods:** Polysialic acid and SIGLEC11 expression was analyzed in human and murine SIGLEC11 transgenic retinas using RT-PCR and immunohistochemistry. Investigation of the *in vivo* therapeutic potential of polySia avDP20 (PCT patent: EP2783691 A1) was carried out by intravitreal injection of SIGLEC11 transgenic mice after laser-coagulation and immunohistochemical whole mount analysis of microglia/macrophage reactivity in retina and RPE/choroid, membrane attack complex (MAC) formation as well as vascular leakage analysis using fluorescein angiography after 48 hours. To study the mechanisms of polySia avDP20 on the complement-phagosome pathway, we analyzed phagocytosis of RPE cell debris and superoxide production of human iPSC-derived microglia as well as complement-mediated lysis of human hepatoma cells.

**Results:** Murine and human retinas contain high levels of polysialic acid and SIGLEC11 is prominently expressed in the human retina. PolySia avDP20-injected SIGLEC11 transgenic mice showed reduced accumulation of reactive phagocytes in the laser lesions compared to vehicle-injected controls. Further, polySia avDP20 treatment effectively reduced the degree of RPE/choroidal MAC deposition and significantly reduced inflammatory vessel leakage in SIGLEC11 transgenic laser-damaged mouse retinas. PolySia avDP20 treatment prevented overt production of superoxide and reduced phagocytosis of iPSdM cells and further interfered with complement-mediated lysis of human hepatoma cells by inhibiting alternative complement pathway activation.

**Conclusion:** We could demonstrate that polySia avDP20 reduces pathological features of AMD-like retinal degeneration by inhibiting innate immune activation and the complement-phagosome pathway. These findings suggest polySia avDP20 as a potential novel therapy option for AMD.

# Functional characterization of novel deleterious *MFSD8* mutations found by whole exome sequencing in early-onset isolated maculopathy

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**Purpose:** Non-syndromic autosomal recessive macular dystrophies are associated with mutations in a number of genes, of which *ABCA4* is the most frequently mutated one. Sequencing of *ABCA4* and whole exome sequencing (WES) was performed to identify the molecular cause of suspected atypical Stargardt disease in an eight-year-old girl.

**Methods:** The entire *ABCA4* locus was enriched with a custom Haloplex panel followed by next-generation sequencing. Next, WES data was generated and analyzed. Mini-gene assays and cDNA sequencing on patient's lymphocytes were used to study the effect of a donor splice variant in *MFSD8*. *MFSD8* expression was assessed in lymphocytes with qPCR. Transmission electron microscopy (TEM) was performed on a patient's skin biopsy. The patient underwent a neurological examination and brain magnetic resonance imaging (MRI).

**Results:** Sequencing of the entire *ABCA4* locus revealed one heterozygous variant. WES revealed two novel heterozygous *MFSD8* variants: c.590del p.(Gly197Valfs*2) and c.439+3 A>C p.(=), occuring in *trans*. *In vitro* mini-gene assays and cDNA sequencing on patient's lymphocytes demonstrated an out-of-frame skip of exon 5 p.(Ile67Glufs*3) resulting from c.439+3A>C. Expression of *MFSD8* in patient's lymphocytes was significantly reduced. TEM on a patient's skin biopsy showed lipopigment inclusions with characteristic and mixed lamellar profiles, as can be seen in cases with neuronal ceroid lipofuscinosis (NCL). The clinical neurological examination of the patient was normal, MRI of the brain showed slight cerebellar atrophy and discrete signs of cerebral cortical atrophy.

**Conclusions:** Two deleterious *MFSD8* mutations were identified in a young patient with an isolated maculopathy. Roosing et al. (2015) reported a combination of mild and severe *MFSD8* mutations in late-onset non-syndromic maculopathy. The combination of two severe *MFSD8* mutations has only been seen in severe variant late-infantile NCL, unlike the presentation here. Through WES and downstream functional characterization we uncovered a potential syndromic maculopathy with poor outcome. This study illustrates the power of WES to refine clinical diagnoses and to anticipate disease progression.

# Novel insights in *KIF11*-related retinopathy

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**Purpose:** Mutations in *KIF11*, encoding a homotetramer spindle motor protein (EG5) of the kinesin family, have been identified as a cause of a rare autosomal dominant syndrome that includes microcephaly, lymphedema, and mental retardation. Patients frequently also present with retinal changes such as chorioretinopathy and/or familial exudative vitreoretinopathy (FEVR). This study aimed at further characterizing the ocular phenotype in patients with *KIF11* mutations.

**Methods:** All patients underwent standardized ophthalmic clinical examination and imaging, including optical coherence tomography (OCT), wide field fundus autofluorescence (AF) imaging and fundus photography. To assess retinal function, best corrected visual acuity and electroretinography (ERG) were performed. Other syndromic features were also assessed.

**Results:** Patient #1 (5 years old) and patient #2 (38 years old) presented with non-syndromic retinal dystrophy. In both, targeted NGS identified novel variants in *KIF11* (p.Ala615del and p.Glu270*, respectively). Subsequent specific evaluation *KIF11*-related syndromic features revealed only a borderline low head circumference. Patient #3 (3 years old) carried the known splice site mutation c.2267+1G>A and exhibited microcephaly with chorioretinopathy, lymphoedema and mental retardation (MCLMR) syndrome. Segregation revealed that the *KIF11* mutations had occurred *de novo* in patients #2 and #3 and in the father of patient #1. The retinal phenotype of all 4 *KIF11* mutation carriers differed considerably. While the typical chorioretinal dysplasia was only present in patient #3, patients #1 and #2 presented with findings similar to a cone rod dystrophy. The father of patient #1 had a FEVR in one eye. His fellow eye with visual acuity of 20/20 appeared funduscopically normal, but showed some outer nuclear thinning in OCT imaging. Visual acuity in the better eye of patients #1–#3 was 20/40, 20/200 and 20/63, respectively. Longitudinal observation in patients #1 and #3 (2 and 4 years follow-up, respectively) revealed growing areas of retinal atrophy. In patient #2, visual acuity had been stable since it was first recorded at the age of 7 years; however, the patient reported worsening of overall visual function, and AF imaging was suggestive for a progressive retinal dystrophy.

**Conclusion:** We detected a progression of retinal atrophy in patients with retinopathy caused by *KIF11*-mutations indicating involvement of this gene not only in ocular development, but also in the maintenance of retinal morphology and function. Moreover, remarkable variability of the ocular phenotype was observed within a family. Even though syndromic manifestations may be subtle, retinal disease may be highly debilitating. Therefore, *KIF11* mutations might be underdiagnosed and should be considered in diagnostic NGS panels for retinal diseases.

# The role of neuroinflammation in the regenerating adult zebrafish retina

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**Purpose:** Neuroinflammation was shown to have a positive impact on stem cell activity in the zebrafish central nervous system after traumatic injury. Since little is known about the inflammatory response of the zebrafish retina upon lesion, we aim to identify general contribution of inflammation to regeneration.

**Methods:** To identify novel marker genes that are differentially expressed after lesion, we studied transcriptome data from injury activated Müller glia. Potential candidate genes were further analysed by performing *In situ* hybridization in combination with immunohistochemistry. Microglia are among the first cells to react to injury in an inflammatory context, and are labelled bympeg1:mCherry. We analyzed microglia for morphological changes as well as cell migration towards the lesion site. Finally, to study the general role of the immune system for regeneration, we performed drug-mediated suppression of the immune system and analysed microglia activity, cell proliferation and the expression of markers for inflammation.

**Results:** We identified matrix metalloproteinase 9 as a novel marker gene for inflammatory processes in Müller glia cells after photoreceptor ablation. In addition, microglia undergo the same morphological changes as described in murine retina and zebrafish telencephalon changing from a ramified to a more amoeboid morphology, by contracting phylopodia and by accumulating at the site of lesion. Under immune suppression, Müller glia proliferation was significantly decreased in comparison to controls. Furthermore, the number of microglia strongly decreased under these conditions, and *matrix metalloproteinase 9* expression was almost not detectable via In *situ* hybridization.

**Conclusions:** MMP9 is a novel marker for an early regenerative and or inflammatory response of Müller glia cells. The dynamics of microglia suggests their involvement in the regeneration response of zebrafish retina. Furthermore, the decreased regenerative response after inhibition of the immune system suggests that inflammation has a positive role in stimulating retinal regeneration, although more details on this process remain to be elucidated.

# The German AugUR study: A population-based prospective study to investigate chronic diseases in the elderly with focus on age-related macular degeneration (AMD)

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**Background:** The majority of patients suffering from chronic diseases is beyond 70 years of age. Typical late-onset disabilities include those affecting the heart, the kidney, cancer, and conditions of the eye such as age-related macular degeneration (AMD). The latter is the main cause of central visual impairment in the elderly. The detailed pathomechanisms of AMD are yet to be deciphered and treatment options are limited. To improve prevention, diagnosis, and therapy of AMD, population-based epidemiological data from the elderly are a prerequisite to understand the various genetic and non-genetic risk factors influencing disease pathology.

**Methods/Design:** We established the German AugUR study (Age-related diseases: understanding genetic and non-genetic influences – a study at the University of Regensburg), a prospective study in the mobile general population of Caucasian ethnicity with at least 70 years of age in and around Regensburg in eastern Bavaria. The study protocol is particularly tailored for the elderly. The program includes a standardized interview-based questionnaire regarding social and life-style factors, quality-of-life, medication history and existing diagnoses of common diseases. The participants undergo medical examinations for cardiovascular or diabetes-related conditions, and general measurements of body shape and fitness. Biobanking of whole blood, serum, plasma, and urine is conducted and standard laboratory analyses are performed. Extensive ophthalmological examinations include testing of central retinal function (visual acuity, photostress test, Amsler Grid) as well as retinal imaging (standardized color fundus photographs of the central retina, confocal laser scanning ophthalmoscopy and spectral domain optical coherence tomography). The presence and extend of AMD is categorized via color fundus photographs into early and late stages.

**Results:** Since 2013, we have recruited 1.133 participants with 1.041 (91.8 %) having gradable fundus images for at least one eye. 554 (48.9%) participants revealed no signs of AMD, 418 (36.9 %) individuals showed large drusen and pigmentary abnormalities corresponding to early AMD findings. A total of 69 (6 %) participants demonstrated late-stage AMD with typical neovascular or atrophic lesions of the central retina.

**Discussion:** AugUR is specifically designed as a research platform to host studies of late onset diseases with a special focus on ophthalmological lesions of AMD development. We further provide the first prevalence estimates for AMD in an elderly German population. With ongoing 3-year-follow-up measurements, this platform will help to unravel the genetic and non-genetic etiology of disease development and progression and to derive further prevalence and incidence data – not only for AMD but for general diseases in the elderly.

# Chemical compound screening in Best vitelliforme macular dystrophy (BVMD)

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**Purpose:** Retinal pigment epithelium (RPE) differentiated from human induced pluripotent stem cells (hiPSC) demonstrated mislocalization and strongly reduced expression of mutated bestrophin-1 (BEST1) in BVMD patients when compared to controls. The overall aim of this study is to establish a platform for chemical compound screening to identify drugs normalizing defective BEST1 folding, thereby enhancing plasma membrane (PM) targeting and surface stability.

**Methods:** To quantify BEST1 cell surface expression, immunofluorescence microscopy is used. To optimize the screening system, a number of transwell coating reagents (matrigel, fibronectin, laminin, gelatin) was tested. Appropriate time points for harvesting of hiPSC-derived RPE cells were determined by transepithelial electrical resistance (TEER). BEST1 immunostaining will be achieved by antibodies specifically designed to exclusively bind to the extracellular loops of BEST1. Fluorescence signal intensities from untreated versus treated cells will be calculated relative to the absolute minimum and maximum signal values of BEST1 immunostaining. The maximum signal will be reflected by fluorescence staining of hiPSC-RPE from healthy control. For minimum background signals, a CRISPR/Cas9 BEST1-deficient hiPSC-RPE line will serve as control. Initially, screening will be performed in a small-scale compound library, commercially available as Spectrum Collection (MicroSource Discovery Systems, Gaylordsville, USA).

**Results:** HiPSC-derived RPE was grown on transwell inserts or coverslips to confluence and optimal times for BEST1 expression and membrane localization were determined. Fully functional maturation was only seen when cells reached full polarization. Cells are best harvested between the sixth and sixteenth week. Coating of transwell inserts with fibronectin, laminin and matrigel revealed similar results for BEST1 localization while gelatin led to a delayed development of TEER and less optimal localization of PM-bound BEST1. Cultivation on coverslips was beneficial in terms of costs and time savings while optimal harvesting time was abbreviated to culture times of only three to five weeks.

**Conclusion:** To date, there is no treatment for BVMD although the molecular pathology of BEST1 mutations has been well established. Currently, several PM proteins are a target for pharmaceutical drug screen, as they are important multifunctional mediators between the cytoplasm and the external milieu. Based on these experiences, we propose a concept for chemical compound screening utilizing hiPSC-derived RPE from BVMD patients ultimately aiming to (partially) rescue BEST1 function.

# Anaphylatoxins C3a and C5a activate the PI3K-AKT-pathway in RPE

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**Background:** Local complement activation is discussed to play a major role in AMD pathophysiology. Cleavage products of the complement cascade, among them the anaphylatoxins C3a and C5a, were found in drusen as well as in extracellular deposits in close proximity to the retinal pigment epithelium (RPE). However little is known about the effect of anaphylatoxins on the RPE. Thus we investigated the effect of anaphylatoxins C3a and C5a on ARPE-19 cells.

**Methods:** Responses to C3a or C5a application in intracellular free $Ca^{2+}$ ($[Ca^{2+}]_i$) were measured by $Ca^{2+}$-imaging in ARPE-19 cells. Using western blot the phosphorylation of AKT and transcription factors was analyzed. The effects of C3a and C5a on protein secretion were measured by Bio-Plex bead analysis.

**Results:** Single application of either C3a or C5a alone increased $[Ca^{2+}]_i$ with significant stronger elevations observed with C5a. Simultaneous application of C3a and C5a had no additive effect on [Ca2+]i that reached the same levels as under single C5a application. When C5a were applied consecutively after C3a, C5a surprisingly reduced $[Ca^{2+}]_i$. The presence of either the PI3K-blocker LY294002 or the AKT-blocker API-2 significantly reduced C5a-induced $[Ca^{2+}]_i$ elevations. Also western blot analysis revealed time-dependent increase in phosphorylation of AKT and of the PI3K-AKT-pathway linked transcription factors CREB, Foxo1 and FoxP3 by C3a and C5a. Protein secretion of IL-8 and VEGF-A were significantly increased in cell supernatants by C3a+C5a co-stimulation. Again application of LY294002 reduced IL-8, VEGF-A and MCP-1 secretion significantly.

**Conclusion:** The anaphylatoxins C3a and C5a increase $[Ca^{2+}]_i$ in ARPE-19 cells as second messenger and activated further downstream the PI3K-AKT-pathway and its linked transcription factors. As a result ARPE-19 cells changed its profile of secreted growth-factors and cytokines. The surprising observation that the two anaphylatoxin receptors at the RPE do not simply react synergistically implies a complex interactive signaling of the two agonists that need to be clarified on the way to understand complement induced RPE-changes contribute to AMD-typical lesions.

# Proteomic changes in ARPE-19 cells stimulated with complement serum and UV-irradiated photoreceptor outer segments (UV-POS)

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**Purpose:** ARPE-19 cells as a model for retinal pigment epithelial (RPE) cells were shown to undergo proinflammatory and proangiogenic changes upon complement stimulation and UV-POS treatment suggesting a role in AMD pathology. The aim of our study was to reveal potential intracellular signaling pathways mediating these functional changes of RPE cells using proteomics and stimulation with complement serum and UV-POS.

**Methods:** For complement stimulation, ARPE-19 cells were incubated in DMEM/F12 medium supplemented with 5 % human complement serum (HCS) for 24 hours. Medium alone or supplemented with either 5 % heat-inactivated HCS or C7-deficient serum were used as controls. Further groups of cells were pre-treated every other day with 10 µg/ml UV-POS for one week to a total of three pre-treatments. Afterwards the cells were washed and incubated in medium containing 5 % HCS or medium alone. Proteins were separated by 2D-gel electrophoresis and differentially occurring protein spots were peptide mapped with MALDI-TOF mass spectrometry.

**Results:** Some protein spots were differentially expressed in the various treatment groups and differed between two independent experimental sets. From the two experimental sets, a total of 27 protein spots were selected and processed for MALDI-TOF analysis that identified a total of 56 different proteins, which could be grouped into 7 functional entities including metabolic (12), structural (16), globular (3), cell-cell and cell-matrix interaction-associated proteins (2), gene expression (4), proteins related to inflammatory processes and angiogenesis (7), and proteins involved in signal transduction (10). 60 percent of the identified proteins participating in signal transduction are associated with cellular and oxidative stress response. Out of two cases, expression of these proteins was observed in response to HCS, UV-POS, or their combination.

**Conclusions:** The results show that complement stimulation and UV-POS uptake regulate the cell proteome. Further experiments are required to clarify how far the identified differentially expressed proteins associated with signal transduction are involved in the mediation of the functional changes of RPE cells in response to complement stimulation and UV-POS.

# Role of PlGF in cellular immune response in CNV

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Age-related macular degeneration (AMD) is a major reason for vision loss mainly due to choroidal neovascularization (CNV). A cellular immune response, driven by mononuclear phagocytes (MP), seems to play a pivotal role in the pathogenesis of AMD. In clinical practice it has been shown that a switch from anti- VEGF-A to a combined VEGF-A with anti-Placenta growth factor (PlGF) treatment is beneficial for some patients. Since MP carry VEGFR1 (Flt-1), a receptor specific for both VEGF-A and PlGF, we hypothesize that a regulation of MP activity by PlGF contributes to the pathophysiology of CNV.

Laser-induced CNV was used in MacGreen (*Csf1r*-EGFP) mice, creating 5 laser spots around the optic nerve. MP were visualized in vivo by SLO auto-fluorescence (AF) and quantified ex vivo in whole-mounts. Differential expression of angiogenic factors and M1/M2 macrophage polarization markers were analyzed by qPCR. Protein expression of PlGF and VEGF-A was detected both in sagittal sections and whole-mounts of the retina. One day after laser (D1), intravitreal injection of anti-VEGF-A + anti-PlGF (aflibercept) or anti-PlGF was performed and macrophage recruitment was analyzed. PBS served as control.

After CNV, PlGF mRNA expression increases at D1 and turns to normal levels at D4 whereas VEGF-A expression did not increase during the early phase (D1) and even decreased at D4. At D14, in sagittal sections or retina whole-mounts, we observed that up-regulation of VEGF-A expression in response to laser impact is limited to the scar area, while PlGF shows a more homogenous distribution. Additionally, comparable to PlGF expression, activated MP were also present in distant areas from the laser-spots. Among macrophages, we found an increase of M1 (pro-inflammatory) markers (CD68 and CD86) up to D4, whereas the M2 marker IL4R did not show significant changes. Intravitreal injection of aflibercept significantly decreased the amount of activated MP and minimized the size of the laser scar at D7 and D14 respectively, whereas anti PlGF could not suppress the macrophage recruitment effectively. Laser scars in PlGF injected eyes were similar to controls.

Thus we show first hints that the interplay of PlGF and microglia plays an important role in the initial phase of CNV after laser. However, anti-PlGF treatment alone is not sufficient to suppress MP recruitment.

# A first step towards efficient drug delivery to the retina through intravitreal injection of DNA based nanoparticles

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**Purpose:** After establishing successful drug delivery via novel lipid DNA-Nanoparticles (NPs) as eye drops to the corneal epithelium, we here tested distribution and delivery of this class of NPs into the back of the eye to treat retinal diseases. A successful delivery via these NPs would offer chances to reduce the concentration of the drugs or reduce the number of injections needed per year. Patients would greatly benefit from both options.

**Methods:** Fluorescently labelled NPs were injected either into the vitreous body of ex vivo pig eyes obtained from the local slaughterhouse. After (30 min, 2, 4, 8, 24 h) the animals were sacrificed and the eyes were embedded into TissueTek and frozen in liquid nitrogen. Subsequently, cryo sections of the eyes were prepared and counterstained with DAPI. Using fluorescence microscopy the NP-diffusion and binding to different structures/tissues (retina, sclera, choroidea and pigment epithelium) was evaluated.
In addition, the NPs were injected into the vitreous body of living rats. After 30 min, 2, 4, 8, 24 h, 3 days and 5 days after injection the rat were killed, the eyes were enucleated and processed as described above.
Finally, using a fluorophotometer (OcuMetrics Fluorotron™ Master) intraocular measurements of NP-concentration and location were performed on rat- and pig eyes in order to analyze diffusion and depot effects.

**Results:** Intravitreal injections into the pig eyes showed good binding to retinal tissue. The in-vivo injections into the vitreous body of living rats proofed excellent binding of the NPs to most of the tissue in the eye instantly, with residence times lasting up to 5 days. Examination of the eyes via fluorophotometer confirmed that larger amounts of the NP were detected inside the eye compared to the control dye.

**Conclusion:** Our results proof good binding of the NPs to retinal tissue after administration by intravitreal injection. Thus, DNA-NPs might be a versatile tool to prolong the residence time of intraocularly injected drugs. Further investigations with medical drugs are necessary to confirm these results.

# Cystathionine γ-lyase deficiency exerts oxidative stress-mediated barrier dysfunction in retina pigment epithelium

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**Abstract:** Cystathionine γ-lyase (CSE / CGL / CTH) is well known for its involvement in trans-sulfuration pathway to produce hydrogen sulfide ($H_2S$), a novel gasotransmitter, together with two other enzymes, cystathionine β-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (MPST / 3-MST). In addition to $H_2S$, CSE also produces cysteine, the rate-limiting substrate for glutathione (GSH) synthesis, along this pathway. CSE role in conferring cytoprotection against oxidative stress has been reported in neurodegenerative disease. Despite its well-known high expression in cardiovascular system, CSE has been detected in eye and CSE deficiency was shown to mediate cataractogenesis in aging mice. In addition to lens, CSE has also been detected in retina, but its role in this part of the eye still remains unexplored. Here, we found that CSE, but neither CBS nor 3-MST, to be required for counteracting hydrogen peroxide ($H_2O_2$) and 4-hydroxynonenal (4-HNE)-mediated oxidative injuries in RPE layer. The increased sensitivity of CSE-silenced ARPE-19 cells to oxidative challenges was correlated with the decrease in total glutathione concentration, GSH/GSSG ratio as well as glutamylcysteine ligase (GCL) and glutathione reductase (GR) activities, the enzymes required for de novo glutathione synthesis and recycling, respectively. Overexpression of CSE and supplementation of N-acetyl cysteine (NAC) alleviated the hypersensitivity of CSE-silenced ARPE-19 towards oxidative challenges. As a consequence to loss of CSE, we found ARPE-19 barrier functions to be impeded, such that the monolayer integrity was breached, due to cytoskeletal actin reorganization and discontinuous peripheral beta-actin localization. We also found that knocking down of CSE led to p21 and p27-induced cellular senescence. Congruent with this finding, we observed a temporal decrease in CSE expression in mouse whole retina with age. Furthermore, RPE isolated from 10-month-old adult mice was found to have lower total glutathione than those from 1-week-old mice. This current study, thus, presents a novel association of CSE-deficiency with RPE pathophysiology.

# Recoverin isoforms as membrane-associated regulators of G-protein-coupled receptor kinase

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**Purpose:** Recoverin and the G-protein-coupled receptor kinase (GRK) play a key role in the feedback mechanism of phototransduction. Recoverin can sense the intracellular concentration of the second messenger $Ca^{2+}$ to inhibit the GRK at high [$Ca^{2+}$] and to release the GRK at low [$Ca^{2+}$]. The released GRK phosphorylates light-activated rhodopsin after photoexcitation. The best known member of the GRK-family is the rhodopsin kinase (GRK1), which is expressed in rods. An inactivating mutation of GRK1 causes Oguchi disease, which is a recessive form of stationary night blindness. Oguchi disease patients have abnormally slow dark adaptation but fairly normal day vision. The expression of a second GRK – the opsin kinase (GRK7) – enables the fairly normal day vision by a partially compensation of the lost GRK 1. In contrast, $^{GRK1-/-}$mice exhibit severe defects in cone recovery because rodents do not express GRK7 in photoreceptor cells. In order to investigate cone specific features of photoresponse recovery and $Ca^{2+}$-mediated feedback mechanism we use zebrafish as a model organism.

**Methods:** In zebrafish, a special feature is the expression of two homologues of GRK1 (1-A & 1-B) and GRK7 (7-A & 7-B) and of four recoverin (zRec1a, zRec2a, zRec1b, zRec2b) isoforms. The interaction of recoverin and GRK isoforms was investigated with Surface Plasmon Resonance Spectroscopy (SPR). SPR enables investigations of protein-protein-interactions in real time by an immobilization of one protein followed by a titration of a second protein. Additionally an enzyme-linked immunosorbent assay (ELISA) was performed to verify a protein-protein interaction by an enzyme-coupled antibody reaction.

**Results:** Recoverin isoforms were expressed in *E.coli* and purified by a Hydrophobic-Interaction-Chromatography except of zRec1b, which is purified by an Ammonium-Sulfate-Precipitation followed by a Size-Exclusion-Chromatography. The GRK homologues were also expressed in *E.coli* followed by an Immobilized-Metal-Ion-Chromatography Purification. Previous experiments showed that based on $Ca^{2+}$-binding all recoverin isoforms are functional after the purification procedure. The interaction of all recoverin isoforms with the GRK7-A were tested by SPR and preliminary results indicate an interaction between the zRec1a and the zGRK7-A.

**Discussion:** The suggested interaction of zRec1a and zGRK7-A has to be verified by additional binding assays like fluorescence spectroscopy and isothermal titration calorimetry (ITC). Furthermore, interactions of the other recoverin and GRK isoforms have to be investigated as well as the biochemical properties of the different recoverins.

# The extracellular RS1 protein is a novel regulator of MAP Kinase signaling in the retina

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**Background:** X-linked juvenile retinoschisis (XLRS) is a hereditary retinal dystrophy. It is caused by mutations in the *RS1* gene resulting in a functional loss of the encoded RS1 protein, termed retinoschisin (Sauer et al., 1998, Molday et al., 2012). The function of RS1 and the molecular mechanisms underlying XLRS pathogenesis are still unresolved, despite the fact that the retinal Na/K-ATPase was identified as a direct RS1 interaction partner (Molday et al., 2008; Friedrich et al., 2011). Na/K-ATPases are membrane spanning ion pumps, maintaining cellular ion homeostasis. Increasing evidence suggests that Na/K-ATPases also act as hormone receptors, influencing intracellular signaling cascades (Aperia et al., 2016). An earlier study on Rs1h-deficient *(Rs1h$^{-/Y}$)* mice revealed increased MAP kinase signaling during XLRS development (Gehrig et al., 2007). Aim of this study was to investigate whether retinoschisin binding to retinal membranes directly affects MAP kinase signaling, and whether the Na/K-ATPase could possibly mediate this effect by forming an RS1-Na/K-ATPase signalosome complex.

**Methods:** Human retinoblastoma (Y79) cells and retinal explants of *Rs1h*$^{-/Y}$ mice were exposed to RS1 and an RS1-mutant, RS1-C59S. Activation of MAP Kinase signaling was followed via immunoblotting against activated MAP kinase ERK1/2, and quantitative real-time RT-PCR against the MAP kinase target gene *C-FOS*. To obtain insight into signaling mechanisms, we investigated an interplay between RS1, the Na/K-ATPase and SRC, a known intracellular Na/K-ATPase signal transducer. By immunoblot analysis, RS1 dependent SRC activation and the influence of the SRC-inhibitor PP2 were examined in Y79 cells and *Rs1h*$^{-/Y}$ retinae. As MAP kinases regulate cellular processes including apoptosis, the effect of RS1 on stress induced apoptosis was addressed by following caspase 3/7 activation in Y79 cells.

**Results:** Exposure to recombinant retinoschisin, but not to recombinant RS1-C59S, significantly reduced ERK1/2 activation and C-FOS expression in Y79 cells as well as *Rs1h*$^{-/Y}$ retinae. SRC activation was observed prior to ERK1/2 activation and both components were deactivated in the presence of PP2, indicating an initiatory role for SRC in this cascade. Finally, RS1 revealed a protective effect against apoptosis in Y79 cells.

**Conclusion:** Our data imply retinoschisin in the regulation of MAP kinase signaling, presumably by modulating the influence of the Na/K-ATPase on SRC. We suggest that disturbances of the RS1-Na/K-ATPase-SRC signalosome complex (e.g. by RS1 deficiency) might be an initial step in XLRS pathogenesis.

# Frequency, phenotypic characteristics and progression of atrophy associated with a diseased Bruch membrane in pseudoxanthoma elasticum

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**Purpose:** To characterize atrophy of the outer retina and the retinal pigmentepithelium in patients with Pseudoxanthoma elasticum (PXE).

**Methods:** The frequency and phenotypic characteristics of manifest atrophy were investigated in 276 eyes of 139 patients using color fundus photography, fundus autofluorescence (AF) imaging with a scanning laser ophthalmoscope and spectral domain optical coherence tomography. Progression rates of atrophy were quantified in eyes with longitudinal AF recordings.

**Results:** Atrophy was present in 90 eyes (32 %, mean age 60, range 32– 88 years). In 19 eyes (7 %, mean age 56, range 37–77 years) atrophy occurred without any signs for an active or fibrotic choroidal neovascularization (CNV). The frequency of both, atrophy and CNV increased with age. In those > 60 years of age, atrophy and/or CNV were almost universally present but varied considerably in severity. Eyes with emerging pure atrophy (n = 13, no signs of CNV) showed pattern dystrophy like changes (100 %), reticular pseudodrusen (82 %), and reduced choroidal thickness. Advanced atrophy was multifocal, reached beyond the arcades and was present nasal to the optic disc. The average expansion rate of atrophy was 3.3 ±1.3 and 1.6 ±1.1 $\mu m^2$/year (mean±SD), in those without or with signs for CNV, respectively.

**Conclusions:** Atrophy is a common finding in PXE patients characterized by early onset and fast progression with subsequent visual loss independent from CNV. This suggests that atrophy is the natural endpoint of Bruch membrane disease. The phenotype shows similarities with geographic atrophy in age-related macular degeneration suggesting common pathogenetic pathways at the level of Bruch membrane.

# The subretinal space as a niche for human retinal disease modeling

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Humanized mouse models represent a powerful tool for basic research and preclinical studies as been shown for human hematopoietic stem cells, hepatic and cancer cells.

Within the eye, the subretinal space between the photoreceptors and the retinal pigment epithelium has a long-standing history as an exceptional niche where transplanted cells can survive and mature beyond levels achieved ex vivo.

Thus, we would like to develop a humanized retinal disease mouse model to monitor and study human disease processes in an in vivo setting.

Here we show that hiPSC-derived retinal organoids can be successfully generated using our modification of the pioneering protocol.

Human retinal organoids analyzed up to day 170 of differentiation develop retinal ganglion [BRN3+, ELAVL3/4], amacrines [CALR+], cone [CRX+, RCVRN+, PNA+, ARR3+] and rod [CRX+, RCVRN+, NRL+] photoreceptors, Müller glia [Sox2+, RLBP1+] and potentially retinal pigment epithelium. Additionally, we dissociated 96 days old human iPSC-derived retinal organoids and transplanted the cells into the subretinal space of wildtype mice. We found that grafted cells survived at least up to 3 weeks and express photoreceptor markers such as CRX, RCVRN and OPN1SW.

Our results open up the possibility to study the mouse subretinal space as a permissive niche for human photoreceptor and retinal disease development in situ and in vivo.

# Arf-like Protein 3 (ARL3) regulates protein trafficking and ciliogenesis in mouse photoreceptors

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**Purpose:** Arf-like protein 3 (ARL3) is a ubiquitous small GTPase expressed in ciliated cells of plants and animals. Germline deletion of *Arl3* in mouse causes multi-organ ciliopathy reminiscent of Bardet-Biedl or Joubert syndromes. As photoreceptors are elegantly compartmentalized and have cilia, we probed the function of ARL3 by generating rod photoreceptor-(prefix $^{rod}$) and retina-specific (prefix $^{ret}$) *Arl3* deletions.

**Methods:** EUCOMM gene trap was inserted in intron 1 of the mouse *arl3* gene. Rod- and retina-specific *arl3* conditional knockout mice were obtained by crossing with Flp-mice followed by mating with iCre75$^{+}$ or Six3Cre$^{+}$ transgenic mice, respectively. ERG and OptoMotry were used to test photoreceptor function, whereas progress of retinal degeneration was evaluated by optical coherence tomography, confocal immunohistochemistry and histology. Adeno-associated virus (AAV) particles were injected in the subretinal space to rescue *arl3*$^{-/-}$ photoreceptors.

**Results:** In predegenerate $^{rod}$*Arl3*$^{-/-}$ mice, lipidated phototransduction proteins (PDE6, GRK1 and transducin) showed trafficking deficiencies to the rod outer segments (ROS), consistent with ARL3's role as a cargo displacement factor (CDF) for lipid binding proteins (PDE6D and UNC119). Transmembrane proteins (rhodopsin and GC1) traffic normally. Its recently discovered GEF, ARL13b, appears unaffected and localizes to ROS of both wild-type and mutant mice. By contrast, $^{ret}$*Arl3*$^{-/-}$ rods and cones expressing Cre recombinase during embryonic development formed neither connecting cilia nor outer segments, and degenerated rapidly as shown by ERG, OCT and histology. Farnesylated inositol polyphosphate phosphatase (INPP5E), reportedly in primary cilia and associated with Joubert Syndrome, is present exclusively in Golgi membranes of wildtype photoreceptors and significantly reduced in $^{ret}$*Arl3*$^{-/-}$ as revealed by immunohistochemistry and neonatal electroporation. Absence of cilia infers participation of ARL3 in ciliogenesis and axoneme formation. Ciliogenesis was rescued and degeneration was reversed in part by subretinal injection of AAV particles expressing ARL3-EGFP before eye-opening; viral expression of ARL3-EGFP preserved ONL thickness and ROS-like structure, also rescued delivery of lipidated cargo to the ROS.

**Conclusion:** The conditional knockout phenotypes permitted identification of two ARL3 functions, both in the GTP-bound form: one as a regulator of intraflagellar transport (IFT) participating in photoreceptor ciliogenesis and second, as a CDF transporting lipidated protein to the outer segment. Surprisingly, a farnesylated INPP5E only trafficked from the endoplasmic reticulum to the Golgi, thereby excluding it from a role in photoreceptor cilia physiology.

# Development of Ganglion Cell-specific promoters for gene therapy

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The loss of retinal ganglion cells (RGCs) is a hallmark of a number of retinopathies, both inherited and multifactorial. These diseases can be caused by mutations in any of a number of nuclear or mitochondrial genes. There are a number of gene therapies being developed that have shown to be effective in preserving RGCs when administered using an AAV vector. Localising expression of any therapeutic to the target cell type (ganglion cell layer, GCL) would represent a significant optimisation of the approach. The packaging capacity of AAV (4.7 kb) imposes a limit on the size of the promoters and genes relevant for AAV-mediated gene delivery. Few tissue-specific promoter sequences have been defined that limited expression to RGCs and that are sufficiently small in size for AAV-guided gene expression. Genes with expression profiles limited to the GCL were examined and candidate promoter sequence conservation was explored. Conservation of sequences across mammalian species was used as a proxy for putative function. Adopting this strategy, ganglion cell promoter 1 (GCP1), demonstrating the key features outlines above was identified. To test its function, GCP1 was engineered into an AAV2 virus expressing EGFP.

Here we demonstrate the effectiveness of GCP1 in localising EGFP expression to the GCL when administered via intravitreal injection. Furthermore, absence of EGFP expression was demonstrated when targeted towards photoreceptors via subretinal injection, verifying the tissue-specific nature of GCP1. Expression of AAV2.GCP1-EGFP was compared to dose-matched expression from a non-specific promoter construct, AAV2.CMV-EGFP. GCP1-EGFP was shown to provide equivalent expression to CMV-EGFP in the GCL. GCP1 thus offers a tissue-specific promoter option for therapies targeted toward RGCs. Its size should allow for deployment within AAV vectors and the potential to target therapeutic gene expression to RGCs without compromising functionality. This represents a valuable addition to the expanding gene therapy toolbox.

# miRNA profile of Müller cells from the diabetic retina implicates a possible role of PDGF-mediated signaling in retinal glia for disease progression

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**Purpose:** Compelling evidence indicates that microRNAs (miRNAs) are modulators of disease progression in diabetic retinopathy (DR). We investigated miRNA profiles of different retinal cell types in a murine diabetes model to improve our understanding of signaling pathways potentially modulated by miRNAs. Focus was set on Müller cells, the major retinal macroglia and likely key players in DR.

**Methods:** Retinae of C57BLKS-Lepr$^{db}$ 24-weeks-old wildtype and diabetic mice were investigated by immunofluorescence labeling, patch clamp recordings of Müller cells and proteomic profiling. Microglia, neurons, Müller and vascular cells were enriched by magnetic activated cell sorting from wildtype and diabetic retinae of 12-weeks-old animals. RNA was extracted and miRNA libraries were generated for subsequent next generation sequencing. Candidate miRNAs and expression of putative target genes were validated by quantitative real time-PCR (qRT-PCR). The effect of platelet-derived growth factor (PDGF) on Müller cell volume regulation was analyzed by micro-fluorimetric measurements.

**Results:** Müller cells gliosis in 24-weeks-old diabetic mice was characterized by a reduced potassium conductance, a disrupted volume regulation and upregulation of cytoskeletal proteins (vimentin, GFAP) as well as Stat3 (transcription factor driving gliotic reactivation). We demonstrated loss of pericytes – a cardinal feature of DR. miRNA sequencing revealed significant regulation of 38 miRNAs in gliotic Müller glia. qRT-PCR largely confirmed these findings. Analysis of putative target genes pointed to predominant modulation of genes involved in angiogenesis (e.g. PDGF pathways) and Müller cell gliosis (e.g. Stat3). We demonstrated that vascular and Müller cells express PDGF and its receptors in control mice, a downregulation of the PDGF pathway in diabetic retinae and that PDGF reestablishes the volume regulation of gliotic Müller cells in the diabetic retina.

**Conclusion:** The early diabetes-associated changes of miRNA profiles in Müller glia – that besides vascular cells contribute to maintain the blood-retina-barrier – support the idea that miRNAs affect disease progression and are consistent with the fact that DR essentially is a vascular disease. One candidate pathway modulated by miRNAs is that of PDGF. We plan to target respective miRNAs to test for effects on PDGF-signaling in Müller cells and the diabetic retina.

# New insights into thyroid hormone associated pathogenesis of age-related macular degeneration

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**Purpose:** The thyroid hormones (TH) thyroxine (T4) and triiodothyronine (T3) are crucial for photoreceptor maintenance. Moreover, evidence is striking that TH-disorders are involved in the pathogenesis of age-related macular degeneration (AMD). However, the transporting mechanisms of TH into photoreceptors are still fragmentary. Thereof, we investigated age-dependent expression patterns of the T4-specific organic anion transporting polypeptide 1c1 (Oatp1c1) and the T3-specific monocarboxylate transporter 8 (Mct8) in the mouse retina for the first time. We aimed to create a scaffold for retinal TH-transport at different developmental stages as a basis for *in vitro* studies addressing TH-associated pathogenesis of AMD as a novel approach.

**Methods:** We quantified Mct8 and Oatp1c1 mRNA expression via Real-Time Reverse-Transcriptase PCR in mouse eyes (C57BL/6) of five different age groups (14-days-old, 21-days-old, 28-days-old, 6-months-old, 24-months-old). In addition, Mct8 protein was localized via immunofluorescence staining and quantified via western blotting in the retinae of the same age groups.

**Results:** On protein level, we found an age-dependent decline of Mct8 in the mouse retina. While Mct8 immunoreactivity in juvenile animals was broadly observed along the retinal pigment epithelium (RPE), the inner nuclear layer and the ganglion cell layer, immunoreactivity visibly declined in adult retinae (6-months-old, 24-months-old). In contrast, we did not find any age-dependent changes in Mct8 mRNA expression. This kind of asymmetry between Mct8 mRNA and protein levels are in line with previous reports of Mct8 in liver and kidney. Interestingly, Oatp1c1 mRNA expression increased significantly in adult animals, suggesting similar increase at protein level. The corresponding immunohistochemical localization of Oatp1c1 is currently in progress. So far, Oatp1c1 was shown to be expressed in the RPE and inner retinal capillaries of adult rats.

**Conclusion:** Our results, along with previous studies, suggest that Mct8 and Oatp1c1 are expressed subsequently during development. While Mct8 is likely to regulate TH-supply of early postnatal photoreceptors, increasing expression of Oatp1c1 in adult mice suggests an involvement in adult photoreceptor maintenance. Since TH-disorders are common in the general population, with a prevalence of up to 10 %, our results can contribute to a deeper understanding and early prevention of TH-associated retinal disorders, especially AMD.

# Development of a NGS exome analysis pipeline on the HPC cluster at the University of Oldenburg – From exome sequencing data to annotated variant calls

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**Purpose:** Whole-exome sequencing (WES) has led to the discovery of many new disease-genes in recent years. However, the large amount of potential candidate variants poses significant challenges even after applying common filtering strategies. Making use of the wealth of publicly available sequence variants and combining them with locally generated patient information offers the potential to improve filtering and significance of WES analyses. Therefore, we establish an analysis pipeline for whole exome sequencing data on the high performance computer cluster at the University of Oldenburg that will allow the integration of local and public sequencing data.

**Methods:** We use scripting languages and the Linux operating system to combine publically available open source software tools with the Broad Institute GATC software suite. By employing the aforementioned tools, we set up an integrated data analysis pipeline that follows and implements the Broad Best Practices workflows. The analysis tasks are submitted by a job management system to the computing cluster. Public sequencing data are obtained via file transfer protocol from servers of the 1000 Genome Project and fed into the analysis workflow.

**Results:** We set up a semi-automated analysis pipeline for locally generated exome data. To foster the discovery of mutations in known and novel disease-genes, we integrate sequencing data obtained from the 1000 Genome Project. This allows for improvements in variant filtering and statistical analysis. So far, whole genome and whole exome datasets of eighteen individuals with European ancestry were obtained from the 1000 Genome Project and integrated into the workflow.

**Conclusion:** Integration of publicly available genomic data together with local patient data can increase the power of detecting potentially disease-causing genetic variants. In this way the disadvantage of only having relatively few patient samples compared to large gene diagnostic centres can be alleviated.

# Genetic risk scores in 60 complex diseases and traits – Search for novel associations with age-related macular-degeneration (AMD)

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With the advances of genome-wide association studies (GWAS), hundreds of different complex diseases and human traits were characterized for their genetic risk structure. As one consequence, thousands of single-nucleotide polymorphisms (SNPs) were implicated to influence disease risk. These studies mostly focused on single entities, associating a defined phenotype with a defined genetic marker profile. Beyond such one-dimensional correlations, SNPs identified by defined phenotype- or trait-driven GWAS may harbor additional information on SNPs simultaneously implicated in several clinical phenotypes due to overlapping functional disease mechanisms. Consequently, the question arises whether common pathways would be reflected by SNPs with shared genetic associations. To directly address this question, we investigated 2,493 SNPs associated with 60 different diseases or traits on a genome-wide significance level for their association with AMD. The SNPs were previously implicated in autoimmune diseases, cardiovascular diseases and different metabolic traits. Initially, we calculated genetic risk scores for the 60 diseases/traits in a total data set of 43,566 subjects with predominantly European descent from the International AMD Genomics Consortium (IAMDGC). Further, we analyzed all GWAS-SNPs to identify potentially new significant associations with AMD.

We found significant genetic associations between AMD and 16 investigated risk scores of different diseases/traits (FDR< 0.01). The implicated risk scores include high-density lipoprotein levels, (OR = 1.02 [1.014-1.021], FDR = $4.07 \times 10^{-15}$) and coronary artery disease (OR = 0.99 [0.987-0.996], FDR = $2.57 \times 10^{-3}$), as well as previously unknown associations like cutaneous malignant melanoma (OR = 1.02 [1.006-1.024], FDR = $4.43 \times 10^{-3}$) and psoriasis (OR = 1.01 [1.009-1.020], FDR = $5.74 \times 10^{-6}$). In total, 57 SNPs revealed a significant association with AMD, of which 28 were novel after exclusion of SNPs in known AMD-risk loci (FDR < 0.01). Interestingly the effect sizes of most of these 28 SNPs increased after adjustment for the AMD risk score (one-sample t-test, two-sided, p=$1.2 \times 10^{-4}$), indicating that these SNPs are independent from known AMD variants.

Taken together, our data show genetic associations between AMD and different diseases, such as cancer, cardiovascular diseases and autoimmune diseases, as well as different metabolic traits, e.g. high-density lipoprotein and total cholesterol levels. This has resulted in the identification of 28 risk associated variants so far not associated by existing AMD GWAS.

# HDAC6 inhibitor tubastatin A is protective against oxidative stress in a photoreceptor cell line

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**Purpose:** Progressive loss of photoreceptor cells is characteristic for retinal degenerative disorders, such as retinitis pigmentosa (RP) and age-related macular degeneration. Recent studies suggest that histone deacetylases (HDACs) are involved in neurodegeneration and that inhibition of their enzymatic activity plays a protective role. Using a pan-inhibitor it was shown that HDAC inhibition could prevent photoreceptor cell death in a mouse model for RP. HDAC6 is unique among the 18 members of the HDAC family since it mainly deacetylates non-histone proteins like α-tubulin, HSP90, cortactin and the redox regulatory protein peroxiredoxin 1 (Prx1), which is involved in the reduction of hydrogen peroxide ($H_2O_2$). It plays a role in cellular stress responses and its inhibition has been implicated to be protective against neurodegenerative diseases. The aim of the present study was to elucidate the role of HDAC6 and the influence of its inhibition by tubastatin A (TST) in 661W cells, a cell line with characteristics of cone photoreceptors, after oxidative stress. The presence of HDAC6 was investigated in retina lysates and slices of C57BL/6 mouse retinae.

**Results:** Immunohistochemistry revealed that HDAC6 is expressed in the retina of C57BL/6 mice and prominent in photoreceptor inner segments as well as in the outer plexiform layer. It is expressed in 661W cells and its inhibition by TST results in hyperacetylation of α-tubulin. In 661W cells oxidative stress, exerted by treatment with $H_2O_2$, caused cytotoxic responses. Interestingly, preincubation with TST promoted cell survival after $H_2O_2$ treatment. TST enhanced the expression of heat shock protein (HSP) 25 and HSP70 by activating the heat shock factor 1 (HSF1). The protective effect, however, is not causally related to the induction of HSF1, as demonstrated by incubation with KRIBB11, an HSF1-inhibitor. Immunoblot analysis revealed that $H_2O_2$ treatment leads to strong overoxidation and thereby inhibition of Prx1. This effect was significantly reduced after preincubation with TST. Thus, HDAC6 inhibition by TST is protective against oxidative stress by regulating the activity of Prx1.

**Conclusion:** HDAC6 inhibition provides a protective means against a stress situation which occurs in retinal degenerative diseases.

# *ARMS2* activates complement on stressed ARPE-19 cells and triggers VEGF secretion which is controlled by factor H

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**Background:** AMD which represents the most common cause of blindness in developed countries is characterized by the formation of drusen at the macula and the degeneration of RPE cells and photoreceptors. Polymorphisms in age-related maculopathy susceptibility 2 *(ARMS2)* gene and in complement factor H are highly associated with the risk to develop AMD. Recently we identified *ARMS2* as complement activator on stressed or apoptotic human cells thereby enhancing the surface opsonization for phagocytosis. If *ARMS2* activates complement on stressed ARPE-19 cells and whether factor H restricts further complement activation and inflammation on these cell surfaces was unclear.

**Methods:** *ARMS2* was recombinantly expressed in *Pichia pastoris* and purified via chromatography. Interaction of *ARMS2* with the positive regulator of complement properdin was analyzed in detail by ELISA, interferometry and pepspot analysis. Cell binding of *ARMS2* and factor H to human retinal pigmented epithelial cell surfaces was determined by flow cytometry. Complement activation on ARPE cells was followed by C3b deposition and release of VEGF measured by ELISA.

**Results:** We identified specific binding of *ARMS2* and factor H to the surface of oxygen damaged human ARPE-19 cells. Bound to the cell surface, *ARMS2* enhanced opsonization of the cell surface with complement C3b. *ARMS2* binds properdin via a distinct binding region in the C-terminal part of *ARMS2*. Surface bound *ARMS2* mediated complement activation resulted in VEGF secretion from ARPE-19 cells, which in turn was downregulated by factor H. In summary complement activation leads to VEGF secretion and this process is controlled by factor H.

# Comparison of retinal pigment epithelium atrophy progression in late-onset Stargardt disease and Age-related Macular Degeneration

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**Purpose:** To describe the distinct courses of retinal pigment epithelium (RPE) atrophy progression in geographic atrophy (GA) secondary to age related macular degeneration (AMD) and late onset Stargardt disease (LO-STGD).

**Methods:** Patients were recruited from the prospective natural history Fundus-Autofluorescence imaging in AMD study (FAM, NCT00393692) and the Departments of Ophthalmology of the University of Nijmegen and the University of Bonn. Longitudinal examinations with fundus autofluorescence (FAF) and near-infrared reflectance (IR) imaging (Spectralis HRA+OCT or HRA2, Heidelberg Engineering) were performed. Areas of RPE atrophy were quantified using a semi-automated software tool (RegionFinder, Heidelberg Engineering). Rates of RPE atrophy progression were calculated and compared using a linear mixed-model (LMEM) approach.

**Results:** A total of 226 eyes (151 patients) with GA secondary to AMD and 66 eyes (38 patients) with RPE atrophy secondary to LO-STGD with a follow-up period of 2.8 years (± 1.98 years) were included in the analysis. Mean age at baseline was 74.02 ± 7.44 years in the AMD and 63.62 ± 10.38 years in the LO-STGD patients cohort ($p < 0.001$). At first presentation square-root transformed RPE atrophy size was 2.30 ± 1.02 mm in AMD and 2.10 ± 1.35 $mm^2$ in LO-STGD eyes ($p = 0.47$). Applying the LMEM revealed that square root atrophy progression was significantly faster in AMD patients than in LO-STGD patients (0.28 ± 0.01 mm/y vs. 0.23 ± 0.03 mm/y, $p = 0.029$).

**Conclusions:** RPE atrophy progression is significantly slower in eyes with LO-STGD as compared to those with AMD. These natural history data underline the relevance of refined phenotyping in elderly patients presenting with RPE atrophy and contribute to the design of future interventional trials.

# Photoreceptor transplantation: Do donor and recipient photoreceptors "fuse"?

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Photoreceptor replacement by cell transplantation has been proposed as a therapeutic approach to treat retinopathies. Pre-clinical studies provided evidence for successful transplantation of photoreceptors into mouse models of retinal degeneration. Donor photoreceptors were suggested to integrate into the host outer nuclear layer (ONL) and mature with the capacity to restore visual responses. A potential "fusion" of donor and host cells, as it was observed in other transplantation paradigms, e.g. in bone marrow transplants, was ruled out, as reporter labeled cells contained a single nucleus and donor cell processes showed no co-labeling when transplanted into fluorescent labeled hosts.

Here, we reinvestigated the potential of fusion events following photoreceptor transplantation by (i) separating cytoplasm from nuclear labeling, (ii) using the Cre/lox fusion assay, and (iii) single cell analysis by flow cytometry and high-throughput microscopy.

Donor photoreceptors were isolated from male photoreceptor-specific GFP reporter mice (Nrl-GFP) and transplanted into female recipients (i). Fluorescent in situ hybridization (FISH) analysis of grafted cells located in the sub-retinal space revealed that virtually all $GFP^+$ cells contained a Y-chromosome identifying them as donor photoreceptors. Interestingly, of all $GFP^+$ photoreceptors located within the host ONL, less than 3 % showed co-staining for the Y-chromosome, suggesting that cytoplasm content but not the nucleus of donor cells is located within endogenous photoreceptors. To further study a potential exchange of cytoplasmic contents between donor and host photoreceptors, (ii) donor cells isolated from a floxed reporter mouse line (Ai9 mice) were transplanted into rod-specific Cre recombinase expressing hosts (B2-$Cre^{+/-}$ mice). Reporter expression was detected in both, cells in the sub-retinal space and photoreceptors within the ONL, showing that Cre recombinase produced by host cells was transported into donor photoreceptors leading to the expression of the reporter protein. Moreover, (iii) combined flow cytometric analysis with high throughput microscopy (ImageStream technology) revealed double positive cells after transplantation of Nrl-GFP photoreceptor precursors into ubiquitously expressing dsRed mouse retinas.

In conclusion, we provide strong evidence that donor photoreceptors transplanted into the adult mouse retina can "fuse" with host photoreceptors resulting in the exchange of cellular content but without translocation of the nucleus by an unknown mechanism. Further studies will be needed to elucidate this observation.

# Development of a mitochondrial assay to evaluate novel complex I therapies

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Leber's Hereditary Optic Neuropathy (LHON) is characterized by mitochondrial dysfunction due to mutations affecting the mitochondrial genome, in particular genes coding for subunits of Complex I of the electron transport chain. Mitochondrial dysfunction leads to a lack of energy production and ultimately the death of the cell – the type of cell affected by or vulnerable to this dysfunction affects the pathology of the disease. For instance, in the case of LHON, retinal ganglion cells (RGCs) are affected. These observations have prompted interest in exploring innovative therapeutics to modulate mitochondrial disorders involving complex I deficiency. The Farrar laboratory has explored candidate gene therapies for complex I deficiency using Ndi1, a yeast gene which is a complex I homologue.

In order to test the efficacy of Ndi1 amongst other candidate therapies, we have developed a robust, empirical assay of mitochondrial function. Previous assays measured the level of NADH oxidation in a sample, both before and after rotenone as a measure of complex I activity. To optimally distinguish between the activity of complex I and the potential therapeutic, the assay was modified with the addition of a second inhibitor which allowed specific measurement of the therapeutic, in this case Ndi1 as well as modified versions of Ndi1. As this is an in vitro assay, it allows large-scale screening of potential therapeutics and ensures only those that show strong evidence of efficacy are then tested in vivo. In combination with other quantitative assays such as Reactive Oxygen Species (ROS) generation this allows detailed evaluation of the health of mitochondria within a sample and thereby demonstrates the benefit of a potential therapeutic modality.

# Primary cilium regulates human iPS cell-derived RPE maturation via regulation of WNT signaling

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**Purpose:** The retinal pigment epithelium (RPE), a ciliated monolayer epithelium tightly associated with photoreceptors, is critical for photoreceptor function and survival. Development and function of the RPE has been shown to depend on wingless (WNT) signaling, a core signaling pathway known to regulate diverse processes during development. Considering that the primary cilium has been shown to regulate WNT signaling, we sought to investigate the role of the primary cilium in RPE development and maturation. We sought to apply this knowledge to improve differentiation of iPSC-derived RPE.

**Methods:** Using a variety of microscopic and expression analyses, we examined ciliogenesis in the developing RPE of cilia mutant mice and during differentiation of human iPSC-derived RPE. We manipulated expression of the primary cilium during iPSC-derived RPE differentiation by treatment with compounds that enhance or disrupt ciliogenesis and assessed for maturation and function.

**Results:** In wild type mice canonical-WNT signaling was found to be active during the development of the RPE. Primary cilia mutant mice develop immature RPE and continually active canonical-WNT signaling in post-natal animals. iPSC-derived RPE can be matured and fully-polarized *in vitro* by manipulation of the primary cilium. In human iPSC-RPE, experimentally enhanced primary cilium activation leads to mature, fully-polarized, and functional RPE through canonical-WNT suppression and PKC-δ activation. Inhibition of primary cilium function in human iPSC-RPE results in a lack of RPE maturation, missing PKC-δ activation, and continued canonical-WNT activation.

**Conclusion:** Our results show a developmental role for primary cilia in RPE maturation, provide insight into retinal degeneration caused by ciliopathies, and sheds light on a mechanism with which to mature any epithelial cell type derived from human iPSCs. Furthermore, our findings elucidate potential targets for treatments and cures of retinal ciliopathies.

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# Mutant Bestrophin-1 (BEST1) is degraded via the endo-lysosomal pathway

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**Purpose:** Bestrophin-1 (BEST1), an integral membrane protein in the basolateral aspect of the RPE, is thought to act as a volume-regulated anion channel. Mutations in BEST1 cause Best vitelliforme macular dystrophy (BVMD) and are assumed to give rise to trafficking problems prompting the protein to remain within so far undefined cellular compartments. The precise molecular pathomechanisms of the disease-associated mutations are still unclear.

**Methods:** Protein half-life was determined in polarized MDCKII cell lines, constitutively expressing wildtype BEST1 and seven disease-associated mutants. Cultivated cells were treated with cycloheximide (CHX, 20 µg/ml) and harvested at various time points. Protein lysates were analyzed by densitometry of Western blot signals. To examine degradation pathways, cells were treated with CHX and a series of inhibitors for the proteasomal, lysosomal and autophagy pathway. BEST1 was localized by immunocytochemistry.

**Results:** Normal BEST1 and BEST1-R218C revealed localization to the plasma membrane (PM) in contrast to intracellular localization of BEST1 mutants T6P, L21V, W93C, L224M, Y227N and F305S. Protein level of wildtype BEST1 remains stable even after 24 h CHX treatment and arrest of protein synthesis, whereas six out of seven mutants degraded within 3 hours and mutant R218C within 12 h. Similar to the majority of integral PM proteins, wildtype BEST1 is degraded via the endo-lysosomal pathway, inhibited by ammonium chloride (20 mM) and chloroquine (10 µM). Unexpectedly, all mutants tested do not reveal degradation via the proteasomal but instead via the endo-lysosomal degradation pathway. Consequently, low temperature (26 °C) for 36 h failed to foster trafficking of mutant BEST1 protein to the cell surface as described for many endoplasmic reticulum (ER)-retained mutants.

**Conclusions:** Our data support the hypothesis that loss of BEST1 function is caused by a decrease in mutant protein stability with instable, temperature-insensitive mutant BEST1 degraded via the endo-lysosomal pathway. As a consequence, a chaperon-mediated rescue as shown for many mutants in disorders like CFTR, Parkinson Disease and Alzheimer disease may not lead to success in BVMD.

**References:** Milenkovic et al. Bestrophin 1 is indispensable for volume regulation in human retinal pigment epithelium cells. *Proc Natl Acad Sci USA* **112**:E2630-9, 2015

# Perception of Haidinger's brushes in macular diseases

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**Purpose:** To optimize the perceptibility of Haidinger brushes (HB) and to investigate its association with visual acuity and macular pigment density.

**Methods:** In this prospective cross-sectional study, each subject underwent best-corrected visual acuity (BCVA) testing, funduscopy, and assessment of macular pigment optical density (MPOD) using the two-wavelength fundus autofluorescence method. Haidinger brush visibility was tested with a rotating linear polarizer and a controllable three-color light-emitting diode (LED) panel as light source. A simple model of macular pigment absorption was used to predict HB visibility as a function of stimulus wavelength and MPOD.

**Results:** All control eyes (n = 92) and 34 % of the 198 eyes of subjects with macular disease (age-related macular degeneration, n = 40; macular telangiectasia type 2, n = 52; Stargardt disease, n = 58; other retinal dystrophies, n = 48) perceived HB when an optimized test setup (464-nm LED light) was applied. The degree of psychophysical perception and the dependency on different wavelengths were in accordance with the absorptance model. In eyes of subjects with macular disease, minimum thresholds of MPOD and BCVA required for HB perception were identified. Subjects with macular telangiectasia type 2 showed lowest values of MPOD and were mostly unable to perceive HB despite relatively preserved BCVA.

**Conclusions:** Macular pigment and a relatively preserved foveal function are necessary for the perception of HB. Haidinger brushes are usually not perceived by subjects with macular telangiectasia type 2, likely due to their characteristic foveal depletion of macular pigment.

# Elucidation of the molecular cause underlying Sorsby fundus dystrophy in a large Belgian pedigree: N-terminal TIMP3 mutation 15 years later

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**Purpose:** Sorsby fundus dystrophy (SFD) is an autosomal dominant retinal dystrophy, caused by mutations in *TIMP3*. TIMP3 is a member of a family of four secreted proteins inhibiting the activity of matrix metalloproteinases. In 2000, Assink *et al.* examined a large Belgian family with SFD. Although linkage was shown with the *TIMP3* locus, mutation screening did not reveal a mutation in *TIMP3*. Here, it was our aim to elucidate the genetic cause of SFD in this family.

**Methods:** We performed microsatellite analysis with additional markers, and subsequently performed Sanger sequencing of the coding region. In addition, we identified a French family with the same mutation. As we assumed a common origin, haplotype reconstruction was done. In 2003, Arris *et al.* proposed a mechanism whereby mutations adding an extra cysteine create proteins that form disulfide-bonded dimers. To test this hypothesis, we performed Western blot analysis on patient derived fibroblasts. The N-terminal domain of TIMP3 alone is sufficient to mediate its metalloproteinase inhibitory activities. To investigate whether the N-terminal mutation affects this inhibitory activity, we performed enzymatic activity assays with MMP2, a target for TIMP3.

**Results:** We identified a known mutation, c.113C>G; p.(Ser38Cys). This is the only mutation located in the N-terminal domain of the TIMP3 protein and segregates with disease in 63 family members. Haplotype analysis in two families revealed a common haplotype of 6,39Mb. TIMP3 comprises six disulfide bonds, established by twelve cysteines. The p.(Ser38Cys) mutation results in an additional cysteine. However, we could not confirm the proposed hypothesis by Arris *et al.* for this mutation using Western blot analysis. However, using an MMP2 activity assay, we revealed a possible decrease in TIMP3 activity.

**Conclusion:** In conclusion, we elucidated the molecular cause underlying SFD in a large Belgian pedigree, previously missed. Our study confirms the genetic homogeneity of SFD. We could not confirm the hypothesis proposed by Arris *et al.* on protein dimer formation, but revealed a possible effect on TIMP3 inhibitory activity.

# The Rhodopsin Schiff base counterion mutation p.E113K can cause two different phenotypes within the same family.

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**Purpose:** The present study focused on an identification of disease-causing mutations in the index patient affected with retinitis pigmentosa (RP) and in several older family members affected with an incomplete form of congenital stationary night blindness (CSNB). So far, very few studies reported RP and CSNB as independent phenotypes within the same family.

**Methods:** The RP-affected index patient, five night-blind relatives, and one healthy individual from a three-generation family were analysed. A complete clinical characterization was carried out in the index patient and one CSNB-affected individual. Whole exome sequencing was used towards a rapid identification of unique genetic alterations in the index patient and two CSNB-affected family members. Genetic variants were verified in family members and 199 unrelated controls using Sanger sequencing.

**Results:** Ophthalmological examinations of the index patient confirmed a typical form of RP. In contradiction to the index patient, clinical investigations of a night-blind family member revealed a non-progressive and incomplete form of CSNB (Schubert-Bornschein type). Whole exome sequencing identified the non-synonymous substitution c.337G>A, p.E113K in the rhodopsin *(RHO)* gene. The mutation co-segregated in all examined family members, including the index patient and five CSNB-affected relatives. The mutation was excluded in the healthy relative and in 199 ethnically matched controls. No other genetic alteration in the coding sequence of *RHO* was found in the investigated family members.
We present the first evidence for the naturally-occurring mutation p.E113K in the Schiff base counterion of RHO in human patients. With the aim to explain the phenotypic differences between the RP-affected index patient and the CSNB-affected family members, genetic variants in a promotor as well as in untranslated regions of *RHO* still need to be analysed in the attempt.

**Conclusions:** Our findings demonstrate that the mutation of the biochemically well-characterized counterion p.E113 in RHO may lead to either RP or incomplete CSNB, even within the same family.

# News from the DRUGSFORD project: The lead compound LP-DF003 preserves retinal morphology and function in three different *in vivo* models for retinitis pigmentosa.

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Retinitis pigmentosa (RP) relates to a genetically heterogeneous group of diseases leading to photoreceptor degeneration and blindness. Many RP subtypes display an excessive accumulation of cGMP in photoreceptors, providing a common target for photoreceptor protection. The DRUGSFORD project has generated new compounds combined with a liposomal drug delivery system, to selectively rebalance cGMP-signalling. The project's first lead compound formulation – LP-DF003 – showed remarkable photoreceptor protective properties in different *in vivo* RP models.
To identify the best *in vivo* application paradigm for compound and DDS combinations, topical, intravitreal, subtenonal, intraperitoneal, and intravenous applications were tested using fluorescently labelled liposomes and scanning laser ophthalmoscopy. The treatment effect was then tested *in vivo* on *rd1, rd2,* and *rd10* mice, the effects were analysed using electroretinography and histology. In rapidly degenerating *rd1* mice, intraperitoneal injection of LP-DF003 significantly slowed down the degenerative process at post-natal day (P) 14. In the more slowly degenerating *rd2* and *rd10* animals, photoreceptor survival was significantly improved at P30. Importantly, P30 ERG recordings in *rd2* and *rd10* animals showed significantly increased cone photoreceptor-driven light responses.
Taken together, LP-DF003 increased photoreceptor viability in three different RP models *in vivo,* with robust improvement of retinal function in two of these models. Since *rd2* and *rd10* animals carry mutations in two different genes (*Prph2* and *Pde6b*, respectively), this suggests the general applicability of LP-DF003 treatment to a variety of different RP subtypes with significant promise for future clinical development.

# Specific quantification of CFHR3 from human sera

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**Purpose:** Deficiencies in the complement regulatory genes *cfhr3* and *cfhr1* are protective against age-related macular degeneration (AMD) but increase the risk for atypical hemolytic uremic syndrome (aHUS). However, the function of the CFHR3 protein has not been deciphered so far. We generated a highly specific monoclonal antibody to determine CFHR3 concentration in human sera.

**Methods:** We used ELISA, genotyping and immunoprecipitation including western blot analyses of standard samples to determine antibody specificity. We measured CFHR3 concentrations in standard serum samples and in samples of patients with rheumatic diseases, AMD as well as aHUS.

**Results:** (I) The monoclonal anti-CFHR3 antibody showed high specificity for the recombinant and serum CFHR3 protein. (II) Exclusively CFHR3 and no other CFHR protein was immunoprecipitated from human serum. Specificity of the monoclonal CFHR3 antibody was confirmed with *cfhr3/1*$^{-/-}$ deficient sera. (III) CFHR3 concentration in human sera of healthy control samples was about 50 µg/mL. (IV) AMD and aHUS sera did not show a highly significant change in systemic CFHR3 compared to controls. (V) Whereas, patients with rheumatoid arthritis (RA) and polymyalgia rheumatic (PR) surprisingly showed 2–3 times higher CFHR3 concentrations in serum than healthy controls.

**Conclusions:** The new monoclonal CFHR3 antibody is highly specific and suitable for determining CFHR3 serum concentrations and for further biochemical analyses. The increased CFHR3 serum levels in RA and PR as compared to controls indicates a role of CFHR3 in these diseases. The combination of our and gene association studies suggests that the involvement of CFHR3 in AMD progression takes place on a local retinal rather than on a systemic level.

# On the interaction between the retinal Na/K-ATPase and RS1

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**Background:** X-linked juvenile retinoschisis (XLRS) is a macular dystrophy caused by mutations in the *RS1* gene encoding for the RS1 protein, termed retinoschisin. Expression of *RS1* is limited to the retina, where retinoschisin is secreted from and binding to photoreceptor and bipolar cells. While it was shown that it is crucial for maintaining the structural integrity of the retina (Weber et al., 2002), its exact role in the molecular pathology of XLRS is yet to be elucidated.
In previous work, the retinal Na/K-ATPase consisting of subunits α3 and β2 was identified as a direct RS1 interaction partner, anchoring RS1 to the plasma membrane (Molday et al., 2007, Friedrich et al., 2011). The aim of the present work is to gain insight into the binding mechanism of Na/K-ATPase / RS1 interaction to provide a basis for a deeper understanding of the role of the RS1-Na/K-ATPase complex in retinal cell biology and XLRS disease.

**Methods:** Human Na/K-ATPase isozymes for the α (α1/2/3) or the β (β1/2/3) subunit, as well as chimeric β subunits were heterologously expressed in Hek293 cells to test the affinity of Na/K-ATPase complexes with varying compositions for RS1 binding. An influence of Ouabain, a steroid hormone known to bind to the Na/K-ATPase α-subunit, on the interaction between RS1 and the Na/K-ATPase was investigated by exposing transfected Hek293 cells to increasing Ouabain concentrations. To identify the interaction site in retinoschisin, RS1 binding was tested in the presence of peptides identical to spike structures in the discoidin domain of RS1, and by using an RS1 mutant exhibiting an amino acid exchange in spike 3 (R141H). RS1 binding was followed by Western blotting.

**Results:** The extracellular domain of the β2 subunit mediated the specific interaction of the Na/K-ATPase with retinoschisin, whereas the α subunit revealed no effects. Interestingly, binding of Ouabain significantly reduced retinoschisin affinity of the Na/K-ATPase. Blocking spike 3 of the RS1 protein inhibited RS1 binding to retinal membranes. Consequently, the RS1 mutant RS1-R141H failed to bind to retinal membranes.

**Conclusion:** Our data imply that the β2 subunit of the retinal Na/K-ATPase mediates RS1 binding, presumably via an interaction of specific sugar residues on β2 and spike 3 in the discoidin domain of RS1.

# Malondialdehyde-acetaldehyde protein adducts present in AMD lesions regulate the sphingolipid metabolism in Weri Rb-1 and ARPE-19 cells

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**Purpose:** To search for novel biomarkers in age-related macular degeneration (AMD), we have previously undertaken an exploratory study and have observed higher levels of sphingomyelin and ceramides in serum samples of late stage AMD patients compared to age-matched controls. Ceramides and related sphingolipids are involved in processes such as cell proliferation and survival. To better understand the role of sphingolipids in the etiology of AMD, we evaluated the effects of lipid peroxidation products in two human cell lines, ARPE-19 and Weri Rb-1.

**Methods:** Bovine serum albumin (BSA) was incubated with malondialdehyde and acetaldehyde to produce MAA-BSA, a lipid-protein adduct present in AMD lesions. MAA specific modifications were corroborated using a monoclonal antibody. ARPE-19 cells were cultured in 6-well-plates until confluency. Weri Rb-1 cells were plated in 6-well-plates ($1.5*10^6$ cells/mL) coated with poly-L-lysine and grown for 48 h. Cells were serum starved for 24 h and treated with 20 or 80 µg/mL MAA-BSA or BSA for another 24 h. RNA was extracted and quantitative PCR performed with the Universal Probe Library from Roche Diagnostics and Taqman reagents. Sphingolipids were quantified by direct flow injection and liquid chromatography coupled to tandem mass spectrometry. To this end, MAA-BSA treated cells were harvested in 0.1 % SDS, and lipids extracted. Cell survival was evaluated by MTT assay.

**Results:** MAA-BSA induced genes related to the oxidative-stress defense system in both cell lines. Weri Rb-1 cells showed a higher rate of changes in the expression of sphingolipid genes compared to ARPE-19, and a lower survival under 80 µg/mL MAA-BSA treatment. The synthesis of ceramides was induced via the so-called *de novo* pathway, and several genes involved in the catabolism of ceramides were also upregulated. Significant changes were observed for Weri Rb-1 in sphingosine kinases (SPHK), with an increase of SPHK1 and a decrease of SPHK2 gene expression.

**Conclusion:** We observed changes in the metabolism of sphingolipids in human cell lines exposed to MAA-BSA adducts that resemble the alteration of sphingolipid levels in serum of AMD patients. Moreover, we found changes in the expression of enzymes regulating the ceramide/sphingosine-1-phosphate rheostat, which is known to determine cell fate.

# Microglia-specific expression of Translocator Protein (18kDa) (TSPO)

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**Purpose:** Translocator protein (TSPO) is an 18-kDa transmembrane protein located on the outer mitochondrial membrane. Reactive microglia exhibit enhanced TSPO expression, making it an accurate marker for neuroinflammatory conditions. Importantly, specific TSPO ligands such as XBD173 are capable of modulating microglial activation and therefore present as attractive pharmacological tools to alleviate neurodegenerative disorders. However, understanding TSPO regulation in health and disease is paramount prior to its use as a target for such interventions.

**Methods:** A 2.8Kb TSPO promoter sequence (-2733/+79) was amplified by PCR and cloned into the pGL4.10-Basic luciferase reporter vector. Plasmids containing 5' unidirectional deletions of the promoter were then generated by PCR. Luciferase assays were performed 24 hours after transfection of BV-2 microglial cells and for an additional 6 hours under lipopolysaccharide (LPS) stimulated conditions.

**Results:** Deletion of the area between -2733 and -845 did not have a significant effect on promoter activity, with as little as 845 bases upstream of the transcription start site being able to reconstitute near maximal activity in this cell line. In addition, deletion of the areas between -845 and -520bp and between -168 and -39bp showed a significant decrease in activity, indicating that these sequences may contain elements that positively regulate TSPO expression. We further analysed TSPO promoter activity during lipopolysaccharide (LPS) activated conditions. LPS significantly induced TSPO promoter activity, with the stimulatory effect of LPS being mediated by the promoter region spanning -593-520bp upstream of the transcription start site. Sequence analysis of these identified regions revealed the presence of many putative transcription factors including two Ets sites, a half site for androgen/progesterone receptors, one Nkx3.1/SP1/SP3 and one AP1 site.

**Conclusion:** These experiments constitute the first functional analysis of microglia-specific TSPO gene regulation in health and disease states, and may help build a framework for further optimization of TSPO-mediated immunotherapy approaches in neurodegenerative diseases.

# Humoral immune response to subretinal AAV8 in non-human primates

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**Purpose:** Knowledge of the humoral immune response to single subretinal administration of clinical grade recombinant adeno-associated virus (rAAV) in non-human primates is a key factor for the development of safe and efficient clinical trial protocols for retinal gene therapy. This study investigated longitudinal changes of anti-drug-antibody (ADA) titres in non-human primates (NHPs, *Macacca fascicularis*) between before and after single administration of rAAV8-pseudotyped virus.

**Methods:** 18 NHPs received subretinal injections in three cohorts (high dose: $1x10^{12}$ vector genomes [vg], low dose: $1x10^{11}$ vg, or vehicle only) and concomitant immunosuppressive therapy equivalent to a clinical trial scenario. Four additional animals received intravitreal injections to mimic *via falsa* application. Baseline samples were compared to those taken 1, 2 and 3 days and 1, 4 and 13 weeks after application of the vector. A sandwich-ELISA was developed and used to quantify ADAs directed against the rAAV8 capsid.

**Results:** ADA titres in all animals of the high dose group stayed constant over the 13-week observation period. Variability was greater in the low dose and the intravitreal control cohort. Mean titres pre- and 4 weeks post-treatment were 50.82 ± 35.67 (mean ± sd) and 47.70 ± 30.24 in all animals receiving vector. The mean titres of low and high dose group did not vary from the ones of the control group, which had received only saline injections.

**Conclusion:** This study provides data relevant for clinical retinal gene therapy trials, where rAAV8 might be used for subretinal delivery of therapeutic transgenes. When mimicking the clinical scenario with clinical grade vector, surgery and concomitant immunosuppression, no induction of anti-drug-antibodies occurred in non-human primates.

# Microglia modulation in degenerative retinopathy

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**Purpose:** Inflammation and glial activation can change the course of diabetic retinopathy (DR). The polycystic-kidney-disease (PKD) rat shows a retinal phenotype similar to DR, in which CD74 serves as a microglial activation marker. Our aim was to analyze the impact of microglia modulation on degenerative retinopathy.

**Methods:** BV2 cells were stimulated with clodronate-coated-liposomes, uncoated liposomes were used as a control. Cell viability was analyzed by an MTT-assay. PKD-rats were intravitreally injected with clodronate-coated-liposomes at 4 and 8 weeks of age and sacrificed at 12 weeks. Sprague Dawley (SD)-rats served as control. Glial activation was assessed by immunofluorescence of activation markers. BV2 cells were stimulated towards M1 and M2 phenotypes and expression of activation markers was determined.

**Results:** BV2 cells were effectively depleted by clodronate in a dose-dependent manner. At a concentration comparable to the concentration used in the animal experiment, the cell number was reduced by 96.4% ($p < 0.0001$). In the deep capillary layer clodronate-injected rats showed an increase of CD74+ microglia compared to uninjected animals ($238 \pm 70$ vs $69 \pm 19$ cells/mm$^2$; $p < 0.01$) while the total number(Iba1+) was unchanged ($384 \pm 38$ vs $295 \pm 87$cells/mm$^2$; n.s.). In SD rats, neither total ($71 \pm 40$ vs $30 \pm 14$ cells/mm$^2$; n.s.) nor activated ($17 \pm 12$ vs $1 \pm 1$ cells/mm$^2$; n.s.) microglia were elevated upon clodronate injection. In BV2-cells, CD74 expression was increased upon both M1 (2.2-fold vs control; $p < 0.001$) and M2 stimulation (2.6-fold vs control; $p < 0.0001$). Glial activation in clodronate-injected SD and PKD rats was confirmed by GFAP-positive Müller cells.

**Conclusion:** Clodronate activates microglia following intravitreal injection. Since short term application and in vitro experiments show microglial depletion, our data on long-term effects indicate a stimulatory effect. In future studies, the functional outcome of the selective elevation and lack of polarization of CD74+ microglia will be analyzed.

# Retina-wide transduction after intravitreal injection of AAV

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**Purpose:** Subretinal injections of Adeno-associated virus vectors (AAVs) are efficient in transducing many retinal cell types including photoreceptors and retinal pigmented epithelial (RPE) cells. Disadvantages of subretinal AAV injections constitute the (i) limited retinal area that can be transduced and (ii) the retinal detachment, which may result in structural changes. We developed a new intravitreal injection method for AAV administration, which leads to widespread transduction of almost the complete retina.

**Methods:** Single stranded AAV vectors with serotype 8 capsids and eGFP under the control of cytomegalovirus (CMV) promoter (ssAAV2/8-CMV-eGFP) were applied. 2 – 4 months old C57/Bl6 mice received an intravitreal injection either with or without prior vitrectomy. Vitrectomy was performed by removing a small portion of the vitreous with a needle and syringe. AAVs were administrated slowly into the remaining vitreous with a 33 gauge blunt-end needle. Fundus imaging and histological analysis were performed 3 – 4 month after injections.

**Results:** Fundus imaging showed that 50 % of the injected eyes without prior vitrectomy had less than 5 % of the retinal surface area transduced. In contrast, half of the vitrectomized eyes had a GFP expressing retinal area larger than 80 %. Eyes with a coverage exceeding 80 % in the central retina were also largely transduced in the inferior, superior, nasal and temporal retina. Retinal flatmount analysis documented the transduction of different cell types, whereas photoreceptors showed a 4 times higher expression level of GFP compared to other transduced cell types. Overall, GFP expression was observed in all retinal layers and RPE cells. ERG measurements suggested that the novel injection method does not lead to obvious functional defects in the retina.

**Conclusion:** Our newly developed technique for intravitreal injection leads to a wide-spread transduction of the mouse retina and allows AAV particles to reach a brought variety of retinal cells. It is suitable to test AAV-based ocular gene therapies in mouse models.

# Role of the splenic monocytes in Aged-related Macular Degeneration (AMD)

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**Purpose:** Are splenic monocytes and the renin-angiotensin system (RAS) involved in subretinal inflammation induced by a light stress and a Laser photocoagulation induced choroidal neovascularisation (CNV) models?

**Methods:** Light-stress model was performed on CX3CR1GFP/GFP mice. Animals were exposed to light (4500 lux) during 4 days. Illumination induced mononuclear phagocytes (MPs) accumulation in the subretinal space. Animals were treated with intraperitoneal injection of PBS or Losartan (125mg/kg). Infiltrated MPs in the subretinal were immunostained with iba1 and angiotensin II receptor 1 (ATR1) antibodies on choroids and retina flatmounts. Infiltrated MPs were counted on the whole subretinal space according to the expression of iba1 +/- ATR1 markers. CNV was induced by 4 laser impacts per eye on control mice, splenectomized mice or angiotensin II infused C57Bl6 mice. Angiotensin II infusion was performed through subcutaneous implantation of osmotic pumps. Seven days after laser impact, the choroids were immunostained with CD102 and iba1 antibodies. CNV area and MPs cells infiltration around laser impact were evaluated for each laser impact of all animals.

**Results:** Immunostaining of choroid from illuminated mice revealed 3 sub populations of infiltrated monocytes: iba1+, ATR1+ and iba1+/ATR1+ cells. Counting revealed a significant decrease quantity of infiltrated iba1+ MPs in illuminated mice treated with Losartan. Furthermore, the proportion of ATR1+ MPs was significantly lower in Losartan than in PBS treated group. On CNV model, we observed that spleen removal had an inhibitory effect on MPs infiltration and choroidal neovascularisation compared to control animals. In contrast, the systemic elevation of angiotensin II through osmotic pump implantation triggered a significant increase of infiltrated MPs around the laser impact and increased the size of the neovascularized area.

**Conclusion:** We described for a first time a sub population of infiltrated MPs that express the receptor for angiotensin II. Using the two best described animal models that mimic features of AMD, we showed that angiotensin II is involved in subretinal inflammation.

# Transplantation of mouse primary and ES-derived rod photoreceptors into a mouse model of complete photoreceptor degeneration

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Photoreceptor degenerations are amenable to cell replacement therapies and, proof-of-principle studies in pre-clinical mouse models resulted in some functional visual repair at the retinal and behavioral level. However, most of the striking achievements were performed in mouse models wherein photoreceptors slowly degenerate and where grafted cells might "fuse" with host photoreceptors. Such condition does not represent an initial scenario in a clinical setting. Currently, little is known about the therapeutic outcome following photoreceptor transplantation into mouse models of severe photoreceptor degeneration. Hence, we address this by transplanting rod photoreceptors into a double mutant harboring the cone photoreceptor function loss 1 (Cpfl1) and the rhodopsin knockout ($Rho^{-/-}$) mutations, tg(Cpfl1; $Rho^{-/-}$), where virtually all photoreceptors are gone at 12 weeks of age.

Donor rod photoreceptors isolated from postnatal day 4 Nrl-GFP retinae or from mouse embryonic stem cell (mES)-derived retinal organoids were enriched using CD73-based magnetic activated cell sorting (MACS) and transplanted into the subretinal space (SRS) of 12–14 week-okd tg(Cpfl1; $Rho^{-/-}$) mice. Additionally, different amount of primary photoreceptors ($2 \times 10^5$, $5 \times 10^5$ and $7.5 \times 10^5$) were transplanted into the SRS to assess graft volume. Transplanted rod photoreceptor survival, maturation and synaptic connectivity were evaluated three weeks after transplantation.

Graft size increased with higher amounts of donor cells and $7.5 \times 10^5$ cells resulted in a volume equivalent to a macula in a mouse retina (~0.0195 $mm^3$). Independent of the source of rod photoreceptors or the amount transplanted cells, grafted photoreceptors survived in the subretinal space expressed rod-specific markers as well as some synaptic markers, reflecting their maturation. However, some of the synaptic markers analysed were not detected or properly expressed.

In conclusion transplantation of rod photoreceptors from primary and mES-derived photoreceptors can survive and mature in the subretinal space of tg(Cpfl1; $Rho^{-/-}$) but lack correct expression of some synaptic proteins. The absence of correct synaptic machinery might impair correct synapse formation hindering functional repair of retinal circuitry. Grafting of $7.5 \times 10^5$ cells is sufficient to generate enough volume that resembles the equivalent of a macula in a mouse retina and, therefore, it will be used as a new standard for future transplantation studies.

# Cre recombinase expression or topical tamoxifen treatment do not affect retinal structure and function, neuronal vulnerability or glial reactivity in the mouse eye

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**Study objectives:** Mice with a constitutive or tamoxifen-induced Cre recombinase (Cre) expression are frequently used research tools to allow the conditional deletion of target genes via the Cre-loxP system. We analyzed whether retinal Cre expression or topical tamoxifen treatment itself would cause structural or functional changes, including changes in the expression profiles of molecular markers and glial reactivity, and influence neuronal vulnerability by using light damage as a model for photoreceptor degeneration.

**Methods:** Transgenic *α-Cre, Lmop-Cre* and the tamoxifen inducible *CAGGCre-ER$^{TM}$* mouse lines were used. Additionally, we characterized the effects of topical tamoxifen treatment itself in wild-type mice. Retinal morphology was studied on semithin sections and morphometric analyses were performed in spider diagrams. *In vivo* ERG analyzes were used to analyze the retinal function. We performed immunohistochemical staining for IBA-1 to visualize microglial cells and GFAP, a Müller cell and astrocyte marker. Light induced damage was performed to examine the influence of Cre expression or tamoxifen exposure on the vulnerability of photoreceptors. Apoptotic cell death was analyzed by TUNEL labeling followed by quantitative analysis. Real-time RT-PCRs were used to analyze whether the expression of Cre or tamoxifen exposure might interfere with the expression levels of certain neuroprotective or immune modulating factors.

**Results:** Cre recombinase expression or tamoxifen treatment do not change retinal morphology and function in the experimental mice compared to their respective controls. In addition, we observed no changes in the morphological appearance of microglia cells. We observed immunoreactivity for GFAP in the RGC layer only, indicating a resting astrocyte population but no Müller cell reactivity. There were no significant differences in the expression levels of several neuroprotective and immune modulating factors in all Cre mouse strains or tamoxifen treated animals compared to their controls. Furthermore, Cre expression or tamoxifen treatment did not alter neuronal vulnerability.

**Conclusion:** The Cre-loxP system and its induction through tamoxifen is a safe and reliable method to delete desired target genes in the neural retina.

# rAAV8 biodistribution and shedding after subretinal injection in non-human primates

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**Purpose:** The aim of this study was to analyse virus distribution and shedding after a single subretinal administration of clinical grade recombinant adeno-associated virus (rAAV8) in non-human primates. This is important for environmental risk assessment in retinal gene therapy trials.

**Methods:** 18 non-human primates *(Macacca fascicularis)* underwent 23G pars plana vitrectomy and subretinal injection in three cohorts (high dose: $1x10^{12}$ vector genomes [vg], low dose: $1x10^{11}$ vg, or vehicle only). Four additional animals received intravitreal injections to mimic *via falsa* biodistribution. Tissues samples were harvested at necropsy (day 91) from the treated eye, draining lymph nodes, salivary gland and spleen, optic nerve, brain and spinal cord, heart, lung, liver, adrenal glands and gonads. Blood, urine, lacrimal and nasal swabs were harvested from each animal prior to dosing and 1, 2 and 3 days and 1, 4 and 13 weeks after application of the vector for DNA extraction and quantification of vector genomes by qPCR.

**Results:** Dose dependent rAAV8 DNA was found in the treated retina. Persistence of rAAV8 in other tissues was most notable after intravitreal injection (*via falsa* control). Shedding was found in all bio fluids. The highest concentrations were found in lacrimal fluid of the high dose group. DNA was not detected in the germ line tissues and apart from sporadic signals detected in a small number of animals in the liver and lung, all remaining tissues were negative. Concerning the biofluids at day 31 only blood samples showed remaining Virus DNA.

**Conclusion:** These data are relevant for clinical retinal gene therapy trials, where trial subjects, investigators and regulators alike are interested to identify environmental risks associated with application of genetically modified organisms. While shedding into biofluids seems to occur in a dose dependent manner, transduction of off-target organs seems minimal.

# The USH1G scaffold protein SANS is part of the cytoplasmic dynein transport module in the photoreceptor inner segments

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**Purpose:** Human Usher syndrome (USH) is the most common form of combined deaf-blindness and a complex disease. To get insights into the molecular function of the *USH1G* protein SANS (scaffold protein containing ankyrin repeats and SAM domain) we aim to identify protein interactions that disclose functional modules related to USH protein complexes in retinal photoreceptor cells.

**Methods:** We identified proteins and complexes interacting with SANS by yeast-2-hybrid screens (Y2H) in retinal cDNA library, affinity proteomics based on tandem-affinity purification, and co-immunoprecipitation from HEK293T cells and retinal tissue, respectively. We validated interactions by complementary pull down- and co-transfection assays. We showed subcelluar localization of complex partners in retinal photoreceptor cells by light and electron microscopy and applied proximity ligation assays (PLA) to demonstrate complexes *in situ*. We applied shRNAs and siRNAs for protein depletion from cells. We analysed transport modules by a fluorescence recovery after photo bleaching (FRAP)-based method in living photoreceptor cells.

**Results:** We demonstrated SANS in protein complexes together with several components of the cytoplasmic dynein motor module, regulatory small GTPases, and rhodopsin. Knock-downs of the complex components by sh/siRNA led to the decrease of the number of primary cilia in starved cells. Immunocytochemistry and PLAs revealed the localization of SANS related protein complexes along microtubule tracks in the inner segment and the periciliary region at the base of photoreceptor cilia. FRAP experiments demonstrated that the apical transport of rhodopsin to the photoreceptor cilium is microtubule dependent *in vivo*.

**Conclusions:** Our data support that the USH1G protein SANS participates at the microtubule-dependent vesicular transport of ciliary molecules, e.g. rhodopsin mediated by cytoplasmic dynein. Defects in this transport module lead to ciliary dysfunction and photoreceptor cell death, which may underlay the retinal degeneration as characteristic for USH1 patients.

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# Generation of an *atf6* knockout Zebrafish Line using TALEN Technology

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**Purpose:** Achromatopsia (ACHM) is an autosomal recessive retinal disorder characterized by color blindness, photophobia, reduced visual acuity and nystagmus. Recently, mutations in the *ATF6* (activating transcription factor 6A) gene have been identified in ACHM patients. ATF6 is part of the Unfolded Protein Response (UPR) and one of the keyregulators responsible for proper protein-folding and cell survival under ER stress conditions. Since *ATF6* is ubiquitously expressed, it is striking that patients harboring mutations in the *ATF6* gene show solely a loss of cone photoreceptor function without any other irregularities including the rod photoreceptor system. To elucidate the pathomechanism of ACHM and the underlying role of ATF6, an *atf6* knockout zebrafish model is being generated to investigate retinal development, photoreceptor function and ER stress in an Atf6 deficient retina.

**Methods:** Transcription activator-like effector nucleases (TALEN) technology is applied to generate the *atf6* knockout zebrafish line. TALEN are engineered proteins that recognize and bind to a specific DNA motif (target sequence) and induce a double stand break via a coupled nuclease. Three different target sites for the *atf6* gene have been identified using the prediction tool TAL Effector Nucleotide Targeter 2.0. TALEN were cloned via Golden Gate Ligation using the TALE Toolbox (Sanjana et al., 2012). To evaluate TALEN activity, *in vitro* transcribed cRNA of three different TALEN pairs were injected into the egg yolk of zebrafish oocytes in the one cell stage, using concentrations of 50-300 pg/larvae. To assess toxicity, the fraction of larvae developing normal and without any morphology abnormalities was determined. The mutation efficiency of each TALEN pair was quantified by PCR and RFLP.

**Results:** We successfully cloned three TALEN pairs. All three tested TALEN were active. TALEN pair 3 showed to be the most active one, achieving a somatic mutation rate of 49 % with a low toxicity of 30 % using a concentration of 300 pg/larvae.

**Conclusion:** Based on the toxicity and somatic mutation rate evaluation, a TALEN pair has been identified that induces a disruption of *atf6* upstream of all functional domain encoding regions. This specific TALEN pair will finally enable the generation of an *atf6* knockout zebrafish line.

# $Ca^{2+}$ sensitive conformational switch in a mutational hot spot region in GCAP1

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**Purpose:** Efficient photoreceptor cell function relies fundamentally on a well-balanced interplay of two second messengers, cGMP and calcium. Calcium sensors like the guanylate cyclase-activating proteins (e.g. GCAP1, GCAP2) regulate the activity of the guanylate cyclase (GC) in a calcium-dependent manner. The GC-GCAP complex is finely tuned in photoreceptors, in fact even single point mutations in either GC or GCAP disturb cGMP homeostasis and lead to severe retinal degenerations. Especially GCAP1 bears a mutational hot spot region around calcium binding site EF3, in which most disease related mutation are localized. Here we investigate the conformational change of GCAP1 triggering cGMP synthesis with a special focus on the region around EF3. Furthermore, the biophysical and biochemical properties of four GCAP1 mutants located in the hot spot region and correlated with human cone dystrophies were investigated.

**Methods:** The conformational change of GCAP1 wild type (WT) was studied at its endogenous cysteine residues by site specific labelling, time-resolved fluorescence spectroscopy and molecular dynamic (MD) simulations. Calcium-binding parameters of GCAP1 WT and mutants were investigated by isothermal titration calorimetry and catalytic parameters were determined by enzymatic assays using the target guanylate cyclase.

**Results:** Fluorescence lifetime and rotational anisotropy measurements reveal a distinct structural rearrangement of GCAP1 in the mutational hot spot region around EF3 upon release of $Ca^{2+}$. We developed a model of a twisted accordion-like movement for GCAP1 upon changing $Ca^{2+}$ concentration in agreement with MD simulations. Calcium-binding studies revealed three functional EF-hand calcium-binding sites in all disease related mutants, but two EF-hands showed a several-fold lower affinity in the mutants than in WT GCAP1. The EF-hand with the highest affinity remained nearly unchanged. All mutations decrease the catalytic efficiency in regulating the target GC.

**Conclusion:** The fluorescence studies indicate that GCAP1 shows a high flexibility in a region around position 106 and a $Ca^{2+}$ sensitive twisted accordion-like movement. Although its cellular cognate GCAP2 has a high structural homology, GCAP1 operates by distinct different switching mechanisms, vital for a proper GC regulation. All studied mutations in the hot spot region of GCAP1 lead to a disturbed regulation of GC. We conclude that the accordion like movement in GCAP1 is a $Ca^{2+}$ sensitive trigger for the regulation of GC and any impairment in this mutational hot spot region can alter cGMP homeostasis in photoreceptors.

# Coding and non-coding copy number variations explaining unsolved retinal dystrophies – A contribution to the elucidation of hidden genetic variation

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**Purpose:** It was our aim to gain insight into susceptibility factors for the formation of copy number variations (CNVs) affecting retinal dystrophy (RD) genes, and to identify and assess the underlying mechanism of coding and non-coding CNVs in RDs.

**Methods:** Genomic architectural features contributing to CNV formation were investigated in regions containing known RD genes listed in RetNet (https://sph.uth.edu/retnet/). Detection of CNVs in a diagnostic cohort of RD patients without a molecular genetic diagnosis after sequence-based mutation screening was performed by SNP arrays, multiplex ligation-dependent probe amplification (MLPA) or qPCR. For breakpoint mapping we used conventional PCR, qPCR, targeted arrays, long- range PCR and sequencing of junctions. Targeted Locus Amplification (TLA) on extracted DNA was used to characterize CNVs at the nucleotide level.

**Results:** Genomic architectural features like gene size, intron length, repetitive elements, sequence motifs, non-B DNA conformations were assessed for all RetNet genes. A hypothetical ranking of RD genomic regions prone to CNV formation was proposed. This was first tested by extensive mining of reported CNVs in known RD genes. Secondly, 18 distinct newly identified CNVs including 15 deletions and three duplications in nine different RD genes *(BEST1, EYS, KCNV2, MERTK, OPA1, PCDH15, PRPH2, SPATA7* and *USH2A)* were further studied here. Eleven of these are novel, including two deletions in *PRPH2* in which no CNVs have been reported previously. Three of these (two in *EYS*, one in *PCDH15*) affect non-coding, putative regulatory regions of their target gene. Fine-mapping of the breakpoints was performed for all CNVs. TLA, a recent strategy based on the crosslinking of physically proximal sequences, was used to map six CNVs at the nucleotide level, for the first time on extracted human DNA instead of living cells. Finally, bio-informatic analyses contributed to the underlying genetic mechanisms of all delineated CNVs studied here.

**Conclusion:** This study proposed a ranking of CNV-prone RD disease genes, which was validated by investigating genomic data of reported and newly identified RD-associated CNVs, of which 11 are novel. In addition, we demonstrated the efficacy of TLA on extracted genomic DNA to characterize CNVs in a hypothesis neutral manner.

# Macular Dystrophy related mutation in the calcium sensor GCAP1

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**Purpose:** Age-related macular degeneration (AMD) is one of the most frequent eye diseases in this time. This disease causes central vision loss along with decreased vision acuity and is facilitated by smoking or solar radiation. Macular dystrophy (MD) shows similar symptoms but is linked to genetic mutations. A novel point mutation in the GUCA1A gene found in MD patients causing a L176F amino acid exchange in the encoded guanylate-cyclase-activating-protein 1 (GCAP1). The aim of this present work was to investigate the cellular and biochemical consequences of the L176F mutation in GCAP1 to deeper understand the resulting MD.

**Methods:** Site-directed mutagenesis was performed to create the L176F CGAP1 mutant using standard cloning techniques. For functional studies the protein was expressed as myristoylated and non-myristoylated form in *E. coli* and purified. Its operational performance as a calcium-sensitive regulator was compared with the wildtype GCAP1 (WT).

**Results:** The mutation is inherited within three unrelated families by an autosomal mode of inheritance. Patients with this mutation showed paracentral and central soctomas in the 30° visual field and a Best Corrected Distance Visual Acuity (BCVA) up to 0.1. Biochemical analysis showed an almost similar apparent affinity of the mutant for the target Guanylate Cyclase (GC), but the $Ca^{2+}$ sensitive regulation of GC was severely affected by the myristoylated L176F. This would cause a high shift in the $Ca^{2+}$ dependent cGMP production of the GC. The Tryptophan-fluorescence spectroscopy indicated that the myristoylated form of the protein shows a difference in the conformational change during an increase of $Ca^{2+}$ concentration.

**Conclusion:** The point-mutation in this region seemed to influence the conformational change of the myristoylated L176F. It shows a different $Ca^{2+}$ affinity which leads to an imbalance of cGMP concentration and calcium homeostasis in the photoreceptor cells. These findings are different from previous results obtained with GCAP1 or guanylate cyclase mutations that correlate with Cone-Dystrophy, Leber's Congenital Amaurosis and RP. Therefore, the less severe dysfunction in GCAP1 operation would be consistent with a delayed onset of macular dystrophy as observed in the clinical studies.

# Efficient generation of 3D retina and cone photoreceptor enriched organoids from pluripotent stem cells

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Recently, protocols for the differentiation of 3D retinal organoids from embryonic stem cells (ESCs) have been developed, which either recapitulate complete eyecup morphogenesis or maximize photoreceptor genesis. Here, we optimized the generation of large 3D stratified retina organoids from mouse ESCs, so that it does not require optic-vesicle or eyecup formation.
Similar to previous reports we observed efficient eyefield induction (82 ± 12 SD % of aggregates, N = 7), but a less efficient optic vesicle evagination limits the yield of retina organoids (35 ± 10 SD % of aggregates form Rax+ evaginations). We demonstrate that aggregate trisection at the eyefield neuroepithelium stage circumvents this limitation and facilitates an efficient and synchronized development of organoids with big, stratified retinal tissue, reminiscent of early postnatal retina in vivo (87 ± 3 SD % of aggregates, N = 4). A given starting aggregate results on average in 1.8 ± 0.6 organoids (N = 12) each with 1.4 ± 0.4 SD mm retinal circumferential length. Temporal gene expression comparative analysis of individual organoids (5 timepoints, 60 organoids, 22 genes) and cell birthdating experiments indicate efficient, reproducible, and temporally regulated retinogenesis in this organoid system.
Further, inhibition of Notch signaling by DAPT treatment at early or late stages of the differentiation protocol enables the generation of organoids enriched with cone or rod photoreceptors, respectively, demonstrating the power of our improved organoid system for future research work in stem cell biology and regenerative medicine.

# *In vivo* imaging of retina regeneration with optical coherence tomography in adult zebrafish

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**Purpose:** Studying regenerative mechanisms in the adult zebrafish is a promising strategy to find new therapeutic targets for retinal diseases. However, working on adult fish requires *in vivo* imaging techniques to follow regeneration in living animals. Optical coherence tomography is a powerful approach to repeatedly and non-invasively image individual fish during retina degeneration and regeneration.

**Methods:** A custom-built spectral-domain-optical coherence tomography (SD-OCT) system was adapted to match the specific zebrafish eye optics. By implementing an adjustable optical telescope, we were able to obtain high resolution images from the zebrafish retina with a field of view of 30° x 30°. Furthermore, a hybrid system that allows simultaneous high-resolution SD-OCT in the near-infrared and single-photon excited fluorescence (SPEF) detection in the green and red visible wavelength range can be used for *in vivo* detection of fluorescently labelled retinal cells. In the fish system, retinal degeneration can be specifically induced (e.g. via extensive light or injection of neurotoxins into the eye) and the degenerative and regenerative processes can be monitored via OCT.

**Results:** We combined live imaging by OCT with subsequent traditional histology in order to correlate the information in OCT images with morphological features in the retina. As an example, the GFP labelled photoreceptor mosaic of UV cones can be detected with our hybrid fluorescence system in both the fluorescence and OCT image. Moreover, the degeneration and regeneration in zebrafish retina after light damage or toxin injection shows a variety of phenotypes in both the *in vivo* image and the end point histological section. Following the morphological progression of damage in individual fish results not only in valuable information on anatomical correlates but will also be useful for follow-up experiments on functional analysis of the regenerated retina.

**Conclusion:** Using our custom-built SD-OCT system we obtain high resolution *in vivo* images of degenerative and regenerative processes in the adult zebrafish retina. As the technique is non-invasive we can repeatedly analyze individual fish and achieve detailed insight into the diversity of morphological processes and continue with experiments on functional analysis.

# Translational read-through for targeting nonsense mutations causing ciliopathies

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**Purpose:** Inherited retinal ciliopathies, such as Usher syndrome (USH) and Bardet-Biedl syndrome (BBS) are a genetically heterogeneous group of diseases with no effective cure available so far. USH is the most common form of inherited combined deaf- and blindness. Cochlea implants can compensate the hearing deficiency, whereas for the ocular phenotype no therapy exists so far. BBS patients show retinal degeneration, malformation of organs, metabolic disorders and mental retardation. Approximately 12% of all ciliopathy cases are caused by nonsense- mutations. In recent years, translational read-through has emerged as a therapy targeting nonsense mutations. Translational read-through inducing drugs (TRIDs) can over-read these mutations restoring full-length proteins. We compared the efficacy of different TRIDs on nonsense-mutations found in *USH1C*, *USH2A* and *BBS1* patients and evaluate the retinal biocompatibility of these promising therapeutics.

**Methods:** To evaluate read-through efficacy, cDNAs carrying disease-causing nonsense mutations were cloned into the bidirectional vector pBI-HA. Read-through efficacy of mutations in *USH1C, USH2A* and *BBS1* were tested in transfected HEK293T cells. We applied TRIDs, such as Ataluren, Amlexanox and NB-designer aminoglycosides. We analysed read-through efficacy by quantitative immunofluorescence microscopy and Western blot. The functionality of the recovered protein was tested via GST-pulldowns, co-localisation, actin-bundling assays and membrane targeting assay, respectively. The biocompatibility of TRIDs was assessed on retinal explants with TUNEL-assay.

**Results:** We observed recovery of protein expression after TRID application for all ciliopathy-related nonsense mutations. We validated the functionality of the recovered proteins in interaction assays using known binding proteins. In particular, we show restored scaffolding function of isoforms of the USH1C protein harmonin by interaction with USH2a and actin-filament bundling. Restoration of BBS1 protein binding to BBS9 was found in co- immunoprecipitations and membrane-targeting assays. TUNEL assays reveal good retinal biocompatibility of all analysed TRIDs.

**Conclusion:** The translational read-though efficacy combined with good retinal biocompatibility reveal the high potential of TRIDs. The restored protein expression might stop or slow down the progression of vision loss in patients suffering from USH and BBS caused by nonsense mutations. Furthermore, other degenerative disorders caused by nonsense mutations could benefit from this gene-based therapy strategy.

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# Control of the nucleotide cycle in photoreceptor cells by the RD3 protein

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**Purpose:** The RD3 (retinal degeneration 3) protein is a conserved protein, expressed in rod and cone photoreceptor cells. A mutation in the *rd3* gene leads to an unstable, non-functional C-terminal truncated protein. This mutation is linked to an early onset of photoreceptor degeneration in patients with Leber's congenital amaurosis type 12 (LCA12). So far two different functions for RD3 are proposed, making the protein suitable to operate as an essential part in functional phototransduction. RD3 is capable of inhibiting the guanylate cyclase (GC), a key enzyme in the phototransduction cascade. Further RD3 is indispensable for the GC transport from the inner to the outer segments in rods. The aim of this project is to get a deeper understanding of the molecular mechanisms leading to retinal degeneration involving the RD3 protein and its interaction partners.

**Methods:** Enzyme assays were used to analyze the regulatory function of the RD3 protein. Here key enzymes of the phototransduction cascade (guanylate cyclase (GC), phosphodiesterase (PDE6) and guanylate kinase (GUK1)) were tested. The guanylate cyclase converts GTP to cGMP, while PDE6 degrades cGMP to 5'-GMP. Guanylate kinase in turn recycles 5'-GMP back to GDP. Via HPLC measurements the different nucleotide levels upon RD3 interaction with enzymes from ROS (Rod Outer Segment) preparations were analyzed and the catalytic activity was determined. A spectrophotometric assay was used to define the effect of RD3 on the GUK1 activity. The interaction will be confirmed via ELISA or SPR (Surface Plasmon Resonance) measurements.

**Results:** It was already shown that RD3 inhibits the GC activity, which could be confirmed during our experiments. In an coupled enzyme assay RD3 reveals in addition to its inhibitory function an activating effect on the GUK1 enzyme, dramatically increasing its activity. A doubling in catalytic activity induced by RD3 could be recorded with protein from ROS preparations. Repetitions of the experiment with a commercial available guanylate kinase showed an increase in the catalytic rate up to a factor of ten.

**Conclusion:** Here we show that RD3 interacts at different points with the nucleotide cycle in photoreceptor cells. Next to its inhibitory function the new described effect of RD3 on GUK1 has to be confirmed and its role in phototransduction and retinal degeneration upon RD3 loss will be discussed.

# Expression and function of Myocyte Enhancer Factor 2c (Mef2c) in the retina

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**Background:** Photoreceptor-specific genes are regulated by a hierarchical network of transcription factors, including the master regulators Cone-rod homeobox (Crx) and Neural retina leucine zipper (Nrl). Recent findings indicate that members of the Myocyte enhancer factor 2 (Mef2) family, especially Mef2d, play an important role in regulating photoreceptor development in concert with Crx. Also, several studies pointed out that Mef2c could play a potential role in the retina, especially in photoreceptor function and disease. It was shown that Mef2c levels are decreased in several mouse models of retinal degeneration. Mef2c was originally described as a muscle-enriched transcription factor but only little is known about its putative function in the retina. Therefore, we studied the expression and function of Mef2c in the retina.

**Methods:** The retina-specific temporal expression of Mef2c in the developing retina was assessed by qRT-PCR. Using immunohistochemical stainings, localization of Mef2c protein was determined in the murine and human retina. A cell culture model for transient knockdown of Mef2c using shRNA plasmids was used to investigate Mef2c-dependent expression of photoreceptor-specific genes. To characterize and overexpress the different Mef2c mRNA isoforms in the retina, murine Mef2c was PCR-amplified from cDNA from 2 month old C57BL/6 mice, cloned and sequenced. Mef2c chromatin immunoprecipitation coupled with massively parallel sequencing (ChIP-seq) was used to identify genomic targets of Mef2c.

**Results:** Mef2c expression steadily increases during postnatal development with highest expression in adult animals. Immunohistochemistry showed consistent Mef2c expression in the outer nuclear layer in both mouse and human retina and nuclear-specific localization in cultured mammalian cells. Knockdown of Mef2c in 661W cells resulted in decreased mRNA levels of Cone arrestin (Arr3) and M-opsin (Opn1mw). Both Mef2c isoforms present in the retina contain the mutually exclusive exon 7α1 as well the β domain and differ only in the presence of the inhibitory γ domain. Mef2c-ChIP showed enrichment of putative target genes Nrl and histone deacetylase 9 (Hdac9).

**Conclusions:** Here we showed that Mef2c is strongly expressed in the adult retina, which indicates a potential role in photoreceptor homeostasis. Future ChIP-seq studies and Mef2c knockdown and overexpression approaches could help to elucidate the function of Mef2c in the adult retina.

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