

# Travel Award Abstracts

## Indian hedgehog (Ihh) secreted by adult choroid endothelial cells` regulates choroidal homeostasis and immune response

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**Purpose:** Patients with atrophic AMD display photoreceptor loss secondary to RPE dysfunction and choroidal dropout. However, the exact etiology of AMD remains unknown due to lack of information on RPE/choroid cell diversity and intercellular crosstalk mechanisms. Here we report the selective high expression of Indian Hedgehog (Ihh) in adult choroid endothelial cells (ECs) and its participation in a homeostatic and immunomodulatory choroid signaling circuit likely relevant to AMD.

**Methods:** Cells from tissue of wildtype RPE/choroid were single-cell sorted and scRNAseq was performed. Bulk RNAseq was used to compare the transcriptomes of purified choroid, retinal, liver, and heart endothelial cells. To study crosstalk between RPE/choroid, adult tamoxifen-induced knockout mice were generated by crossing *Ihh*<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup> with *Cdh5-Ert2Cre*<sup>+</sup> transgenic mice, and cDNA of *eGFP* was inserted into exon 1 of mouse *Gli1* gene to generate *Gli1*<sup>+/eGFP</sup> knock-in animals. Photoreceptor function was measured by ERG and OKT, and morphology of RPE/choroid and photoreceptors was evaluated by immunohistochemistry. In-vitro assays, including flow-cytometry and RT-qPCR, were used to examine crosstalk between EC-derived Ihh and stromal, *Gli1*<sup>+</sup> perivascular cells.

**Results:** We report the first single cell RNAseq analysis of adult mouse RPE/choroid tissue, identifying 13 main cell types including 3 subtypes of ECs. By combining these results with a transcriptomic analysis of tissue-specific ECs, we found a marked enrichment of Indian Hedgehog expression in choroid ECs, particularly in those located in close apposition to the RPE. Using reporter mice, we identified the target of choroidal Hedgehog (Hh) signaling as a large population of stromal, *Gli1*<sup>+</sup> perivascular mesenchymal stem cell (MSC)-like cells. EC-specific *Ihh* KO and *Gli1*<sup>eGFP/eGFP</sup> mice displayed loss of mast cells, altered inflammatory response, visual impairment and retinal degeneration indicating a novel *Ihh*/*Gli1*-dependent signaling circuit in adult choroid regulating choroidal homeostasis, immune response and retinal function.

**Conclusion:** Our study uncovers the cellular and molecular landscape of the adult mouse choroid and a Hh-mediated homeostatic and immunomodulatory choroid signaling circuit, thus providing possible new therapeutic targets for atrophic AMD and choroidal disorders.

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## A Novel Rat Model for Mast Cell Involvement in Geographic Atrophy and an Ex Vivo Assay for Evaluating Drug Efficacy to Quiesce Mast Cells

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**Purpose:** Blindness from geographic atrophy (GA) remains unaddressed because we lack a distinct animal model. To date, there is no proven drug treatment for GA. Degranulation of resident mast cells (MCs) are considered as the trigger of ocular inflammation and subsequent pathological events in choroid. In patients with GA, the number of degranulated MCs was significantly elevated (Bhutto, BJO, 2016), and choroidal macrophages were increased in sphericity and decreased in volume, signals of activation (McLeod, IOVS, 2016). The purpose of this study was to develop a rat model to demonstrate GA-like changes by continuously stimulating MCs, and a short acute *ex vivo* assay to promptly evaluate drugs to stabilize choroidal MCs.

**Methods:** A MC stimulator, compound 48/80, in a hydrogel slow release pellet was implanted subconjunctivally in the rat. Degranulation of MCs and retinal pigment epithelium (RPE) loss as well as retino/choroidal thickness was evaluated after implantation. Electroretinogram (ERG) was also performed to evaluate residual retinal function. For *ex vivo* assays, two commercially available MC stabilizers, ketotifen fumarate or cromolyn sodium were administered simultaneously with 48/80 to the rat isolated choroid as a preliminary study to evaluate the potential of preventing MC degranulation and subsequent macrophage activation.

**Results:** MC degranulation was apparent at 3 days after 48/80 implantation. By 6 weeks, significant loss of RPE was detected. Additionally, reduction in retino/choroidal thickness and ERG amplitude were significant by 8 weeks. *Ex vivo*, 48/80 stimulation led to significant choroidal MC degranulation. MC degranulation was inhibited by treatment with both ketotifen and cromolyn. Macrophage volume was decreased with 48/80 and restored with either drug. Also, macrophage sphericity was increased with 48/80 and decreased with both treatments.

**Conclusions:** Inducing choroidal MC degranulation in a slow release fashion was sufficient to recapitulate two features of GA: RPE loss and retino/choroidal thinning, within 8 weeks in our rat model. From *ex vivo* assays, MC quiescence by drugs prevented macrophage activation (increased sphericity and decreased volume) indicating cytokine release from MC was prevented. Administration these MC stabilizers in our rat model might prevent GA-like changes from occurring and hence might have the potential for treating GA.

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## Macrophage microRNA-150 promotes pathological angiogenesis as seen in age-related macular degeneration

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**Purpose:** Age-related macular degeneration (AMD) is a leading cause of blindness in older adults. We and others have shown that aged macrophages exhibit functional changes in their inflammatory status and their ability to maintain cholesterol homeostasis and thereby promote AMD. However, the role of microRNAs (miRs), which modulate immune processes, in regulating these AMD-promoting functional shifts in aged macrophages is underexplored. The goal of this study was to identify the miRs that coordinate the transcriptomic changes in aged macrophages that contribute to AMD pathogenesis and cause blindness.

**Methods:** To identify miRs that skew macrophages towards the AMD-promoting phenotype, we compared macrophages from 6-8-week-old mice and 18-month-old wild-type mice by miR microarray. We then examined the phenotype associated with miR-150 overexpression by RNA-Sequencing and pathway analysis for enriched gene ontology (GO) processes, process networks, and pathway maps. To validate our findings, we quantified miR-150 levels in the peripheral blood mononuclear cells (PBMCs) of human AMD patients (N=43) and control subjects without AMD (N=63). We considered  $P < 0.05$  to be statistically significant.

**Result:** MiR-150 had the highest fold change in aged macrophages with ~9-fold higher expression in old peritoneal macrophages ( $P < 0.0001$ ), ~3-fold higher expression in old splenic macrophages ( $P < 0.01$ ), and ~3-fold higher expression in old bone marrow-derived macrophages ( $P < 0.05$ ). Macrophages overexpressing miR-150 exhibited an altered transcriptome enriched for the pathway map of aberrant lipid trafficking and metabolism in AMD pathogenesis ( $P = 4.4 \times 10^{-5}$ ;  $FDR = 5.5 \times 10^{-3}$ ). AMD patients had significantly higher PBMC miR-150 levels compared to control subjects ( $P < 0.0001$ ). A gender- and age-adjusted binary logistic regression model confirmed that a ten-fold increase in PBMC miR-150 levels was associated with a 29.0-fold increased odds of having AMD (95% CI: 5.9-141.5). Mechanistically, miR-150 directly regulates stearyl-CoA desaturase-2 expression, which subsequently coordinates macrophage-mediated inflammation and pathologic angiogenesis, as seen in AMD, independently from vascular endothelial growth factor (VEGF).

**Conclusion:** We show that miR-150 is upregulated in AMD-promoting macrophages and implicate miR-150 as pathogenic in AMD, providing novel molecular insights into diseases of aging. Our results identify not only potential therapeutic targets for modulating the transition to the aged macrophage phenotype but also a potential biomarker for monitoring AMD progression.

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## Endothelial colony forming cell modulation of the choroidal vasculature.

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**Purpose:** Progressive atrophy of the choriocapillaris is a feature of age-related macular degeneration (AMD). Enhancing repair of the choriocapillaris could improve declining oxygenation of the outer retina and slow or reverse disease progression. Endothelial colony forming cells (ECFCs) are well-characterised progenitors with proven vasoreparative potential in the ischaemic retina. ECFC interaction with choroidal vasculature is unknown, thus this study aims to investigate the potential of ECFCs to modulate the choroidal vasculature.

**Methods:** Choroidal explants were established from C57BL/6J mice sacrificed at different ages (postnatal (P) days 8,10,11,13) and grown and imaged for 5 days (N=16). ECFCs were isolated from cord blood and characterised morphologically and by flow cytometry of surface antigens, such as CD34. Choroidal explants from P8 mice (N=8) in the presence/absence of ECFCs were established. Since ECFCs, *in vivo*, need to function in hypoxic conditions, a novel hypoxia-responsive miRNA termed miR-X was identified via microRNA array analysis of ECFCs. miR-X over-expressing ECFCs were incorporated into the choroidal explant model to assess its potential in improving the pro-angiogenic effect elicited by the cells. Human antigen-specific CD31 antibody differentiated between the human ECFCs and the murine choroidal sprouts by immunocytochemistry.

**Results:** Choroid explants from P8 mice sprouted faster and had a larger mean sprouting distance on day 5 than mice at P11 and P13 ( $p<0.001$ ). Furthermore, P8 explants grown in co-culture with ECFCs had significantly increased sprouting distance vs. control explants ( $p<0.0001$ ). ECFC-conditioned media did not evoke this response in choroidal explants. ECFC mediated pro-angiogenic effect was further enhanced when cells overexpressed miR-X, vs. no cell, non-transfected cells, and control-mimic cell controls ( $p<0.0001$ ). ECFCs integrated with the choroidal vasculature, with ECFC containing vascular sprouts exhibiting increased branching and filopodia.

**Conclusions:** P8 explants sprout faster and of greater length than explants from older mice. ECFCs enhanced vascular network formation in choroidal explants, whilst miR-X overexpression, further enhanced the angiogenic response, thus demonstrating that these cells have potential to enhance repair of the atrophic choroidal vasculature. Future research effort is exploring their vasoreparative properties *in vivo*.

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## Outer Plexiform Layer Remodeling in Aging and Age-related Macular Degeneration in SD-OCT.

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**Purpose:** To assess changes in outer plexiform layer (OPL) of the retina in aging and age-related macular degeneration (AMD) using spectral-domain optical coherence tomography (SD-OCT).

**Methods:** Comparative study of 76 eyes with non-advanced dry AMD, 14 drusen non-AMD, 12 geographic atrophy (GA), 22 old and 25 young normal controls. OPL, outer nuclear layer (ONL) and inner nuclear layer (INL) thicknesses were assessed in 12 quadrants of three annuli (inner 400-1000  $\mu\text{m}$ , middle 1000-2000  $\mu\text{m}$  and outer 2000-3000  $\mu\text{m}$ ) of central 3 mm perifoveal region. Generalized linear models were performed.

**Results:** Significant thinning of the OPL was noted in the central 3 mm area in aging ( $35.7 \pm 1.7 \mu\text{m}$  in old vs  $44.8 \pm 1.6 \mu\text{m}$  in young ( $p < 0.001$ )) specially in the temporal and superior quadrants of the outer and middle annulus. Further thinning of the OPL and extension of the thinning into the inner annulus as small drusen developed. In the early stages of AMD, the OPL thinning were confined to the nasal and temporal quadrants of the inner annulus with the mean thickness of  $28.5 \pm 1 \mu\text{m}$  in AMD vs  $35.2 \pm 1.9 \mu\text{m}$  in controls in the temporal quadrant of the inner annulus ( $p$  value  $< 0.006$ ). OPL thinning was also present in the nasal quadrant of the inner annulus in the non-involved eye of patients with unilateral AMD. ONL thickness was significantly decreased in the superior quadrant of the inner and middle annulus in intermediate vs early AMD ( $74.9 \pm 2.8 \mu\text{m}$  in intermediate vs  $87.5 \pm 3.8 \mu\text{m}$  in early AMD in the superior quadrant of middle annulus ( $p = 0.003$ )). INL did not show any significant change.

**Conclusion:** Changes in the OPL thickness happen in aging and may precede drusen in AMD. These findings suggest that pathologic neurosensory retina changes in AMD are more prominent than that seen by clinical examination.

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**Genome-wide association study reveals variants in *CFH* and *CFHR4* associated with systemic complement activation: implications in age-related macular degeneration.**

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**Purpose:** To identify genetic variants associated with complement activation, which could help to select age-related macular degeneration (AMD) patients for complement inhibiting therapies.

**Methods:** A genome-wide association study (GWAS) on serum C3d/C3 levels as a measure of systemic complement activation followed by replication and meta-analysis was performed in 2,245 AMD patients and controls. The GWAS was performed in 1,548 AMD patients and controls. After genotype imputation and quality control, 9,972,920 variants were included in the analysis. For replication and meta-analysis, 697 additional individuals were genotyped for the lead SNPs in the associated signals. A model for complement activation including the identified genetic and non-genetic factors was built, and the variance explained was estimated. Haplotype analysis was performed for eight SNPs across the *CFH/CFHR* locus. Association with AMD was performed for the variants and haplotypes found to influence complement activation.

**Results:** Associations with complement activation were identified at the *CFH/CFHR* locus. Complement activation was independently associated with rs3753396 located in the *CFH* gene ( $P_{\text{discovery}}=1.09 \times 10^{-15}$ ,  $P_{\text{meta}}=3.66 \times 10^{-21}$ ,  $\beta=0.141$ ,  $SE=0.015$ ) and rs6685931 located in the *CFHR4* gene ( $P_{\text{discovery}}=8.18 \times 10^{-7}$ ,  $P_{\text{meta}}=6.32 \times 10^{-8}$ ,  $\beta=0.054$ ,  $SE=0.010$ ). A model including age, AMD disease status, body mass index, triglycerides, rs3753396, rs6685931, and previously identified SNPs, explained 18.7% of the variability in complement activation. Haplotype analysis revealed three haplotypes (H1-2 and H6 containing rs6685931, and H3 containing rs3753396) associated with complement activation. Haplotypes H3 and H6 conferred stronger effects on complement activation compared to the single variants ( $P=2.53 \times 10^{-14}$ ,  $\beta=0.183$ ,  $SE=0.024$  and  $P=4.28 \times 10^{-4}$ ,  $\beta=0.144$ ,  $SE=0.041$  respectively). Association analyses with AMD revealed that SNP rs6685931 and haplotype H1-2 containing rs6685931 associated with a risk for AMD development, while SNP rs3753396 and haplotypes H3 and H6 were not associated with AMD.

**Conclusions:** SNP rs3753396 in *CFH* and SNP rs6685931 in *CFHR4* are associated with systemic complement activation levels. The stronger effects of haplotypes H3 and H6 on complement activation suggest that other variants at the *CFH/CFHR* locus also influence this trait. SNP rs6685931 in *CFHR4*, and its linked haplotype H1-2, conferred also a risk for AMD development, and therefore could be used to identify AMD patients that would benefit most from complement inhibiting therapies.

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