Alterations in Circulating Leukocyte Populations in Diabetic Retinopathy

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Purpose: Diabetic retinopathy (DR) is the highest cause of blindness in the global working population. Inflammation is highly implicated in DR pathogenesis although the underlying mechanism remains poorly-defined. The aim of this study was to investigate the immunophenotype of circulating leukocytes in DR patients.

Methods: Peripheral whole blood was collected from 13 healthy controls (age-, gender-matched) and 39 type 1 DR patients, including 13 mild non-proliferative DR (NPDR), 14 non-active PDR (nAPDR) and 12 active PDR (aPDR). Immunophenotype was examined by flow cytometry. The immune markers investigated in this study include lymphocytes (CD4 and CD8 T cells, CD19 B cells), monocytes (CD14+CD16+, CD14+CD16−, and CD14+CD16+), neutrophils (CD16+HLA-DR+) and NK cells (CD56); chemokine receptor expression (CCR2 and CCR5); cell adhesion molecules (CD66a and CD157); inhibitory molecule (CD305); angiogenesis marker (TIE2) and cell activation markers (HLA-DR, CD62L).

Results: The percentages of lymphocytes (CD4+, CD8+, CD19+), non-classical monocytes (CD14+CD16+), and HLA-DR+ cells were significantly reduced, and the percentage of neutrophils (CD16+HLA-DR+) was significantly increased in DR compared to those in healthy controls. CD4+ cells from DR patients also expressed significantly lower levels of CD66a and CD157. The neutrophil-lymphocyte ratio (NLR), in particular, the CD16+HLA-DR+/CD4+ and CD16+HLA-DR+/CD19+ ratio was significantly higher in DR compared to that in healthy controls. The reduction in CD4 and CD8 T cells and the increment in CD16+HLA-DR+ neutrophils appear to be associated with the severity of DR, i.e., the orders of CD4% and CD8% T cells were aPDR<NPDR<Control, and the percentage of CD16+HLA-DR+ neutrophils was aPDR>NPDR<Control. There were no significant differences between the percentages of classical (CD14+CD16+) and intermediate (CD14−CD16+) monocytes as well as populations positive for CD56, CCR2, CCR5, CD66a, CD305, TIE2 and CD62L between DR and controls.

Conclusions: Our results suggest that DR in type-1 diabetes may be related to increased innate immunity and reduced adaptive immunity and the neutrophil/CD4 ratio may be a useful marker to predict the severity or progression of DR. Further understanding the functional alterations of the immune cells may uncover the immune mechanisms of DR and identify potential targets for therapy.

Commercial Relationships: Gideon Obasannu, None; Nuala-Jane Lavery, None; Jose R. Hombrebueno, None; Aisling Lynch, None; Mei Chen, None; David Armstrong, None; Noemi Lois, None; Heping Xu, None

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Our hypothesis predicts that blood vessels within the retina were also significantly increased compared to controls, who had fewer Ly6Chi monocytes at that time.

**Conclusions:** We show that splenic monocytes are recruited in the retina after ischemic injury and that the content of splenic myeloid cells correlates with the level of infiltrating cells. These results put the spleen on the spotlight for targeting diabetic complications.

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**Presentation Time:** 8:30 AM–10:15 AM

**Association between Vascular Density and Loss of Protective RAS during Early NPDR by Fractal Dimension**

Krishnan Radhakrishnan, Ruchi Vyas, Matthew Murray, Douglas Bryant, Yaqian Duan, Maria B. Grant, K.V Chalam, Patricia A. Parsons-Wingerter.

**Purpose:** Our hypothesis predicts that blood vessels within the retina increase in density during early-stage nonproliferative diabetic retinopathy (NPDR), based on previous results of a small retrospective study (IOVS 51(1):498). For our new prospective study, the fractal dimension (Df) is being used to assess the remodeling of arteries and veins during progression of early NPDR. In complex structures such as branching vascular trees, Df is a sensitive measure of space-filling capacity. The renin-angiotensin system (RAS) is implicated in DR pathogenesis and the function of circulating angiogenic cells (CACs), a critical bone marrow-derived population instrumental in vascular repair.

**Methods:** Arterial and venous branching patterns were extracted from images of 6 normal controls and 3 early NPDR subjects (2 moderate, 1 mild) acquired by Heidelberg Spectralis® OCT following fluorescein angiography (FA). The vascular branching patterns were analyzed by NASA’s VESsel GENeration Analysis (VESGEN) software, in which skeletonized representations were generated automatically to yield Df by the box-counting method. For binary 2D images, Df varies between limiting Euclidean dimensions of 1 and 2. Peripheral blood of diabetics and controls was collected for CD34+ CAC isolation. The gene expression of RAS in CACs was assessed by qPCR for Mas receptor to Ang-(1-7). The vasoreparative function of the CACs was measured by migration ability toward CXCL12 (SDF-1).

**Results:** By Df, venous and arterial densities were 1.370 ± 0.006 and 1.329 ± 0.016 for early NPDR, compared to 1.318 ± 0.012 and 1.320 ± 0.036 for control. The space filling capacity in early NPDR measured by Df, a sensitive parameter, therefore demonstrated a pronounced increase for veins, but not for arteries. Mas receptor mRNA in CACs was increased in diabetics without DR but reduced with onset of NPDR, indicating possible loss of compensation of protective RAS during early DR. Migratory dysfunction of CD34 cells was further associated with DR.

**Conclusions:** As assessed by the fractal dimension, the space-filling capacity of veins, but not arteries, was greater in early NPDR than in control. Larger patient populations will be examined as we complete our ongoing longitudinal study. Results further suggest the protective RAS axis within diabetic CACs is lost early in DR and is associated with increased vascular remodeling as evidenced by VESGEN analysis.

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**Presentation Time:** 8:30 AM–10:15 AM

**CXCL8 can increase retinal microvascular endothelial cell (hRMEC) migration rate and its receptor signals may be linked to VEGF-induced migration**

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**Purpose:** Increased interleukin 8 (IL-8/CXCL8) and VEGF levels in the vitreous correlate with diabetic retinopathy (DR) progression and angiogenesis. We have previously reported CXCL8 induction by diabetes-relevant stimuli in human retinal microvascular endothelial (hRMEC) and Müller cells. There is evidence in the cancer literature that CXCL8 and VEGF angiogenic signals may be linked, but this has not been investigated in the context of DR. We aim to compare CXCL8 and VEGF’s effects on hRMEC migration and the potential of CXCL8 receptor inhibitors to reduce their effects.

**Methods:** hRMECs were grown in attachment factor coated 12-well plates and stimulated with vehicle, 25ng/mL VEGF, or 10ng/mL human recombinant CXCL8, in the presence or absence of 30min pre-treatment with 50ng/mL of Reparixin (a CXCL8 receptor inhibitor). A magnetic stencil migration assay was used to quantify migration at 0, 6, 24, 33 and 48hr post-stimulation. Data
was recorded by a blind observer and plotted as percentage of the area devoid of cells at time zero filled at each time-point. Two-way ANOVA was used to determine statistical significance (P<0.001**; N=6 per group).

**Results:** At 6hr, migration was not significantly different between the groups. At 24hrs, the filled area in VEGF- and CXCL8-stimulated cultures was 20%** and 23%** larger than vehicle-treated cultures, respectively. By 33hrs, the mean increase in filled area compared to vehicle was 31%** for both. Reparixin pre-treatment significantly reduced VEGF- or CXCL8-induced migration by 16%** at 24hr and by 22%** at 33hrs, to levels similar to vehicle-treated cultures. There was no significant difference in filled area between vehicle- and Reparixin-treated cells at any time point. No cell morphology change was evident in the cultures.

**Conclusions:** CXCL8, which is highly upregulated in the vitreous of patients with DR, increased the rate of hRMEC migration, while the CXCL8 receptor inhibitor, Reparixin, limited hRMEC migration induction by both CXCL8 and VEGF independently. This suggests that signals from CXCL8 and VEGF receptors may be linked in hRMEC, as has been postulated for tumor and other endothelial cells. These findings support the notion that targeting the CXCL8 signaling pathway may be beneficial in DR and other pro-angiogenic retinal diseases.

**Commercial Relationships:** Dolly A. Padovani-Claudio, None; Yueli Zhang, None; John S. Penn, None

**Support:** Knights Templar Eye Foundation Grant

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**Presentation Time:** 8:30 AM–10:15 AM

**Purpose:** Diabetic retinopathy (DR), is the most common causes of vision impairment in the working-age population. Disease severity is intimately coupled with retinal neovascularization during progression to proliferative disease (proliferative diabetic retinopathy; PDR), but to date it has not been possible to study this process due to limitations in the available animal models. As such, there is a gap in our knowledge about what causes the switch to proliferative disease. Unlike other animals, retinal neovascularization may be induced in adult zebrafish, but whether diabetes could drive retinal neovascularization in zebrafish is not known. The purpose of this project was to test the hypothesis that recently characterized diabetic pdx1-mutant zebrafish develop PDR, thus constituting a long-sought-after model of this common and severe disease.

**Methods:** Pdx1-mutant zebrafish, crossed with fluorescent reporters for detection of endothelial cells, pericytes, smooth muscle cells, glial cells and macrophages were used. These fish were investigated by immunohistochemistry, qPCR, western blot and by electron microscopy at 3, 6, 9 and 12 months of age.

**Results:** Diabetic, pdx1-mutant fish exhibited clear signs of PDR already at 3 months of age, including increased vessel dilation and tortuosity, hypersprouting and disruption of endothelial tight junctions and contact with perivascular cells. These phenotypes became more severe over time leading to significant disruption of the retinal vasculature in 12-months old pdx1-mutant fish compared to controls. Robust retinal neovascularization was intimately coupled to a breakdown of the blood-retinal barrier (BRB) and disease severity was proportional to the degree and duration of hyperglycemia. Also progressive degeneration of photoreceptors were observed, the degree of which correlated to the severity of the neovascular phenotypes.

**Conclusions:** Retinal neovascularization, leakage and photoreceptor degeneration, is induced in long-term diabetic pdx-1 mutant zebrafish, resembling human PDR. Zebrafish therefore provide a strong alternative to mouse models for research into mechanisms underlying retinal angiogenesis under pathological situations in adults, and may be used to identify new targets for treatment of PDR in the future.

**Support:** Vetenskapsrådet (Sweden), Jeansson stiftelse (Sweden), Swedish society for medical research (Sweden), H2020-MCSA-RISE (Europe).

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**Purpose:** Efforts have been made to identify biomarkers to quantify characteristics of various disease processes and to establish therapeutic endpoints. In this study, we aimed to develop and validate a method of combining multiple factors into a composite biomarker using linear discriminant functions. The purpose of a composite biomarker is to provide a useful tool to accurately classify disease-state proliferative diabetic retinopathy (PDR) from disease-free subjects without complicated data interpretation.

**Methods:** Vitreous samples collected from control subjects (n=28) and those with PDR (n=35) were subjected to ELISA or multiplexing to detect levels of 4 distinct factors that have been classically associated with PDR: VEGF-A, PLGF, PAI-1 and IL-8. All four factors were analyzed individually and in pairs in a linear discriminant function to produce a final composite biomarker. Comparison of success rates for correct diagnosis of disease condition, PDR vs. control was performed with training and test sets using the randomized k-fold stratified validation method.

**Results:** In a discriminant function analysis using one proangiogenic factor (VEGF-A or PLGF) or one pro-inflammatory factor (PAI-1 or IL-8), the results for successful classification of PDR was approximately 82.52% in a 7-fold stratified test data set. In a two-factor linear discriminant function analysis, the pair combination of one proangiogenic and one pro-inflammatory factor into a multi-cytokine biomarker demonstrated approximately 90% success in distinguishing between PDR and control. The derived linear discrimination function of the combination of VEGF-A and IL-8 was tested against independent published data demonstrated a 90% success classification rate in identifying PDR from control.

**Conclusions:** Our study provides a useful method for identification of disease process in unknown subjects by applying linear discriminant analysis of 4 distinct biomarkers that have been widely accepted to be associated with PDR. The application of multiple factors to construct a composite biomarker provides stronger evidence in identifying disease processes than a single biomarker alone. This methodology may be extended to plasma/serum biomarkers, which ultimately could allow a simple blood test to be used for diagnosis of a specific eye disease.
Acrolein (ACR) is a highly reactive unsaturated aldehyde.

Charlie Norwood V A, None; Namrata Nandakumar, None; Gianna C. Teague, None; Megan E. Baldwin, Opthea Pty Ltd (S); Kameran Lashkari, None

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Presentation Time: 8:30 AM–10:15 AM

Hypoxia is essential for hyperglycemia to induce permeability and angiogenesis in retinal endothelial cells via GLUT1

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Purpose: Most diabetic patients develop diabetic retinopathy (DR) regardless tight glycemic control, suggesting other factor, in addition to hyperglycemia (HG), is essential for progression of DR. Hypoxia is the most likely factor particularly, it is followed by advanced DR. We hypothesize that in diabetes, hypoxia sensitizes retinal endothelial cells (RECs) to HG via activation of glucose transporter-1 (GLUT1) to induce intracellular glucose transport and metabolic switch from oxidative phosphorylation (OXPHOS) to glycolysis.

Methods: Human retinal endothelial cells (HRECs) were treated with HG (30 mM D-glucose) or osmotic control (5mM D-glucose+25 mM L-glucose) for 3 days with or without low oxygen (1%O2) for additional 1-2 days. Expression of GLUT1 was examined by Western blotting. Barrier function of HRECs was assessed in the presence or absence of GLUT1 inhibitor (STF31, 10 M in DMSO) by Electric Cell-substrate Impedance Sensing (ECIS) that measures transcellular electrical resistance (TER). Expression/organization of Zonula Occluden-1 (ZO-1) was evaluated by immunofluorescence. Bioenergetic profiles of HRECs was assessed by Seahorse XF analyzers that measures oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). The length of capillary/tube formation in HRECs was measured under the joint effect of hypoxia and HG versus the effect of hypoxia or HG alone and in the presence or absence of GLUT1 inhibitor. All data were summarized as means ± SD. Group differences were evaluated by ANOVA followed by Tukey Posthoc test.

Results: Joint treatment of HRECs with hypoxia and HG significantly upregulated GLUT1 compared to normoxia/HG (2.3±0.7 vs 0.8±0.2 respectively, P<0.05). Hypoxia/HG also decreased TER, disrupted ZO1 expression/organization and enhanced capillary formation compared to the effect of normoxia/HG (P<0.05). Effects of hypoxia were prevented by GLUT1 inhibitors. Bioenergetics profile of HRECs demonstrated higher ECAR/OCR ratio (fold change of 1.5±0.2) under joint treatment with hypoxia and HG compared to hypoxia or HG (P<0.05) alone suggesting that HRECs preferentially use glycolysis rather than OXPHOS under hypoxia/ HG condition.

Conclusions: Hypoxia is required to induce the permeability and angiogenic effects of hyperglycemia via GLUT1, suggesting GLUT1 as a therapeutic target to prevent progression of DR.

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Potential role of tumor-necrosis factor (TNF) in the diabetes-induced loss of platelet-endothelial cell adhesion molecule-1 (PECAM-1) from retinal endothelial cells

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Purpose: PECAM-1 is an endothelial surface molecule that plays important roles in endothelial cell junctions, barrier function, leukocyte transmigration, intracellular signaling, and cell survival. We have found a loss of PECAM-1 from the retinal microcirculation of diabetic rats; however, the mechanisms for this loss have yet to be identified. We hypothesize that elevated levels of TNF may be a contributing factor.

Methods: Male Wistar rats injected with streptozotocin (30 mg/kg/day for 3 consecutive days) were used as a model of type 1 diabetes, with vehicle-injected, age-matched rats serving as controls. Upon euthanasia 8 weeks later, retinas and plasma were obtained for analysis of PECAM-1 (immunostaining, Western blots) and TNF (Western blots). Additionally, rat retinal microvascular endothelial cells (RRMECs) were grown under normoglycemic (NG; 5 mM glucose) or hyperglycemic (HG; 25 mM) conditions for 6 days and analyzed for PECAM-1 and TNF. Finally, RRMECs under normal glucose were cultured for 24 hours along with TNF at concentrations of 0.5, 10, 20, and 50 ng/ml.

Results: We found a significant loss of PECAM-1 from the retina in the in vivo model of diabetes, using both immunostaining (a 44% decrease in the superficial vasculature and an 86% decrease in the deep capillary layer; N=9 CON, 8 STZ; p<0.05) and Western blotting (61% decrease; N=13 CON, 13 STZ; p<0.05). A similar decrease in PECAM-1 was found in the in vitro model of hyperglycemia (65% decrease; N=3 NG, 3 HG; p<0.05). TNF protein levels increased by 70% in the plasma of diabetic rats (N=6 CON, 5 STZ; p<0.05) and by 74% in RRMECs (N=3 NG, 3 HG), even though the TNF protein levels decreased in the retinal tissue as a whole (by 36%, N=7 CON, 7 STZ; p<0.05). In vitro, TNF added to normoglycemic RRMEC cultures caused a decrease in PECAM-1 levels at concentrations of 20 and 50 ng/ml, but not at lower concentrations of 0-10 ng/ml.

Conclusions: Elevated TNF concentrations may play a role in the loss of PECAM-1 from the diabetic retinal microcirculation, as we find in retinal endothelial cell culture.

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Presentation Time: 8:30 AM–10:15 AM

Accumulation of Acrolein-conjugated Protein in the Vitreous Fluid of Proliferative Diabetic Retinopathy

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Purpose: Acrolein (ACR) is a highly reactive unsaturated aldehyde that readily forms conjugates with proteins, and has been recognized as an exogenous pollutant causing pulmonary disorders. However, it has recently been shown that ACR is also produced endogenously as a result of polyamine metabolism mediated by amine oxidases
and plays a role in cell toxicity and oxidative stress. We previously reported that semicarbazide-sensitive amine oxidase (SSAO) increased and correlated with oxidative stress in the vitreous of patients with proliferative diabetic retinopathy (PDR); however, its role in the pathogenesis of PDR has remained unclear. In this study, we measured the concentration of acrolein-conjugated protein, Ne-(3-formyl-3,4-dehydropiperidino) lysine adduct (FDP-Lys), in the vitreous fluid samples of proliferative diabetic retinopathy (PDR) and sought to explore the link between SSAO and ACR in eyes with PDR.

**Methods:** Vitreous samples were collected from 12 eyes of 12 patients with PDR (7 males and 5 females; mean age, 58.2±4.1 y/o), who underwent pars plana vitrectomy for prolonged vitreous hemorrhage and tractional retinal detachment involving macular lesions. For a control, vitreous samples were obtained from 9 eyes of 9 patients with non-diabetic ocular diseases (non-PDR): epiretinal membrane and idiopathic macular hole (4 males and 5 females; mean age, 68.4±3.1 y/o). Vitreous levels of FDP-Lys and semicarbazide-sensitive amine oxidase (SSAO) were measured by ELISA.

**Results:** The FDP-Lys was detectable in all the PDR and non-PDR vitreous samples and was significantly elevated in the vitreous fluids of PDR patients (11.2±0.8 nmol/ml) compared with those of non-PDR patients (8.8±0.3 nmol/ml; P<0.05). In addition, protein levels of SSAO showed approximately 8.0-fold increase in the vitreous fluids of PDR patients when compared with those of non-PDR patients (9.4±1.4 ng/ml vs 1.2±0.2 ng/ml; P<0.01). Furthermore, the level of FDP-Lys showed a moderate correlation with SSAO concentration in the vitreous samples of PDR patients (r=0.67, P<0.05).

**Conclusions:** The current data showed that ACR-conjugated protein was increased and correlated with SSAO in the vitreous fluid of patients with PDR, suggesting that SSAO plays a role in the production of ACR.

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**Presentation Time:** 8:30 AM–10:15 AM

**miR-15a/16 inhibits TGF-beta3 and SMAD2/3 signaling and maintains retinal endothelial barrier**

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**Purpose:** A key risk factor of diabetic retinopathy is hyperglycemia, and molecular mechanisms underlying hyperglycemia and diabetic retinopathy in association with microRNA are still poorly understood. There are increasing amounts of evidence on microRNA as a mediator of pathological mechanisms in diabetic retinopathy. The purpose of this study was to test the hypothesis that miR-15a/16 inhibit TGF-beta3 and SMAD2/3 signaling, thus maintaining retinal endothelial barrier.

**Methods:** Human primary retinal endothelial cells (REC) were maintained in normal (5 mM) glucose or transferred to high glucose medium (25 mM) for 3 days. REC were transfected with miRNA mimics (hsa-miR-15a-5p and -16-5p, at a final concentration of 30 nM) 48 hours before cell harvest. Western blot analyses were performed to measure the levels of proteins of interest. A permeability assay was performed to measure solute flux through the monolayer of cultured REC. In addition, retinal lysates of miR-15a-transgenic mice were analyzed for the changes of protein levels.

**Results:** We demonstrated that overexpression of miR-15a/16 resulted in decreased TGF-beta3 signaling and VEGF levels in REC cultured under high glucose conditions. In addition, levels of the tight junction proteins ZO-1 and occludin were elevated in REC overexpressing miR-15a and -16. Overexpression of miR-15a in REC played a role in reducing cellular permeability, potentially through inhibition of TGF beta3 signaling in high glucose conditions. Using miR-15a-transgenic mice, we demonstrated the regulatory role of miR-15a on TGF-beta signaling and key tight junction proteins in vivo.

**Conclusions:** miR-15a/16 played a role in 1) inhibiting TGF beta3, SMAD2/3, and VEGF signaling and 2) increasing levels of ZO-1 and occludin in REC cultured in high glucose conditions. Additionally, miR-15a overexpression resulted in decreased levels of endothelial permeability in vitro. Our in vivo data confirmed the regulatory roles of miR-15a on TGF beta3 signaling and the tight junction proteins in the retina. Therefore, we suggest that miR-15a/16 maintains retinal endothelial barrier by reducing TGF-beta3 signaling and increasing the levels of tight junction proteins.

**Commercial Relationships:** Eun-Ah Ye, None; Jena J. Steinele, None

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**Presentation Time:** 8:30 AM–10:15 AM

**Hypoxia stimulates TNFalpha-converting enzyme activation through endoplasmic reticulum stress: implication for retinal neovascularization**

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**Purpose:** Retinal neovascularization (RNV) is a crucial component of ischemic retinopathies such as retinopathy of prematurity and diabetic retinopathy. Inflammation has been shown to play a key role in RNV. Tumor necrosis factor alpha-converting enzyme (TACE) is a key regulator of inflammation through ectodomain shedding of a number of cytokines, cytokine receptors, growth factors, and adhesion molecules. TACE has been shown to critically contribute to pathological neovascularization. The goal of this study was to identify the molecular mechanism(s) involved in TACE activation during RNV. Specifically, we have tested the hypothesis that endoplasmic reticulum (ER) stress-mediated up-regulation of the ER proteins iRhom leads to TACE activation in response to hypoxia.

**Methods:** TACE mRNA and protein expression were measured in human retinal endothelial cells (HREC) in response to 2% O2 (1-12 hours) by quantitative PCR (qPCR) and Western blotting analysis, respectively. TACE activity was evaluated by fluorimetric assay. Induction of ER stress was assessed by evaluating expression of GRP78 by qPCR and Western blotting analysis. Blocking and activation of ER stress were performed by treating HREC with 4-phenyl butyric acid (PBA; 1 mM) and thapsigargin (1-10 mM), respectively. iRhom1/2 gene silencing was achieved by transfection of the cells with specific siRNAs.

**Results:** Hypoxia promoted a significant elevation of TACE mRNA and proteins levels as well as an increase in TACE activity in HREC. Treatment of the cells with hypoxia was associated with induction of ER stress as evidenced by a significant increase in GRP78 expression, and correlated with up-regulation of ER residing iRhom1 on both mRNA and protein levels. Silencing of iRhom1 with iRhom1 specific siRNA reverted hypoxia-induced activation of TACE. Furthermore, inhibition of ER stress with PBA ameliorated hypoxia-mediated elevation of both iRhom1 and TACE expression. In addition, ER stress activator thapsigargin promoted an increase in iRhom1 as well as TACE expression.

**Commercial Relationships:**
Upregulation of Lysyl Oxidase Pro-peptide (LOX-PP) Promotes HG-induced Apoptosis in Retinal Endothelial Cells

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**Purpose:** Retinal vascular cell loss by apoptosis is a characteristic feature of diabetic retinopathy. It is unclear whether high glucose (HG)-induced LOX-PP overexpression compromises survival of retinal endothelial cells (RRECs) through disruption of AKT signaling. In this study, we examined if LOX-PP is involved in inducing apoptosis under HG condition in these cells.

**Methods:** To examine whether HG alters LOX-PP protein expression, RRECs were grown in normal (N; 5mM glucose) or HG (30mM glucose) medium for 7 days. In parallel, cells were grown in N medium and exposed to purified LOX-PP for 24 hours prior to harvest. Cells were harvested after 7 days and Western Blot (WB) analysis was performed to determine changes in LOX-PP protein expression, AKT activation, and caspase-3 activation. In parallel, cells were analyzed for apoptosis using a differential dye staining assay to investigate the effect of LOX-PP on cell survival.

**Results:** WB analysis showed significant LOX-PP upregulation (155±19% of control; p<0.05) in cells grown in HG medium compared to cells grown in N medium. As expected, cells grown in HG showed a significant increase in the number of apoptotic cells (218±44% of control; p<0.05) compared to cells grown in N medium. Interestingly, cells grown in N medium and exposed to purified LOX-PP alone resulted in increased apoptosis compared to cells grown in N medium (179±29% of control; p<0.05). Additionally, cells grown in HG medium exhibited decreased AKT phosphorylation (54±16% of control; p<0.05) and increased caspase-3 activation (132±10% of control; p<0.05) compared to those of cells grown in N medium. Importantly, cells grown in N medium and exposed to LOX-PP also showed decreased AKT phosphorylation (74±12% of control; p<0.05) and increased caspase-3 activation (138±9% of control; p<0.05) compared to cells grown in N medium.

**Conclusions:** Findings from this study indicate that HG-induced LOX-PP overexpression may compromise AKT activity in RRECs and thereby induce apoptosis, a characteristic of early stage diabetic retinopathy.

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The hiPSC-RPE cell lines derived from type 1 diabetic (T1D, 04511.WTS), type 2 diabetic (T2D, 08002.DMS, 08102.DMS, 08203.DMS) and normal control (CTRL, EURCCS_10802, EURCCS_10902, EURCCS_10212) patients were grown for 4-6 weeks under high or low glucose, and high or low insulin and subjected to cytokine stimulation (H2O2, TNFα) or autophagic stimulation (AICA ribonucleotide, 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR), starvation or resveratrol. The barrier function of the epithelium was assessed with permeability assays, gene expression with PCR, morphology with immunofluorescence (IF) and electron microscopy (EM), MMP2 and MMP9 activity with zymography and autophagic activity with Western blotting (WB).

**Results:** All the cell lines acquired RPE specific morphology, and gene expression profile under high and low glucose and high and low insulin. There were no statistically significant alterations in barrier function or tight junction morphology or IF staining in any cell lines in above mentioned stimuli. The proMMP2 was reduced after H2O2 stimulation and proMMP9 was shown to be upregulated under TNFα exposure. Whereas AICAR and resveratrol induced cell type independent but glucose and insulin concentration dependent variation on the p62 or LC3 expression.

**Conclusions:** The glucose and insulin concentration, as well as cytokine and autophagy stimulation induced alteration on cellular functions, but these were similar in all cell lines. This data thus suggest that the hiPSC-RPE T1D and T2D cells function similarly in respect of morphology, barrier function, autophagic or matrix metalloproteinase activity when compared to hiPSC-RPE WT cells.

**Commercial Relationships:** Kati M. Juuti-Uusitalo, Alexa Klettner, Elisabeth Richert, Mostafa Kiamehr, Arto Koistinen, Katriina Aalto-Setälä, Kai Kaarniranta, Heli Skottman.

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**Purpose:** In diabetic retinopathy the high blood glucose induces alterations in retinal function and can lead to visual impairment. In previous studies with immortalised retinal pigment epithelial (RPE) cell lines diabetic stimuli i.e. high glucose, cytokines and hypoxia showed alterations in the barrier properties. Here we studied the effects of high glucose and high insulin concentration on the functionality of normal control or diabetic human induced pluripotent stem cell (hiPSC) RPE cells.

**Methods:** The hiPSC-RPE cell lines derived from type 1 diabetic (T1D, 04511.WTS), type 2 diabetic (T2D, 08002.DMS, 08102.DMS, 08203.DMS) and normal control (CTRL, EURCCS_10802, EURCCS_10902, EURCCS_10212) patients were grown for 4-6 weeks under high or low glucose, and high or low insulin and subjected to cytokine stimulation (H2O2, TNFα) or autophagic stimulation (AICA ribonucleotide, 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR), starvation or resveratrol. The barrier function of the epithelium was assessed with permeability assays, gene expression with PCR, morphology with immunofluorescence (IF) and electron microscopy (EM), MMP2 and MMP9 activity with zymography and autophagic activity with Western blotting (WB).

**Results:** All the cell lines acquired RPE specific morphology, and gene expression profile under high and low glucose and high and low insulin. There were no statistically significant alterations in barrier function or tight junction morphology or IF staining in any cell lines in above mentioned stimuli. The proMMP2 was reduced after H2O2 stimulation and proMMP9 was shown to be upregulated under TNFα exposure. Whereas AICAR and resveratrol induced cell type independent but glucose and insulin concentration dependent variation on the p62 or LC3 expression.

**Conclusions:** The glucose and insulin concentration, as well as cytokine and autophagy stimulation induced alteration on cellular functions, but these were similar in all cell lines. This data thus suggest that the hiPSC-RPE T1D and T2D cells function similarly in respect of morphology, barrier function, autophagic or matrix metalloproteinase activity when compared to hiPSC-RPE WT cells.

**Commercial Relationships:** Kati M. Juuti-Uusitalo, Alexa Klettner, Elisabeth Richert, Mostafa Kiamehr, Arto Koistinen, Katriina Aalto-Setälä, Kai Kaarniranta, Heli Skottman.
Signal interaction between Norrin and VEGF promotes blood-retinal barrier restoration

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Purpose: Norrin signaling through FZD4, LRP5/6, and TSPAN12 receptors, is required for vascularization and formation of the blood-retinal barrier (BRB). Here, we examined how VEGF and Norrin signaling interact to alter BRB properties. Our overall hypothesis is that Norrin can reverse VEGF-induced permeability by inducing the stabilization of the tight junction (TJ).

Methods: Endothelial permeability in Vitro was tested by measures of transendothelial electrical resistance (TEER) and solute flux of 70kDa RITC-dextran in primary bovine retinal endothelial cells (BREC). The signal transduction crosstalk between Norrin and VEGF signaling pathways was assessed by Western blot and qRT-PCR.

Norrin effect was corroborated in two animal models of diabetes: intravitreal injection of VEGF and 5-month STZ-diabetic rats. Data was analyzed with t-test, one- or two-way ANOVA with at least an n of 3.

Results: VEGF decreased TEER ∼30-40% at 24h that was maintained for 72h, while Norrin addition simultaneous with or after VEGF addition restored TEER to control values at 72h. Similar results were found measuring the permeability of 70 kDa FITC-dextran across BREC monolayers. VEGF promoted Norrin signaling by the induction of TSPAN12 translocation to the cell membrane at 24h in a MEK/ERK dependent manner and increased mRNA content at 72h. Norrin induced βCatenin stabilization and its translocation to nuclear fractions. Chemical inhibitors and transfection of inhibitory or constitutively active forms of βCatenin revealed that this signaling is necessary but not sufficient to induce barrier properties after VEGF. Moreover, transfection of disheveled mutant that promotes planar cell polarity, led to a decrease of VEGF-induced permeability. VEGF and Norrin both increased the expression of the TJ protein Claudin-5 but only Norrin promotes its localization at the TJ. The effect of Norrin was further demonstrated in vivo with intravitreal injection of Norrin, VEGF or both. VEGF increased permeability of Evans blue while Norrin/VEGF completely restored the barrier properties to control levels. Likewise, 5-month STZ-diabetic rats showed increased permeability to FITC-dextran that was completely restored after 24 hours of Norrin injection.

Conclusions: These results suggest that Norrin signaling acts in coordination with VEGF to promote proper induction of the BRB.

Commercial Relationships: Monica Diaz Coranguez, None; Chengmao Lin, None; Stefan Liebner, None; David A. Antonetti, None

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Program Number: 2519 Poster Board Number: A0053
Presentation Time: 8:30 AM–10:15 AM

Diabetes-induced increase in immunoglobulin-laden exosomes

We used C57BL/6 (WT) and Apolipoprotein E knockout (ApoE-KO) mice either fed with a normal diet (ND) or a 10% w/v fructose diet (FD) in drinking water from 2 months of age. Confluent primary retinal pigmented epithelial cells were cultured from WT or ApoE-KO mice and exosomes were isolated from media. The concentration of the isolated exosomes was measured by Nanoparticle Tracking Analysis (NTA). Immunogold labeling of exosomes showed that the isolated exosomes were exosome size and shape (40-200nm) that are secreted into extracellular environment, were shown to contribute to the pro-inflammatory changes seen in several tissues. The present study aims to elucidate the role of plasma exosomes in activation of the complementary pathway during DR pathogenesis.

Methods: Plasma exosomes were isolated from control or STZ-induced diabetic C57/B6 mice, Wistar control rats, and control and type II diabetic patients via ultracentrifugation or the ExoQuick method, and further purified via density gradient separation. Static Light Scattering (SLS) was used to quantify the exosomes. Classical complement activation was measured in vitro by C1 activation assay and C1s activity was determined by Western blot.

Results: In this study we found that most immunoglobulin binds to exosomes in plasma. SLS quantification of plasma exosomes showed significantly higher number of vesicles in diabetic mice (1.14 x10^{11}/mL) when compared to control (0.735x10^{10}/mL)(n=9, p<0.05).

Exosome makers, CD63 and ALIX, were used to confirm results and were analyzed via western blot (n=9 p<0.05).

Importantly, diabetic mouse plasma had higher exosome-bound IgG levels when compared to control (n=9, p<0.05).

Immunoglobulin bond to exosomes caused activation of the classic complement complex, C1, which is the first step in downstream complement protein recruitment (C4 and C2), formation of C3 and subsequent C5 convertase, and release of pro-inflammatory anaphylatoxins. Stimulation with pro-inflammatory anaphylatoxins (C3a/C5a) for 3 and 6 hours induced significant VEGFA (C5a: 30% increase relative to control) and IL1β (C3a: 30% increase relative to control) upregulation in human retinal endothelial cells via their receptors (n=6, p<0.05).

Conclusions: Diabetes-induced increase in immunoglobulin-laden exosomes in circulation can activate classical complement pathway and induce a pro-inflammatory response in retinal endothelial cells. This finding suggests that exosomes can contribute to the development of DR.

Commercial Relationships: Chao Huang, None

Support: NIH Grant EY025383

Program Number: 2521 Poster Board Number: A0055
Presentation Time: 8:30 AM–10:15 AM

Retinal dysfunction in early stages of Metabolic Syndrome established on a new experimental mouse model

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Purpose: Diabetic retinopathy (DR) is the most serious ocular complication associated with T2DM, which is a metabolic syndrome (MS), and one of the leading causes of blindness. Although animal models of DR have contributed to the knowledge of this disease, these models have some limitations in to reproduce completely the retinopathy. We proposed to analyze in early stages of the MS, markers of retinal vascular integrity and neuronal functionality.

Methods: We used C57BL/6 (WT) and Apolipoprotein E knockout (ApoE-KO) mice either fed with a normal diet (ND) or a 10% w/v fructose diet (FD) in drinking water from 2 months of age. Time-dependent kinetic studies were done from 4 to 6 months of age analyzing lipid profile, glucose tolerance test (GTT) and insulin tolerance test (ITT). For GTTs, WT or ApoE-KO with ND or FD-fed mice (n=8/group) were fasted 5 h prior to glucose injection (2g/kg, i.p.). For ITTs, mice (fasted 5 h) were injected with regular human insulin (0.75U/kg, i.p.). Blood samples were taken from the tail vein at time 0 (basal value) and at 0.5; 1 and 2 h after injections.

Retinal functionality was assessed by ERG at 2, 3 and 4 month of

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treatment in mice dark-adapted (>15 hours). Retinal histology and immunofluorescence analysis were performed in flatmounts and cryosections, vascular permeability and leakage were quantified by Evans Blue extravasations. GraphPad Prism was employed for statistical analysis.

**Results:** After 2 month of treatment, ApoE-KO FD showed dyslipidemic profile, altered GTT and ITT vs. other groups. At this time, the ERG a- and b-wave did not show changes but the oscillatory potential amplitudes were significantly decreased in retinas of these mice vs. ApoE-KO ND (p<0.05). In addition, ApoE-KO after 4 month of FD evidenced increased vascular permeability and leakage vs. ApoE-KO ND and WT ND or FD (p<0.05). At this early stage of the MS, the GFAP expression levels were observed just in astrocytes but not in Müller glial cells, demonstrating non-reactive gliosis in retinas of ApoE-KO FD.

**Conclusions:** The results showed that ApoE-KO after 2 months of FD presented metabolic alterations mimicking some features of human MS at its initial stages, accompanied by a partial retinal neuronal dysfunction that precedes the vascular changes. Thus, this model could offer the opportunity to investigate DR at an early stage of the MS, which shows increasing prevalence worldwide.

**Commercial Relationships:** Maria C. Paz, None; Pablo Barcelona, None; Paula V. Subirada Caldarone, None; Magali E. Ridano, None; Gustavo A. Chiabrando, None; Claudia Castro, None; María C. Sanchez, None

**Support:** SECyT-UNC, CONICET, AGENCIA NACIONAL

**Program Number:** 2522 **Poster Board Number:** A0056 **Presentation Time:** 8:30 AM–10:15 AM

**Analysis of Sphingolipid Composition of Human Vitreous from Control and Diabetic Individuals**

Lynda A. Wilmott, Megan Stiles, Timothy Lyons, Maria C. Mandal, Pierce K. Nazary, Paul A. Rutledge, Catherine Martin, Megan Stiles, Timothy Lyons, Claudia Castro, Maria C. Sanchez, None  

**Purpose:** Sphingolipids have a fundamental role in many cellular processes, and they have been implicated in insulin resistance and diabetes mellitus (DM) and its complications, including diabetic retinopathy (DM-DR). The majority of retinal sphingolipid research has been limited to rodent models, so little is known as to how bioactive sphingolipids influence human DM and DM-DR. In this study, our aim was to determine the sphingolipid composition of the human vitreous in control, DM, and DM-DR samples.

**Methods:** We conducted an observational clinical study on post-mortem human vitreous samples from non-diabetic (controls; n=3; age: 73.7 ± 13.0 yrs, mean ± SD), type II diabetic (n=4; age: 73.8 ± 3.6 yrs), and diabetic with retinopathy (n=5; age: 62.6 ± 9.4 yrs) to identify changes in retinal sphingolipid composition. Samples were subjected to sphingolipid extraction using previously established protocols, and individual sphingolipid species were confirmed and quantified using a triple quadrupole mass spectrometer. For quantitation, vitreal sphingolipid species were compared to a target lipid ion peak using an internal standard (C12 Ceramide) and normalized.

**Results:** The total Ceramide (Cer) and sphingomyelin (SM) levels (pmol/mg protein) appeared to be increased in diabetic vitreous. Total Cer and certain individual Cer species (C22:0, C24:0, C24:1, C26:0, and C26:1) exhibited increased in diabetic versus control vitreous significantly (DM vs. control; p<0.05). Cer species C22:0, C24:1, and C26:0 were increased in DM-DR versus control (p<0.05). Interestingly, total Hexosyl-Ceramide (Hex-Cer) and certain species were higher in DM than DM-DR or control, including C18:0, C20:0, C22:0, and C26:0 (p<0.05). However, certain Hex-Cer species were significantly decreased in DM-DR vs DM: C24:1 and C26:1 (p=0.01 and p=0.05, respectively). Finally, the di-hydro Sphingosine-1-phosphate (dh-S1P), showed greater levels in both DM and DM-DR samples vs controls (p<0.05), but there was no significant difference between DM and DM-DR.

**Conclusions:** Our results suggest that changes in sphingolipid composition are characteristic of the diabetic human vitreous. Further studies using animal models will be conducted to identify potential causes and consequences, and to investigate novel therapeutic targets.

**Commercial Relationships:** Lynda A. Wilmott, None; Megan Stiles, None; Timothy Lyons, None; Nawajes A. Mandal, None

**Support:** NIH Grant EY022071

**Program Number:** 2523 **Poster Board Number:** A0057 **Presentation Time:** 8:30 AM–10:15 AM

**Glucose Effect on Cell Viability and VEGF Secretion in Retinal Endothelial Cells**

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1 Biology, University of Texas at San Antonio, San Antonio, TX; 2 Biomedical Sciences, The University of Texas Rio Grande Valley School of Medicine, Edinburg, TX.

**Purpose:** The objective of current study is to investigate the effect of glucose on retinal endothelial cell viability and VEGF secretion.

**Methods:** 20,000 cells per well were treated without glucose or with 5.5mM (euglycemic), 18.5mM and 30mM (hyperglycemic) glucose for 24 hours. Viable cells were counted using Trypan blue dye exclusion method. ELISA was used to measure VEGF secretion from cells into the cell medium.

**Results:** The number of viable cells incubated with 5.5mM glucose (physiological control) increased by 53.7% after 24 hours. In comparison, cells treated with 18.5mM glucose decreased by 2.8% while cells treated with 30mM glucose decreased by 20% after 24 hours of incubation. Cells without glucose treatment (0mM control) decreased by 33.3%. In contrast to the decrease of viable cell numbers after treatment with high glucose, there is an increase in VEGF secretion (pg/mL) to the cell medium with increase in glucose concentration from 5.5mM to 18.5, and 30mM. The amount of VEGF secreted per cell also increased with increasing glucose concentrations.

**Conclusions:** Our results show that viability of retinal endothelial cells and VEGF release are highly responsive to changes in glucose concentration. Such glucose-induced changes in retinal endothelial cells may negatively impact the integrity of the microvasculature in the diabetic retina leading to angiogenesis and microaneurysms.

**Commercial Relationships:** Brandi S. Betts-Obregon, None; Jessica Buikema, None; Andrew T. Tsir, None

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**Program Number:** 2524 **Poster Board Number:** A0058 **Presentation Time:** 8:30 AM–10:15 AM

**The role of insulin in diabetic retinal homeostasis**

lilly khamsy, Laura Kowalczik, Tatiana Favez, Catherine Martin, Francine F. Behar-Cohen 1, 2  
1 HOJG, Lausanne, Switzerland; 2 Ophthalmology, University of Lausanne, Lausanne, Switzerland.

**Purpose:** Type 2 diabetic insulin-resistant patients have high insulin circulating levels and insulin treatment is potentially aggravating...
the risk of retinopathy and macular edema. But the exact direct role of insulin on hydro-ionic retinal homeostasis remains incompletely understood. The aim of this study is to analyze the effect of insulin on human retinal pigment epithelium (RPE) cells in hyperglycemic conditions in vitro and after intravitreal injection in Goto Kakizaki (GK) type 2 diabetic rats.

Methods: ARPE19 cell line and human induced pluripotent stem cells derived into RPE (iPSc-RPE) were used to evaluate tight junction (TJ) rearrangement in hyperglycemic conditions in presence of insulin in vitro. Cell viability and proliferation were analyzed. Immunofluorescence of Zona occludens-1 staining was performed after 7 days of treatment in high glucose (40mM) and high insulin (10nM, 100nM) conditions following 24h FBS starvation. In parallel, transepithelial resistance (TER) measurements was performed. In vivo, insulin was injected in the vitreous of GK rats and 150kDa FITC dextran was injected intravenously one hour prior to sacrifice (n=6). Glycemia levels were recorded.

Results: ARPE19 and iPSc-RPE cell monolayer form TJ thus maintaining a concentration gradient between apical and basal environments. We validated the presence of functional insulin receptors in our cell cultures. Insulin (10nM) induces cell proliferation. We consistently see opening of TJ in high glucose (40mM) and high insulin (10nM) conditions after 7 days of treatment compared with baseline conditions in which ARPE19 cells exhibit intact cobblestone appearance (glucose 25mM, insulin 0nM). Paradoxically TER increased in high glucose and high insulin conditions in which opening of TJ was seen. B27 supplement starvation in iPSc-RPE cultures reduced TER. Interestingly, higher insulin (100nM) levels did not induce opening of TJ, suggesting involvement of a pathway dependent on insulin receptor regulation. In vivo, insulin injected intravitreally in GK diabetic rats induced retinal vessels vasodilation and leakage. No glycemic changes resulted from intravitreal injection of insulin.

Conclusions: Our findings reveal that insulin in combination with high glucose impairs TJ arrangement in RPE. In vivo, high levels of insulin in the vitreous may be responsible for aggragation of macular edema.

Commercial Relationships: Lilly Khamsy, None; Laura Kowaleczuk, None; Tatiana Favez, None; Catherine Martin, None; Francine F. Behar-Cohen, None

Program Number: 2525 Poster Board Number: A0059
Presentation Time: 8:30 AM–10:15 AM
DEVELOPMENT OF A NOVEL APPROACH FOR ASSESSMENT OF MITOCHONDRIAL RESPIRATION IN DIABETIC RETINA

Anand R. Saripalli1,2, Yan Levitsky1, Elahe Crockett1, Evangelyn Alocilja1, Julia V. Busik1, Denis Proshlyakov1, 1Physiology/Chemistry/Medicine, Michigan State University, East Lansing, MI; 2Molecular and Integrative Physiology, University of Michigan, Ann Arbor, MI; 3Engineering, Michigan State University, East Lansing, MI.

Purpose: Despite recent success in treatment approaches, diabetic retinopathy (DR) remains a leading cause of progressive vision loss and blindness. It is well accepted that mitochondrial dysfunction contributes to the development of DR, however the mechanism(s) of mitochondrial damage are not well understood. Recent studies demonstrate that there is an intricate connection between ceramide and mitochondrial function. We have previously demonstrated that activation of acid sphingomyelinase (ASM) and ceramide production is an important early event in the pathogenesis of DR, however the role of ceramide-induced mitochondrial dysfunction in DR is unknown due to the lack of sufficiently sensitive tools. This study aims to design the tools for evaluation of the role of mitochondria in ceramide-induced damage in diabetic retinas.

Methods: Using differential centrifugation and magnetic nanoparticles (MN) conjugated to anti-translocase of the outer membrane of mitochondria-22 (anti-TOM-22) antibodies, mitochondria were isolated from mouse livers, retinas, and retinal pigment epithelium (RPE) cell cultures in normal (5.5mM) or high (25mM) glucose for 24 hours. Mitochondrial respiration was measured using a NeoFox fluorescent oxygen sensor in both conventional and microfluidic chambers. Glucose oxidase bound MNs were used for calibration of the microfluidic device.

Results: The conventional NeoFox oxygen sensor requires mitochondrial isolation from ~700mg of tissue or about 0.05mg of total protein to achieve the detection limit. The microfluidic device reduced tissue requirements to ~7mg or about 0.0027mg of total membrane protein making it possible to analyze mouse retinal mitochondrial metabolism from a single animal. We have demonstrated that MNs can be used to concentrate mitochondria in the microfluidic chamber. Using this approach we found a 3.6 ± 0.2 fold increase in mitochondrial outer membrane permeability in RPE cells cultured in 25mM glucose compared to control. The increase was corrected by ASM inhibitor desipramine.

Conclusions: MN-based mitochondria isolation coupled with a microfluidic sensor for respiratory and electrochemical activity shows potential for identification of complex-specific changes in the mechanism of ceramide-induced mitochondrial dysfunction in the diabetic retina.

Commercial Relationships: Anand R. Saripalli, None; Yan Levitsky, None; Elahe Crockett, None; Evangelyn Alocilja, None; Julia V. Busik, None; Denis Proshlyakov, None.

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Program Number: 2526 Poster Board Number: A0060
Presentation Time: 8:30 AM–10:15 AM
ACE2/ACE-Imbalance in Circulating CD34+ Cells of Individuals with Diabetic Retinopathy

Charles A. Garcia1, Shriniidh Joshi1, Julio Quiroz-Olvera1, Miguel Duran-Mendez1, Wendy A. Cantu-Delgado1, Samuel Gomez1, Stephen Bartelmez1, Yagna Jarajapu1, Ophth-Herman Eye Ctr, Univ of Texas Houston Med Sch, Houston, TX; 2Pharmaceutical Sciences, North Dakota State University, Fargo, ND; 3Retinal Vascular Center, Houston, TX; 4BetaStem Therapeutics, Sausalito, CA.

Purpose: Endothelial dysfunction and reduced microvascular repair are the hallmarks of diabetic retinopathy (DR). Bone marrow derived CD34+ cells have the propensity of vascular regeneration. Diabetes is associated with decreased number and vasoreparative dysfunction of CD34+ cells. Angiotensin-converting enzyme (ACE) and ACE2 are primary enzymes of the vascular-detrimental and protective axes of the renin-angiotensin system (RAS), respectively, and are expressed in the vascular endothelium and CD34+ cells. Activation of ACE2 was shown to stimulate vasoreparative functions in CD34+ cells. We tested the hypothesis that balance between ACE and ACE2 shifted towards detrimental axis in CD34+ cells of individuals with DR.

Methods: Peripheral blood was obtained from healthy (control) and diabetic individuals with DR. Mononuclear cells (MNCs) and plasma were separated and lineage- (Lin-) and CD34+ cells isolated. Activities of ACE and ACE2 were determined in plasma and lysates of Lin and CD34+ cells by using enzyme-selective fluorogenic substrates and inhibitors. Proliferation and migration of cells

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were determined by using BrdU-ELISA or by chemotaxis assay, respectively.

Results: RESULTS: DR was associated with decreased number of Lin$^+$ (2±0.2)x10$^3$, P<0.04) and CD34$^+$ (0.6±0.1)x10$^3$, P<0.02) cells (n=6) compared to control group (Lin$^+$ (25±4)x10$^3$; CD34$^+$ (2±0.4)x10$^3$), expressed as per million MNCs. Abundance of both populations is negatively correlated with HbA1C levels (r=0.9, P=0.01). ACE and ACE2 activities were not different in MNCs. Decreased ACE2 and increased ACE activities were observed in plasma, and CD34$^+$ cell-lysates resulting in reduced ACE2/ACE (DR vs control: Plasma - 0.34±0.07 vs 0.8±0.08, P<0.05; CD34$^+$ - 0.4±0.07 vs 1.8±0.2, P<0.01, n=6). Proliferation of Lin$^+$ or CD34$^+$ cells from DR-group was lower in basal conditions or in response to stromal-derived factor-1α (SDF) or vascular endothelial growth factor (VEGF) compared to control (P<0.05 to 0.001, n=4-8). SDF- or VEGF-induced migration of both populations was impaired in DR-group compared to control (n=4-8).

Conclusions: DR decreased the number of circulating Lin$^+$ and CD34$^+$ cells and impaired vascular repair due to imbalance in the protective and detrimental axles of RAS. Future trials would offer a unique, minimally invasive treatment for vascular regeneration to those with blinding DR and other diabetic diseases.

Commercial Relationships: Charles A. Garcia, None; Shrinidh Joshi, None; Julio Quiroz-Olvera, None; Miguel Duran-Mendez, None; Wendy A. Cantu-Delgado, None; Samuel Gomez, None; Stephen Bartelmez, None; Yagna Jarajapu, None

Program Number: 2527 Poster Board Number: A0061
Presentation Time: 8:30 AM–10:15 AM

Diabetes hinders resolution of inflammation and reestablishment of the inner blood-retinal barrier following retinal ischemia-reperfusion injury

Sumathi Shanmugam, Cheng-mao Lin, Dejuan Kong, Heather Hager, David A. Antonetti, Steven F. Abcouwer. Ophthalmology and Visual Sciences, University of Michigan, Kellogg Eye Center, Ann Arbor, MI.

Purpose: Diabetic retinopathy may be caused by an imbalance of retinal damage and repair processes. The mouse intraocular pressure-induced ischemia-reperfusion (IR) model exhibits neurodegeneration, neuroinflammation, leukostasis followed by leukocyte infiltration and vascular permeability; which all normally resolve between 3 and 4 weeks after injury. We utilized the IR model to test the hypothesis that negative effects of diabetes on retinal repair processes impede resolution of inflammation and restoration of the inner blood-retinal barrier (iBRB) following retinal injury.

Methods: IR injury was unilaterally produced by injection of saline into the anterior chamber for 90 min, while contralateral Sham eyes received a needle puncture only. To examine the effects of diabetes, IR injury was produced in control and diabetic mice after 4 weeks of streptozotocin-induced diabetes. Vascular permeability was assayed by measuring the retinal accumulation of circulating FITC-BSA. Retinal microglia and infiltrating leukocytes were quantified by flow cytometry with CD45, CD11b, Ly6C and Ly6G markers. Microglial proliferation and migration was assessed by immunofluorescence of Iba1 and Ki67 antigen in retinal sections.

Result: Whereas IR produced 45% less (p<0.01) vascular leak in diabetics than controls at 2 days following injury, diabetic mice exhibited a 2.4-fold higher (p<0.05) leak at 4 weeks after IR. In normal mice, microglia responded to IR by transiently proliferating and migrating from plexiform layers toward the ganglion cell layer; while infiltrating leukocytes exhibited a temporal progression with granulocytes and Ly6C$^+$ inflammatory monocytes predominating at 1 day and Ly6C$^+$ reparative monocytes peaking at day 7. There were no significant differences in accumulation of granulocytes or Ly6C$^+$ monocytes between control and diabetic mice. Surprisingly, at 2 weeks following IR diabetic mice exhibited a persistent 2.3-fold increase in microglial population (p<0.01) that was not observed in controls. The numbers of Ly6C$^+$ monocytes were also more than 2-fold greater (p<0.05) in retinas of diabetic mice at both 2 and 4 weeks following IR.

Conclusions: Lack of iBRB recovery and extended microglial and Ly6C$^+$ monocyte responses suggests that impaired repair processes can contribute to diabetic retinopathy pathology.

Commercial Relationships: Sumathi Shanmugam, None; Cheng-mao Lin, None; Dejuan Kong, None; Heather Hager, None; David A. Antonetti, None; Steven F. Abcouwer, None
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Program Number: 2528 Poster Board Number: A0062
Presentation Time: 8:30 AM–10:15 AM

C57BL/6JBonTac is an Ideal Mouse Model for Diabetic Retinopathy Research: Why Selecting Proper Wild-Type Control Mouse Matters in Validating Preclinical Studies of Diabetic Retinopathy


Purpose: This study validates the use of the C57BL/6JBonTac mouse model as a potential control in studies that investigate diabetic retinopathy (DR). C57BL/6J (B6J) mouse strain has been used as an inbred control in diabetes mellitus (DM) and DR studies. However, B6J mice display impaired glucose intolerance and reduced insulin secretion due to a spontaneous mutation in the nicotinamide nucleotide transhydrogenase (Nnt). It’s been reported that Nnt mutation enhances glucose-dependent ROS production, increases oxidative damage and impairs β-cell mitochondrial metabolism. Ample evidence indicates that mitochondrial dysfunction plays a major role in developing DM with the accompanying risk for developing DR. Arguably, using B6J mice in studies that focus on mitochondrial dysfunctional diseases, such as DM and DR, would be especially concerning, given the direct effect of Nnt’s loss of function on the mitochondrial NADP status and the overall redox balance. Against such a genetic background, the development of DM and DR is likely going to occur. It is therefore prudent to explore the use of other animal models that don’t display the Nnt mutation, DM or DR.

Methods: We examined 14 intervals throughout the growth of the C57BL/6JBonTac mouse that doesn’t carry the Nnt mutation, using fluorescein angiography (FA), optical coherence tomography (OCT), electoretinography (ERG) and trypsin digestion (TD) studies.

Results: FA revealed that all mice exhibited healthy retinas characterized by dense capillary network, normal retinal blood vessels and vasculature that exhibited the absence of vascular leakages or hemorrhages. OCT scans exhibited normal comparable thickness of healthy retinal layers throughout the areas of section scans. ERG revealed that visual responsiveness was characterized by a- and b-waves that remained normal in waveform and amplitude. TD sections displayed healthy vasculature that presented vigorous retinal angiogenesis and normal endothelial to pericyte ratios, while ghost pericytes and acellular capillaries were absent.

Conclusions: This report identifies the C57BL/6JBonTac mouse model as a potential control in DM and DR studies. Our study offers a word of caution concerning the use of Nnt-mutated B6J mouse as a model to investigate diseases that are pertinent to metabolism or mitochondrial pathophysiology.

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Commercial Relationships: Michael DeNiro, None; Areej AlQahtani, None; Ewa Goljan, None; Alaa AlHazmi, None; Futwan AlMohanna, None

Program Number: 2529 Poster Board Number: A0063
Presentation Time: 8:30 AM–10:15 AM

Monocytes from patients with diabetic retinopathy are characterized by increased VEGF transcription during early macrophage differentiation

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Purpose: Type 2 diabetes (T2DM) affects a growing part of the population in all developed countries. More than 60% of people with T2DM will develop a form of Diabetic Retinopathy (DR) after 20 years of diabetes. Diabetic patients demonstrate robustly elevated intravitreal levels of inflammatory cytokines. Infiltrating monocyte (Mo)-derived inflammatory Macrophages (MPs) account for most of the production of these cytokines in animal models of DR. We hypothesized that pre-activated circulating inflammatory monocytes found in T2DM are an important pathogenic factor in DR.

Methods: T2DM and control patients were enrolled and diagnosed for diabetic retinopathy. Four groups were defined as follows: (i) controls; (ii) diabetic patients with no retinopathy; (iii) patients with NPDR; and (iv) PDR patients. Patients with known history of nephropathy and periodontitis were excluded. Plasma and monocytes were collected from all groups. Fresh monocytes were allowed to differentiate into MPs for 18h and RNA and culture supernatant were collected. Fresh Monocytes and monocyte-derived MPs were then evaluated for their ability to produce cytokines known to be elevated in the vitreous of DR patients including IL-1β, IL-6, IL-8, CCL2, and VEGF.

Results: Seventy-two subjects were included. The 3 diabetic groups were evenly distributed for age, BMI, blood glucose and HbA1c. Circulating monocytes demonstrated low expression of cytokines with minor differences between groups. In contrast, upon differentiation MP increased their expression of all tested cytokines. After 18h the expression of VEGF was higher in patients with NPDR or PDR than in diabetic patients with no retinopathy.

Conclusions: Our data support a model in which circulating monocytes in DR patients have a greater propensity to produce inflammatory cytokines when they differentiate into inflammatory macrophages compared to T2DM patients. Understanding how monocyte derived-inflammatory cytokine influence DR and treatment outcomes might be new cues to propose innovative treatments of these pathologies.

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Evaluation of Zucker Diabetic Fatty and ZSF1-Obese Rats as Potential Models for Diabetic Retinopathy
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Purpose: The most common animal model for assessing diabetic retinopathy is the Streptozotocin-induced rodent model. This model involves injection of STZ followed by glucose monitoring to confirm the diabetic state. Development of ocular lesions takes several weeks and study duration may be longer, during which time the health of the animals may be declining due to the diabetic state. This study was designed to monitor ZDF and ZDF-1 obese rats as a potential spontaneous model of diabetic retinopathy.

Methods: Fifteen rats (2 ZDF rats and 13 ZSF1-obese rats) were evaluated for ocular changes indicative of spontaneous development of diabetic retinopathy. Additionally, 4 ZSF1 lean rats were evaluated as controls. Adult animals aged 39-48 weeks were used on the study. Over a period of 11 weeks, the animals were evaluated approximately biweekly via opthalmic examinations and optical coherence tomography (OCT). Slit lamp biomicroscopy was used to assess the anterior chamber and lens. Indirect ophthalmoscopy was used to assess the fundus and vitreous. OCT was conducted using the Heidelberg Spectralis to collect IR+OCT scans.

Results: One ZDF animal was noted with focal retinal degeneration, while the second ZDF animal was noted with irregular retinal pigmentation. Ophthalmic findings for all other animals were limited to lens capsule and lens nucleus opacities. Once the opacities reached the point where visualization of the fundus was blurred, the animals were released from study. For each rat, there were no substantial changes in OCT scans over the course of the evaluation period. There were no differences in OCT scans between the three rat strains.

Conclusions: After 11 weeks of monitoring, there was no spontaneous development of diabetic retinopathy lesions that were detectable by OCT. Ophthalmic examinations demonstrated retinal changes in both ZDF animals. The ZSF1-obese strain does not appear to provide a spontaneous model of diabetic retinopathy that would be useful in the contract research setting; however, future evaluation using fluorescein angiography may provide useful data for characterizing retinal changes. Further evaluation of more ZDF animals is warranted.

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on human retinal microvascular endothelial cells (HRMEC) and human CD14+ monocytes. 

Methods: HRMEC were cultured on transwell filters for 7 days. HRMEC phenotype was visualized by immunofluorescence staining and confocal microscopy for markers PECAM and VWF. Primary CD14+ monocytes were maintained in culture and CD14 surface expression monitored using flow cytometry. Hyperglycemia was mimicked by exposure of cell cultures to high glucose (25.5 mM), or mannitol as an osmotic control (20.5 mM) for 4 days. RAGE was activated by treatment with AGE-modified BSA or BSA alone for 4 days. Cytokines were profiled using luminex and qPCR. Cellular phenotype was characterized using immunofluorescence and flow cytometry.

Results: Treatment of HRMEC with high glucose for 4 days did not produce release of inflammatory cytokines. However, treatment with AGE-BSA induced production of IL-1β, IL-4, IL-6, IL-8, G-CSF, IFNγ and TNFα (p < 0.05) into the apical and basolateral compartments. At the RNA level, expression of ICAM and VCAM were also increased by AGE-BSA treatment only (p < 0.05).

AGE-BSA treatment of HRMEC produced limited breakdown of the cellular barrier as evidenced by interrupted ZO-1 staining. However, endothelial cell phenotype markers (PECAM and VWF) were preserved. In response to RAGE activation, CD14+ monocytes also released inflammatory cytokines and adopted an M1-like phenotype.

Conclusions: RAGE activation constitutes a strong pro-inflammatory stimulus in both HRMEC and CD14+ monocytes, whilst short term glucose over-load does not. Pro-inflammatory effects of hyperglycaemia are likely mediated by glucose-dependent AGE formation, which occurs over weeks-months in vivo. Future efforts will focus on improving our understanding of how inflammation and glucose-induced cellular stress interact to produce phenotypes related to Diabetic Retinopathy.

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