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Aqueous humour concentrations of TGF-β, PLGF and FGF-1 and total retinal blood flow in patients with early non-proliferative diabetic retinopathy

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Abstract

Purpose—To correlate angiogenic cytokines in the aqueous humor with total retinal blood flow in subjects with Type 2 diabetes with non-proliferative DR (NPDR).

Methods—17 controls and 16 NPDR patients were recruited into the study. Aqueous humor was collected at the beginning of cataract surgery to assess the concentration of 14 angiogenic cytokines. Aqueous humor was analyzed using the suspension array method. Six images were acquired to assess total retinal blood flow (TRBF) using the prototype RTVue™ Doppler FD-OCT (Optovue, Inc., Fremont, CA) using a double circular scan protocol, 1 month post-surgery. At the same visit, forearm blood was collected to determine glycosylated hemoglobin (A1c).

Results—TGF-β1, TGF-β2 and PLGF were increased while FGF-1 was reduced in NPDR compared to controls (Bonferroni corrected, $p<0.003$ for all). TRBF was significantly reduced in the NPDR group compared to controls (33.1±9.9 vs. 43.3±5.3, $p=0.002$). Aqueous FGF-1 significantly correlated with TRBF in the NPDR group ($r = 0.71$, $p = 0.01$; $r^2 = 0.51$). In a multiple regression analysis, A1c was found to be a significant predictor of aqueous TGFβ1 and FGF-1 ($p=0.018$ and $p=0.020$, respectively).

Conclusion—Aqueous angiogenic cytokines (TGF-β1, TGF-β2 and PLGF) were elevated in conjunction with a reduction in TRBF in patients with NPDR. Non-invasive measurement of TRBF may be useful for predicting aqueous FGF-1 levels and severity of vasculopathy in DR.

Keywords

Total retinal blood flow; aqueous humor cytokines; angiogenesis; non-proliferative diabetic retinopathy; optical coherence tomography; retinal blood flow; fibroblast growth factor; type 2 diabetes mellitus; vasoregulatory

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INTRODUCTION

Diabetic retinopathy (DR) is a sight threatening complication of diabetes mellitus and is currently the leading cause of blindness in working age adults. Following 20 years of diabetes, nearly all patients with Type 1 and Type 2 diabetes mellitus will suffer from some form of retinopathy.(Klein & Klein 2010, Yau et al. 2012) Using non-invasive measurement techniques, animal and human studies have reported alterations in retinal blood flow before any clinical symptoms of DR.(Bursell et al. 1996, Grunwald et al. 1986, Takagi et al. 1996) Although perturbations in retinal blood flow are hypothesized to be an early hallmark of DR, the roles of diabetes-induced changes that modulate retinal blood flow in early DR are still unknown. To assess the pathogenesis of DR as well as to provide clinical biomarkers of DR severity, we set out to examine the role of angiogenic cytokines during diabetes-induced microvascular disturbances in early DR.

The role of angiogenic cytokines in the progression DR has been reported by many groups. (Clermont et al. 1997a, Clermont et al. 1997a, Noma et al. 2009, Noma et al. 2010) Moreover, previous studies have demonstrated that angiogenic cytokines are elevated in the ocular fluid of eyes affected by DR (Chiang et al. 2012, Dong et al. 2013, Oh et al. 2010) and diabetic macular edema(Jonas et al. 2012, Lee et al. 2012)(Jonas et al. 2012) compared with control eyes. Recently, one study reported that the elevated VEGF levels in the aqueous humor correlates with retinal blood flow in patients with central retinal vein occlusion. (Yamada et al. 2015) However, the use of multiplex bead analysis allows for simultaneous analysis of multiple cytokines in small volumes, provides broader insight into the mechanisms involved in retinal hemodynamic alterations in early DR.

An established technique that has been used to investigate volumetric retinal blood flow in DR is bidirectional laser Doppler velocimetry with simultaneous vessel densitometry. However, this technique is currently limited to large vessels and one single measurement site. Subsequently, Doppler spectral Fourier-domain optical coherence tomography (Doppler FD-OCT) blood flow (i.e. Optovue RTVue) has been developed to evaluate volumetric total retinal blood flow (TRBF). The FD-OCT system uses low coherence light to measure the total retinal blood flow of all vessels around the optic nerve head.(Wang et al. 2008) This system has been proven to reliably and consistently measure retinal blood flow in both healthy (Tayyari et al. 2014) and subjects with DR (Wang et al. 2009a). Thus, the Optovue system is a convenient method for measuring blood flow perturbations in DR and can be used clinically to assess DR severity.

We set out to evaluate the levels of angiogenic factors in the aqueous humor in NPDR to ascertain if changes in these locally produced factors vary in patients with NPDR. Further, we investigate the relationship between angiogenic cytokines and TRBF in patients with NPDR. Non-invasive quantification of TRBF may be a novel biomarker for biochemical alterations in early DR.

METHODS

Sample

All eligibility was determined and confirmed by an ophthalmologist during slit-lamp examination. Participants must be eligible for phacoemulsification. Subjects were excluded from the study if they had any clinical evidence of ocular disease (other than DR) and/or a history of ocular surgery or laser procedure. Subjects with a history of glaucoma in a first-degree relative were excluded from the study. Subjects taking medications with known effects on blood flow (except for well controlled systemic hypertension) and subjects with rheumatologic diseases were excluded. None of the subjects smoked or had any respiratory diseases. This study was approved by the University Health Network Research Ethics Board, University of Toronto Research Ethics Board and Kensington Eye Institute Research Ethics Board. Informed consent was obtained from each participant after thorough explanation of the nature of the study and its possible consequences, according to the tenets of the Declaration of Helsinki.

Aqueous Humor Collection

At the time of surgery, paracentesis was made in the peripheral cornea next to the limbus and undiluted samples of AH (80-120μl) was obtained from all participants. The AH was collected into an attached tuberculin syringe and then subsequently deposited into a 1.5ml micro-tube and placed on dry ice. The AH sample was immediately stored in a −80 degree Celsius refrigerator until analysis was performed. The aqueous samples were thawed at room temperature, vortexed, and then spun at 13,000× g for 5 minutes to remove any precipitates. Aqueous humor samples were then separated into aliquots of 30ul.

Measurement of Angiogenic and Hemodynamic factors using Multiplex Bead Analysis

Multiple cytokines were quantified simultaneously using MILLIPLEX® MAP technology to discover biomarker patterns in small sample volumes (Eve Technologies Corp, Calgary, AB, Canada), using the BioPlex 200 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Capture bead kits (R&D, Minneapolis, MN & Millipore, St. Charles, MO, USA) were used to determine fibroblast growth factor (FGF-1 & FGF-2), angiopoietins (ANG-2), interleukin-8 (IL-8), leptin, stromal-derived factor-1, epidermal growth factor (EGF), transforming growth factor-beta (TGF-β1 and TGF-β2), granulocyte colony stimulating factor (G-CSF) platelet-derived growth factors (PDGFs), vascular endothelial growth factor (VEGF), erythropoietin, endothelin-1 (ET-1), insulin (Millipore, St. Charles, MO, USA). The sensitivities of the markers range from 0.2 – 17.0 pg/ml. Individual analyte values and other assay details are available on Eve Technologies' website or in the Milliplex protocol. The tests were performed in accordance with the manufacturer's instructions. An analyte was considered "detectable" if the levels exceeded the minimal detectable levels identified in manual.

Quantifying Total Retinal Blood Flow Using FD-OCT

Four weeks post-surgery, the study eye was dilated with 1% tropicamide and 2.5% phenylephrine eye drops and images were then acquired. Doppler OCT images were taken using the commercially available known as the RTVue Doppler OCT (Optovue Inc,

Freemont, CA). The assessment included 6 sets of two concentric circular scans (each 3.4 and 3.75 mm in diameter, centered on the optic nerve head).

Non-invasive OCT technology has applied Doppler FD-OCT to extract information to calculate the volumetric blood flow. A computer algorithm chooses candidate vessels followed by manual refining by human operators, using the Doppler OCT of Retinal Circulation (DOCTORC) grading software. In the post-analysis process, the operator compares the initial automated processing with a colour fundus image to help accurately determine the distribution of vessels. The operator also adjusts the vessel classification and evaluates the Doppler signal size and intensity in order to precisely calculate total blood flow. Since this software is semi-automated, it takes an experienced technician 30-50 minutes per eye for analysis. This method has been previously described and has been recently validated.(Tan et al. 2012, Tayyari et al. 2014, Wang et al. 2008)

Finally, the TRBF is corrected by axial length. In Doppler OCT, the scan is actually based on a model eye with axial length of 24 mm and is found to vary with different eye lengths. Longer axial lengths decrease the magnification of the fundus image, reduce the transverse diameter of the vessel and increase the apparent Doppler angle, making the velocity and flow appear artifactually low. However, this magnification can be corrected. As described by Srinivas et al, (Srinivas et al. 2015) corrected TRBF can be obtained the formula: corrected TRBF= oTRBF*(axial length/default axial length)^2. Here, oTRBF is the original total blood flow before eye length correction and the default axial length, which is a value of 24 mm. The axial length–corrected TRBF was used in the statistical analyses.

Briefly, the RTVue system has a light source of 840nm with a bandwidth of 50nm, an axial resolution of 5μm in tissue and a transverse resolution of 20 μm.(Wang et al. 2011) The Doppler OCT is based on the principle that moving particles, such as a red blood cell inside a blood vessel, causes a Doppler *frequency shift (Δf)* to the scattered light. The Doppler shift (Δf) is proportional to the *flow velocity* (V) component to the axis of the probe beam. The Doppler shift (Δf) and angle α is measured from the difference between 2 concentric circular OCT scans. The measured Doppler shift, the incident angle and vessel area are used to calculate blood flow in each vessel. Total retinal blood flow was measured by summing the flow of all the detectable veins. Previously, Feke et al confirmed that retinal flow in arterials is equal to the retinal flow in veins due to a steady state system.(Feke & Riva 1978)

Statistical analysis

All analyses were performed with Statistica System 11.0 software (SAS Institute Inc., Cary, North Carolina, USA). All results are presented as the mean ± standard deviation (SD). The mean fold changes of the detected biomarkers were calculated between NPDR and control subjects. Differences in total retinal blood flow and aqueous humor factor components between healthy and NPDR participants were assessed with Student T-test. A Bonferroni correction was applied to each P value according to the number of comparisons in this study (corrected P value of 0.05/14 = 0.003). The correlation between TRBF and aqueous humor cytokines was determined with Pearson Bivariate Correlation test. In all analyses, two-tailed $p<0.05$ indicated statistical significance.

RESULTS

The demographic data of subjects are shown in Table 1. A total of 18 controls and 16 NPDR patients were initially recruited for the study. NPDR participants had early signs of retinopathy including microaneuryms, as confirmed by the ophthalmologist.

Retinal Blood Flow Findings

Table 2 lists the mean (±standard deviation (SD)) corrected TRBF for the control and NPDR groups. The mean TRBF for controls was 43.3±5.3 ul/min (95% CI: 40.2-46.3 ul/min) and 33.1±9.9 ul/min (95% CI: 27.1-39.1 ul/min) for NPDR patients. NPDR patients had significantly lower TRBF (~22%) than control subjects (p=0.002). Compared to controls, NPDR patients had significantly reduced superior and inferior retinal blood flow rates (~23%; p=0.01 and p=0.02 respectively).

Aqueous Humor Findings

Table 3 lists the results of our analysis of 14 candidate biomarkers in the aqueous humor samples of healthy control and NPDR subjects using suspension array analysis. Due to low aqueous sample volumes, only 11 controls and 8 NPDR samples were analyzed for aqueous TGFβ1 and TGFβ2.

Correlations between TRBF and Aqueous Humor Cytokines

Aqueous FGF-1 was significantly correlated with the TRBF across all participants with a correlation coefficient of r= 0.59, p = 0.001, r^2 = 0.34. Within the NPDR group, aqueous FGF-1 remained significantly associated with TRBF with a correlation coefficient of r = 0.71, p = 0.013, r^2 = 0.51, Figure 1. The results also revealed a significant association between aqueous TGFβ1 and TRBF with a correlation coefficient of r =−0.64 (p=.005) across all subjects. In the NPDR group, however, aqueous TGFβ1 was not associated with TRBF (r=−0.65, p=0.11).

Aqueous VEGF, ANG-2, ET-1, insulin factors were not significantly correlated with TRBF (p>0.114 for all). Forearm A1c was associated with TRBF across all patients (r=−0.42, p=.023).

Multiple Regression Analysis

A multivariate model was fitted for aqueous TRBF and included predictor variables ANG-2, ET-1, FGF-1 and TGF-β1 in the NPDR group (F=5.37, df=4,7 and p=.027). The only significant predictor for TRBF was aqueous FGF-1 (b*= 0.806, p=0.003). Additionally, a multivariate model was fitted for aqueous FGF-1 (F=5.42, df= 3,21 and p=0.006) and TGF-β1 (F=2.81, df=5,24 and p=0.0387). The only significant predictor for aqueous FGF-1 and TGF-β1 was A1c (p=0.020 and p=0.018, respectively).

DISCUSSION

Although retinal blood flow abnormalities are an early hallmark of DR, the precise mechanism for blood flow dysfunction has not been clear. Using the Doppler OCT, we

report a reduction in TRBF in patients with NPDR compared to controls. In patients with NPDR, aqueous TGF-β and PLGF levels were significantly elevated while aqueous FGF-1 levels was decreased compared to controls. Our results reveal a strong association between aqueous FGF-1 with TRBF in patients with NPDR. Although alterations in retinal hemodynamics have previously been found to be associated with increased aqueous humor growth factor expression in advanced retinal ischemic diseases (Noma et al. 2009, Noma et al. 2010, Yamada et al. 2015) this is the first study to identify a role of FGF-1 in impaired retinal blood flow in early DR. Overall, measurement of TRBF using Doppler OCT may be useful for predicting aqueous FGF-1 levels and severity of hemodynamic disturbances in early DR.

We observed a significant increase in TGF-β and PLGF in the aqueous humor of patients with NPDR compared to controls. Although not significant after Bonferroni corrections, additional angiogenic cytokines ANG-2, HGF and EGF tended to increase in the NPDR compared to controls ($p<0.023$). Similar angiogenic cytokines have been reported in earlier aqueous humor studies of DR.(Chiang et al. 2012, Dong et al. 2013, Oh et al. 2010) Despite the lack of correlation of these angiogenic cytokines with TRBF, the aqueous profile from these eyes showed marked similarity to those with PDR, and was significantly distinct from the controls. These results lend further support that angiogenic growth factors not only have a role in PDR but mediate early vascular changes in early DR.

To the best of our knowledge, this is the first study to demonstrate an association between FGF-1 and retinal blood flow perturbations in DR. As administration of FGF-1 and FGF-2 lowers blood pressure,(Cuevas et al. 1991) both FGF-1 and FGF-2 have shown to play a part in the maintenance of vascular tone. In non-ocular models, reduced plasma FGF levels have been found to correlate with a reduction in peripheral blood flow in Type 2 diabetic patients. (Alrouq et al. 2014) In experimental models, FGF has been shown to stimulate vasodilation and improve blood flow perfusion in animal models of ischemia through NO mediated mechanisms.(Wu et al. 1996) Moreover, basal FGF signaling plays a protective role with ongoing FGF stimulation required for the maintenance of vascular system integrity.(Cuevas et al. 1998, Hatanaka et al. 2012, Murakami et al. 2008) Since diabetes has been found to impair the expression of endogenous FGFs, (Yeboah et al. 2007) low FGF expression may explain the decreased retinal blood flow in early DR. These findings suggest FGF-1 may be a contributing factor for the observed reduction in TRBF and may predisposing for diabetic vascular diseases.

Decreases in retinal blood flow have been found in animal and humans with early diabetes. (Grunwald et al. 1986, Nagaoka et al. 2010, Sinclair et al. 1982) Using FD-OCT, Wang et al report reduced TRBF in in patients with DM without retinopathy compared to normal controls.(Wang et al. 2009a) The range of TRBF values found in this study is within the previously reported TRBF range.(Shahidi et al. 2014, Tayyari et al. 2014, Wang et al. 2009b) This and other corroborating studies (Bursell et al. 1996, Clermont et al. 1997b, Wang et al. 2009a) provide evidence that impaired retinal blood flow is a characteristic in early DR. A potential mechanism for this retinal blood flow change is associated with the vascular endothelial dysfunction which is with abnormal synthesis or action of vasodilators and increased expression and action of vasoconstrictors resulting in a shift of homeostasis to a

net vasoconstrictive effect in early diabetes. We have shown that the expression of ET-1 is increased while FGF-1 is decreased in the NPDR group compared to controls. Thus, an increased ET-1 and reduced FGF-1 production in the ocular tissue of diabetic patient may explain the decreased retinal blood flow in patients with early DR.

The simultaneous alteration in retinal hemodynamics and increase in angiogenic cytokine expression found in this study is consistent with the hypothesis that hyperglycemia-induced activation of several activated pathways may mediate diabetes-induced retinal vascular dysfunction in diabetes. There is sufficient evidence in animal and human studies that show that hyperglycemia initiates the development of DR and increase the synthesis and activity angiogenic cytokines (Joussen et al. 2001) and ultimately lead to retinal vascular abnormalities. As shown in our multiple regression analysis, a significant determinant of aqueous FGF-1 and TGF-β1 levels is A1c. A1c is a well-known contributor to metabolic pathways involved in DR progression.(Stratton et al. 2001) Longer studies that will allow the development of retinal blood flow changes and evaluation of the role of cytokines and growth factors, as well as inflammatory factors, will be required to reach firm conclusions.

The present study has its limitations. One limitation is that the cytokines released in the aqueous humor may differ due to the underlying cataract condition. However, we attempted to mitigated this issue as we compared the level of cytokines amongst all patients who underwent the same cataract procedure. This reduced the likelihood of differences in cytokine levels between groups. Another limitation of this study included growth factors and cytokine and did not consist of soluble receptor analysis. Soluble receptors may play an important role in modulating cytokine activity. For instance, a soluble form of the FGFR1 has been found in the vitreous (Hanneken & Baird 1995) and binding of FGF1 and FGF2 may inhibit the biological activity, acting in an agonistic manner. Furthermore, our study was limited to a 4 week follow-up TRBF measurement period in which TRBF may have undergone fluctuations due to the surgical procedure. Measurement of retinal blood flow 4 weeks post-surgery would more accurately reflect the homeostatic condition of the retinal vasculature due to diabetes rather than change secondary to cataract surgery.

Overall, low TRBF was associated with low aqueous FGF-1 levels in patients with NPDR. Additionally, aqueous angiogenic factors (TGF β1, TGF β2 and PLGF) were elevated in conjunction with a reduction in TRBF in patients with NPDR. This study provides evidence of angiogenic factors are involved in early retinal blood flow abnormalities in DR and provides a link between aqueous biomarkers and retinal vascular disorders in diabetes. This study suggests that measurement of TRBF using FD-OCT may be useful for determining aqueous FGF-1 levels and severity of vascular dysfunction in early DR.

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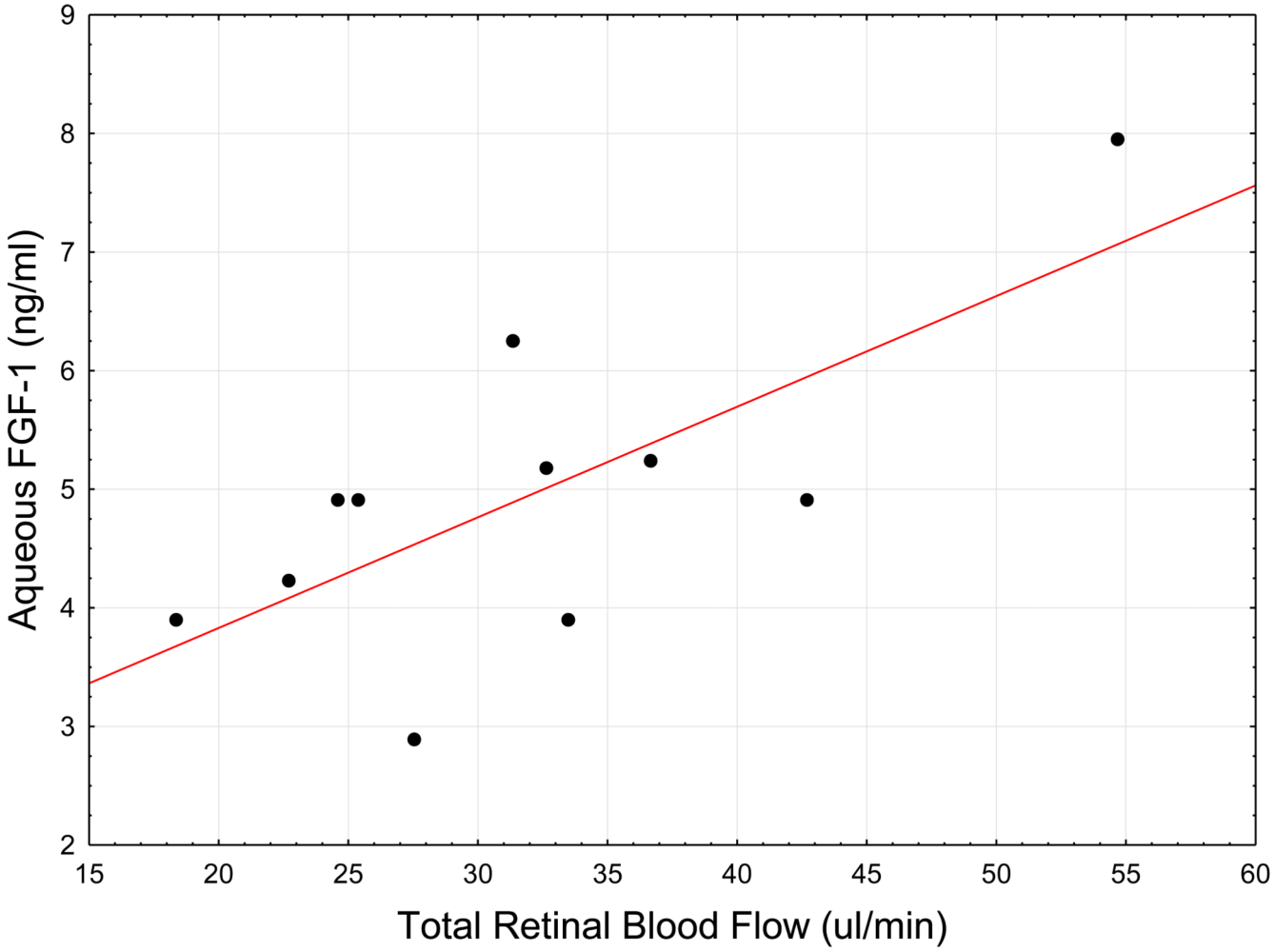

Figure 1.

Scatter-plot of corrected Total Retinal Blood Flow (TRBF) and Aqueous humor cytokines Fibroblast growth factor-1 (FGF-1) in NPDR subjects. The correlation between TRBF and FGF-1 was found to be r = 0.71, p = 0.01; r^2 = 0.51, FGF-1 = 1.9632+0.0933*x. Pearson Bivariate Correlation analysis was used to determine the relationship between aqueous humor cytokines

Table 1

Subject demographics of study population. Sample size per group (n), group mean age (standard deviation, SD), known duration of diabetes (years), systolic and diastolic blood pressure (BP), mean ocular perfusion pressure (MOPP), intraocular pressure (IOP) and glycosylated hemoglobin (A1c) as a function of group.

Variable	Control	NPDR	P-value
Sample size (n)	17	15	--
Age (years)	68.8 ± 6.35	67.5 ± 10.1	0.658
Duration (years)	0	12.0	--
Baseline Systolic (mmHg)	128.1± 17.2	134.5 ± 19.1	0.329
Baseline Diastolic (mmHg)	76.9 ± 9.03	76.9 ± 8.10	0.997
Heart rate (bpm)	76.7 ± 10.5	62.9 ± 18.4	0.029
MOPP (mmHg)	48.1 ± 5.10	51.1 ± 5.13	0.106
IOP(mmHg)	14.6 ± 2.03	14.9 ± 1.72	0.657
A1c (%)	5.49 ± 0.33	7.13 ± 0.74	<0.001 *

* Indicates significant different from controls, $p<0.05$.

Table 2

Corrected Total Retinal Blood Flow as a Function of Group. Doppler FD-OCT was used to extract information to calculate the volumetric blood flow using the RTVue Doppler OCT four weeks post-surgery. Total retinal blood flow as investigated in the healthy control group and patients with Type 2 diabetes and non-proliferative diabetic retinopathy.

	Control	NPDR	P-value
Sample	17	15	
Total Blood Flow (ul/min)	43.3±5.3	33.1±9.9	0.002*

** P < 0.05 compared to controls.

Table 3

List of Aqueous Humor Cytokines as a Function of Group. Aqueous humor cytokines included fibroblast growth factor (FGF-1 & FGF-2), angiopoietins (ANG-2), interleukin-8 (IL-8), leptin, stromal-derived factor-1, epidermal growth factor (EGF), transforming growth factor-beta (TGF-β1 and TGF-β2), granulocyte colony stimulating factor (G-CSF) platelet-derived growth factors (PDGFs), vascular endothelial growth factor (VEGF), erythropoietin, endothelin-1 (ET-1), insulin. The arrows indicate an increase (↑) or decrease (↓) in aqueous cytokine expression in comparison to healthy controls.

		Control	NPDR	P-Value	↑/↓
	N	**17**	**15**		
		ng/ml	**ng/ml**		
	Angiogenic				
1	ANG-2	12.8±5.7	21.3±10.9	0.012	↑
2	EGF	0.25±0.09	0.17±0.06	0.011	↓
3	FGF-1	7.49±1.67	4.93±1.33	<0.001*	↓
4	FGF-2	22.90±12.37	16.20±4.68	0.235	--
5	G-CSF	6.69±19.09	0.93±0.78	0.287	--
6	HGF	108.88±41.32	236.84±203.40	0.023	↑
7	IL-8	1.13±0.54	2.25±2.33	0.081	--
8	Leptin	32.23±28.56	54.09±35.99	0.091	--
9	PLGF	0.51±0.22	0.95±0.45	0.002*	↑
10	VEGF-A	73.1±36.7	81.5±35.4	0.529	--
11	TGFβ1	2.4±1.9	14.7±6.6	<0.001*	↑
12	TGFβ2	1977.8±607.1	4314.8±768.9	<0.001*	↑
	Vasogenic				
13	ET-1	2.3±0.8	3.1±1.3	0.051	--
14	Insulin	5.9±1.7	7.2±2.8	0.167	--

*Bonferroni corrected, P-value <0.003