Acta Ophthalmologica

Acta Ophthalmologica 2016

# Proteome analysis of retinal glia cells-related inflammatory cytokines in the aqueous humour of diabetic patients

Stela Vujosevic,[1] Alessandra Micera,[2] Silvia Bini,[1] Marianna Berton,[1] Graziana Esposito[2] and Edoardo Midena[1,2]

[1]Department of Ophthalmology, University of Padova, Padova, Italy
[2]Fondazione G. B. Bietti, IRCCS, Roma, Italy

**ABSTRACT.**

***Purpose:*** **Retinal glia cells (RGC) activation and release of inflammatory cytokines have been associated with development of diabetic retinopathy (DR). In this study, we evaluated by protein array the presence of aqueous humour (AH) cytokines secreted by RGC in patients with diabetes without DR and with mild DR.**

***Methods:*** **This is a cross-sectional, case–control study. Thirty-five subjects (diabetics and controls) underwent full ophthalmic examination and AH samples collection before cataract surgery at the Department of Ophthalmology University of Padova. AH samples were analysed for total protein concentration (Bradford method) and RGC-related inflammatory cytokines using glass chip protein arrays.**

***Results:*** **Twelve diabetic patients without DR, 11 diabetic patients with mild DR and 12 non-diabetic controls were included. There was no significant difference in total protein concentration among the 3 groups. Interleukin IL-1$\beta$, IL-3, interferon gamma (IFN-$\gamma$), (IFN-$\gamma$)-induced protein (IP)-10 and monocyte chemotactic protein (MCP)-2 were significantly increased in diabetics versus controls. IFN-$\gamma$, IL-1$\alpha$, IL-3 and MCP-2 were significantly increased in diabetics without DR versus controls, whereas granulocyte–macrophage colony-stimulating factor (GM-CSF), IFN-$\gamma$, IL-10, IP-10, regulated and normal T cell expressed and secreted (RANTES), and soluble tumour necrosis factor receptor (sTNF-R)II were significantly increased in diabetics with mild DR versus controls. Macrophage inflammatory protein (MIP-1$\beta$), GMCSF, RANTES and sTNF-RII were significantly increased in diabetics with mild DR versus diabetics without DR ($p < 0.05$ at least for all).**

***Conclusions:*** **Differences in expression profile of AH cytokines between diabetics, without and with mild DR, and controls have been documented. Retinal neuroinflammatory biomarkers of RGC activation evaluated in AH by protein array analysis could guide in detecting specific phenotypes with potential for personalized management.**

**Key words:** aqueous humour – diabetic retinopathy – inflammation – microglia – Muller cells – proteomic – retinal glia cells

Acta Ophthalmol. 2016: 94: 56–64


doi: 10.1111/aos.12812

## Introduction

Diabetic retinopathy (DR) is a frequent complication of diabetes mellitus (DM). Persistent hyperglycaemia leads to the activation of multiple cellular pathways involved in the pathogenesis of DR, resulting in the increase in inflammation, oxidative stress and vascular dysfunction. (Kern 2007; Tang & Kern 2011; Zong et al. 2011; El-Asrar 2012; Rangasamy et al. 2012) DR has been considered a 'chronic, low-grade inflammatory disease of the retina'. (Yang et al. 2009) One of the first signs of inflammation in DM is the activation of retinal glial cells (RGC). (Bringmann et al. 2009; Grigsby et al. 2014) RGC include microglia and macroglial cells (Müller cells and astrocytes). Activated RGC release cytotoxic substances responsible for recruitment of leucocytes, blood-retinal barriers breakdown, direct glial dysfunction and neuronal cell death. (Bringmann et al. 2009; Yang et al. 2009; Bringmann & Wiedemann 2012) An increasing body of scientific evidence documented that cytokines and chemokines (chemotactic cytokines) released by activated RGC include colony-stimulating factors: macrophage colony-stimulating factor (M-CSF) and granulocyte–macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-1 $\alpha/\beta$, IL-3, IL-6, IL-8, IL-10, IL-12, IL-15, IL-18, tumour necrosis factor (TNF-$\alpha$), interferon gamma (IFN-$\gamma$), interferon gamma (IFN-$\gamma$)-induced protein (IP)-10, transforming growth factor-$\beta$ (TGF-$\beta$),

vascular endothelial growth factor (VEGF), lymphotoxin, macrophage inflammatory protein 1 (MIP-1), MIP-2, MIP-3, monocyte chemotactic protein (MCP)-1, regulated and normal T cell expressed and secreted (RANTES), matrix metalloproteinases (MMPs) and other reactive oxygen species (glutamate, proteases, leukotrienes). (Martinez-Moczygemba & Huston 2003; Kremlev et al. 2004; Maurer & von Stebut 2004; Langmann 2007; Hanisch & Kettenmann 2007; Bringmann & Wiedemann 2012; Grigsby et al. 2014; Karlstetter et al. 2015).

Different studies have evaluated changes in concentrations of inflammatory and angiogenic cytokines in the aqueous humour (AH) or vitreous of patients with diabetes, focusing mostly on diabetic macular oedema or proliferative DR. (Funatsu et al. 2002, 2005a; Demircan et al. 2006; Funatsu et al. 2009; Adamiec-Mroczek et al. 2010; El-Asrar et al. 2011; Sohn et al. 2011) Changes in cytokine profile in the AH of diabetic patients with DR and/or macular oedema include proteins such as IL-6, VEGF, IL-12, IL-8, IP-10, MCP-1, platelet-derived growth factor (PDGF) and intracellular adhesion molecule type 1(ICAM-1) (Funatsu et al. 2002, 2005b; Oh et al. 2010; Sohn et al. 2011; Cheung et al. 2012; Chiang et al 2012; Gverovic et al. 2012; Jonas et al. 2012), while the profile of vitreous samples includes IL-6, TNF-$\alpha$, IL-1$\beta$, VEGF, ICAM-1 and MCP-1. (Funatsu et al. 2005a; Demircan et al. 2006; Funatsu et al. 2009; Adamiec-Mroczek et al. 2010; El-Asrar et al. 2011) Methods used for the determination of inflammatory markers in eye fluids include classical techniques (enzyme-linked immunosorbent assay: ELISA and Western blotting) (Rungger-Brandle et al. 2000; Funatsu et al. 2002, 2005a; Demircan et al. 2006; Murugeswari et al. 2008; Patel et al. 2008; Funatsu et al. 2009; Noma et al. 2009; Yang et al. 2009; Oh et al. 2010; El-Asrar et al. 2011; Ibrahim et al. 2011; Gverovic et al. 2012; Zhou et al. 2012) and newer, larger-scale methods such as multiplex bead assays and proteomic approaches using protein array chips. (Funatsu et al. 2005a; Grus et al. 2007; Sharma et al. 2009; Forooghian et al. 2010; Sohn et al. 2011) Reported herein are the results of a proteomic approach to analyse the specific cytokine profile of aqueous humour samples collected from diabetic patients without or with early DR and compared to non-diabetic subjects in order to evaluate early intraocular inflammatory biomarkers related to RGC activation.

# Materials and Methods

### Patients

Patients presenting for cataract surgery at the Department of Ophthalmology, University of Padova (from January to September 2012), were prospectively recruited in this study. The enrolled study population consisted of three groups: a control group of subjects without DM, and study groups of patients with type 2 diabetes mellitus (DM) with mild non-proliferative DR or without DR.

The major inclusion criterion was the indication for cataract surgery, and, in diabetic groups, a diagnosis of type 2 DM (according to the World Health Organization criteria: glycated haemoglobin (HbA1c)>6.5% in two occasions, or glycemia ≥126 mg/dl after at least 8 hr of fasting in two occasions, or glycemia ≥200 mg/dl after 2 hr from an oral glucose tolerance test to be confirmed by a fasting test, or casual glycemia ≥200 mg/dl in presence of typical symptoms (polyuria, polydipsia, weight loss) (American Diabetes Association, 2012) absence of DR or presence of mild non-proliferative DR. Exclusion criteria for all groups were as follows: presence of macular oedema; intraocular pressure ≥22 mmHg or a history of glaucoma; other ocular diseases of vascular, degenerative or inflammatory nature not related to DM; steroids, non-steroidal anti-inflammatory drugs, anti-VEGF treatment or any eye surgery in the 3 months before intervention; presence of a neurodegenerative disease (e.g. Alzheimer's, Parkinson's, dementia); in diabetics, presence of DR other than mild non-proliferative DR.

Informed consent was obtained from each patient, and the research was carried out in accordance with the Declaration of Helsinki regarding experimentation involving human tissue. Local Ethics Committee approval for the study was obtained.

### Study design

The study was open-label, cross-sectional, case–control series. Each subject underwent a complete ophthalmologic examination, including slit-lamp biomicroscopy, tonometry, ophthalmoscopic examination and colour photographs of the fundus with grading of DR on colour photographs according to internationally established criteria (Wilkinson et al. 2003) and spectral domain optical coherence tomography (OCT) examination of the studied eye in order to exclude the presence of macular oedema. The cut-off for presence of macular edema was central retina thickness ≥300 $\mu$m.

### Sample collection and preparation for analysis

All patients underwent standard preoperative preparation for phacoemulsification surgery and intraocular lens insertion: disinfection of periocular skin with povidone-iodine 10% (ESOJOD, ECOLAB, Agrate Brianza, Italia), instillation of sterile lidocaine 4% (Alphaintes, Napoli, Italia), irrigation of the conjunctival sac with povidone-iodine 5% (Oftasteril, Alphaintes) and abundant washing out of the eye with balanced salt solution.

Aqueous humour (150–200 $\mu$l) was aspirated from the anterior chamber of the eye, via a paracentesis under microscope using a 30-gauge needle and an insulin syringe (1 ml), and avoiding any contact with the intraocular tissues. Immediately after the aspiration of AH, the first operator carried out the subsequent surgical procedure, while a second operator handled the sample.

The collected sample was divided into two 50 $\mu$l of aliquots which were placed into 1.7 ml numbered conical test-tubes and stored at a temperature of −20°C. Each of these contained 50 $\mu$l of modified RIPA buffer (25 mM Tris buffer, 150 mM NaCl, 0.1% Tween-20, 1 mM EDTA, 0.1% SDS, 10 mM NaF, 1 mM PMSF, pH 7.5) and to which 50 $\mu$l T-protein extraction reagent (T-PER) supplemented with a cocktail of protease inhibitors (code #0078510; Pierce Biotechnology, Rockford, IL, USA) was added. The excess material was placed into a third test-tube with 3uL of protease inhibitors.

After gently mixing, the tubes were labelled with 'PT: ID code' (patient: identification code of the study subject). Within an hour from collection, the samples were stored at −70°C. After sample collection was complete,

the two sample sets (RIPA and T-PER preparations) were shipped to the G.B. Bietti Foundation Lab for planned analysis.

#### Quantitative determination of total protein

Quantitative determination of proteins allowed the evaluation of the potential variations in total protein concentrations among and within different groups, and the normalization of the samples before proteomic analyses.

The AH samples were prepared for RIPA and T-PER sampling as described above. For RIPA sampling, quantification of total protein was performed using the Bradford method. In brief, 2 $\mu$l of AH were diluted with 18 $\mu$l of double-distilled water (DDW; water DirectQ5; millipore.com) and mixed with 200 $\mu$l of a prediluted solution (code 500-0006; BioRad, Milan, Italy). After incubating for 5 minutes at room temperature, 3 $\mu$l of each sample and of a reference standard curve (0–1000 $\mu$g/ml BSA; Sigma-Aldrich, Milan, Italy) was read by a digital spectrophotometer with the appropriate Bradford option (Nanodrop ND1000 UV–Vis Spectrophotometer; Thermo Scientific, Wilmington, DE, USA). The protein concentration was calculated based on the linearized standard curve provided by the programme.

For T-PER sampling, 3 $\mu$l samples were directly evaluated by digital spectrophotometer the A280 option with comparison to a standard (IgG), as provided by the manufacturers. Both evaluations were carried out after blank options, both against DDW and the appropriate buffer.

After the analyses of total protein, and before the subsequent protein analyses, the AH underwent sonification (VibraCell, Sonics, CT, USA) before being clarified by centrifugation to separate cellular proteins from DNA and RNA. Data were used to evaluate possible variations in total protein content among groups and to normalize the samples before loading in the specific assay.

#### Inflammatory profile analysis with protein array

The protein array was conducted using the RayBiotech technology established by the manufacturer (Norcross, GA, USA). Glass chips were used in this study. Normalization, positive/negative and internal controls were carried out according to the manufacturer's instructions.

#### Chip-based arrays

Array chips were incubated with prediluted AH samples and labelled with a biotin-conjugated cocktail of antibodies, followed by a cy3-conjugated streptavidin complex, according to the manufacturer's instructions. Both diabetics and control groups were processed in parallel. After an overnight incubation at 4°C, the array slides were washed and exposed to a biotinylated antibody mixture followed by a cy3-streptavidin labelling solution. All steps were performed under orbital shaking (Certomat II, Sartorius AG), and all the hybridization/washing solutions were provided by the kit. As a final point, the glass-slides were washed once with MilliQ water, spin-dried and acquired with a GenePix 4400 Microarray scanner (Molecular Devices LLC, Sunnyvale, Silicon Valley, CA, USA). To obtain appropriate Cy5 (background signal) and Cy3 (specific signal) images, the slides were scanned over previously validated acquisition parameters and the images/arrays (blocks) were uniformly adjusted for size, brightness and contrast at the moment of acquisition. Using the SPOT tool, the specific area (corresponding to each cytokine on the array) was manually spotted and automatically adjusted, according to prefixed acquisition parameters applied to all glass-slides of the study. The fluorescence intensity data (FI) of each spot were calculated by the GENEPIX PRO 6.0 pro software (Molecular Devices) that provides background-subtracted FI data (F532-B532, N factor) as of a value for spot volume, representing the product of the area and the highest pixel value contained in that area. The fluorescence signals were acquired with the GenePix 4100 microarray scanner (Molecular Devices LLC, Sunnyvale, CA, USA) equipped with the GENEPIX PRO 3.0 software (Axon Instruments, Foster City, CA, USA), and expressed as a ratio (pathological/reference signal).

#### Array data analysis

Fluorescence signals were analysed automatically by the GenePix 4100 microarray scanner. Normalization was carried out using the GENEPIX software according to the manufacturer's instructions, and fluorescence signals were analysed and compared by NIH and STATVIEW softwares. In the array approach, all comet tails were ignored and only median signal values obtained using the same setting were used for the identification of any biomarker variation. An inter- and intra-assay coefficient of variability limit of $\leq$10% was set for the study, and a $\geq$1.5-fold increase or $\leq$0.65-fold decrease in signal intensity was considered to guarantee specific signals above background. In order to minimize intra- and interassay variability, a single tester handled all the material and followed all the phases of the experiment.

#### Statistical analysis

Results were reported as mean $\pm$ standard deviation. The difference in mean protein concentration among the 3 groups was compared using an analysis of variance (ANOVA) test. The difference in cytokine concentration, expressed in terms of intensity of the fluorescence signal, among groups was determined using ANOVA. In order to control the number of false-positive results (false discovery rate-FDR) due to multiple testing, Benjamini–Hochberg procedure was applied. (Benjamini & Hochberg 1995) We considered up to 10% of significant results being false positives. Tukey–Kramer post hoc analysis was applied to significant results after Benjamini–Hochberg procedure. Firstly, all diabetics were compared to controls. Secondly, diabetics were subdivided in those without DR and with DR and separately compared to controls. Volcano plots were used to show logarithm of fold changes versus negative logarithm of statistical significance (p-values) of cytokines' expression in AH of diabetics (all diabetics, diabetics without DR and diabetics with DR) versus controls and diabetics with DR versus diabetics without DR. (Cui & Churchill 2003).

## Results

#### Patient cohort and baseline characteristics

Thirty-five subjects were included in the study, and 35 eyes were evaluated. The study population consisted of 12

subjects without DM and 23 patients with DM. In the DM group, there were 12 patients with no DR and 11 patients with mild non-proliferative DR. The average age of the cohort ranged from 62 to 75 years. All patients with diabetes had DM type 2 and the duration of diabetes ranged from 7.7 to 21.8 years (Table 1). Mean HbA1c was 7.8 ± 0.6%. There was a significant difference in DM duration between diabetics with DR and those without DR (17.6 ± 10.8 years versus 7.7 ± 5.1 years, $p < 0.05$) (Table 1). There were no significant differences in age ($p = 0.21$, ANOVA test) and baseline intraocular pressure ($p = 0.23$, ANOVA test) among the 3 groups. (Table 1). There were no significant differences in mean blood pressure among the three groups ($p = 0.33$, ANOVA test).

### Protein concentration

Total protein concentration in the samples was not different among groups. There was no significant difference in mean protein concentration among the controls (0.44 ± 0.07 mg/ml), diabetics without DR (0.45 ± 0.10 mg/ml) and diabetics with DR (0.43 ± 0.05 mg/ml), $p = 0.83$ (ANOVA test).

### Array signal intensities

The intensity of the fluorescence signal was significantly greater in diabetics (without and with DR) versus controls for the following cytokines: IFN-γ ($p < 0.001$); IL-1$\beta$ ($p = 0.03$); IL-3 ($p = 0.02$); IL-10 ($p = 0.02$); IP-10 ($p = 0.009$); MCP-2 ($p = 0.02$); and borderline significance for TNF-$\alpha$ ($p = 0.04$) (ANOVA followed by Benjamini–Hochberg procedure for multiple testing) (Table 2, Fig. 1A).

In diabetic patients without DR, the intensity of the fluorescence signal was significantly greater versus controls for the following cytokines: IFN-γ ($p = 0.01$), IL-1$\alpha$ ($p = 0.03$), IL-3 ($p = 0.002$) and MCP-2 ($p = 0.01$). There was a borderline statistical significance for IL-1$\beta$ ($p = 0.05$) versus controls. The intensity of macrophage inflammatory protein (MIP)-1$\beta$ ($p = 0.04$) signal was significantly decreased in these patients compared to controls (ANOVA followed by Benjamini–Hochberg procedure for multiple testing and Tukey–Kramer post hoc test for multiple comparisons) (Table 3; Fig. 1B).

The intensity of the signal was significantly greater in diabetic patients with mild DR compared to controls for granulocyte–macrophage colony-stimulating factor (GM-CSF) ($p = 0.01$), IFN-γ ($p < 0.001$) IL-10 ($p = 0.03$), IP-10 ($p = 0.03$), regulated and normal T cell expressed and secreted (RANTES) ($p = 0.03$), and soluble tumour necrosis factor receptor (sTNF-R)II ($p = 0.01$) (ANOVA followed by Benjamini–Hochberg procedure for multiple testing and Tukey–Kramer post hoc test for multiple comparisons) (Table 3; Fig. 1C).

When diabetic patients with and without DR were compared, the intensity of the signal was significantly greater in patients with DR compared to patients without DR for MIP-1$\beta$ ($p < 0.001$), GMCSF ($p = 0.03$), RANTES ($p = 0.03$) and sTNF-RII ($p = 0.04$); IL-3 was significantly reduced in patients with DR compared to those without DR ($p = 0.03$) (ANOVA followed by Benjamini–Hochberg procedure for multiple testing and Tukey–Kramer post hoc test for multiple comparisons) (Fig. 1D).

**Table 1.** Baseline patient characteristics.

| Parameters | Controls (N = 12) | Diabetics without DR (N = 12) | Diabetics with DR (N = 11) |
|---|---|---|---|
| Mean age ± SD (years) | 75.4 ± 6.2 | 73.9 ± 9.0 | 68.9 ± 11.4 |
| Sex (M:F) | 5:7 | 4:8 | 6:5 |
| Mean duration ± SD DM (years) | n.a. | 7.7 ± 5.1 | 17.6 ± 10.8 |
| DM treatment | | | |
| Oral hypoglycaemics + insulin | n.a. | 1 | 2 |
| Oral hypoglycaemics | n.a. | 8 | 2 |
| Insulin | n.a. | 0 | 6 |
| Diet | n.a. | 2 | 1 |
| No treatment | n.a. | 1 | 0 |
| Mean intraocular pressure±SD (mmHg) | 16.3 ± 2.3 | 17.5 ± 2.4 | 15.8 ± 2.5 |

Mean ± standard deviation; DM, diabetes mellitus; DR, diabetic retinopathy; F, female; M, male; NA, not applicable; T2DM, type 2 diabetes mellitus.

# Discussion

In this study, we evaluated, by proteome analysis, inflammatory cytokines related to RGC activation in the aqueous humour of diabetic patients without DR or with mild DR (and compared to non-diabetic subjects). The evaluated biomarkers include IL-1$\alpha$, IL-1$\beta$, IL-3, IL-6, IL-8, IL-10, GM-CSF, IFN-γ, IP-10, RANTES, MCP-1, MCP-2, MIP-1 chemokines and TNF-$\alpha$. (Hanisch & Kettenmann 2007; Langmann 2007; Bringmann & Wiedemann 2012; Grigsby et al. 2014) A general increase in RGC-related inflammatory cytokines in the AH was detected in diabetics without and with mild DR versus non-diabetic subjects. In particular, in diabetics without DR, IFN-γ, IL-1$\alpha$, IL-3 and MCP-2 were significantly increased compared to non-diabetic controls. IFN-γ was approximately threefold increased and MCP-2 more than 10-fold increased. In diabetics with mild DR, IFN-γ was increased when compared to non-diabetic subjects, similarly as in diabetics without DR. IP-10 was approximately threefold increased and IL-10 approximately fivefold increased, only in diabetics with mild DR versus non-diabetic subjects, whereas RANTES, GM-CSF and sTNF-RII were increased only in the DR group compared to both non-diabetic subjects and diabetics without DR. MIP-1$\beta$ was significantly increased, whereas IL-3 was significantly decreased in patients with DR versus diabetic patients without DR. The significance of above-mentioned inflammatory biomarkers in DM was previously reported in the literature. (Rothwell & Luheshi 2000; Yoshida et al. 2003; Kowluru & Odenbach 2004; Krady et al. 2005; Cardona et al. 2006; Demircan et al. 2006; Aveleira et al. 2010; Oh et al. 2010; Pereira Tde et al. 2010; Zhang et al. 2011; Cheung et al. 2012; Liu et al. 2012; Garcia et al. 2013; Grigsby et al. 2014) IL-1$\beta$ is

**Table 2.** Fluorescence intensities as determined by glass chip array analysis in diabetics versus controls.

| | Controls (N = 12) | | Diabetics (N = 23) | | ANOVA |
|---|---|---|---|---|---|
| Cytokine | Mean | SD | Mean | SD | p-value* |
| GMCSF | 128.3 | 245.0 | 580.3 | 862.8 | 0.09 |
| **IFN-γ** | 154.8 | 324.0 | 567.0 | 260.3 | **< 0.001** |
| IL-1α | 310.0 | 302.6 | 636.3 | 609.5 | 0.09 |
| **IL-1β** | 199.8 | 385.7 | 767.7 | 808.3 | **0.03** |
| **IL-3** | 880.8 | 180.6 | 1155.2 | 352.2 | **0.02** |
| IL-6 | 894.2 | 625.3 | 932.3 | 570.1 | 0.86 |
| IL-6R | 323.2 | 398.7 | 540.1 | 478.8 | 0.19 |
| IL-8 | 764.8 | 563.8 | 938.0 | 436.1 | 0.32 |
| **IL-10** | 135.0 | 273.8 | 474.0 | 452.1 | **0.02** |
| IL-12p40 | 338.0 | 427.5 | 192.3 | 470.7 | 0.37 |
| IL-12p70 | 226.2 | 352.8 | 93.5 | 255.9 | 0.21 |
| IL-15 | 863.3 | 342.6 | 958.8 | 505.8 | 0.56 |
| **IP-10** | 676.5 | 529.5 | 1534.6 | 995.4 | **< 0.01** |
| MCP-1 | 6464.3 | 3517.0 | 7189.1 | 4923.0 | 0.65 |
| **MCP-2** | 30.5 | 338.1 | 328.8 | 360.3 | **0.02** |
| MCSF | 161.1 | 58.2 | 360.6 | 421.8 | 0.11 |
| MIP-1α | 100.5 | 277.0 | 326.3 | 812.5 | 0.36 |
| MIP-1β | 644.5 | 233.0 | 595.0 | 370.0 | 0.68 |
| MIP-1δ | 361.8 | 439.0 | 421.9 | 503.7 | 0.73 |
| RANTES | 76.2 | 135.7 | 309.2 | 591.1 | 0.19 |
| TGF-β1 | 514.7 | 522.7 | 262.0 | 301.1 | 0.07 |
| TNF-α | 827.5 | 477.5 | 1258.9 | 616.3 | 0.04 |
| sTNF-RI | 283.5 | 221.0 | 403.2 | 461.6 | 0.40 |
| sTNF-RII | 160.3 | 371.3 | 481.3 | 560.2 | 0.08 |

GM-CSF, granulocyte–macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; IP-10, interferon gamma-induced protein 10; MCP, monocyte chemotactic protein; M-CSF, macrophage colony-stimulating factor; MIP, macrophage inflammatory protein; RANTES, regulated and normal T cell expressed and secreted; sTNF-R, soluble tumour necrosis factor receptor; TNF, tumour necrosis factor; SD, standard deviation. Bold characters indicate significant results.

* In bold character ANOVA p-value statistically significant after Benjamini–Hochberg procedure with false discovery rate (FDR) = 10%.

considered a trigger of the neuroinflammatory cascade in DM. ( Rothwell & Luheshi 2000; Liu et al. 2012) IL-1β and TNF-α increase caspase 3 activity, thus inducing endothelial cell damage. (Kowluru & Odenbach 2004; Aveleira et al. 2010; Zhang et al. 2011) Therefore, the release of inflammatory mediators and neurotransmitters may contribute to early neuronal cell death in the retina in DM. (Pereira Tde et al. 2010) MCP-2 (in this study increased even in diabetics without clinical signs of DR) is a monocyte chemotactic factor and higher levels of this chemokine suggest an early inflammatory response. (Oh et al. 2010) MIP-1, IL-1 and IL-3 have a role in angiogenesis as established in experimental ischaemic mouse models. (Yoshida et al. 2003; Krady et al. 2005; Demircan et al. 2006; Grigsby et al. 2014) However, in humans, plasma level of IL-3 was not associated with DR. (Hang et al. 2014) GM-CSF is an important regulator of macrophage, granulocyte and dendritic cell behaviour and function. (Cheung et al. 2012) A decrease in a specific chemokine fractalkine, secreted by RGC, determines neurotoxicity and an increase in inflammatory IL-1β and IFN-ɣ in DR, thus inducing a modulation of RGC function and morphological abnormalities. (Cardona et al. 2006; Garcia et al. 2013).

The analysis of ocular cytokine profile of diabetic patients typically occurs via the sampling of vitreous or aqueous humour from the eye. (Funatsu et al. 2001, 2005a,b; Demircan et al. 2006; Patel et al. 2006; Maier et al. 2008; Funatsu et al. 2009; Adamiec-Mroczek et al. 2010; Oh et al. 2010; El-Asrar et al. 2011; Sohn et al. 2011; Cheung et al. 2012; Gverovic et al. 2012; Jonas et al. 2012; Zhou et al. 2012) Nevertheless, most of the studies investigated subjects who had advanced stages of DR, in many cases already in the proliferative form or associated with DME. (Funatsu et al. 2002, 2005a,b; Demircan et al. 2006; Patel et al. 2006; Funatsu et al. 2009; Adamiec-Mroczek et al. 2010; Oh et al. 2010; El-Asrar et al. 2011; Sohn et al. 2011; Jonas et al. 2012; Zhou et al. 2012) This may partially explain differences with our results because we have evaluated patients in earlier stages of DR.

Although some evidence suggests that proteins in vitreous samples may be different to those in aqueous samples (Ecker et al. 2011), Funatsu et al. found that levels of VEGF and IL-6 were highly correlated between AH and vitreous and also to the severity of DR. (Funatsu et al. 2005b) Recently, there has been an increased interest in the AH cytokines determination. (Funatsu et al. 2002, 2005b; Oh et al. 2010; Sohn et al. 2011; Cheung et al. 2012; Gverovic et al. 2012; Jonas et al. 2012) The cytokine profile in AH samples from patients with diabetes in other studies is variable, although this may be partly due to differing limits of detection of the various methods used for protein analysis. (Cheung et al. 2012) Different studies reported that IL-6 is commonly increased in the AH of patients with DM, irrespective of the presence of DR. (Funatsu et al. 2002; Funatsu et al. 2005b; Cheung et al. 2012; Jonas et al. 2012) Data about TNF-α are controversial, as some reported increase (Hanisch & Kettenmann 2007; Grigsby et al. 2014), while Cheung et al. reported decrease in TNF-α in diabetics with DR versus controls. (Cheung et al. 2012) In diabetics with DR, commonly increased inflammatory mediators include IP-10, MCP-1, IL-8, ICAM-1 and TGF-β. (Oh et al. 2010; Sohn et al. 2011; Jonas et al. 2012) In diabetic patients with DR versus non-diabetic controls, IL-10, IL-12 and IFN-ɣ were found to be decreased. (Cheung et al. 2012) The results from this study (and others) show that investigation of inflammatory processes in the diabetic eye using AH samples is a feasible approach, and confirmation of the robustness of aqueous sampling in terms of achieved results would be important as less invasive then vitreous sampling. Although both AH and vitreous sampling are invasive techniques, AH sampling has a very limited risk to the intraocular tissues. As consequence, AH sampling may be justified to be

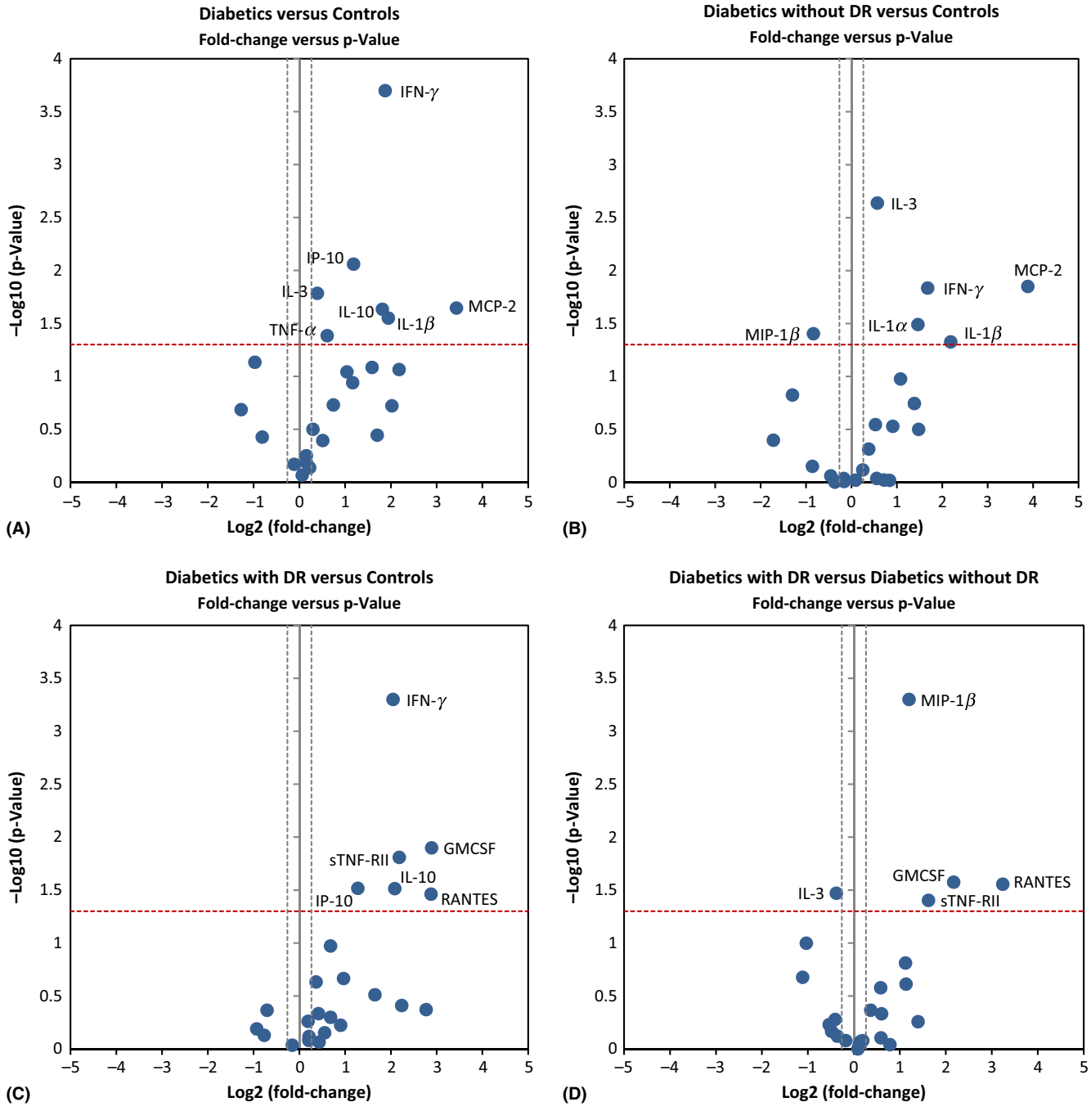


**Fig. 1.** Volcano plots showing logarithm of fold changes of cytokine expression in the aqueous humour versus negative logarithm of statistical significance (p-values) in patients with (A), diabetes mellitus (both without and with diabetic retinopathy) versus controls; (B), diabetes mellitus and no diabetic retinopathy versus controls; (C), diabetes mellitus and diabetic retinopathy versus controls; and (D), diabetes mellitus and diabetic retinopathy versus diabetes mellitus without diabetic retinopathy.

used to characterize the intraocular phenotype in diabetic eyes.

Histopathological and morphological studies evidenced RGC involvement (in particular microglia) in animal models and humans with DR. In a rat model of DM, RGC density was increased, and changes in cellular morphology indicated that the microglia was activated. (Zeng et al. 2000) In human histopathological and clinical studies, an increase in number of microglia cells (or cells aggregate) in the retina was reported. Zeng et al. showed on histopathology a moderate increase in number of perivascular microglia cells with hypertrophy in all retinal layers in background DR. (Zeng et al. 2008) Vujosevic et al. showed, in a clinical study using SD-OCT, that eyes with DM had an increased number of hyperreflective spots compared to controls, in inner and outer retina, and this number increased as DR progressed. The hyperreflective spots were believed to correspond to aggregates of activated microglia which migrate from inner to outer retina. (Vujosevic et al. 2013) Moreover, a significant increase in the thickness of the inner nuclear layer, mostly composed by the nuclei of bipolar and Müller cells (with consequent activation

**Table 3.** Fluorescence intensities as determined by glass chip array analysis in diabetics without and with diabetic retinopathy.

| | Controls (N = 12) | | Diabetics – DR (N = 12) | | Diabetics +DR (N = 11) | | ANOVA |
|---|---|---|---|---|---|---|---|
| Cytokine | Mean | SD | Mean | SD | Mean | SD | p-value† |
| **GMCSF** | **128.3** | 245.0 | 210.8 | 382.7 | **949.7***,# | 1055.0 | **< 0.01** |
| **IFN-γ** | **154.8** | 324.0 | **495.3*** | 194.9 | **638.8**** | 304.1 | **< 0.001** |
| **IL-1α** | **310.0** | 302.6 | **855.2*** | 739.8 | 417.5 | 353.4 | **0.03** |
| **IL-1β** | **199.8** | 385.7 | **909.0*** | 894.9 | 626.4 | 722.3 | 0.06 |
| **IL-3** | **880.8** | 180.6 | **1307.3**** | 361.0 | **1003.1**# | 280.3 | **< 0.01** |
| IL-6 | 894.2 | 625.3 | 1063.0 | 637.9 | 801.6 | 485.0 | 0.55 |
| IL-6R | 323.2 | 398.7 | 607.0 | 426.5 | 473.3 | 536.4 | 0.33 |
| IL-8 | 764.8 | 563.8 | 995.2 | 540.0 | 880.8 | 314.6 | 0.52 |
| **IL-10** | **135.0** | 273.8 | 376.3 | 301.6 | **571.6*** | 561.9 | **0.04** |
| IL-12p40 | 338.0 | 427.5 | 186.3 | 428.8 | 198.3 | 528.6 | 0.68 |
| IL-12p70 | 226.2 | 352.8 | 68.7 | 254.0 | 118.4 | 266.5 | 0.42 |
| IL-15 | 863.3 | 342.6 | 920.2 | 499.5 | 997.4 | 531.2 | 0.78 |
| **IP-10** | **676.5** | 529.5 | 1431.5 | 804.5 | **1637.8*** | 1184.0 | **0.03** |
| MCP-1 | 6464.3 | 3517.0 | 5755.5 | 3234.0 | 8622.7 | 5978.0 | 0.27 |
| **MCP-2** | **30.5** | 338.1 | **449.8*** | 255.6 | 207.8 | 417.3 | **0.02** |
| MCSF | 161.1 | 58.2 | 420.7 | 502.0 | 300.6 | 334.8 | 0.21 |
| MIP-1α | 100.5 | 277.0 | 179.7 | 364.1 | 473.0 | 1096.0 | 0.38 |
| **MIP-1β** | **644.5** | 233.0 | **360.8*** | 378.9 | **829.2**### | 151.9 | **< 0.001** |
| MIP-1δ | 361.8 | 439.0 | 264.0 | 437.8 | 579.8 | 533.3 | 0.26 |
| **RANTES** | **76.2** | 135.7 | 59.2 | 280.1 | **559.2***,# | 718.1 | **0.01** |
| TGF-β1 | 514.7 | 522.7 | 209.2 | 285.0 | 314.9 | 319.9 | 0.17 |
| TNF-α | 827.5 | 477.5 | 1193.5 | 697.0 | 1324.3 | 546.9 | 0.11 |
| sTNF-RI | 283.5 | 221.0 | 253.7 | 381.5 | 552.8 | 501.1 | 0.13 |
| **sTNF-RII** | **160.3** | 371.3 | 235.8 | 209.1 | **726.8***,# | 693.5 | **0.01** |

DR, diabetic retinopathy; Diabetics-DR, diabetics without DR; Diabetics+DR, diabetics with DR; GM-CSF, granulocyte–macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; IP-10, interferon gamma-induced protein 10; MCP, monocyte chemotactic protein; M-CSF, macrophage colony-stimulating factor; MIP, macrophage inflammatory protein; RANTES, regulated and normal T cell expressed and secreted; sTNF-R, soluble tumour necrosis factor receptor; TNF, tumour necrosis factor. Bold characters indicate significant results.
*$p < 0.05$; **$p < 0.01$ versus controls. #$p < 0.05$; ###$p < 0.001$ versus Diabetics – DR.
† In bold character ANOVA p-value statistically significant after Benjamini–Hochberg procedure with FDR = 10%.

due to hypertrophy of Müller cells), was described in patients with non-proliferative DR. (Vujosevic & Midena 2013) Further clinical investigation into the role of RGC in DR is therefore warranted.

In this study, a significant increase in the cytokines produced by the RGC would confirm the key role of these cells, not only in the presence of microvascular lesions, but also in the preclinical stage of DR. For this reason, RGC activation would entail the triggering of the inflammatory process responsible for retinal damage, both vascular and nervous, confirming again the central role of these cells in the pathogenic process of DR.

A strength of the present study therefore lies in its search for inflammatory biomarkers of RGC origin in the AH from the earliest stages of DM. Moreover, the fact that there was no significant difference in total protein concentration in the AH among all three examined groups suggests that the increase in RGC-specific proteins depends on posterior–anterior flow of these proteins from the posterior (where they are released) to the anterior chamber. (Inoue et al. 1994) One of the major limitations of this study is the relatively small number of enrolled patients. Because of the small patient population, statistical significance was not reached (although absolute numbers were increased or decreased) for some cytokines, whose expression has already been shown to be altered in DR. These include increases in TNF-α and MIP-1α, and decrease in TGF-β, a potent neuroprotective factor even in diabetics without DR. (Bringmann et al. 2009).

Recently, there is a growing body of scientific evidence that activated retinal microglia cells show different phenotypes (with either inflammatory or anti-inflammatory response) potentially regulated by different gene expression. (Grigsby et al. 2014; Karlstetter et al. 2015) Treatments that target RGC may reduce the release of several pro-inflammatory cytokines, as well as suppress microglial activation, proliferation and migration, and may protect against microglia-mediated neurotoxicity. (Karlstetter et al. 2011; Scott et al. 2014; Karlstetter et al. 2015) In this way, a delay in onset or improvement of DR might be obtained.

In conclusion, the analysis of AH samples using a proteomic approach shows that patients with DM have an increase in RGC-related inflammatory profile when compared to normal subjects. Hyperglycaemia leads to early activation of RGC in the retina. These cells release specific mediators that can be detected in the AH. The small sample size of the present study requires confirmation in a larger series of patients. Notwithstanding, these differences in the expression profile might help in identifying new DR phenotypes, with potential for more personalized follow-up and treatment of diabetes-related retinal changes.

# References

Adamiec-Mroczek J, Oficjalska-Mlynczak J & Misiuk-Hojlo M (2010): Roles of endothe-

lin-1 and selected proinflammatory cytokines in the pathogenesis of proliferative diabetic retinopathy: analysis of vitreous samples. Cytokine **49**: 269–274.

American Diabetes Association (2012): Diagnosis and classification of diabetes mellitus. Diabetes Care **35**: S64–S71.

Aveleira CA, Lin CM, Abcouwer SF, Ambrósio AF & Antonetti DA (2010): TNF-α signals through PKCζ/NF-κB to alter the tight junction complex and increase retinal endothelial cell permeability. Diabetes **59**: 2872–2882.

Benjamini Y & Hochberg Y (1995): Controlling the false discovering rate: a practical and powerful approach to multiple testing. J Roy Stat Soc **57**: 289–300.

Bringmann A & Wiedemann P (2012): Müller glial cells in retinal disease. Ophthalmologica **227**: 1–19.

Bringmann A, Iandiev I, Pannicke T, Hollborn M, Wiedemann P, Osborne NN & Reichenbach A (2009): Cellular signaling and factors involved in Muller cell gliosis: neuroprotective and detrimental effects. Prog Retin Eye Res **28**: 423–451.

Cardona AE, Pioro EP, Sasse ME et al. (2006): Control of microglial neurotoxicity by the fractalkine receptor. Nat Neurosci **9**: 917–924.

Cheung CM, Vania M, Ang M, Chee SP & Li J (2012): Comparison of aqueous humor cytokine and chemokine levels in diabetic patients with and without retinopathy. Mol Vis **18**: 830–837.

Chiang SY, Tsai ML, Wang CY et al. (2012): Proteomic analysis and identification of aqueous humor proteins with a pathophysiological role in diabetic retinopathy. J Proteomics **75**: 2950–2959.

Cui X & Churchill GA (2003): Statistical tests for differential expression in cDNA microarray experiments. Genome Biol **4**: 210.

Demircan N, Safran BG, Soylu M, Ozcan AA & Sizmaz S (2006): Determination of vitreous interleukin-1 (IL-1) and tumour necrosis factor (TNF) levels in proliferative diabetic retinopathy. Eye **20**: 1366–1369.

Ecker SM, Hines JC, Pfahler SM & Glaser BM (2011): Aqueous cytokine and growth factor levels do not reliably reflect those levels found in the vitreous. Mol Vis **17**: 2856–2863.

El-Asrar AM (2012): Role of inflammation in the pathogenesis of diabetic retinopathy. Middle East Afr J Ophthalmol **19**: 70–74.

El-Asrar AM, Nawaz MI, Kangave D, Geboes K, Ola MS, Ahmad S & Al-Shabrawey M (2011): High-mobility group box-1 and biomarkers of inflammation in the vitreous from patients with proliferative diabetic retinopathy. Mol Vis **17**: 1829–1838.

Forooghian F, Kertes PJ, Eng KT, Agrón E & Chew EY (2010): Alterations in the intraocular cytokine milieu after intravitreal bevacizumab. Invest Ophthalmol Vis Sci **51**: 2388–2392.

Funatsu H, Yamashita H, Noma H, Mimura T, Yamashita T & Hori S (2002): Increased levels of vascular endothelial growth factor and interleukin-6 in the aqueous humor of diabetics with macular edema. Am J Ophthalmol **133**: 70–77.

Funatsu H, Yamashita H, Sakata K, Noma H, Mimura T, Suzuki M, Eguchi S & Hori S (2005a): Vitreous levels of vascular endothelial growth factor and intercellular adhesion molecule 1 are related to diabetic macular edema. Ophthalmology **112**: 806–816.

Funatsu H, Yamashita H, Noma H, Mimura T, Nakamura S, Sakata K & Hori S (2005b): Aqueous humor levels of cytokines are related to vitreous levels and progression of diabetic retinopathy in diabetic patients. Graefes Arch Clin Exp Ophthalmol **243**: 3–8.

Funatsu H, Noma H, Mimura T, Eguchi S & Hori S (2009): Association of vitreous inflammatory factors with diabetic macular edema. Ophthalmology **116**: 73–79.

Garcia JA, Pino PA, Mizutani M, Cardona SM, Charo IF, Ransohoff RM, Forsthuber TG & Cardona AE (2013): Regulation of adaptive immunity by the fractalkine receptor during autoimmune inflammation. J Immunol **191**: 1063–1072.

Grigsby JG, Cardona SM, Pouw CE, Muniz A, Mendiola AS, Tsin AT, Allen DM & Cardona AE (2014): The role of microglia in diabetic retinopathy. J Ophthalmol **2014**: 705783.

Grus FH, Joachim SC & Pfeiffer N (2007): Proteomics in ocular fluids. Proteomics Clin Appl **1**: 876–888.

Gverovic AA, Karaman K, Znaor L, Sapunar A, Buško V & Puzović V (2012): IL-12 concentrations in the aqueous humor and serum of diabetic retinopathy patients. Graefes Arch Clin Exp Ophthalmol **250**: 815–821.

Hang H, Yuan S, Yang Q, Yuan D & Liu Q (2014): Multiplex bead array assay of plasma cytokines in type 2 diabetes mellitus with diabetic retinopathy. Mol Vis **20**: 1137–1145.

Hanisch UK & Kettenmann H (2007): Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nat Neurosci **10**: 1387–1394.

Ibrahim AS, El-Remessy AB & Matragoon S (2011): Retinal microglial activation and inflammation induced by amadori-glycated albumin in a rat model of diabetes. Diabetes **60**: 1122–1133.

Inoue M, Azumi A, Shirabe H, Tsukahara Y & Yamamoto M (1994): Laser flare intensity in diabetics: correlation with retinopathy and aqueous protein concentration. Br J Ophthalmol **78**: 694–697.

Jonas JB, Jonas RA, Neumaier M & Findeisen P (2012): Cytokine concentration in aqueous humor of eyes with diabetic macular edema. Retina **32**: 2150–2157.

Karlstetter M, Lippe E, Walczak Y, Moehle C, Aslanidis A, Mirza M & Langmann T (2011): Curcumin is a potent modulator of microglial gene expression and migration. J Neuroinflammation **29**: 8–125.

Karlstetter M, Scholz R, Rutar M, Wong WT, Provis JM & Langmann T (2015): Retinal microglia: just bystander or target for therapy? Prog Retin Eye Res **45**: 30–57.

Kern TS (2007): Contributions of inflammatory processes to the development of the early stages of diabetic retinopathy. Exp Diabetes Res **95**: 103.

Kowluru RA & Odenbach S (2004): Role of interleukin-1beta in the pathogenesis of diabetic retinopathy. Br J Ophthalmol **88**: 1343–1347.

Krady JK, Basu A, Allen CM, Xu Y, LaNoue KF, Gardner TW & Levison SW (2005): Minocycline reduces proinflammatory cytokine expression, microglial activation, and caspase-3 activation in a rodent model of diabetic retinopathy. Diabetes **54**: 1559–1565.

Kremlev SG, Roberts RL & Palmer C (2004): Differential expression of chemokines and chemokine receptors during microglial activation and inhibition. J Neuroimmunol **149**: 1–9.

Langmann T (2007): Microglia activation in retinal degeneration. J Leukoc Biol **81**: 1345–1351.

Liu Y, Biarnés Costa M & Gerhardinger C (2012): IL-1β is upregulated in the diabetic retina and retinal vessels: cell-specific effect of high glucose and IL-1β autostimulation. PLoS ONE **7**: e36949.

Maier R, Weger M, Haller-Schober EM et al. (2008): Multiplex bead analysis of vitreous and serum concentrations of inflammatory and proangiogenic factors in diabetic patients. Mol Vis **14**: 637–643.

Martinez-Moczygemba M & Huston DP (2003): Biology of common beta receptor-signaling cytokines: IL-3, IL-5, and GM-CSF. J Allergy Clin Immunol **112**: 653–665.

Maurer M & von Stebut E (2004): Macrophage inflammatory protein-1. Int J Biochem Cell Biol **36**: 1882–1886.

Murugeswari P, Shukla D, Rajendran A, Kim R, Namperumalsamy P & Muthukkaruppan V (2008): Proinflammatory cytokines and angiogenic and anti-angiogenic factors in vitreous of patients with proliferative diabetic retinopathy and eales' disease. Retina **28**: 817–824.

Noma H, Funatsu H, Mimura T, Harino S & Hori S (2009): Vitreous levels of interleukin-6 and vascular endothelial growth factor in macular edema with central retinal vein occlusion. Ophthalmology **116**: 87–93.

Oh IK, Kim SW, Oh J, Lee TS & Huh K (2010): Inflammatory and angiogenic factors in the aqueous humor and the relationship to diabetic retinopathy. Curr Eye Res **35**: 1116–1127.

Patel JI, Tombran-Tink J, Hykin PG, Gregor ZJ & Cree IA (2006): Vitreous and aqueous concentrations of proangiogenic, antiangiogenic factors and other cytokines in diabetic retinopathy patients with macular edema: implications for structural differences in macular profiles. Exp Eye Res **82**: 798–806.

Patel JI, Saleh GM, Hykin PG, Gregor ZJ & Cree IA (2008): Concentration of

haemodynamic and inflammatory related cytokines in diabetic retinopathy. Eye (Lond) **22**: 223–228.

Pereira Tde O, da Costa GN, Santiago AR, Ambrósio AF & dos Santos PF (2010): High glucose enhances intracellular Ca2 + responses triggered by purinergic stimulation in retinal neurons and microglia. Brain Res **1316**: 129–138.

Rangasamy S, McGuire PG & Das A (2012): Diabetic retinopathy and inflammation: novel therapeutic targets. Middle East Afr J Ophthalmol **19**: 52–59.

Rothwell NJ & Luheshi GN (2000): Interleukin 1 in the brain: biology, pathology and therapeutic target. Trends Neurosci **23**: 618–625.

Rungger-Brandle E, Dosso AA & Leuenberger PM (2000): Glial reactivity, an early feature of diabetic retinopathy. Invest Ophthalmol Vis Sci **41**: 1971–1980.

Scott IU, Jackson GR, Quillen DA, Klein R, Liao J & Gardner TW (2014): Effect of doxycycline versus placebo on retinal function and diabetic retinopathy progression in mild to moderate nonproliferative diabetic retinopathy: a randomized proof-of-concept clinical trial. JAMA Ophthalmol **132**: 1137–1142.

Sharma RK, Rogojina AT & Chalam KV (2009): Multiplex immunoassay analysis of biomarkers in clinically accessible quantities of human aqueous humor. Mol Vis **15**: 60–69.

Sohn HJ, Han DH, Kim IT, Oh IK, Kim KH, Lee DY & Nam DH (2011): Changes in aqueous concentrations of various cytokines after intravitreal triamcinolone versus bevacizumab for diabetic macular edema. Am J Ophthalmol **152**: 686–694.

Tang J & Kern TS (2011): Inflammation in diabetic retinopathy. Prog Retin Eye Res **30**: 343–358.

Vujosevic S & Midena E (2013): Retinal layers changes in human preclinical and early clinical diabetic retinopathy support early retinal neuronal and Müller cells alterations. J Diabetes Res **2013**: 905058.

Vujosevic S, Bini S, Midena G, Berton M, Pilotto E & Midena E (2013): Hyperreflective intraretinal spots in diabetics without and with nonproliferative diabetic retinopathy: an *in vivo* study using spectral domain OCT. J Diabetes Res **2013**: 491835.

Wilkinson CP, Ferris FL 3rd, Klein RE et al. (2003): Proposed international clinical diabetic retinopathy and diabetic macular edema disease severity scales. Ophthalmology **110**: 1677–1682.

Yang LP, Sun H, Wu L, Guo XJ, Dou HL, Tso MO, Zhao L & Li SM (2009): Baicalein reduces inflammatory process in a rodent model of diabetic retinopathy. Invest Ophthalmol Vis Sci **50**: 2319–2327.

Yoshida S, Yoshida A, Ishibashi T, Elner SG & Elner VM (2003): Role of MCP-1 and MIP-1alpha in retinal neovascularization during postischemic inflammation in a mouse model of retinal neovascularization. J Leukoc Biol **73**: 137–144.

Zeng X, Ng Y & Ling E (2000): Neuronal and microglial response in the retina of streptozotocin-induced diabetic rats. Vis Neurosci **17**: 463–471.

Zeng HY, Green WR & Tso MO (2008): Microglial activation in human diabetic retinopathy. Arch Ophthalmol **126**: 227–232.

Zhang W, Liu H, Al-Shabrawey M, Caldwell RW & Caldwell RB (2011): Inflammation and diabetic retinal microvascular complications. J Cardiovasc Dis Res **2**: 96–103.

Zhou J, Wang S & Xia X (2012): Role of intravitreal inflammatory cytokines and angiogenic factors in proliferative diabetic retinopathy. Curr Eye Res **37**: 416–420.

Zong H, Ward M & Stitt AW (2011): AGEs, RAGE, and diabetic retinopathy. Curr Diab Rep **11**: 244–252.

Received on April 29th, 2015.
Accepted on June 23rd, 2015.

*Correspondence:*
Edoardo Midena MD, PhD
Department of Ophthalmology
University of Padova
Via Giustiniani 2, 35128 Padova
Italy
Tel: + 39498212110
Fax: + 39498755168
Email: edoardo.midena@unipd.it

This research, as GB Bietti Foundation is concerned, was supported by the Ministry of Health and Fondazione Roma. The Authors would like to thank Fabiano Cavarzeran ScD for statistical analyses of data.