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NEURORETINAL ACTIVATION IN DIABETES MELLITUS

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SUMMARY

Study premises: Diabetic retinopathy (DR), one of the leading causes of blindness in developed countries, represents the most common microvascular complication of diabetes mellitus. Recent studies have shown that the alteration of glial cells and the resulting loss of neuronal cells occur before the vascular lesions become clinically detectable.

Study purpose: The purpose of the study is to find the early biomarkers of glial activation in the aqueous humour of diabetic subjects, both in presence and the absence of clinically detectable DR signs.

Materials and methods: During cataract surgery, samples of the aqueous humour have been collected in 34 patients, divided as follows: 12 healthy subjects, 11 diabetic patients without diabetic retinopathy and 11 patients with the non proliferative diabetic retinopathy (5 without macular edema-ME and 6 with ME). Before intervention, every patient has undergone a complete ophthalmic examination and the spectral domain optical coherence tomography (SD-OCT) (Spectralis HRA+OCT, Heildeberg Engineering). In 34 samples, the quantification of total proteins has been carried out using the Bradford method, of GFAP, AQP1 and AQP4 using the ELISA test, and of 40 inflammatory cytokines using the protein array. In addition, the segmentation of the retinal layers has been carried out using the SD-OCT scans.

Results: Mean concentration of GFAP, AQP1 e AQP4 was significantly increased in diabetics versus controls ($p<0.05$). GFAP showed an approximate 0.8 fold increase, AQP1 1.1 fold increase, whereas AQP4 about 24 folds increase in diabetic patients versus controls. When we separately evaluated DR-no ME eyes vs DR-ME eyes, there was a significant decrease in GFAP, AQP1 e AQPR in DR-ME eyes versus DR-no ME eyes, (Tukey Kramer post hoc $p<0.05$). Following cytokines were increased in diabetic patients (with or without DR) compared to healthy subjects: GFAP, AQP1, AQP4, IFNy, IL-1a, IL-1b, IL-3, IL-4,

IL-10, IL-11, IL-17, TNF- α, TNF-ß, MCP1, MCP2, Eotaxin, Eotaxin 2, RANTES, sTNFRII, GM-CSF, IP-10, MIP1a, MIP1b. The mean macular thickness of RNFL was significantly higher in diabetic patients with DR and ME compared to diabetics without ME (both with and without DR) and healthy subjects, and the same significance was observed in the inner and the external rings and in the superior, inferior and temporal sectors. The mean thickness of RNFL was significantly reduced in diabetics with DR and without ME compared to healthy subjects.

Conclusions: In the aqueous humour of diabetic subjects, 23 different protein biomarkers of glial activation have been found already in the subclinical stage of DR. They can be used in future as risk markers which predict the onset of such microvascular complications and can be an extremely useful therapeutic target for its prevention and care.

RIASSUNTO

Presupposti dello studio: La retinopatia diabetica (RD), una delle principali cause di cecità nei paesi sviluppati, costituisce la più comune complicanza microvascolare del diabete mellito. Recenti studi hanno dimostrato che l'alterazione delle cellule gliali e la conseguente perdita di quelle neuronali si verificano prima che le lesioni vascolari siano clinicamente rilevabili.

Scopo dello studio: Lo scopo dello studio è quello di ricercare biomarkers precoci di attivazione gliale nell'umore acqueo di soggetti diabetici non solo in presenza di segni clinicamente rilevabili di RD, ma anche in loro assenza.

Materiali e metodi: In corso di intervento di cataratta, sono stati raccolti i campioni di umore acqueo di 34 pazienti così suddivisi: 12 soggetti sani, 11 pazienti diabetici senza retinopatia diabetica e 11 con retinopatia diabetica non proliferante (di cui 5 senza edema maculare e 6 con edema maculare-ME). Prima dell'intervento, tutti i pazienti sono stati sottoposti a visita oftalmologica completa e tomografia a coerenza ottica di tipo spectral domain (SD-OCT) (Spectralis HRA+OCT, Heildeberg Engineering). Nei 34 campioni è stata effettuata la quantificazione delle proteine totali con metodo Bradford, di GFAP, AQP1 ed AQP4 con test ELISA e di 40 citochine infiammatorie con protein array. E' stata, inoltre, effettuata la segmentazione degli strati retinici sulle scansioni SD-OCT.

Risultati: I valori medi delle concentrazioni di GFAP, AQP1 e AQP4 nell'umore acqueo sono risultati significativamente più elevati nei soggetti diabetici rispetto ai controlli sani ($p<0.05$). L'incremento di GFAP e' stato di circa 0.8 volte, di AQP1 di 1.1 volte e di AQP4 di circa 24 volte nei soggetti diabetici rispetto ai controlli. Le concentrazioni di GFAP, AQP1 e AQP4 sono risultate significativamente ridotte nei soggetti diabetici con ME rispetto ai diabetici senza ME, (Tukey Kramer post hoc, $p<0.05$). La concentrazione nell'umore acqueo, è risultata significativamente maggiore nei pazienti diabetici (con e senza RD) rispetto ai soggetti sani per le seguenti citochine: GFAP, AQP1, AQP4, IFNy, IL-

1a, IL-1b, IL-3, IL-4, IL-10, IL-11, IL-17, TNF- α, TNF-ß, MCP1, MCP2, Eotaxin, Eotaxin 2, RANTES, sTNFRII, GM-CSF, IP-10, MIP1a, MIP1b. Lo spessore maculare medio di RNFL è risultato significativamente maggiore nei pazienti diabetici con RD e ME rispetto ai diabetici senza ME (con e senza RD) ed ai soggetti sani; lo stesso rapporto è stato osservato negli anelli interno ed esterno e nei settori superiore, inferiore e temporale. Lo spessore maculare medio di RNFL è risultato significativamente ridotto nei diabetici con RD e senza ME rispetto ai soggetti sani.

Conclusioni: Sono stati riconosciuti nell'umore acqueo di soggetti diabetici 23 diversi biomarkers proteici di attivazione gliale presenti sin dallo stadio subclinico della RD. Questi potranno essere utilizzati in futuro come marcatori di rischio per l'insorgenza di tale complicanza microvascolare e costituire degli utili bersagli terapeutici per la sua prevenzione e cura.

ABSTRACT

Background: Diabetic retinopathy (DR), one of the leading causes of blindness in developed countries, is the major microvascular complication of diabetes mellitus. Recent studies have demonstrated that the alteration of glial cells and the consequent loss of retinal neuronal cells occur before the vascular lesions are clinically detectable.

Purpose: To find early biomarkers of glial activation in the aqueous humor (AH) of diabetic patients both in presence and in absence of clinically detectable signs ofDR.

Materials and methods: During cataract surgery, 34 patients' AH samples were collected as follows: 12 healthy subjects, 11 diabetic patients without DR and 11 diabetic patients with nonproliferative diabetic retinopathy (5 without macular edema-ME and 6 with ME). Before surgery, full ophthalmic examination and Spectral-Domain Optical Coherence Tomography (SD-OCT) (Spectralis HRA+OCT, Heildeberg Engineering) were performed in all eyes. The samples were analyzed for the quantification of total proteins by Bradford method, of GFAP, AQP1 and AQP4 by ELISA and of 40 inflammatory cytokines by protein array. Segmentation of retinal layers was also performed.

Results: Mean concentration of GFAP, AQP1 e AQP4 was significantly increased in diabetics versus controls (324.44±262.54 pg/µg vs 182.34±114.44 pg/µg for GFAP; 105.72±15.69 pg/µg vs 50.92±20.36 pg/µg for AQP1; and 852.03±103.24 pg/µg vs 33.58±21.20 pg/µg for AQP4, $p<0.05$). GFAP showed an approximate 0.8 fold increase, AQP1 1.1 fold increase, whereas AQP4 about 24 folds increase in diabetic patients versus controls. When we separately evaluated DR-no ME eyes vs DR-ME eyes, there was a significant decrease in GFAP, AQP1 e AQPR in DR-ME eyes versus DR-no ME eyes, (Tukey Kramer post hoc $p<0.05$). GFAP and AQP1 showed even a slight fold decrease versus controls. AQP4/AQP1 concentration showed weak and non significant correlation

(Tau=0.21, p=0.3) between these biomarkers, despite the trend in increase. Following cytokines were increased in diabetic patients (with or without DR) compared to healthy subjects: GFAP, AQP1, AQP4, IFNy, IL-1a, IL-1b, IL-3, IL-4, IL-10, IL-11, IL-17, TNF- α, TNF-ß, MCP1, MCP2, Eotaxin, Eotaxin 2, RANTES, sTNFRII, GM-CSF, IP-10, MIP1a, MIP1b. RNFL mean thickness was significantly higher in diabetic patients with DR and ME compared to diabetics without ME (both with and without DR) and healthy subjects (respectively 37.3 µm vs 24.3µm vs 26µm 5µm vs 26.8µm), and the same significance was observed in the inner (33.4µm vs 22.0µm vs 25.0µm 24.3µm) and the external (54.7µm vs 36.7µm 34.6µm 38.1µm) rings and in the superior (40.3µm vs. 26.1µm vs 29.2µm vs 29.5µm), inferior (44.3µm vs 27.2µm vs 29.1µm vs 30.1µm) and temporal (26.3µm vs 16.8µm vs 18.9µm vs 18.0µm) sectors. RNFL mean thickness was significantly reduced in diabetics with DR and without ME compared to healthy subjects.

Conclusions: 23 different biomarkers of glial activation have been recognized in the AH of diabetic patients even with subclinical DR. These proteins could be used in the future as risk markers of occurrence of DR and could provide useful therapeutic targets for its prevention and therapy.

INTRODUCTION

1.1. Impact of diabetes mellitus on global health

1.1.1. Epidemiology of diabetes mellitus

Diabetes and its complications constitute one of the major health problems in the economically developed countries, and the exponential growth of its prevalence in the recent years has prompted the experts to describe it as a 'global epidemic'.[1] Based on the data from 2010, its prevalence in the world is confirmed by approximately 284 million individuals with diabetes (about 6.4% of the global population), and the estimates show that in 2030 the number will rise to 439 million (about 7.7% of the global population); such increase will mainly concern the adult subjects in the developing countries, with an increase of 73% compared to the 20% increase in the developed countries.[2, 3]

The prevalence of diabetes rises with age, reaching the 15.8% in the population over 65 years, and 18.8% in those over 75 years of age, without significant differences between the two sexes. [4] The recent estimates of the WHO (World Health Organization), moreover, show that diabetes is responsible for 2% of world deaths, causing more than 1 million deaths a year (a value, however, which is considered an underestimate).[5] If we focus on the situation in Italy, the ISTAT (The National Institute for Statistics) has estimated in 2008 the existence of known diabetes in 2.9 million of individuals (about 4.8% of the population), with an increase in the future years in accordance with the global trends. Such prevalence results greater in the South and in the islands, with about 5.8% individuals afflicted, followed by the center with 5.3% and the North with 3.9%.[5] The 2008 estimates show that in the Veneto region there are about 230 thousand subjects with known diabetes and that there is an average annual increase of 4-5% of the total number of patients. [6]

1.2.1. Epidemiology of diabetic retinopathy

Diabetic retinopathy (DR), one of the most common and severe diabetic vascular complications, constitutes the first cause of visual impairment and blindness in the adult working-age population in the developed countries.[1] It afflicts about 93 million people in the world (about 2-3% of the global population) and is responsible for about 2.4 million cases of blindness. [7] In Europe, it afflicts about 3% of individuals, with greater prevalence among people aged 60 and over in France and Germany.[7, 8] Italy and the Veneto region conform to these values. [9]

According to the global estimates in 2012, 34.6% of diabetic patients present retinopathy, 7% of which in a proliferative stage, and 6.8% with macular edema. The prevalence is greater for Type 1 diabetes mellitus (DM) and the insulin-treated Type 2 DM, and rises with the duration and the values of HbA1c, arterial pressure and cholesterolemia. [7, 9]

While in patients with Type 1 DM the clinical symptoms of DR are present in 15-20% of the cases 5 years after the diagnosis, in 50-60% of the cases 10 years after the diagnosis, and virtually in all the patients 30 years after the diagnosis, in patients afflicted by Type 2 DM such symptoms are present in 15-30% at the moment of diagnosis, in 50-70% 10 years after the diagnosis and in 90% after 30 years. This can be explained by the fact that Type 2 DM is generally diagnosed much later compared to Type 1 DM , when its negative effects have had a longer period to act unobserved.[10]

In the last thirty years, however, the incidence and the progression risk of such complications during the course of DM have diminished from values superior to 90% to values inferior to 50%, thanks to stricter controls of the glycemia, the pressure and the cholesterolemia: recent American studies show that the

percentage of Type 2 diabetic patients who reach normative values for these three parameters has risen from 30% to 50% between the years 2000 and 2006. Thanks to all this, the percentage of diabetic patients confronted with proliferative retinopathy, macular edema and visual deficit has significantly diminished in time.[11]

Calculations show that if proliferative retinopathy is not treated, 50% of patients with retinal new-vessels are destined to become blind within 5 years, and 50% of patients with new-vessels of the optic disc become blind within 2 years.[11] The three greatest risk factors for the appearance and the progression of DR are hyperglycemia, hypertension and dyslipidemia; to which one can add the duration of diabetes, pregnancy, genetic factors, cigarette smoke, alcohol, obesity and anemia. Antonetti et al. have recently suggested an association, uncertain as it might be, with serum prolactin levels, adiponectin and homocysteine, nonalcoholic steatohepatitis and the nocturnal apnea syndrome.[11]

A singular aspect is represented by the fact that myopia, especially if high, appears to be a protection factor with respect to DR: the reduced blood flow in the myopic eye would, in fact, reduce the hematic extravasation of inflammatory molecules.[12]

Even though retinopathy is the main ocular diabetic complication, it is not the only one. There are numerous studies, in fact, which demonstrate that, among the diabetic population, there is an increase in cataract incidence and its fast progression; such concept is valid for the cortical and subcortical posterior forms, while the association with nuclear forms appears to be without foundation. [10, 13] In addition, there is an increase in the incidences of glaucoma, retinal hemorrhages, transient visual blurring and refraction defects.[14]

1.2. Retinal glial cells and the physiology of aqueous humour

1.2.1. Retinal glial cells

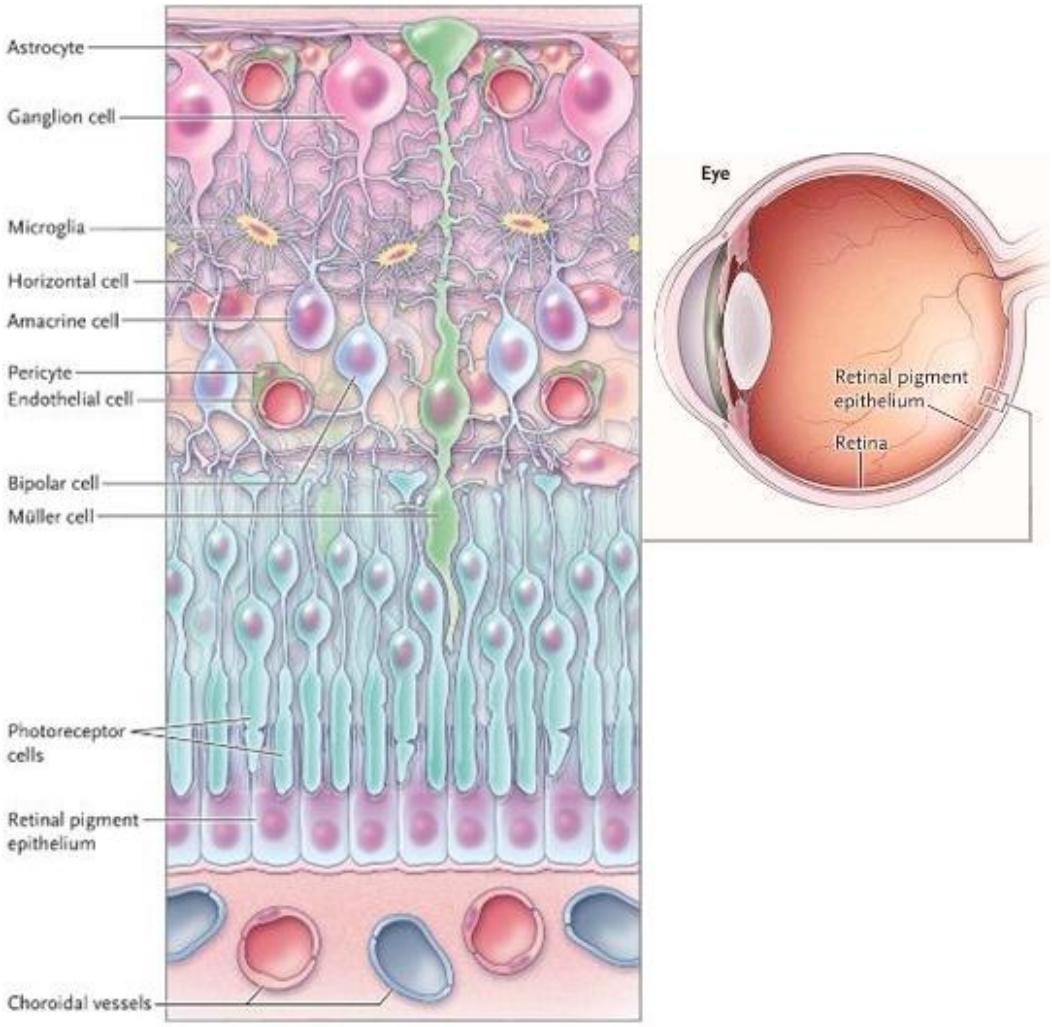


Figure 1. *Schematic representation of the most important retinal cell types* [11]

The retina, which, with the exception of the optic disc, covers the entire inner surface of the eyeball, is a membrane assigned to transform the light stimuli into bioelectric impulses. It is structurally divided into an outer wall, constituted by the retinal pigment epithelium, and an inner one, represented by a multilayered nervous tunic (the neuroretina).[15, 16]

The neuroretina is composed of five main types of neuronal cells (ganglion, bipolar, horizontal, amacrine and photoreceptor cells), glial cells (Müller cells,

astrocytes and microglia) and vascular cells (pericytes and endothelial cells).[16, 17]

The retinal glial cells comprise the macroglia, which in turn comprises the Müller cells and astrocytes, and the microglia. They function as neuronal structural support cells and represent connection elements between the neurons and the vascular cells, hence regulating neuronal nutrition, development and metabolism; they contribute, moreover, to the local inflammatory response. [17] Consequently, it is possible to understand how the glia, though it represents only one part of the retina, can influence the physiology of neurons and retinal vessels. Each cell underclass it includes, moreover, is markedly distinct from the others in distribution, morphology and physiology. [18]

Retinal blood vessels are surrounded in its entirety by the glial cells' processes: the deepest ones (located at the inner and the outer margins of the inner nuclear layer) are surrounded exclusively by the Müller cells, while the surface vessels (located at the interface between the vitreous humor and the retina, and in the ganglion cell layer) are surrounded both by Müller cells processes and the astrocytary ones. In both cases, glial and endothelial cells are separated by the basement membrane. [17]

1.2.1.1. Müller cells

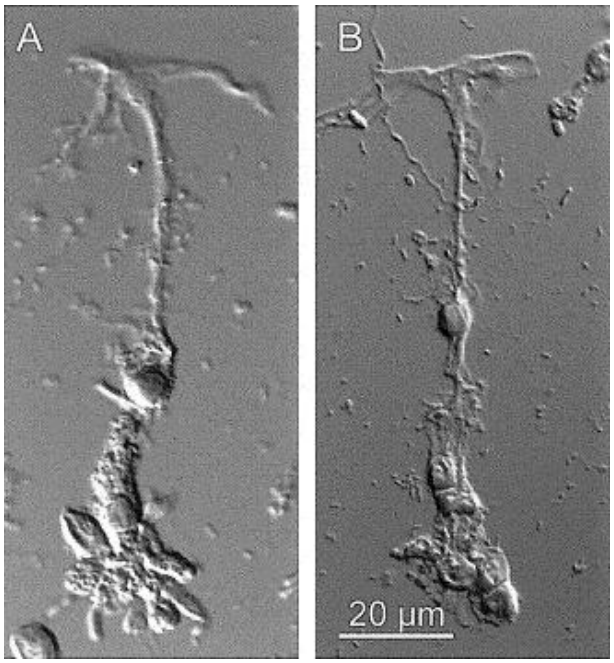


Figure 2. *Müller cell in the human retina, as seen with an optical microscope. (A) Photographed immediately after isolation; (B) thawed after 12 months of cryopreservation.*[19]

Müller cells, present exclusively at the retinal level representing the main glial component of the area, extend almost through the entire thickness of the nervous retina, from the photoreceptor inner segments to the border with the vitreous. They fill out almost completely the extracellular space between the neuronal elements, and they constitute a great part of the total retinal volume, enveloping and separating different neurons, with the exception of synaptic junctions. Their cellular bodies, located in the inner nuclear layer (INL), develop a thick fiber directed towards the outside and one directed towards the inside. The first one, developing lateral processes which branch out in the outside plexiform layer (OPL), ends in the external limiting membrane (ELM), from which, for a very brief time, it develops microvilli in the space between the inner segments of rods and cones (called the fiber basket); their aim is to increase the cellular surface and to regulate the exchange of metabolites and ions between the cells and the subretinal space. The second fiber extends into the retinal nerve fiber layer

(RNFL), terminating in a process which comes into contact with the limiting glial cells in order to form the inner limiting membrane (ILM) between the retina and the vitreous humour. [16-18, 20-22]

Müller cells are connected to blood vessels, astrocytes and neurons, and thanks to the fusion of the basal lamina on which they rest with perivascular cells and with the vascular endothelium, they participate in the formation of the blood-retinal barrier and regulate its characteristics. They express different ion channels and co-transporters which rapidly remove ions, carbon dioxide and other metabolites released by neurons into synaptic spaces, which appear to be responsible for the association between neuronal activity and the blood flow regulation. These cells maintain the stability of the retinal extracellular space and cell plasma membranes through the regulation of retinal ion concentration (in particular potassium), the deactivation and the recycling of neurotransmitters, detritus removal, the regulation of glucose uptake through GLUT transporters, the glycogen storage and its conversion into lactate, the participation in the glutamate/glutamine cycle and the protection from glutamate excitotoxicity, and the retinoic acid synthesis starting with retinol. Finally they contribute to the development, metabolism and synaptic functioning of neurons, as well as to the mechanical support and the inflammatory response of the retina.[16-18, 20-22]

1.2.1.2. Astrocytes

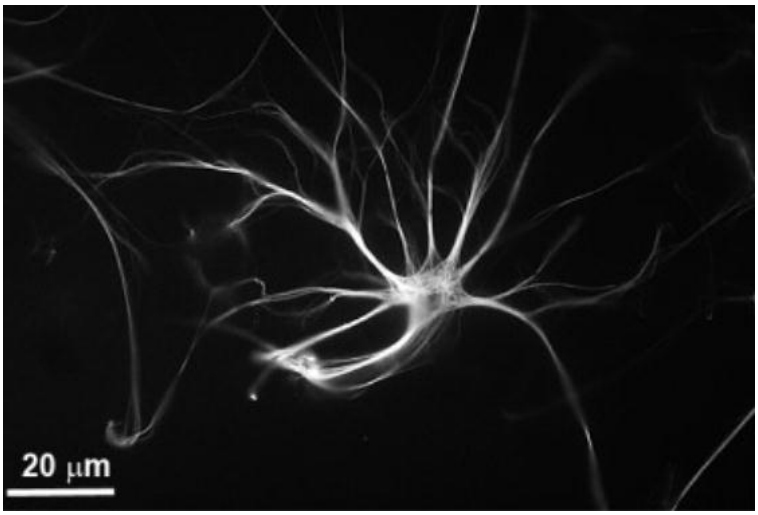


Figure 3. *Human retinal astrocyte as seen on fluorescence microscope.*[23]

The astrocytes, getting their name because they are characteristically star-shaped, are ubiquitous components of the central nervous system (CNS). At the retinal level, their cellular bodies are located inside the RNFL, while their processes envelop the neuronal cells of the ganglion cell layer (GCL).

These cells almost certainly originate in a migratory process starting from the optic nerve, and, maintaining the same function, they constitute support elements for the axons of the ganglion cells. Their close association with the blood vessels of the inner retina hints at their participation in the constitution of the blood-retinal barrier and in its regulation. The endfeet of their processes come in contact with the adjacent capillaries, probably to allow the substance transport between the local circulation and the neurons. [16-18, 20]

It is generally considered that the astrocytes carry out significant roles in the regulation of synaptic functions, e.g. in the extracellular ion regulation (particularly potassium), glycogen deposition, neurotransmitter capture and inactivation, the contribution to the inflammatory response and the regulation of ganglion axon growth. The astrocytes usually contain the particular proteins of the intermediate filaments: the immature ones are composed mainly of vimentin, while

subsequently the cells become rich in glial fibrillary acidic protein (GFAP). Different ionic channels for potassium, sodium and calcium are located on their surface, in order to maintain the equilibrium in the extracellular matrix, and they are able to produce proinflammatory cytokines and to replace the damaged nerve tissue. [16-18, 20]

1.2.1.3. Microglial cells

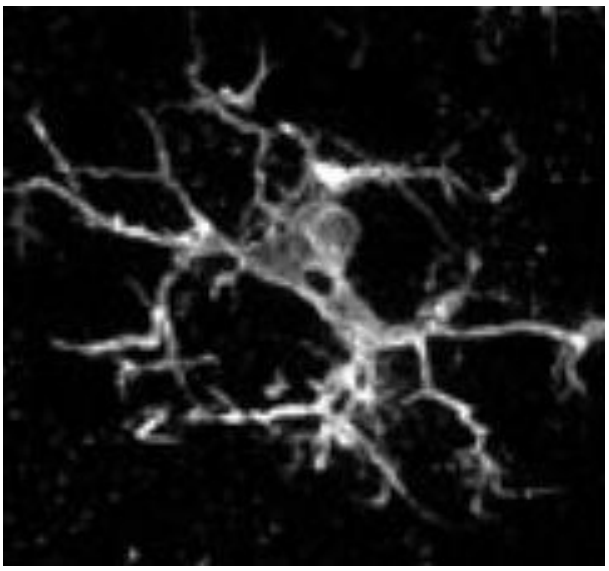


Figure 4*. Human microglial cell as seen on fluorescence microscope.*[24]

Retinal microglial cells belong to the mononuclear phagocyte system (monocytes-macrophages) which, derived from bone marrow, migrate into CNS through blood, representing the most important component of the retinal innate immune system. These cells are situated both in the inner plexiform layer (IPL) and the outer one (OPL), and are endowed with branching processes, usually distributed in parallels on the retinal plane, which give them their star form.[18]

The microglial cells usually monitor the extracellular matrix using their processes, but they do not migrate through retinal layers; furthermore they transiently come in contact with neural synapses, probably to monitor their functioning. When activated, they undergo a morphological transformation into amoeboid cells with retracted and thickened branches, alter the expression of their surface molecules

and become mobile, migrating into damaged areas of the retina where they participate in the phagocytosis of the degenerating neurons and the cellular detritus, in the secretion of growth factors and cytokines and in the active remodeling of neurons and synapses. [16, 18, 20, 25, 25] The microglia, therefore, helps contrast the retinal stress, but if the stress perseveres, they can cause a damaging and persistent inflammatory response.[21]

1.2.3. Characteristics and physiology of the aqueous humour

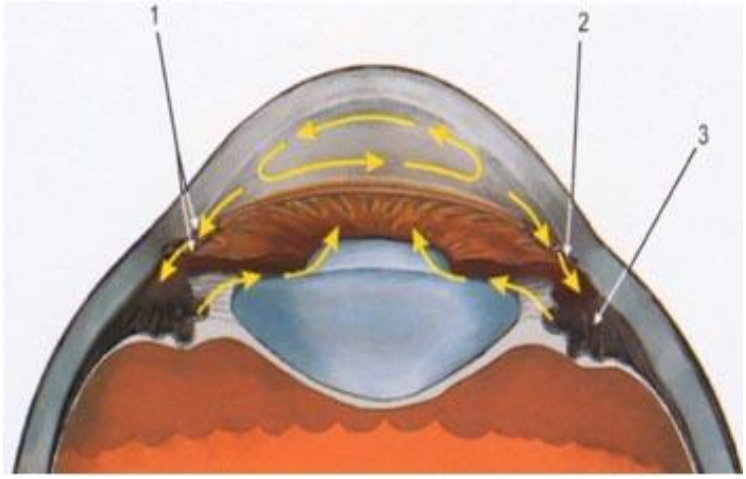


Figure 5. *Schematic representation of the hydrodynamics of the aqueous humour. Produced by the nonpigmented ciliary epithelium (3), it leaves the anterior chamber through the trabecular meshwork and Schlemm's canal (1) and in minimal part through the uvea (2).*[26]

The aqueous humour (AH) is a clear, transparent liquid located in the anterior and the posterior chambers of the eye: while most of it (the four fifths) is located in the anterior chamber, a fifth is found in the posterior chamber where it is produced, at a velocity of about 2.2µl/min during the day and of about 1.2µl/min during the night, by the nonpigmented ciliary epithelium. However, as demonstrated by the qualitative and quantitative differences of proteins, ions and other solutes with respect to the plasma, the AH is not formed exclusively for a simple process of diffusion and filtration, but also for the active secretion operated by intraocular tissues, in particular by the ciliary epithelium. From the posterior chamber, the AH flows into the anterior chamber passing between the

iris and the crystalline lens, and from here a major part (85%) is drained, through the trabecular meshwork, into the Schlemm's canal, to enter the blood flow through the episcleral venous plexus (trabecular pathway or the usual one); a small quantity (15%), however, reaches the choroid venous vessels through the uveal trabeculae (sclerouveal pathway).[15, 16]

This ocular fluid plays an important role in maintaining certain functions, e.g. the ocular refraction, form and pressure; in addition, it supplies nutritive substances and the oxygen to the cornea, the crystalline lens and the trabecular meshwork, and it removes waste substances; finally, it contributes to the constitution of the local immune system.[27]

From the physical point of view, the AH presents a specific weight which varies from 1003 to 1012, a refraction index between 1132 and 1137, and inferior viscosity and superior surface tension compared to the plasma. [26]

From the chemical point of view, the AH is constituted by a complex mix of electrolytes, organic solutes, growth factors, cytokines and proteins which satisfy the metabolic needs of the nonvascularized tissues of the anterior eye segment. Among all of its components, it is important to remember sodium, potassium, chlorine, bicarbonate, glucose, urea, ureic acid, creatinine, enzymes such as cholinesterase, amylase, hyaluronidase, glycosidase and aldolase, as well as hyaluronic, ascorbic and lactic acids. The protein component is minimal, and it seems to derive from plasmatic filtration through fenestrated capillaries in the ciliary body.[16, 26]

1.3. Classification of diabetic retinopathy

In order to arrive at a successful treatment capable of significantly reducing the risk of severe visual loss, there is a strong need for an international classification system which would allow the proper assessment of the severity of DR and of the diabetic macular edema (DME). The classification suggested by the Early

Treatment of Diabetic Retinopathy Study (ETDRS), based on the photographs of the ocular fundus, has resulted useful when applied in clinical trials, researches and publications, but is rather difficult to use in the everyday clinical practice. This is the reason for which, after several attempts by the single countries, the American Academy of Ophtalmology (AAO) has in 2001 initiated a project, concluded in 2003, which has enabled the creation of a new classification system of the severity of DR. [28]

According to this international classification, diabetic retinopathy is divided in 5 stages: absent, non proliferative mild, moderate, severe and proliferative (Table I). It may or may not be associated with macular edema which, if present, is classified in 3 stages: mild, moderate and severe (Table II): [28]

Table I. *International classification of diabetic retinopathy (AAO).*[28]

<table>
<tr><td colspan="2">DR ABSENT</td><td>Absence of retinal lesions</td></tr>
<tr><td rowspan="3">DR NON PROLIFERATIVE</td><td>MILD</td><td>Microaneurysms only</td></tr>
<tr><td>MODERATE</td><td>More than only microaneurysms, but less lesions compared to severe NPDR</td></tr>
<tr><td>SEVERE</td><td>At least one of the following:
More than 20 intraretinal hemorrhages in each of the 4 retinal quadrants
Definite venous beading in at least two quadrants
Intraretinal microvascular anomalies in at least 1 quadrant
No sign of proliferative retinopathy</td></tr>
<tr><td colspan="2">DR PROLIFERATIVE</td><td>At least one of the following:
Neovascularization
Vitreous/preretinal hemorrhage</td></tr>
</table>

Table II. *International classification of diabetic macular edema (AAO).*[28]

MACULAR EDEMA ABSENT		Absence of retinal thickening or hard exudates in the posterior pole.
MACULAR EDEMA	**MILD**	Presence of thickening or hard exudates in the posterior pole but distant from the center of the macula.
	MODERATE	Presence of thickening or hard exudates close to the center of the macula but not in the center.
	SEVERE	Presence of thickening or hard exudates in the center of the macula.

While in the early stages of the non proliferative form there are few visual symptoms, when we move into the advanced stages and into the proliferative form there can be significant visual loss and compromission of the central vision in the case of DME. [29]

- Non proliferative diabetic retinopathy (NPDR):

The mild form of non proliferative diabetic retinopathy is characterized by the sole presence of microaneurysms which, though usually asymptomatic, may burst and cause intraretinal haemorrhages. Moving from the mild form into the moderate and severe ones, flame and dot-and-blot haemorrages, hard exudates, venous caliber changes and intraretinal microvascular anomalies (IRMA) appear, the last one considered a premonitory sign of the progression into the proliferative form. Microinfarctions at the nerve fiber level, known as cotton wool spots, indicate the presence of retinal ischemia caused by the obstruction of precapillary arterioles.

- Proliferative Diabetic Retinopathy (PDR):

Once ischemia develops, it may cause NVD - neovascularization of the disk, or NVE - neovascularization elsewhere, possibly followed by the new-vessel ruptures and the formation of retinal and vitreous haemorrhages. This may be followed by membrane constitution on the surface of the retina, the contraction of which may cause retinal detachment or the traditional macular edema, and consequently blindness. The most feared complication is constituted by the neovascular glaucoma: it is caused by the growth of new-vessels from the pupil

area to the chamber angle and may lead to blindness. Another possible complication is constituted by the rubeosis iridis.

Diabetic macular edema (DME):

Macular edema may appear in any stage of diabetic retinopathy. The ETDRS has established that it may be considered clinically significant when one of the following occurs: 1) it presents a retinal thickening at or within 500μm of center of macula; 2) when there are hard exudates present in the same area associated with adjacent retinal thickening; or 3) when there is retinal thickening greater than 1 or more disc diameter in size present which is within 1 disc diameter from the center of the macula.[30]

1.4. Metabolic pathways implicated in retinal damage during the course of diabetes mellitus

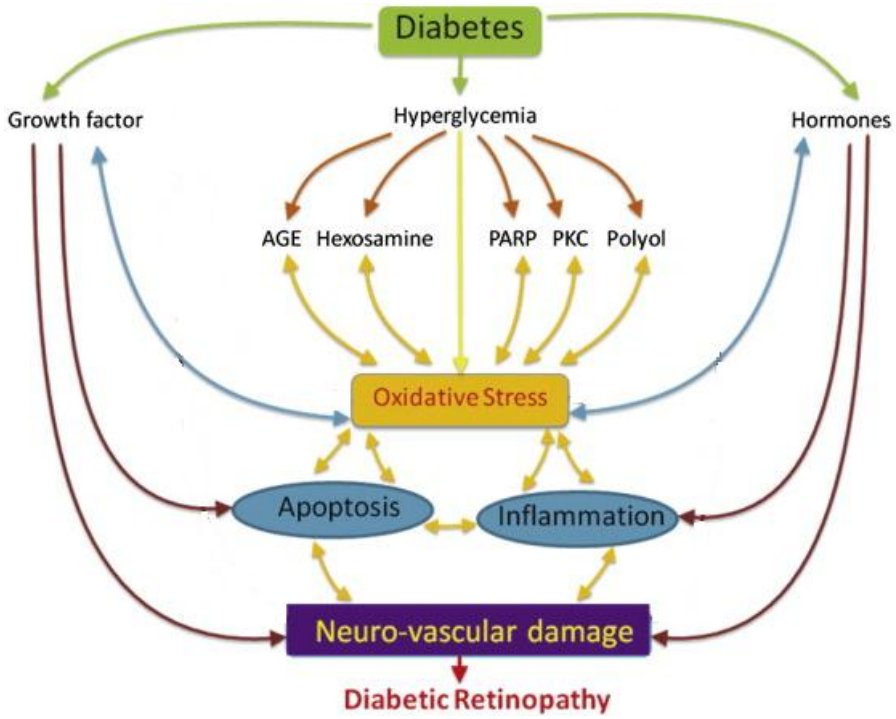


Figure 6. *Metabolic pathways implicated in retinal damage during the course of diabetes mellitus. They damage both the neural and the vascular components of the retina.*[31]

Even though the clinical studies "Diabetes Control and Complications Trials" (DCCT) and "UK Prospective Diabetes Study" (UKPDS) have recognized hyperglycemia as the motivating factor for diabetic retinopathy, the mechanism through which the glucose provokes microvascular damages has not been entirely clarified.[32] There are different metabolic pathways involved: inside the cells, high glucose levels appear to stimulate polyol pathways, advanced glycation endproducts (AGEs), protein kinase C (PKC), poly-ADP-ribose polymerase (PARP), hexosamines, renin-angiotensin-aldosteron system (RAS), reactive oxygen species (ROS), inflammation and growth factors. [31, 32]

- Polyol Pathway

Usually a small fraction of glucose is metabolized through this pathway controlled by aldose-reductase enzymes (AR), which reduce glucose to sorbitol, and by sorbitol-dehydrogenase (SDH), which reduces sorbitol into fructose. In hyperglycemic conditions, such fraction increases, and, using the NADPH (nicotine adenine dinucleotide phosphate) as a cofactor, provokes a glutation decrease and an increase in oxidative stress, which in turn cause retinal damage.[31, 33-35]

- AGEs Pathway

Non enzymatic glycosylation consists in a chemical reaction which causes the glucose to bind to proteins, lipids and nucleic acids, and which, through a series of complex reactions, causes the formation of AGEs. Goh et al. have noticed in retinal vessels, in vitreous humour and in human serum, an increase of AGEs levels associated with the gravity of DR.[36] Stitt et al. have moreover demonstrated that the accumulation of AGEs in retinal pericytes, very important for microvascular homeostasis, is implicated in endothelial damage and in blood-retinal barrier damage.[37] Finally, the Yamagishi group has shown how the

AGEs on one part increase, through ROS generation (free radicals/reactive oxygen species), the levels of VEGF (vascular endothelial growth factor), of MCP-1 (monocyte chemotactic protein-1) and of ICAM-1 (intercellular adhesion molecule-1) and, on the other, how they activate NF-kB (nuclear factor-kappaB) and NADPH-oxydase with the consequent ROS increase and the apoptosis of pericytes and other cells.[38-43] The AGEs disturb microvascular homeostasis through the interaction with their own receptors (RAGE), and they hold a central role in the inflammation, the neurodegeneration and the microvascular dysfunction.[31]

- Protein Kinase C Pathway (PKC)

Hyperglycemia induces the PKC pathway activation by increasing diacylglycerol (DAG) synthesis and other mechanisms. This leads to an increase in blood flow and vascular permeability, to the reduction in density of the basement membrane, to an expansion of extracellular matrix, to cellular apoptosis, angiogenesis, leucocyte adhesion and cytokine activation. [31, 32]

- Poly-ADP-ribose polymerase (PARP) activation

Drel et al. have noticed that when the nuclear enzyme PARP is activated in diabetic animal retina, it causes the DNA to rupture and it exacerbates the oxidative and nitrosative stress. In fact, the PARP activation provokes NAD+ (nicotinamide adenine dinucleotide) consumption, G3PD (glyceraldehyde 3-phosphate dehydrogenase) inhibition and NFkB activation, all elements that induce an increase in ROS and nitrogen production, responsible for the retinal damage. [31, 44]

- Hexosamine pathway

Giacco et al. have demonstrated that the hexosamine content in retinal tissue, both human and animal, is higher in diabetic subjects.[45] This is an extremely important result if we consider that Nakamura group had shown that the hexosamines alter the insulin signaling, contributing to the onset of DR.[46]

- Renin-angiotensin system (RAS)

The renin-angiotensin system, which regulates blood pressure and fluid balance, appears altered in diabetic patients, leading to microvascular dysfunctions.[47] Various studies have analyzed the components of these systems at the retinal level (Ang I -angiotensin I-, Ang II -angiotensin II-, prorenin, renin, ACE -angiotensin-converting enzyme-, AT1R -angiotensin type 1 receptor-, AT2R -angiotensin type 1 receptor-) and they have registered an increase in prorenin, renin and Ang II levels in the vitreous of the patients with proliferative diabetic retinopathy and with diabetic macular edema. [48-50] In addition to being a growth factor and to promoting cellular differentiation, apoptosis and extracellular matrix deposition, Ang II reinforces the harmful effects of the AGEs inducing RAGEs expression (AGEs' receptors) in hypertensive eye: this points to a connection between the two systems.[31]

- Oxidative stress

Hyperglycemia causes the oxidative stress and determines cellular damage at the level of the retina. In the diabetic subject's retina, the sources of reactive oxygen species have not been completely clarified yet, even though different studies evidence an increase in lactate, pyruvate, tricarboxylic acid and nitrotyrosine, in the consumption of cytosolic NADPH and in the stimulation of the glycolitic, AGEs, polyol, hexosamine and PKC pathways.[50, 51] Ulterior studies point at the participation of lipoproteic metabolism, the excess of excitatory amino acids, the alteration of growth factors, the activation of NADPH oxidase, the xantine-oxidase and the decrease of glutathione and antioxidant enzymes. [51-

56] Apart from creating a vicious circle responsible for the production of ulterior EOS, the oxidative stress activates the other metabolic pathways responsible for the retinal damage. It is not entirely clear, however, if it has a primary role in the pathogenesis of diabetic complications in the early stages, or if it is only consequential of the tissue damage.[31]

- Inflammation

Different molecular and cellular alterations typical of inflammation processes have been found out in the retinas of diabetic animals and patients. Different evidence supports the idea according to which the DR is a manifestation of a chronic low-level inflammation in which different effectors, e.g. the cytokines and the leukocytes, are responsible for retinal damage.[31] Diabetic retinopathy presents many similarities to chronic inflammatory diseases: increase in vascular permeability, edema, infiltration of inflammatory cells, tissue destruction, neovascularization and retinal expression of inflammatory cytokines. Recent publications present strong evidence of an important inflammatory involvement even in the early stages of DR.[57-60] In addition, it has been demonstrated that, in both animals and patients with diabetes, the leukocytes, including monocytes-macrophages (microglia), neutrophils and a certain number of lymphocytes adhere to retinal vascular endothelium. This temporally coincides with the compromission of the blood-retinal barrier, with the capillary occlusion and with the death of endothelial cells. The expression of adhesion molecules, particularly of ICAM-1 (intracellular adhesion molecule-1) and VCAM-1 (vascular cell adhesion molecule-1), appears increased in the diabetic subject's retina and is connected to the increased leukocyte adhesion and the leukostasis.[61]

If, in the healthy subject, the neurons express molecules which negatively regulate microglial activation, in diabetic subjects such regulation does not take

place, and the activated microglia produces cytokines and inflammatories. In addition, the high complement and the acute phase protein levels as well as the low levels of complement inhibitors represent a key moment in the damaged neurons' phagocytosis.[11, 21]

At the retinal level of diabetic mice and patients, different alterations have been identified: an increase in iNOS (inducible nitric oxide synthase), NO (nitric oxide), COX2 (cyclooxygenase-2), lipoxygenase, eicosanoids (including prostaglandins and leukotrienes), ICAM-1 and VCAM-1 on the surface of endothelial cells, PDGF (platelet-derived growth factor), VEGF, in IL-1, IL-6, IL-8, IL-13, MCP-1 (monocyte chemotactic protein-1), MIP-1α (macrophage inflammatory protein-1α), IP-10 (interferon gamma-induced protein 10), endothelin-1, TNF-α (tumor necrosis factor-α), RANTES (regulated normal T-cell expressed and secreted) and SDF-1α (stromal cell-derived factor-1). Furthermore, there was an increase in complement components such as C3, C5b and C9, and in molecules such as prothrombin, α1-antitrypsin, antithrombin III, coagulation factor XIII, Ang II, RAGEs (AGEs receptors) and NF-kB, while the PEDF (pigment epithelium-derived factor) resulted decreased. All these alterations have a proinflammatory effect and they entail an increase of leukocyte adhesion and leukostasis, as well as a damage of the blood-retinal barrier and death of endothelial cells.[11, 58, 62-67]

This data, therefore, suggests that inflammation contributes to the development and the progression of retinopathy, as it is demonstrated by the fact that the intravenous administration of glucocorticoids and anti-VEGF improve the clinical picture.[11]

- Growth factors, neurotrophic factors and hormones

Altered levels of certain molecules have been found in the diabetic subject, among which insulin, IGF-1 (insulin-like growth factor-1), aldosterone, adrenomedullin, GH (growth hormon), endothelin, VEGF, PEDF, FGF (fibroblast growth factor) and TGF-β (transforming growth factor-β). These elements are all responsible for both the structural changes of retinal vascularization (angiogenesis, ischemia, and increase in vessel permeability) and the progression of DR.[31] Neurotrophic factors such as insulin, PEDF, CNTF (ciliary neurotrophic factor), NGF (nerve growth factor), GDNF (glial cell line-derived neurotrophic factor) and BDNF (brain-derived growth factor), all of which appear altered in the diabetic subject, are responsible for retinal neuronal damage.[31]

1.5. Clinical Progression of Retinal Damage during the Course of Diabetes Mellitus

In the preclinical stage of DR, one witnesses the damage of endothelial cells, responsible for the integrity of the blood-retinal barrier, which are confronted by an accumulation of sorbitol with the consequent loss of osmotic equilibrium and of tight intercellular junctions; this leads to an increase in vascular permeability and the accumulation of extracellular fluid.[26, 68] The noteworthy rarefaction of intramural pericytes, which have the capacity to synthesize TGF-β and to inhibit the proliferation and migration of endothelial cells, leads not only to the failed regulation of capillary perfusion and the partial weakening, but also to a neovascularization typical of the proliferative form.[68, 69] The thickening of the basement membrane and the increase of deposition of the extracellular matrix components are probably caused by the glycosylation of its constitutive molecules and/or by the reduction in production of proteoglycans, which

contribute to the hemodynamic alteration of retinal capillaries.[26, 68] The hematic and hemorheological alterations, on the other hand, consist in the increase in viscosity, caused by the reduced deformability of red blood cells, and in hypercoagulability, caused by the increase in platelet aggregation, fibrinogen, certain coagulation factors (VII and VIII), α2-globulin, and by the reduction of antithrombin III.[26] At this stage, first the autoregulation mechanisms are triggered, which subsequently they oppose the hypoxia caused by vascular wall lesions and other hemorheological alterations: among these, the first sign of compensation is constituted by vasodilation, more evident when found in macular circulation. With the advancing of the illness, nevertheless, the autoregulation capacities are no longer sufficient for the compensation of retinal metabolic necessities, and the already precarious conditions of the vessel wall are further aggravated. This leads to an ulterior thickening and structural alteration of the basement membrane, to the reduction of endothelial cells with tight junction rupture and to the disappearance of intramural pericytes.[26, 39, 70]

The clinical stage of the illness therefore begins with the appearance of microaneurysms, hard exudates, vascular alterations and capillary occlusions. The microaneurysms are capillary dilatations caused by the swelling of their weakened walls or by endothelial gems which attempt to revascularize the retina; they can be identified as small red dots, are usually temporal with respect to the macula and can be saccular or fusiform in structure. Hard exudates, identified as yellowish white deposits in the external plexiform layer, are accumulations of lipids, proteins and microphages (microglia), which phagocytize such material and are the result of their extravasal precipitation, through capillary incontinence, beginning in the blood flow. The intraretinal hemorrhages are caused by the rupture of weakened capillaries and have different morphologies depending on their topographic localization: the surface ones are typically flame-shaped, as they are located between the axons within the nerve fiber layer, while the deeper

ones are dot-and-blot shaped due to the cell disposition just about perpendicular to the retinal surface. The pre-capillary arteriolar occlusion may cause retinal microinfarctions with consequent formation in the nerve fiber layer of cotton wool spots; the latter, blurred and off-white in colour, are caused by the swelling of nerve fiber terminations which follows the axonal flow halt determined by retinal ischemia. Capillary occlusions may also cause the intraretinal microvascular anomalies (IRMA), usually at the medium to extreme peripheral level of the retina, on the border with ischemic areas; they are represented both by retinal new-vessels and the dilatation of preexistent vessels, and would constitute a compensation attempt implemented through the opening of shunts with not yet occluded capillaries. Venous alterations are represented by irregularities and lumen duplications, and can assume a characteristic rosary-bead shape.[26, 70, 71]

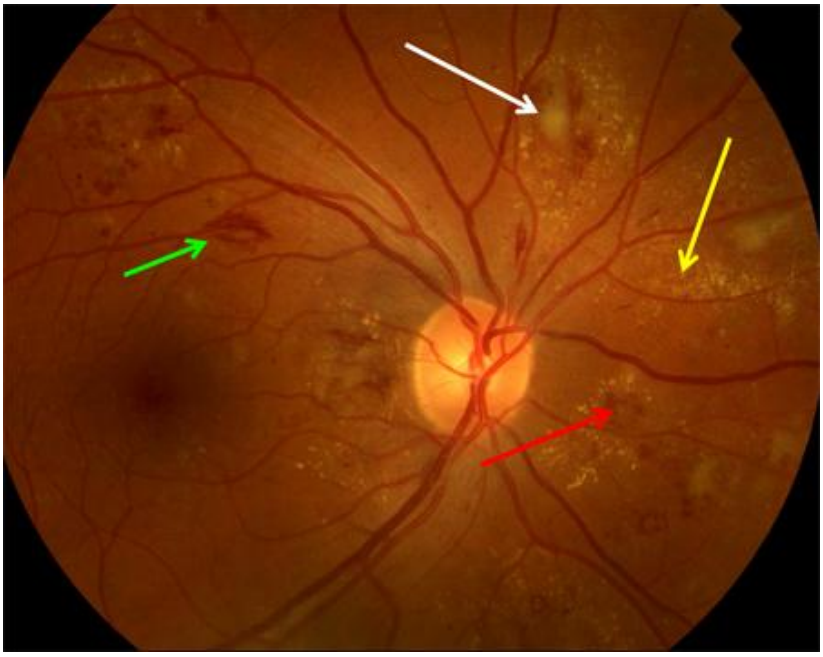


Figure 7. *Photograph of the ocular fundus of a patient afflicted by NPDR. Hard exudates (yellow arrow), microaneurysms (red arrow), cotton wool nodules (white arrow) and retinal hemorrhages (green arrow) are visible.*[72]

From the established ischemic areas, VEGF, IGF-1 and FGF are liberated, and they determine a vascularization of the no longer perfused retinal zones. It initially concerns only the retinal thickness, but later on it can concern also the vitreous space, and more frequently the optic disc, due to the natural interruption of the inner limiting membrane. Newly formed vessels present an anomalous wall constituted only by the endothelium, without a valid junction system or pericytes, and therefore tend to bleed frequently. Initially the new-vessels lack connective support, but in a brief time they become enveloped by a fibrous support tissue. Precisely for this reason the new-vessels bleed easily, but after the constitution of the fibrous component, there is a tendency towards closure and complications, e.g. the retinal detachment. [26]

In each DR stage, the macular region may be involved, with edema, hemorrhages and hard exudates, which can lead to diabetic maculopathy. Macular edema, of particular danger to the visual prognosis when it strikes the foveal region, may be diffused through the entire thickness of the retina, or it may converge into lodges at the external plexiform layer level provoking the cystoid macular edema. It is caused by the loss of function of the blood-retinal barrier, with significant leakage of fluids, proteins and lipids into the sensory retina. Ischemic maculopathy is an ulterior aspect of the macular compromission, caused by capillary occlusion surrounding the fovea and leading to an extremely bad visual prognosis.[26, 30, 70]

1.6. Physiopathology of Retinal Damage during the Course of Diabetes Mellitus

For a long period of time, DR has been considered primarily a microvascular pathology caused by the direct effects of hyperglycemia and by the metabolic

pathways it activates.[73] Some recent studies have nevertheless demonstrated that retinal neurodegeneration, the result of a negative balance between the neuroprotector and neurotoxic factors, is present even before the development of microvascular damages detectable clinically in ophthalmoscopic examination, which proves its participation in their appearance. It would therefore constitute an early event in the pathogenesis of DR and would anticipate the onset of microvascular alterations.[11, 21, 73-76]

Already in 1961, Wolter et al. have observed in autoptic reports of diabetic subject the atrophy of ganglion cells and the degeneration of the inner nuclear layer, advancing the hypothesis according to which DR would not be solely a vascular pathology, but would precociously involve the neurons and the glia.[77] In 1962, the Bloodworth group described, in a study of 295 cadaver eyes, the degeneration of the inner plexiform layer and the ganglion cell layer, evidencing the presence of pyknosis and nuclear fragmentation, characteristics which are today widely recognized as typical of apoptosis.[78] In 1986, Scott et al. identified, in a study of the optic nerve in rats treated for 12 months with streptozotocin (a substance which induces diabetes), a reduction in the number of nerve fibers and an increase in the number of glial cells. [79] Lastly, in a study from 1998, the Barber group demonstrated, both in mice and in diabetic subjects, a high level of caspase-3 apoptosis mediated by the retinal neuronal cells, without noticing a similar significance in endothelial cells.[80]

Neuronal damage and microvascular damage are interdependent and they strengthen one another’s effects: while the vascular hyperpermeability and occlusion compromise the integrity of neuronal and glial cells due to the penetration of inflammatory and cytotoxic molecules into the retina, the loss of function of the glial barrier and its increased expression of inflammatory cytokines and free radicals compromise the vascular functionality.[21] This relationship is explained by the fact that the vascular and the neuroglial components of the

retina come together to form the so-called ‘neurovascular unity’ of the retina, which is altered during the course of DM. It refers to the physical and biochemical relationship which exists between the neurons, the glia and the capillaries and to the close interdependence of these structures which permits the regulation of neuronal energy homeostasis and of neurotransmission. In addition, the interaction between the glial cells, neurons, endothelial cells and pericytes promotes the formation of the blood-retinal barrier which controls the passage of fluids and blood metabolites inside the nervous system parenchyma.

In the internal retina, the neurovascular unity includes astrocytes, Müller cells, amacrine and ganglion cells, all placed in proximity of capillaries, a source of oxygen and nutrients, inside the GCL and on the borders of INL: the close connection of these structures is shown by the retinal blood flow regulation by the glial cells and by cellular metabolites. The neurovascular unity of the external retinal, instead, sees the photoreceptors and Müller cells paired metabolically in order to support the visual function through the diffusion of oxygen and nutrients by the choroidal vessels through the EPR.[11]

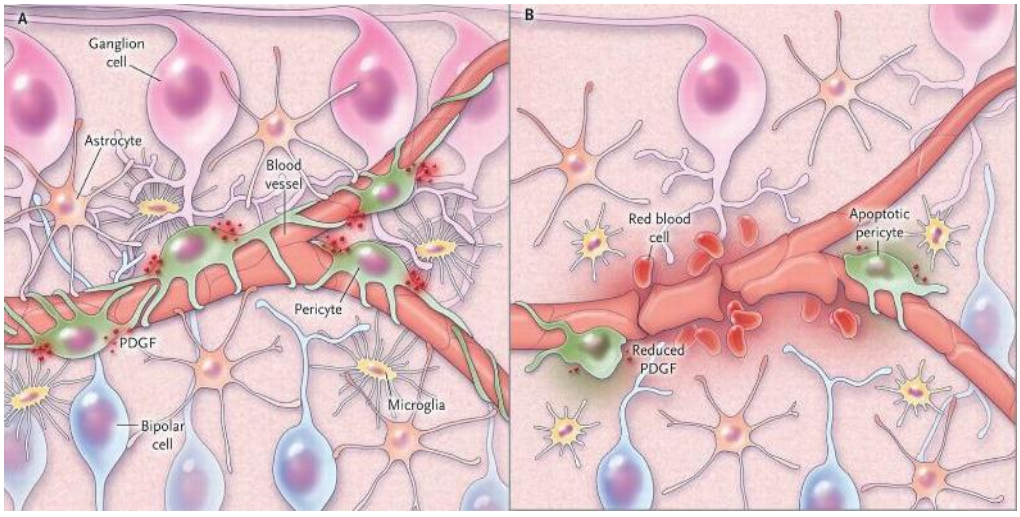


Figure 8. *Alteration of neurovascular unity during the course of diabetes. Image A shows the normal structure of neurovascular unity: pericytes and glial cells promote the formation of the blood-retinal barrier, creating favorable surroundings for neuronal functioning: the projections of microglial cells monitor this process. Image B shows the altered cell communication during the course of diabetes when there is an increased production of VEGF by the glial cells, an increased of inflammatory cytokines and the reduction of PDGF in pericytes; this contributes to the alteration of the blood-retinal barrier and, in certain cases, to angiogenesis.*[11]

The idea according to which nervous damage precedes the vascular one is confirmed in the functional electrophysiological and psychophysical studies, which show that the alterations are present even before the microvascular damage becomes visible at the ophthalmoscopic exam. Such alterations consist in contrast sensitivity loss, altered colour perception and a failure of dark adaptation. It has been observed that in diabetic mice the oscillatory potentials in electroretinography (ERG), probably caused by neurotransmission in the internal retina, have extended peak latencies and/or reduced amplitudes, suggesting a compromised function of the internal retina. The motive for such alterations is unknown, but given that the oscillatory potentials are probably generated by the synaptic activity between amacrine cells and bipolar or ganglion ones, it might be traced to a neuronal transmission degeneration or to the combined loss of amacrine and ganglion cells.[81]

Finally, it is necessary to remember that the particular structure of the retina is explanation enough for its sensitivity to diabetic disease: the demyelination of retinal axons, the reduced blood volume and the scarce number of cellular mitochondria are the key elements responsible for its vulnerability to hyperglycemic conditions.[11]

1.6.1. Damage of retinal neuronal cells

During the course of DR, apoptic cells have been observed in all retinal layers, suggesting the involvement of different types of neurons. [82] Numerous studies have, in fact, evidenced that diabetes, through metabolic pathway alterations or through neuronal support mechanisms, provokes the functional deficit and the loss of different types of cells: ganglion, bipolar, amacrine, horizontal and

photoreceptor. The data generally demonstrates an early dysfunction of the inner retina, followed by subsequent effects on the outer retina.[83]

It is well known that, among nerve cells, ganglion cells are the ones most affected by DM and that they are the first to face apoptosis, as shown by the increase in Bax (Bcl2-associated X protein), caspase-3, caspase-9, Bad (Bcl2 antagonist of cell death), cytochrome c and AIF (apoptosis-inducing factor) in the inner retinal layers.[73] This is explained by the fact that they present a high metabolic activity and the noteworthy axon length exposes them to different types of stress, e.g. hypoxia, free radicals, mechanic compression, photooxidative damage.

The retinal vascularization does not appear great in proportion, because there is the need for structural transparency for the proper photoreceptor functioning: the capillaries are in fact very thin, they have an elevated blood flow velocity and scarce intervascular connections. In addition, ganglion cells have a high tendency for the accumulation of metabolic end products such as free radicals, and, in spite of having greater antioxidant capacity compared to other types of neurons, they are nonetheless more vulnerable compared to Müller or endothelial cells. [84, 85]

Meyer-Rüsenberg et al. have recently demonstrated that in diabetic subjects there is a dendritic structural remodeling at ganglion cell levels, which consists in an increase in length, density and number of terminations, and in a structural alteration of the axons which face a significant swelling.[86]

In a study on diabetic mice from the same period, Gastinger et al. proved for the first time that even the amacrine cells are subjected to apoptosis, and that those which use dopamine and acetylcholine as neurotransmitters present a reduction in activity of enzymes tyrosin-hydroxylase and acetylcholinesterase. The same study evidenced a greater loss and susceptibility to apoptosis of cholinergic amacrine cells in the peripheral retina compared to the central one.[82]

Park et al. have observed that even the photoreceptors face apoptosis during the course of diabetic pathology, with the consequent alteration in colour distinguishing and a reduced blue-yellow contrast sensitivity. Given that the latter characteristic is also common in Parkinson's disease, it would appear that it caused by a dopamine deficit.[82, 87] Photoreceptor apoptosis is exacerbated by the iNOS hyperexpression on behalf of the bipolar cells through a vicious circle involving glutamate-mediated exitotoxicity.[88]

1.6.2. Damage of retinal glial cells

Certain animal models have demonstrated that an important role in retinal neurodegeneration is carried out by glial cells, which constitute the earliest and the main target of chronic hyperglycemia. Metabolic and morphological alterations of these cells imply progressive neuronal suffering and loss, due to their important role in the mediation between retinal vessels and the neurons.[89, 90]

A great number of studies show that the low-level chronic inflammation contributes to retinal dysfunctioning during the course of diabetes mellitus.[58, 62, 91] The high levels of neuroretinal inflammatory cytokines and of microvascular adhesion molecules would in fact lead to the accumulation of leukocytes and to the formation of neocapillaries in the retina. Some studies have found an altered cytokines expression not only at the vascular level, but also at the neuroglial one: if it is true that the retinal microglia carries out a primary role in this response, there is strong evidence that Müller cells and the astrocytes also express inflammatory cytokines in hyperglycemic conditions.[92]

The Carrasco group has proven that both the apoptosis and the glial activation precede microvascular lesions, although it is still not known which one of these two events appears first.[93, 94] As a matter of fact, a study led by Tretiach et al. would corroborate the hypothesis according to which the retinal

neurodegeneration and, more particularly, the Müller cell damage would activate certain metabolic pathways which participate in microangiopathic processes and in malfunctioning of the hemato-retinal barrier, crucial elements of the pathogenesis of diabetic retinopathy.[95]

Since both glial cells and the vascular endothelial cells are found in close association, Barber et al. suggested that the reactivity of the first ones is a direct consequence of the infiltration of glucose and the inflammatory blood agents into the nervous system parenchyma, and that, in its turn, the increased vascular permeability is supported by the release of certain glial factors with the consequent integrity loss by the hemato-retinal barrier.[96]

The Müller cells and the astrocytes, besides supporting the synaptic activity, maintain the integrity of the blood-retinal barrier and regulate the vasoconstriction related to neuronal activity. They react, however, in different ways with regard to hyperglycemic damage: while by now it is clear that, during diabetes, the first ones face gliosis, functional activation and cell proliferation (with consequent increase of their numbers, even though there is an increase in cell apoptosis), few studies have analyzed the alterations that afflict the astrocytes, though it would appear that there is both loss and a functional alteration of these cells, previous to the events relative to Müller cells. [83]

An interesting element consists in the fact that the Ly group has evidenced that glial alterations and tissue hypoxia are stronger in the peripheral retina than in the central one, suggesting a greater vulnerability with respect to diabetes. This may be explained with a structural difference between the two regions, even though it is necessary to carry out further studies in order to explain such phenomena.[83]

1.6.2.1. Alteration of Müller cells

Müller cells result particularly susceptible to diabetes-induced damage and are recognized today as key elements in the onset and the progression of diabetic retinopathy.[97]

These cells react to hyperglycemic condition by facing a reactive gliosis process characterized by three non-specific responses: hypertrophy, cellular proliferation and an increase in intermediate filament proteins nestin, vimentin and GFAP (glial fibrillary acidic protein). The reaction already possesses both cytoprotective and cytotoxic effects with respect to retinal neurons: mostly in the initial stages of diabetic illness, it is neuroprotective and may be interpreted as an effort to limit the extension of tissue damage; successively, however, it contributes to neurodegeneration and impedes tissue restoration and normal neuroregeneration. [98]

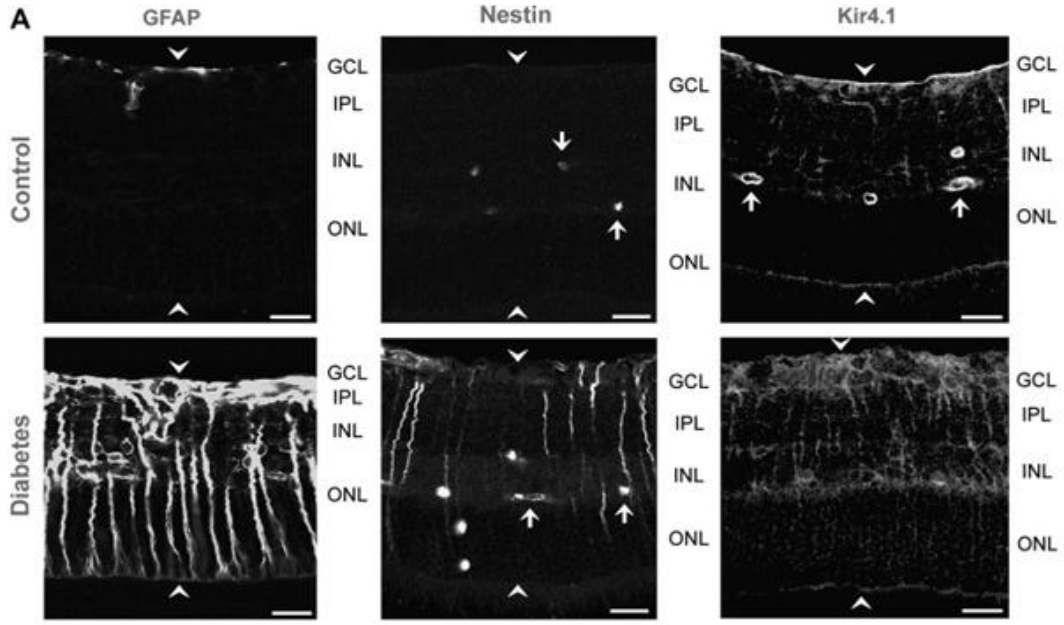


Figure 9. *Müller cell gliosis in rat's retina 6 weeks after the onset of diabetes. In controls, GFAP is expressed only by astrocytes in GCL, while nestin is localized exclusively in blood cells inside the vessels (arrows), and Kir4.1is found prevalently around blood vessels (arrows) and on the inner and outer limiting membrane's level (arrow heads). In diabetics, GFAP is expressed by Müller cells throughout the entire retinal thickness, while nestin is localized both there and in leukocytes infiltrated in the ONL and Kir4.1 is redistributed on the entire length of Müller cells. GCL: ganglion cell layer, IPL: inner plexiform layer, INL: inner nuclear layer, ONL: outer nuclear layer.*[98]

The neuroprotective effect involves different mechanism which entail the alteration of potassium channels, excess glutamate re-uptake, antioxidant release (e.g. glutathione, pyruvate, α-ketoglutarate, mettallothionein, lysozyme, ceruloplasmin, and heme-oxygenase), production of growth factors and neurotrophic factors (in particular GDNF, FGF, VEGF, PEDF and TGF-β), cytokine and erythropoietin secretion, ascorbate reduction and the dedifferentiation of Müller cells in stem cells.[98]

Nevertheless, with the protraction of detrimental stimuli, Müller cells increase the expression of growth factors, which become harmful, and secrete proinflammatory cytokines, which recruit the monocytes/macrophages (microglial cells) and neutrophiles in the damaged area, which in turn release the oxygen reactive species and cytotoxic cytokines.[99] Other alterations involve those of aquaporins, carbonic anhydrase and glutamine-synthetase and of the production of proteins involved in glycolysis; this comprises the damages of glia-neuron interaction and acid-base and ion homeostasis, contributing to the development of edema, neuronal hyperexcitability, glutamate toxicity and the increase in sensitivity with respect to stress stimuli, along with neuronal dysfunction and loss.[98]

Müller cells, having the ability to dedifferentiate themselves into cells which show similar characteristics to pluripotent retinal progenitors, or into stem cells, cause an instability in the removal of neurotransmitters and in the homeostasis of potassium and water, with the consequent functional decoupling with respect to neurons. Müller cells' lack of support, moreover, may have an additional effect on neuronal dysfunction and loss, increasing the susceptibility to stress stimuli in pathological retina: these cells have a leading role in the regulation of osmotic equilibrium, one of the main mechanisms involved in the development of diabetic macular edema, regulating the water and potassium flows through cellular membranes through AQP4 and Kir4.1,.[98, 100]

Müller cells may reenter the proliferative cycle, developing glial scarring, which impedes tissue repair and neuroregeneration through inhibitory molecule expression on the surface of reactive glial cells,.[99]

Diabetes, furthermore, induces the activation of the apoptotic cascade in these cells, as well as their swelling caused not only by the alteration of potassium channels but also by the alteration of proinflammatory enzymes. [101, 102]

Finally, it is necessary to consider that the compromission of the hemato-retinal barrier consequent to the malfunctioning of Müller cells entails an increase of vascular permeability as well as the extravasation into the perivascular space of numerous sieric components, which stimulate the proliferation of the cells.[100]

1.6.2.2. Astrocyte alteration

Rungger-Brändle et al. have noticed that, during the course of diabetes mellitus, the retinal astrocytes, even though gliosis alters them morphologically so that they divide and extend their processes, reduce in number through apoptosis, contrary to what happens to the Müller cells.[103] In spite of the fact that the two types of cells share certain functions, the opposite reaction to hyperglycemic conditions emphasizes the differences inherent in the process of their activation and in their metabolism: it has been observed, in fact, that the expression of sodium bicarbonate cotransporters is different and that the elective glycogen accumulation in Müller cells expresses a different reworking of this molecule. [104, 105] Since the astrocytes are preferentially in contact with ganglion cells and retinal vessels, it is moreover possible that they are influenced by their extracellular surroundings in a different way compared to Müller cells, which in turn extend through the entire retina. [103]

A recent study carried out by the Bringmann group indicates that astrocyte alterations usually take place early in course of diabetic pathology and are accompanied by the inner retinal hypoxia and by the dysfunction of ganglion

cells, while the Müller cell gliosis and the compromission of nerve cells appear later.[98] Since astrocytes are usually recognized as the modulators of neuronal and vascular function, they seem to carry out a significant role in the development of tissue hypoxia, in vascular alterations and in the dysfunction of ganglion cells. Their close proximity to retinal vessels and to the ganglion cell layer, as well as their role in the formation of blood vessels, neurovascular coupling and the modulation of pathological neovascularization, makes them critical modulators of early retinal changes during the course of diabetes.[83] Mogi et al. have in fact demonstrated that the reduced astrocyte communication and the diminished expression of proteins forming the retinal tight junctions, characteristic for hyperglycemic conditions, seem to be connected to neuronal dysfunction.[106]

Ly et al. have recently observed that, in the periferal retina of mice, already four weeks after the onset of diabetes, a connexin reduction can be found. This would not only reflect a reduction in the formation of tight junctions and the consequent compromission of astrocyte communication, but also the reduction of the apoptosis-facing astrocytes. They have, moreover, evidenced that the early astrocyte loss is accompanied by the reduced GFAP expression by these cells.[83]

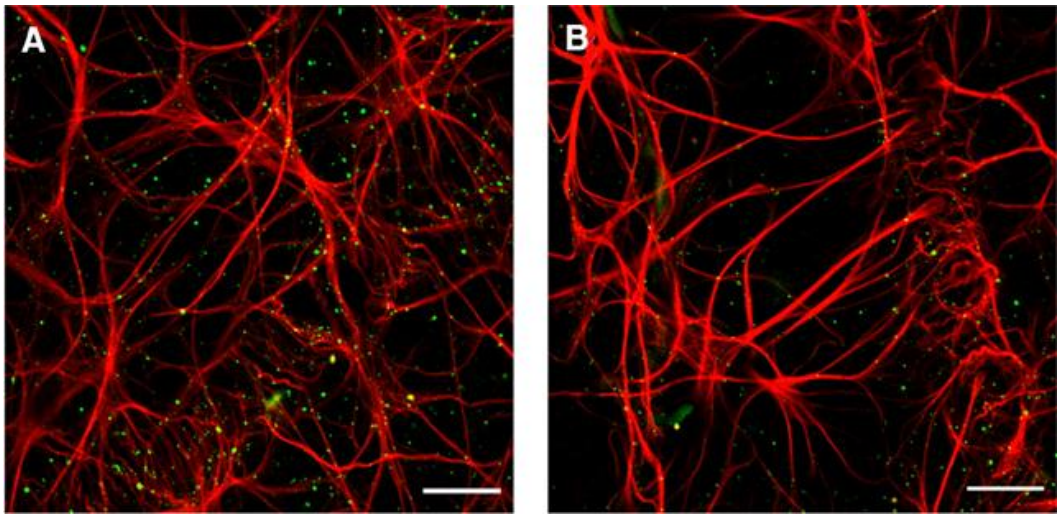


Figure 10. *Alteration in the number and expression of GFAP and connexin in rat with early diabetes (4 weeks after the onset). The astrocytes, seen with the fluorescence*

microscope, have been marked with antibodies for connexin-43 (in green) and for GFAP (in red): notice the reduction in cell number and expression of the two proteins in diabetics (B) compared to healthy controls (A). The bar corresponds to 50 µm.[83]

1.6.2.3. Alteration of microglial cells

In the healthy retina, the microglial cells are not dormant as it was once thought, but, thanks to their processes, they constantly supervise the different retinal regions even without actively penetrating the retinal layers. The insults which cause neuronal apoptosis and degeneration induce the recruitment of microglial precursors originating in blood, as well as their activation and rapid migration towards the damaged region, with concomitant transformation from a branched form to an amoeboid one. There is a consequent accumulation of microglial cells around the dying cells in nuclear layers and in subretinal space, as well as a depletion of these cells in the rest of the retina. [107]

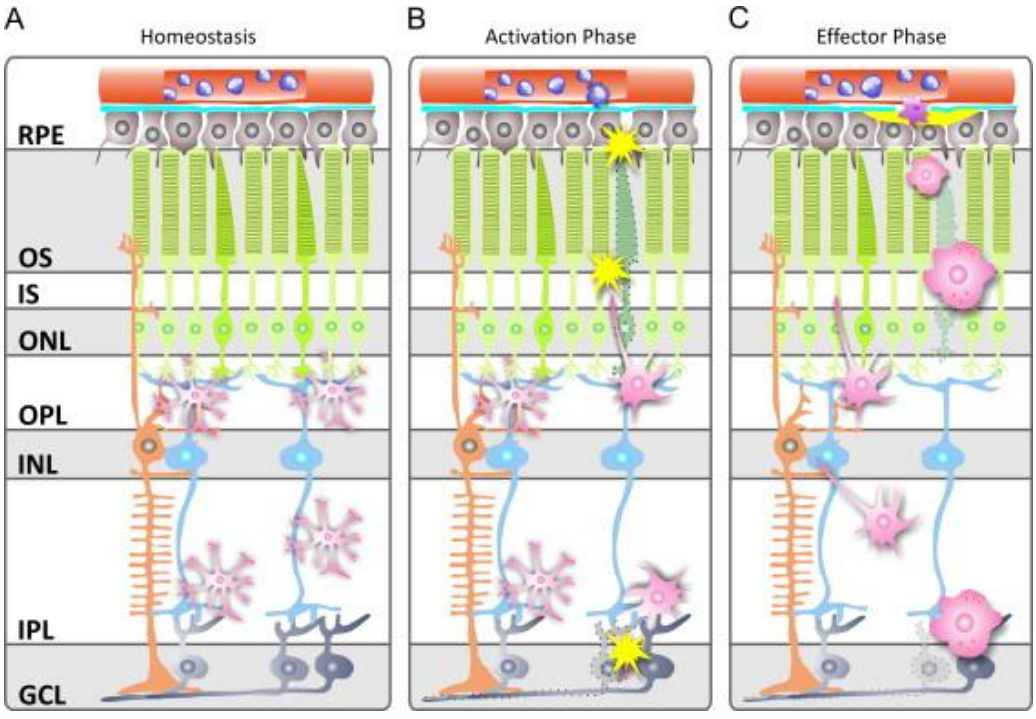


Figure 11. *Schematic representation of the three phases of microglial activation. (A) In the neuroretina the microglia populates chiefly the inner and the outer plexiform layers. (B) Retinal insults rapidly activate the microglia. (C) Microglial cells which are activated and/or derived from blood precursors migrate into lesion sites where they acquire a amoeboid morphology. Such cells may have a protective or a damaging effect based on the immunological phenotype and on the surrounding cytokines. RPE: retinal pigment epithelium; OS: photoreceptor outer segment; IS: photoreceptor inner segment; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer.* [107]

Certain studies have shown that the retinal inflammation during the course of diabetes mellitus is a relatively early event, and that is precedes the vascular dysfunction and the neuronal degeneration. 108] Microglial activation has been recognized as the main responsible for the initial inflammatory response, even though the exact mechanism through which it releases inflammatory cytokines remains in the dark.[109]

The activated microglia secretes cytokines and other proinflammatory molecules used for the phagocytosis and the destruction of damaged cells, as well as for the triggering of reparation processes which lead to the formation of glial scars. If it remains in an activated state, however, the cytokines may damage the neighbouring cells, particularly the neuronal and the vascular ones, leading to the onset of other retinal pathologies, such as the retinal degeneration and the glaucoma.[109] In compliance with this thesis, numerous histopathological studies done on both animals and humans have evidenced the activation of microglial cells, as well as the presence of different inflammatory molecules secreted by the microglia, usually associated with neuronal and endothelial death. [103, 110-113]

Despite the fact that the mechanisms at the base of microglial activation remain unknown, it has been supposed that the glycated albumin, accumulated in the inner retinal layers due to increased vascular permeability, and probably partially produced on local level, can provoke these cells to produce inflammatory molecules. This would induce the TNF-α secretion in microglia as well as the localized secretion of proinflammatory cytokines, growth factors and other bioactive molecules through the connection to different cellular types and various metabolic pathways, carrying out important roles in the onset and the progression of diabetic retinopathy.[109, 114]

M-CSF (macrophage colony-stimulating factor), expressed by neurons, astrocytes, endothelial cells and the very microglial cells, seems to have a key role in the regulation of the activation, the proliferation and the migration of the microglia: Liu et al. have demonstrated that even at the onset of diabetic pathology there is an increased retinal expression of M-CSF and M-CSF-R, its specific receptor present on microglial cells. The same group has observed that M-CSF exclusively induces a microglial proliferation, whose activation through glycated albumin results necessary in order to create an inflammatory response. [114] In addition, Langmann et al. have observed that the M-CSF-R hyperexpression entails a hyperproliferation of these cells and an increased expression of di iNOS, IL-1β, MIP-1α, IL-6 and M-CSF on their part. [115]

The Zeng group, investigating microglial activation around retinal capillaries, has detected the so-called 'microglial perivasculitis', supposing that it originates in the infiltration of hematic inflammatory molecules through vascular walls, but also in a local primary process. The same group has evidenced the nearness of perivasculitis to neuronal cells, especially to the ganglion ones, underlining the ways in which the inflammatory reaction afflicts these cells from the very beginning. The release of cytokines and microglia molecules such as TNF-α, IL-1β, NO and VEGF provoke the diffusion of the inflammatory process through the entire retina, exacerbating the increase in vascular permeability and in neuronal damage, thus creating a vicious circle. [110]

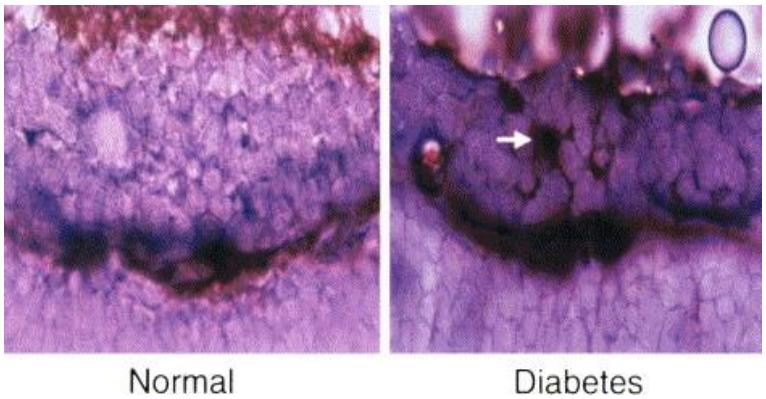


Figure 12. *Activation of the retinal microglia in a rat 4 weeks after the diabetes onset. Microglial cells are evidenced with the specific marker OX-42 (brown). In diabetics, there*

is an increase in brown stains and in digitiform protrusions inside the ganglion cell layer (marked by the arrow) [90]

1.7. Biomarkers of glial activation during the course of diabetic pathology

1.7.1. Aquaporins

The aquaporins (AQPs) are a family of integral membrane proteins with low molecular weight (about 30kDa/monomer) arranged in tetramers, which mediate the transcellular flow of free water and in some cases of gas and solutes. The isoforms 3, 7 and 9 are called ‘aquaglyceroporins’ because of their ability to transport also glycerol and other small solutes, such as ions, urea and lactate. The aquaporins have a crucial task in the preservation of the ion- and osmotic equilibrium inside the CNS, neuroretina included, in response to different osmotic gradients and hydrostatic pressures. [116]

Tenckhoff et al. have demonstrated that the human retina expresses the mRNA of all the 13 isoforms (AQP0-12) identified in mammals. [117] The immunohistochemical studies carried out so far, however, have confirmed the expression of only few aquaporins in the eye of a healthy mammal (mouse, pig, horse, rabbit and man):

- AQP0: in the lens epithelium and in neuroretina (at bipolar cell, amacrine cell and ganglion cell levels);
- AQP1: in the endothelium of the cornea and the iridocorneal trabecular meshwork, in the epithelium of the cilliary body, the lens and the iris, in the RPE and in the neuroretina (in amacrine cells and photoreceptors);
- AQP3: in the conjunctival and the corneal epitheliums and in RPE;
- AQP4: in the ciliary epithelium, the lachrymal gland epithelium and the neuroretina (in Müller cells and astrocytes);

- AQP5: in epitheliums of lachrymal glands, the conjunctiva and the lens.
- AQP6: in the neuroretina (Müller cells);
- AQP9: in the neuroretina (amacrine and ganglion cells).

It is therefore understood that so far only some isoforms (AQP0, AQP1, AQP4, AQP6, AQP9) have been detected in the neuroretinal level of a healthy mammal.[118-130] The presence of these proteins has been confirmed in most cases inside the human eye as well. [131-141] Among the various aquaporins, AQP1 and AQP4 have received the greatest attention, because it has been demonstrated that, in the CNS, they not only facilitate the bidirectional flow of free water through the cerebral parenchyma, but that they also modulate the neuronal excitability and potentiate the astrocyte migration; lastly, they seem to be involved in the production of aqueous humour. [116]

In normal conditions, the fluids that accumulate inside the neuroretina and in subretinal space are reversed into the blood via transcellular transport osmotically coupled with the flow of various molecules, especially ions, at the level of the retinal pigment epithelium, of Müller cells and of astrocytes. The aquaporins facilitate the transcellular water flow: AQP1 in RPE and AQP4 in astrocytes and Müller cells. The last two cell types regulate the flow of potassium released by active neurons, which would, if present in excess on the extracellular level, induce neuronal hyperexcitability.

Above all, Müller cells express different channels for this ion, the most important ones being the Kir2.1 (inwardly rectifying potassium 2.1), expressed in the parts of the membrane contiguous to neuronal cells, and the Kir4.1 (inwardly rectifying potassium 4.1), expressed in the parts surrounding the blood vessels, in both retinal membranes and in the parts facing the vitreous (i.e. in terminal parts of thick fibers). Kir2.1 mediates a unidirectional potassium flow directed at the inside of Müller cells, while the Kir4.1 mediates bidirectional currents between the extraretinal tissue and the cells themselves. The co-localization of Kir4.1 and

AQP4 around the vessels, at the retinal limiting membrane level and at the border with the vitreous, and the co-localization of Kir2.1 and AQP4 at the border with the neuronal cells, indicate the coupling of the free water transport with the potassium transport, through which Müller cells maintain the homeostasis inside the inner retinal layers. [102, 140, 142, 143] It is, therefore, supposed that the aquaporins and the potassium channels cooperate at maintaining the balance of this ion inside the retina: during elevated neuronal activity, these molecules would mediate the potassium and water uptake by the Müller cells, as well as the successive outflow into the vitreous and into the vessels. Nagelhus et al. have moreover observed that the microvilli are the only parts of these cells in which the two types of channels show a clear difference of expression, a fact which seems to have great functional importance: the combination of a low water permeability and a high potassium permeability would, in fact, protect the subretinal space from the inappropriate changes in volume. [142] Recent studies conducted on mice have demonstrated that diabetes alters not only the expression but also the localization of aquaporins.

The Iandiev group has observed that, in healthy mice, the AQP4 expressed by Müller cells and astrocytes, is present in the most internal layers of the retina, mainly around retinal vessels (both surface and deep ones), while the AQP1, expressed by amacrine cells, photoreceptors and RPE, is located in more external retinal layers. In diabetic mice, however, a strong additional expression of AQP1 has been noticed inside the Müller cells in the internal layers, as well as the aquaporin conversion from isoform 4 to isoform 1 in the portion of the membrane surrounding the surface vessels in the retina, while the AQP4 remains expressed in the portions adjacent to more profound vessels. The significance of this change remains uncertain, although the presence of a similar alteration in the retina after a transitory ischemia suggest that it may well mirror the conditions of scarce profusion in diabetic mice.[126] Considering the total expression levels of

the two aquaporins in diabetic mice, various studies agree on the increase of AQP1, while there are contrasting opinions with respect to AQP4. [116, 121, 144, 145] While the Zhang and Cui groups claim that AQP4 has increased, probably in response to the edema, the Curtis group and the Fukuda group affirm that there is a reduction of this channel, and the Gerhardinger group has not noticed any variation.[97, 121, 146-148]

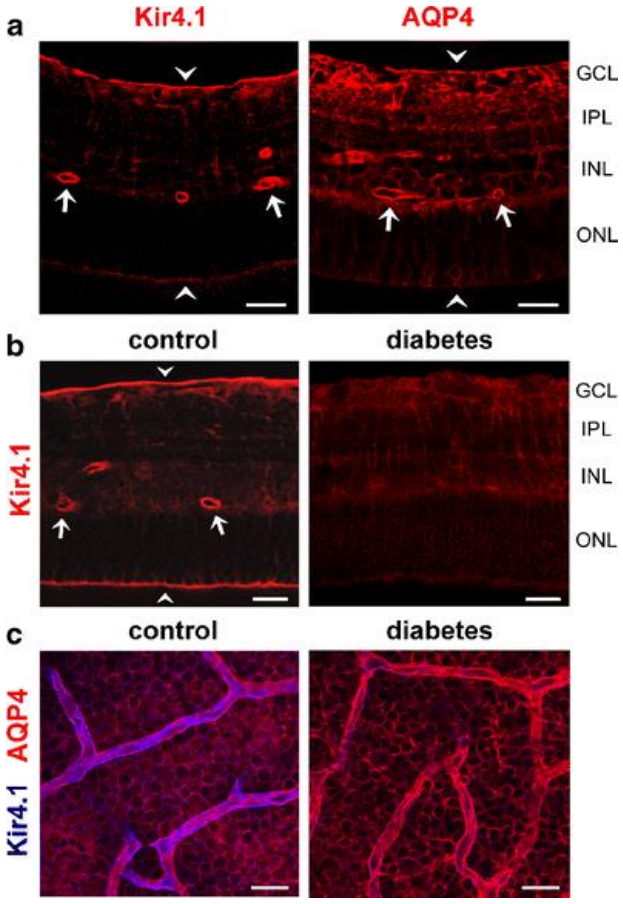


Figure 13. *The alterated expression of AQP4 and Kir4.1 provoked by diabetes in the mouse retina. (a) In controls Kir4.1 and AQP4 are expressed at the membrane level by the Müller cells which surround the vessels (arrows) and in both limiting membranes (arrow heads); AQP4 is moreover localized in Müller cell membranes in both plexiform layers and in the ganglion cell layer. (b) In diabetic mice, the expression of Kir4.1 around the vessels and the limiting membranes results absent. (c) Deep vascular plexus inside the inner nuclear layer. The vessels are surrounded by AQP4 and Kir4.1 in controls and only by AQP4 in diabetic animals. GCL: ganglion cell layer; IPL: inner plexiform layer; INL inner nuclear layer; ONL: outer nuclear layer.* [102]

The Bringmann and the Iandiev groups have observed that, when Müller cells' damage is present, in the initial phases there is a failure in the coupling of water

and potassium channels in the inner layers caused by the redistribution and the down-regulation of Kir4.1 at the levels of the vessels, the limiting membranes and those confining the vitreous, while there are unchanged expressions of AQP4 and Kir2.1 at the interface with neuronal cells. This causes an unchanged potassium uptake by the Müller cells but a compromission of its release, with consequent accumulation and increase of the osmolarity of cytoplasm and the glial swelling which could contribute to the neuronal death and the retinal edema. [100, 102, 126] The AQP1 hyperexpression could therefore be explained as a compensation attempt for the osmotic gradient between the blood and the retinal tissue, a hypothesis that seem to be supported by the contemporaneous increase in GFAP and the creation of apoptotic ganglion cells. [116, 126]

The reduced Kir4.1 expression in Müller cells would therefore be the responsible for the massive retinal neuronal loss. In addition, ion imbalances caused by the potassium channel alteration would contribute to the glutamate accumulation in the extrasynaptic ambient due to its failed removal, and this would lead to the compromission of the normal negative membrane potential.[100]

The Cui group, however, has recently observed an increased AQP4 expression in diabetic mice 48 hours after the streptozotocin injection. The same group has highlighted the ways in which the suppression of this channel's expression worsens the retinopathy through the aggravation of the inflammatory response and the increase of proinflammatory molecules such as IL-1β, IL-6, ICAM-1 and VEGF, and the increase of leukostasis. [147]

It has been recently suggested that even the AQP4-AQP1 shift in the astrocytes may lead to the imbalance of water and other solutes between the astrocytes and the ganglion cells, with consequent increase of the apoptosis of the latter. [121]

The Iandiev and Holborn group have observed that AQP6 is selectively expressed in the membranes of Müller cells surrounding the synapse at the OPL level. [122, 144] The second group has furthermore noticed a reduced

expression of this channel in diabetic mice, which is similar to what happens in the blue-light-induced damage. Given that the AQP6 has a low permeability with respect to the water, and that it prevalently functions as a passage channel for ions and hydrophilic molecules such as glycerol and urea, it has been speculated that it regulates the ion concentrations on the level of the OPL synapses: its reduced expression would therefore imply an altered synaptic activity and the consequent alteration in retinal functionality. [144]

In addition, Hollborn et al. have evidenced the expression in diabetic mice of AQP5, AQP9, AQP11 and AQP12 inside the RPE, usually not located there. AQP5, AQP11 and AQP12 could potentiate the water transport through the RPE, in an attempt to contrast the subretinal edema. The AQP9, able to transport water, ions and small solutes like lactate and glycerol, could facilitate the elimination of the excess lactate; it has to be noted, however, that its expression at the neuroretinal level remains unchanged during the course of diabetes.[144]

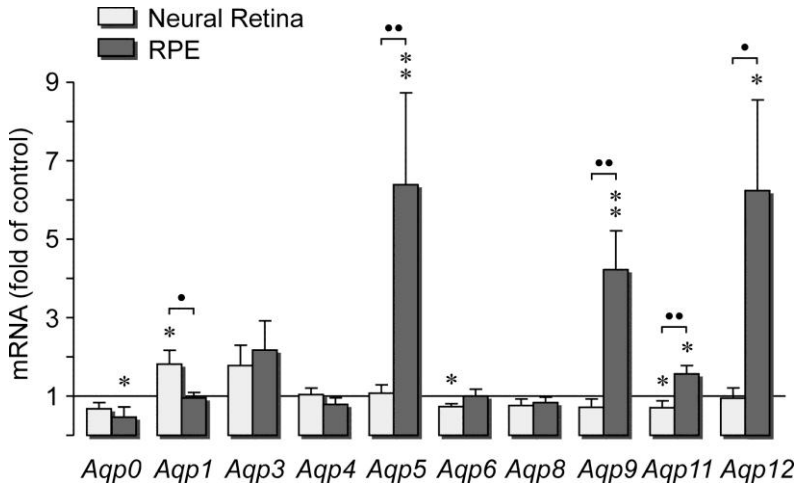


Figure 14. *Variation of mRNA expression of different aquaporins in the diabetic rat retina determined by the RT-PCR. Significant difference between diabetic rats and the controls: *p<0.05; **p<0.01. Significant difference between the neuroretina and the RPE: •p<0.05; ••p<0.01.RPE: Retinal pigment epithelium.* [144]

Lastly, Fukuda et al. have demonstrated an increased AQP0 expression in the ganglion cells' axons in the diabetic mice retina. The fact that such alteration has not been registered either during the course of ischemia or during the course of

glaucoma, seems to suggest that it is specific to diabetes, even though further studies need to be conducted in order to confirm this theory. [121]

1.7.2. Glial Fibrillary Acidic Protein (GFAP)

The GFAP (glial fibrillary acidic protein) is usually expressed only by astrocytes, and is therefore detectable only in RNFL and GCL, while it results completely absent in Müller cells. [83, 97-99, 101] The GFAP increase, a common marker for reactive gliosis, is an early sensor for retinal stress preceding the microvascular damage. [99, 149]

While it has been ascertained by now that Müller cells face an increased GFAP production during the course of diabetes, there are some works which express contrasting opinions regarding the GFAP expression in the astrocytes. [92, 150]

In past, the Lieth group had shown that there was an increased production of such protein in both cell types, while the Rungger-Brändle group had not found significant differences in the astrocyte numbers and the GFAP production in diabetic mice compared to the healthy ones.[103, 151]

Subsequently, however, Barber et al. have demonstrated that in diabetic mice there is an increased GFAP production only in Müller cells, and that this event is preceded by its reduced expression on the part of the astrocytes: in this way, the immunoreactivity of this protein is no longer limited exclusively to the RNFL and GCL layers, but it extends through the entire thickness of the neuroretina. The same authors have detected that the remarkable reduction of this protein in astrocytes disappears soon after the administration of insulin. [96]

The data presented by the Barber group is considered the most correct today, since he used immunohistochemical techniques applied to fresh histological samples and not to frozen ones (in which the tissue structure is changed): this has allowed for a more accurate cellular distinction.[96]

In addition, the recent demonstration by Kumar et al. of the GFAP increase and cellular hyperplasia in astrocytes in the early stages of diabetic retinopathy can be explained by the fact that the apoptosis and the dysfunction of these cells are still not enough to reduction of the levels of this protein. [152]

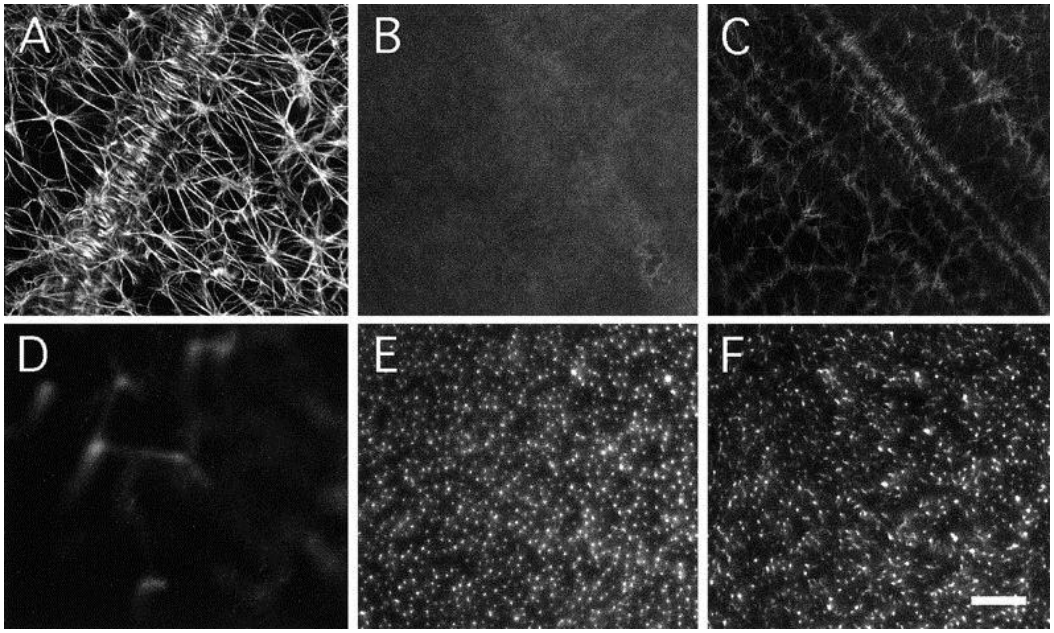


Figure 15. *Variation of GFAP expression in astrocytes and Müller cells in rat retina 4 months after the onset of diabetes, with or without the 48 hour insulin treatment.(A) Intensely GFAP immunofluorescent astrocytes in the healthy mouse. (B) The immunofluorescence for GFAP is almost imperceptible in the diabetic mouse. (C) The immunofluorescence for GFAP is elevated 48 hours after the insulin treatment. (D) Müller cells in the healthy mouse do not have immunofluorescence for GFAP. (E) Müller cells in the diabetic mouse are intensely immunoreactive for GFAP. (F) after the 48 hour insulin treatment, the immunoreactivity for GFAP is reduced. The bar corresponds to 50 μm.*[96]

1.7.3. Inflammatory Molecules

Müller cells, astrocytes and microglia are the cells of the local innate immune system. Under stress, they become active and produce proinflammatory cytokines and growth factors in order to reconstruct the tissue homeostasis, but in chronic pathologies like diabetic retinopathy, the persistent inflammatory response leads to cellular death or damage.

Liou et al. have recently confirmed that the microglia faces the activation already at beginning of diabetic pathology, producing a wide range of proinflammatory cytokines, such as IL-1β, IL-3, IL-6, TNF-α, and other inflammation mediators like

ROS, glutamate, VEGF, lymphotoxins, metalloproteinase and NO. These mediators induce the expression of adhesion molecules (I-CAM and V-CAM) in vascular cells for lymphocytes and monocyte-macrophages, cellular apoptosis, leukocyte infiltration and the weakening of the hemato-retinal barrier. [59]

The Langmann group has detected that the microglial cells are the responsible for an increased retinal production of iNOS, IL-1β, MIP-1α, IL-6 and M-CSF.[115] In addition, Shelton et al. have evidenced an increase in IL-1β, IL-6, IL-8, IL-13, IP-10, ICAM-1 and NO in Müller and endothelial cells during the course of diabetes, confirming their participation in the inflammatory process. [153]

Led by the fact that IL-1β is the main cytokine to trigger the neuroinflammatory cascade, Liu et al. have also considered its role in the amplification of the inflammation itself. This study has, in fact, highlighted that the IL-1β secretion begins in the vascular endothelium as the direct consequence of chronic hyperglycemia, which stimulates the endothelial, macroglial and microglial cells via the autocrine and the paracrine pathways, which do not respond only with activation signals, but also with the strengthening of IL-1β synthesis, thus exalting the inflammatory process. [154]

Besides the already mentioned inflammatory mediators, it is well known that many other mediators are expressed inside the retinal tissue during the course of diabetes, since it is clear by now that inflammation has an essential role in diabetic retinopathy. [62] It is generally thought that the microglial cells also indirectly intervene in their production, due to their ability, via the production of mentioned molecules, to behave as chemioattractants towards the other leukocytes, especially the neutrophils and lymphocytes, which can strengthen the inflammatory process. The analysis of retinal tissue, aqueous humor and the vitreous have in fact detected the presence of other molecules besides those already mentioned, such as TNF-α, MCP-1, RANTES, IL-10, IL-12, MCP-1, MIP-1β, PDGF e SDF-1α.[62, 155, 156]

1.7.4. Glutamate and GABA (gamma-aminobutyric acid)

By now it determined that glial cells have an essential role in the regulation of nerve activity and in the synaptic transmission in the retina thanks so the release of so-called gliotransmitters, which have both an excitatory and an inhibitor effect on the surrounding neurons. Among the various neuroactive molecules released, glutamate and GABA have the task ofmodulating the neuronal activity in cellular products: the excitation is mediated especially by glutamate, while GABA causes neuronal inhibition. [157, 158]

In the healthy retina, Müller cells have an important role in the degradation of the glutamate and GABA neurotransmitters, contributing to the proper functioning of synaptic terminations: the glutamate, released on the extracellular level by the neurons, is caught by Müller cells through the GLAST transporter (glutamate aspartate transporter); here it is rapidly converted into glutamine and led back to the neurons as a precursor of glutamate and GABA. [158]

Glutamate, despite being the main excitatory mediator in the retina and though it permits the nerve signal transmission from the photoreceptors to bipolar cells and then to ganglion cells, can result damaging if present in excessive doses at the extracellular level: it in fact provokes the so-called excitotoxicity, i.e. a nerve damage caused by hyperexcitation. It is the result of the hyperactivation of post-synaptic receptors NMDA (n-methyl-d-aspartate) which provoke the opening of the calcium channels on neuronal membranes; the consequent high intracellular levels of this ion trigger a signal cascade leading to caspase-3 or AIF-mediated cell death, and, stimulating the phospholipase, promote the release of cytotoxic fatty acids into neurons and the extracellular space. [159, 160]

The excessive glutamate concentration in the retina is probably caused both by the insufficient glutamine synthetase activity and the reduction in GLAST transporters. [161] It would moreover seem that the high extracellular glutamate

levels induce the neuronal and glial swelling, with the consequent reduction of extracellular space and neuronal hyperexcitability. [157]

A second fundamental function of Müller cells consists in the inactivation of the inhibitory neurotransmitter GABA (gamma-aminobutyric acid). After its release by the inhibitory interneurons, GABA is channeled into this type of glial cells and then deviated into the Krebs cycle through the degradation carried out by GABA-transaminase. The Ishikawa group has detected an increase in GABA levels in diabetic rats' Müller cells, caused either by the reduction of GABA-transaminase activity or, more probably, by the compromission of the citric acid cycle: such an increase would concur with the neuronal dysfunction. [162]

1.7.5. The Renin-Angiotensin System (RAS)

In the standard renin-angiotensin system (RAS), responsible on the systemic level for the control of blood pressure, salt absorption and aldosterone formation, the angiotensinogen is cleaved by the enzyme renin in order to form Ang I, which is activated by Ang II of the ACE enzyme. Ang II acts through two receptors AT1R and AT2R, the first of which seem to mediate most of the RAS functions. [163]

In the recent years, many studies have shown that alongside the systemic RAS, there are local RAS in different organs including the retina, where, besides exercising nerve and vascular functions, it contributes to the regulation of the aqueous humour pressure. Mice models have evidenced the presence of all RAS components in the retina and a higher Ang I and Ang II concentration in the anterior uvea, the retina, the retinal pigment epithelium and the choroid with respect to the plasma, confirming the presence of a local system. Since it has been observed that the most conspicuous RAS component is found on the level of capillaries, neurons, the inner retinal layers' glia, it is generally thought that

these are the most probable sources of the local RAS. The presence of renin and angiotensin in the glia and in the neurons points at their involvement not only in neuromodulation, but also in angiogenesis, given their close relationship with the vessels.[111, 163, 164]

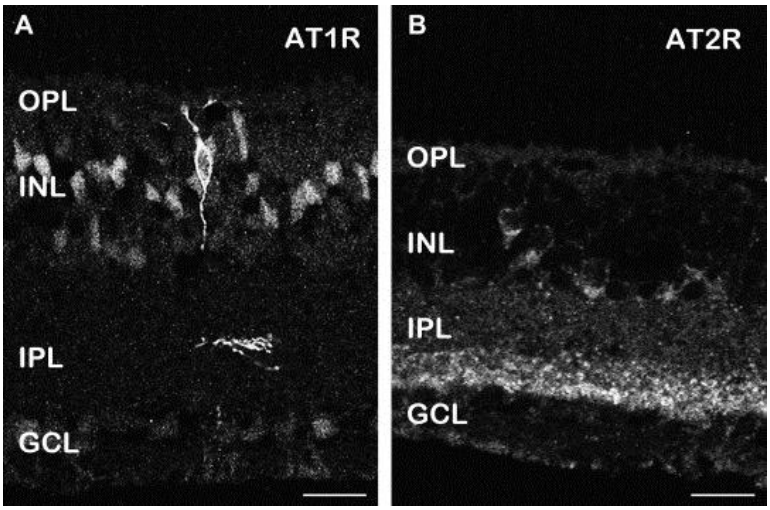


Figure 16. *A section of mice retina marked with AT1R and AT2R antibodies. (A) AT1R is prevalently localizes in glial and bipolar cells, while (B) AT2R is expressed by amacrine cells. OPL: outer plexiform layer; INL: inner nuclear layer: IPL: inner plexiform layer; GCL: ganglion cell layer.*[165]

With respect to the receptors, Phipps et al. have recently analyzed the localizations of two receptor types, with the result that AT1R was detected in astrocytes, Müller cells, bipolar cells and endothelial cells, while AT2R was detected in amacrine cells. [163] The Downie group has subsequently recognized a different function for each of the two receptors: AT1R would appear to be implicated in the neovascularization of hypoxia-induced retinopathies, while the role of AP2R is much less clear, though it would appear to oppose or compensate AT1R functions, and above all, it could be implicated in the nerve tissue survival and reparation.[166]

In the course of diabetic retinopathy, the Ang I, Ang II, renin and ACE levels result increased. Ang II provokes, through AT1R, the alteration of the hemato-retinal barrier, stimulates the release of VEGF and PEDF, favours the leukocyte

adhesion, modulates the pericyte activity, the expression of tight junctions and the neovascularization. [165] Ozawa et al. have noticed that the Ang II hyperproduction leads to the hyperactivation of AT1R and subsequently of ERK (extracellular-signal-regulated kinase), which in turn induces a diminished expression of synaptophysin, a protein contained inside the synaptic vesicles and fundamentally important for the neurotransmitter release, through its degradation mediated by UPS (ubiquitin-proteasome system). They have moreover demonstrated that Ang II induces ROS production, which in turn stimulates the production of proinflammatory cytokines, the inhibition of BDNF and the activation of ERK, inducing cellular apoptosis. [167]

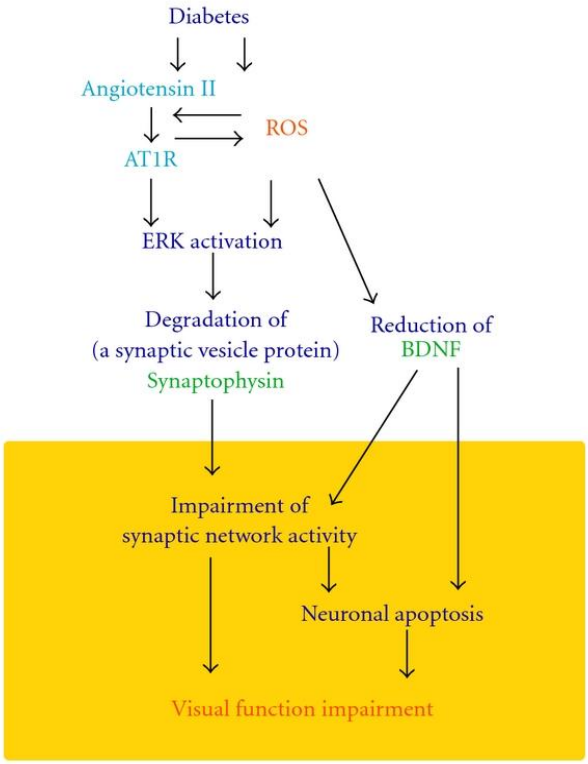


Figure 17. *Retinal neurodegeneration and visual function compromission mediated by Ang II. AT1R: angiotensin II type receptor, ROS: reactive oxygen species; BDNF: brain-derived neurotrophic factor.* [167]

As already mentioned, the role of AT2R is less clear: a hypothesis claims that Ang II provokes the activation of GABAergic amacrine cells with consequent increase in GABA release and the subsequent inhibitory effect on the bipolar

cells in rods.[165] The hyperactivation of AT1R and AT2R would therefore have an overall inhibitory effect on neuronal transmission, which would lead to apoptosis.[31]

1.7.6. Reactive Oxygen Species (ROS)

Oxidative stress is another key factor of the neuroretinal damage during the course of diabetes mellitus: it has been, in fact, observed in diabetic mice that the administration of lutein, an antioxidant able to reduce local ROS, allows for the normalization of the electroretinography (ERG) and the levels of synaptophysin, as well as for the prevention of neuronal prevention.

The ROS cause the NO reduction in diabetic subject's retina, a consequent increase in peroxynitrite and the subsequent reduction of the NGF and BDNF, responsible for the reduced neuronal growth and apoptosis, as well as the compromission of synaptic activity.

The oxidative stress also provokes the damage of Müller cells, through the down-regulation of Kir4.1 and AQP4 channels and the reduction of MMP-7 (matrix metalloproteinase-7) which converts the pro-NGF, toxic for the neurons, into the neuroprotector NGF. [167]

1.7.7. Advanced Glycation End-products (AGEs)

AGEs (advanced glycation end products) and ALEs (advanced lipoxidation end-products) concur at the onset of retinal damage during the course of diabetes mellitus. While it is generally acknowledged that the AGEs contribute to the dysfunction of the hemato-retinal barrier, to the thickening of the capillary basement membrane and to microvascular degeneration, the contribution of ALEs is less clear, although it is known that both of them accumulate especially inside Müller cells and that they are connected to the ROS generation and the activation of proinflammatory and proapoptotic pathways. [97]

RAGE (AGEs receptor) is expressed in an ubiquitous way in almost all retinal cells, but it would appear to be more represented in the glial cells of the inner retinal layers, and especially in Müller cells.[92, 168]

The chronic retinal exposition to hyperglycemia determines the hyperproduction of the AGEs and ALEs and Curtis et al. have observed that the inhibition of the latter protects against the Kir4.1 and AQP4 malfunctioning, suggesting that the dysfunction of Müller cells might be connected to their intracellular accumulation.[97]

Different studies have demonstrated that RAGE hyperexpression reaches its maximum levels in Müller cells during the diabetic pathology. The RAGE activation by the ligands such as AGEs and S100B, a calcium-tying protein, leads to the alteration of many retinal cells, including Müller cells and the microglia. This entails an inflammatory and oxidative response which induces, as well as the well-known microvascular dysfunction, also the neurodegeneration of the retina mediated by the secretion of growth factors and cytokines, the strengthening of pro-oxidative and inflammatory factors, the alteration of the extracellular matrix, the activation of Müller cells and the microglia, and cellular apoptosis.[97, 168]

Zong et al. have furthermore demonstrated that S100B, produced mainly by Müller cells and astrocytes, is significantly increased in hyperglycemic conditions. GFAP, S100B and RAGE, have resulted significantly incremented during diabetes in Müller cells, a fact probably connected to gliosis. This group has therefore shown that the RAGE hyperexpression induced by hyperglycemia carries out an important role in the activation of Müller cells and the subsequent cytokine production in the context of diabetic retinopathy.[92, 168]

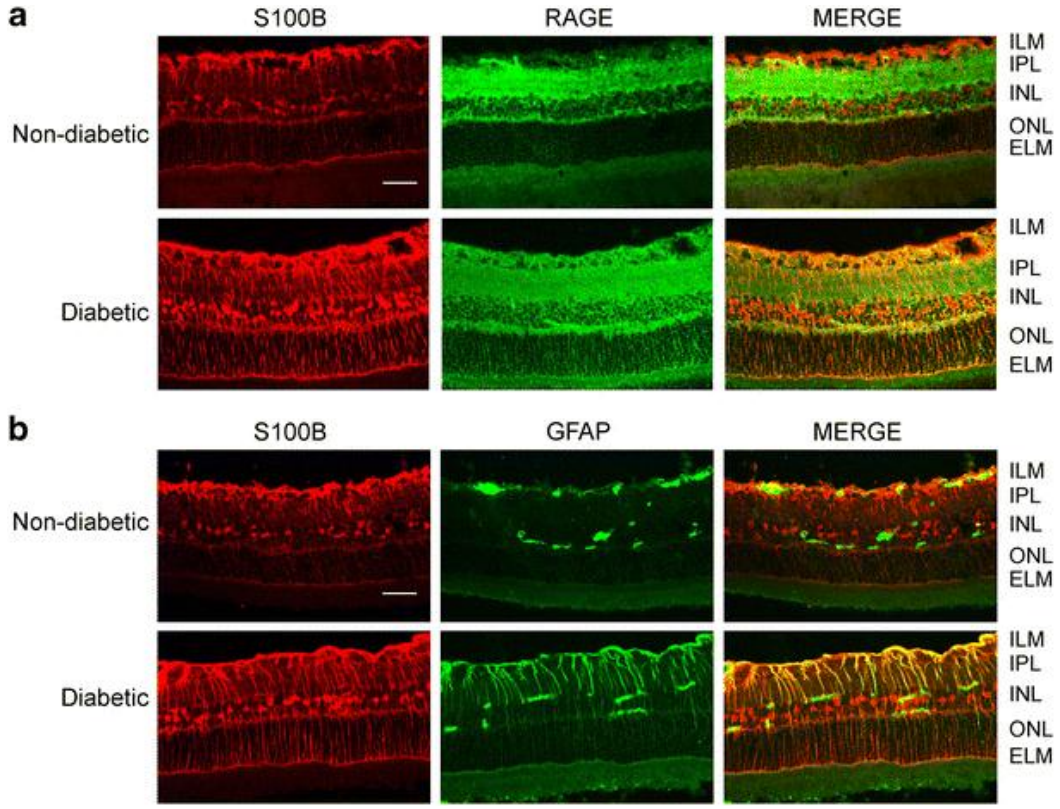


Figure 18. *Increased S100B, RAGE and GFAP expression in a diabetic mouse. The three molecules are evidenced by fluorescent antibodies: S100B in red (a and b), RAGE in green (a), GFAP in green (b). MERGE: union of the two precedent images; ILM: inner limiting membrane; IPL: inner plexiform layer; INL: inner nuclear layer; ONL: outer nuclear layer; ELM: external limiting membrane.*[92]

1.7.8. Neuroprotective Factors

The balance between neurotoxic and neuroprotective factors is crucial for the determination of retinal neurodegeneration in the diabetic patient. Among different neuroprotective factors produced by the glial cells, one must remember PEDF (pigment epithelium-derived factor), VEGF and the neurotrophins.

VEGF, which promotes the neovascularization and the increase in vascular permeability, is expressed by endothelial cells, neurons and glial cells, especially Müller cells, and its effect is contrasted by PEDF and thrombospondin-1. VEGF moreover regulates the survival and the proliferation of neurons and glial cells, having a potent anti-apoptotic and neuroprotective effect. Such effect is lacking during diabetic pathologies probably because, despite a significant increase in VEGF, the high peroxynitrite levels block the anti-apoptotic pathway by inhibiting

survival proteins and stimulating the pro-apoptotic pathway. [21, 157] The deletion of VEGF gene in glial cells furthermore reveals the importance of their communication with vascular cells in the angiogenetic process. [11] This molecule, along with other pro-angiogenetic cytokines and high glutamate levels, increases the release of matrix metalloproteinase by the endothelial cells and Müller cells: this, on one part, damages the tight junctions of the endothelium and of the RPE by means of the proteolytic occludin degradation, and, on the other, entails the loss of contact inhibition which usually prevents endothelial proliferation. [100]

PEDF results reduced during diabetic pathology probably because the hypoxia and VEGF increase the matrix metalloproteinase activity which in turn degrades the PEDF. Expressed by retinal neurons, glial cells (Müller cells in particular), vascular endothelium and EPR, it constitutes one of the most important angiogenesis inhibitors and it protects the neurons from the neurodegeneration caused by glutamate. [21, 157]

Among the neurotrophines, molecules involved in the neuroretinal development and the nerve cell regeneration, the most important ones to remember are BDNF, NGF, GDNF and CNTF.[160] The neurotrophins possess high affinity for tyrasine kinase receptors (Trks), which mediate cell survival and growth, while they have a low affinity for the neurotrophic receptor P75 ($p75^{NTR}$), member of the TNF (tumor necrosis factor) family, which regulates apoptosis and reduced cellular growth. [169]

NGF is secreted and synthesized by glial cells, and Müller cells in particular, starting with proNGF, which is proteolytically cleaved by the enzyme furine on the intracellular level and by MMP7 on the extracellular level. While NGF promotes the survival of neuronal cells through the link with TrkA and $p75^{NTR}$ receptors, proNGF induces their apoptosis due to its greater affinity towards $p75^{NTR}$.

The inflammation and the oxidative stress observed during the course of diabetes induce a ROD increase in the neuroretina which leads to a reduced NO biodisponibility, despite its increased production, and to the formation of peroxynitrite. High peroxynitrite levels cause the pro-NGF hyperproduction on the part of activated Müller cells and they prevent its maturation into NGF through MMP-7 inhibition: this leads to pro-NGF accumulation and NGF reduction with consequent death of neuronal and endothelial cells and as well as the compromission of the hemato-retinal barrier. The neurodegeneration is promoted also by receptor alterations: although TrkA expression remains unchanged, its activity is compromised, while the hyperexpression of $p75^{NTR}$ causes the prevalence of apoptosis. [169-171] The increase of pro-NGF in the intraocular tissue would hence signify an attempt by the cells to stimulate the inhibited pathway, even though it results inefficient due to the alteration of receptor pathways. [170]

BDNF is expressed in many retinal cells, including Müller cells and neuronal cells, and is fundamental for the survival of ganglion and amacrine cells and for the synaptic functionality, since BDNF protects the neurons from cellular death via the tyrosine-kinase receptor TrkB. [172] BDNF levels are regulated also by the neuronal synaptic activity, suggesting that their maintenance might involve the preservation of synaptophysin. It has been stated that in the diabetic subject's retina the BDNF levels result reduced both in ganglion cells and in the Müller cells. [173]

GDNF is usually expressed by photoreceptors, to a lesser extent when compared to the Müller cells and the astrocytes. It induces a FGF increment in Müller cells, and favours photoreceptor survival, protecting the ganglion cells from glutamate-mediated apoptosis through the increased uptake of the latter by GLAST. Zhu et al. have recently demonstrated that in hyperglycemic conditions there is an hyperexpression of GDNF and its receptors GFRα1 and GFRα2 on the part of

Müller cells, suggesting that it might be a protective reaction directed toward the nerve and glial cells. [98, 174]

Less known is the role of CNTF, which promotes the survival of photoreceptors and ganglion cells. The levels of such molecule, produced by the Müller cells, the asctrocytes and the RPE, increase the rate of retinal degeneration, which is quite possibly also what happens in diabetic pathology.[98]

1.8. Spectral Domain OCT (SD-OCT)

Figure 19. *Photo of SD-OCT HRA+OCT (Heidelberg Engineering, Heidelberg, Germany).*

1.8.1. Comparison between Time Domain OCT (TD-OCT) and Spectral Domain OCT (SD-OCT)

The optical coherence tomography (OCT) is a non invasive technique which provides high resolution images of cross-sections of the retina in vivo. Compared to CAT scans and ophtalmoscopy, it has a greater spatial resolution, an inferior acquisition time, and it does not need direct contact with the ocular surface. [175]

Its use allows to obtain more precise distinctions of retinal layers compared with the histological method, thanks to the system's high definition. [20]

The OCT is based on the 'low coherence interferometry' technology, initially applied in ophthalmology for the in vivo measurement of the ocular axial length. The procedure is analogous to the CAT scan, but, because it uses light waves in place of sound waves, it allows to measure the intensity and the delay time of the light reflected echo pulses. [20]

The oldest type of Time Domain OCT (TD-OCT) emits, via a superluminescence diode, a low-coherence light beam directed towards the retina with the wavelength similar to the infrared (843 nm) one. This beam crosses a partially-reflecting mirror which splits it in two beams: the reference beam and the sample one. While the sample beam is directed towards the analyzed eye and is reflected by the ocular tissues according to their distance and their characteristics of density and thickness, the reference beam is reflected by a longitudinally oscillating mirror. Both beams return to the partially-reflecting mirror around the same time, recombine in the interferometer are transmitted to photosensitive detector. The constructive interference between the light impulses reflected by retinal structures and the reference mirror happens only if these impulses reach the interferometer at the same time and only then it is possible to detect the resulting signal and intensity.

The interferometer, therefore, detects, elaborates and memorizes the delay schemes of the echoes coming from the retina and this operation is repeated in the whole duration of the scan. The obtained bidimensional image (tomogram) represents a cross-section of the tissue analyzed by the light beam, and the difference, in terms of the frequencies absorbed by the tissues, is translated into a number to which a colour or a shade of grey is assigned according to the difference in reflectivity of the layers at different depths: the highly reflective structures are visualized in brilliant colours like white or red (nerve fiber layer and RPE), the low reflective structures are represented with darker colours like black or blue (photoreceptor layer and the inner and the outer nuclear layers), while the moderately reflecting structures appear grey-white or green (inner and outer plexiform layers). The intensity of the signal depends on the optical properties if the examined tissues' structures and on different preretinal factors, e.g. media opacity. [20, 175-178]

The newer Spectral Domain OCT (SD-OCT), or Fourier Domain OCT, uses instead a fixed reference mirror and a spectrometer which catches the incident signals. The information provided by the spectrometer is converted into the depth scans of the Fourier-transform, an operation which consists in a series of formulas able to break down any wave into the sum of harmonics that compose it. Measuring thus all the light echoes simultaneously, and not sequentially as it occurs for the TD-OCT, the SD-OCT increases significantly the quantity of data acquired with each scan, with an important reduction in movement artifacts, an increase of signal-ground noise ratio, a greater image resolution, a more precise definition of retinal layers, a greater acquisition speed and a possibility to precisely map the optic nerve head.

By using the appropriate software, SD-OCT can moreover elaborate 3D images and compare them to the photographic, angiographic and autofluorescence images. [175, 179, 180]

Comparing the two OCT types, many differences can be detected which explain the SD-OCT advantages. The TD-OCT realizes 400 A-scans (monodimensional scans) per second, and the B-scan (bidimensional) images have an axial resolution of 10 μm. This definition, however, can lead to failed diagnoses of the milder retinal alterations and it is not likely that it can distinguish the RPE-Bruch's membrane complex, which causes the exclusion of the retinal thickness from the measuring process, with subsequent underestimation of the same. The SD-OCT, however, has a greater repeatability of measurement compared to TD-OCT, even though its different models, using its own calculation algorithms, result in different values for retinal thickness, so that the two are not interchangeable.[184]

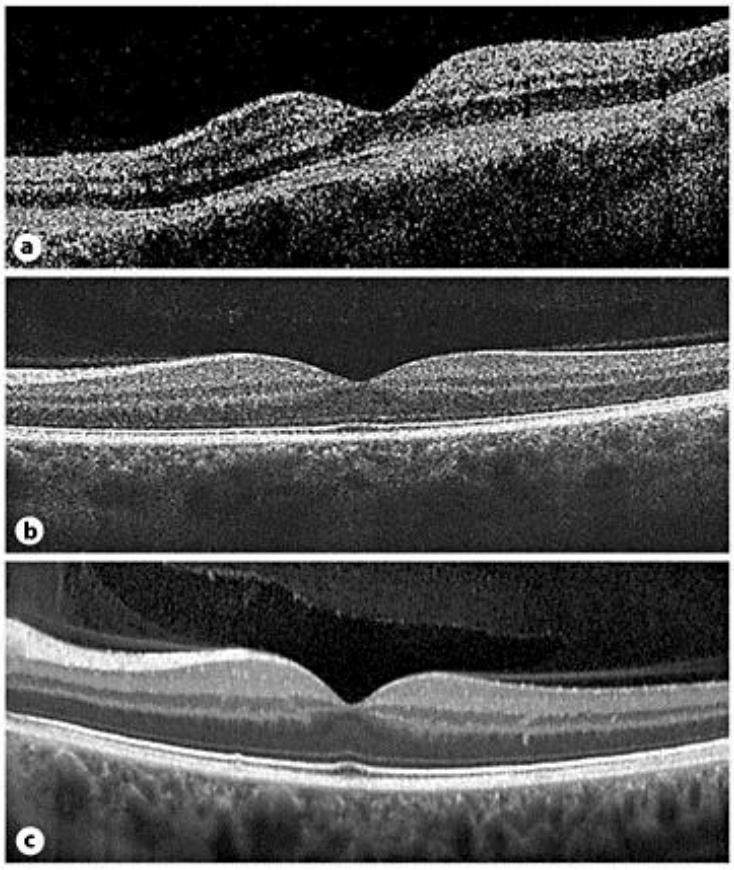


Figure 20. *OCT images of the same non-pathologic macula made by (a) first generation TD-OCT (Stratus OCT), (b) second generation TD-OCT (Cirrus HD-OCT) and (c) SD-OCT (Spectralis HRA+OCT). Note the progressive improvement in image definition.* [185]

In order that the OCT may be used for the qualitative evaluation of the structural retinal pathologies caused by other ocular pathologies, e.g. diabetic retinopathy, it is necessary to carry out a segmentation of different retinal layers, which permits the individuation and the evaluation of thicknesses. There are, however, two problems regarding the automatic segmentation: the first one consists in the fact that the pathologic retina usually presents substantial alterations which can affect the results of the procedure, and the second one consists in the fact that the transparency loss of the dioptric media creates an unclear image with spotted areas which hamper with the procedure. For these reasons, there is usually the need for an operator to manually correct the automatic segmentation, thus correcting the errors caused by accumulations of liquids in the retina. [186, 187]

1.8.2. The importance of OCT during the course of diabetic pathology

By now, it has been generally acknowledged that the OCT constitutes a valid instrument for the diagnosis of diabetic macular edema. Forooghian et al. have demonstrated that measurements of macular thickness and volume when carried out by SD-OCT result significantly more precise compared those measured by TD-OCT. [185, 188] Beside the central retinal thickening, in the case of macular edema, the OCT can also show the intraretinal cysts, subretinal fluids and signs of vitreofoveal traction. [175]

A number of studies have evidenced that the OCT is able to detect the retinal thickness' anomalies already in diabetic patients with no signs of retinopathy and in those with mild, moderate or severe retinopathy without clinically significant edemas, reporting, however, contrasting results. Most of these studies have used TD-OCT.

Schaudig et al. have described an increased macular thickness of the superior quadrant in diabetic subjects with DR compared to those without DR and to healthy controls. [189] The Pires and the Sànchez-Tocino groups have also detected a thickening in certain macular areas in the early stages of DR. [190, 191] Conforming to this data, Oshitari et al. have noticed an increase in macular thickness and a reduction in RNFL thickness in patients with initial stages of diabetic retinopathy, suggesting that they might be caused by neuronal degeneration and the increase of vascular permeability. [192] The Lattanzio group has moreover evidenced a macular thickening in diabetic patients without retinopathy when compared to healthy controls. [193] Sng et al. have instead detected an increase in the foveal and the external temporal macular area thickness in patients with moderate or severe retinopathy without DME, while there was no significant difference between diabetic patients with mild and normal retinopathy. [194]

By contrast, the Massin, Alkuraya and Bressler group have not reported significant differences in macular thickness in patients without or with mild retinopathy with respect to healthy controls. [195-197]

On the other hand, in two independent studies, the Biallosterski and the Nilsson groups have noticed a significant reduction in macular thickness in diabetic patients with mild retinopathy, suggesting that it may be caused by the loss of nerve tissue. [198, 199] Asefzadeh et al. have observed that macular thickness is significantly inferior in subjects without or with mild retinopathy compared to subjects which present one of the more advanced forms of this condition**.**[200] Lastly, the Verma group has noticed a reduction of the macular thickness in diabetic patients without retinopathy and a proportional reduction of retinal sensitivity, detected via microperimetry. [181]

Such differences in the measurements of total macular thickness may be explained in various ways: for example, certain works have completely excluded subjects with macular edema, while others have not; some have considered exclusively subjects affected by type 1 diabetes, others only the type 2 diabetic subjects, while certain works have considered both types; usually the used instruments were different; and, lastly, in many cases, the studied population was not divided according to the characteristics which affect the measurements, such as age, gender, ethnicity and myopia.

Van Dijk et al. have recently noticed, this time using the SD-OCT on patients with mild retinopathy and afflicted by type 1 diabetes mellitus, a thinning of GCL in pericentral macular area and in RNFL in more peripheral macula. GCL is afflicted in an earlier stage, and is compromised in direct proportion to the duration of diabetes, while RNFL is afflicted only subsequently, since the axonal degeneration follows the cellular body degeneration. [201, 202] Contemporarily, Cabrera et al. have observed that the RNFL and the GCL+IPL appear thinner in diabetic subjects with or without mild diabetic retinopathy, resulting therefore

more susceptible to the initial damage. [184] The Peng group has also detected the RNFL thinning in the superior quadrant of diabetic patients without retinopathy. [203] Finally, a recent study by Araszkiewicz et al. has evidenced a reduced thickness of RNFL, GCL and of the total macula in diabetic patients with RD and without DME, compared to patients without RD. [204] In accordance with these results, two studies which have used SLP (scanning laser polarimetry) have pointed at RNFL thinning especially in superior quadrants of diabetic patients. [205, 206]

Lastly, two recent works presented at the ARVO (Association for Research in Vision and Ophthalmology), which have used the most recent SD-OCT technology, have evidenced certain morphological alterations in vivo on the retina of diabetic subjects: in the first study, a significant increase of ILM, IPL, INL thicknesses has been observed as well as a decrease of GCL and RNFL thickness in patients with non proliferative retinopathy, while the second one has detected a thickening of the INL+IPL complex and the presence of microaggregates in ILM and RNFL, which indicate an activated microglia.[207, 208]

Considering the concepts explored so far, it can be understood how in diabetic patients the OCT has shown itself to be a very useful instrument not only regarding the patients with diabetic retinopathy but also regarding the ones who do not present such complication, demonstrating the ways in which neuronal damages precede the vascular damage.

PURPOSE OF THE STUDY

The objective of this study is to investigate the eventual differences, both qualitative and quantitative, in the expression of specific proteins inside the aqueous humour sampled both in healthy subjects and in diabetic patients with or without retinopathy. The purpose is to identify the possible biomarkers of glial activation, implicated in the degenerative neovascular process and to put them in relation with the stage of the retinal compromission. We have chosen to analyze the aqueous humour because the collection of samples results less invasive compared to the analyses of the vitreous (the object of study of most published papers), while the information provided by its analysis is equally useful for the understanding of the onset and progression of diabetic retinopathy.

MATERIALS AND METHODS

3.1. Bibliographic research

As regards the selection of molecules to investigate, a bibliographic research has been conducted on PubMed, with no year limit, inserting and associating in different ways the key words: "*diabetic retinopathy, diabetes, neurodegeneration, retinal degeneration, neural apoptosis, neuroprotection, physiopathology, ganglion cell, glia, Müller cell, astrocyte, microglia, glial activation, inflammation, aqueous humor, vitreous, tear fluid, ELISA, protein array, protein, proteomics, aquaporin, GFAP, cytokine, neurotrophins, renin angiotensin system, reactive oxygen species, glutamate, advanced glycation end products, oxidative stress, biomarkers".*

A list of proteins has therefore been defined as the object of study of the aqueous humour, based on which a more elaborate study has been conducted.

3.2. Study Population

34 eyes of the same number of patients of legal age have been studied at the Eye Clinic of the University of Padua, from January till July 2012.

The study population has been selected in such a way that it forms four groups:

- Control group: 12 subject not affected by diabetes mellitus
- Study group: 22 subjects affected by type 1 or 2 diabetes mellitus. This group has been further divided in three subgroups according to the presence of non proliferative diabetic retinopathy (NPDR) and macular edema (ME):
    - 11 diabetic patients without clinical signs of NPDR.
    - 11 diabetic patients with clinical signs of NPDR of which 5 without ME and 6 with ME.

In every subject the cataract diagnosis was present, with indications for the phacoemulsification and the intraocular lens implantation intervention. An informed consent was obtained from each patient and the research has been carried out in accordance with the Declaration of Helsinki regarding the experimentation involving human tissue, and has been approved by the Local Ethics Committee.

The diagnostic criteria used for the inclusion of patients in the group of diabetics, valid for both sexes and for every age, have been established in 2011 by the WHO (World Health Organization): glycated hemoglobin>6.5% in two occasions, or glycemia ≥126 mg/dl after at least 8 of fasting in two occasions, or glycemia ≥200 mg/dl after 2 hours from a oral glucose tolerance test to be confirmed by a fasting test, or casual glicemia ≥ 200 mg/dl in presence of typical symptoms (polyuria, polydipsia, weight loss). [209]

The study has not taken into consideration patients affected by neurodegenerative disease (e.g. Alzheimer's, Parkinson's, dementia), neoplasia in the central nervous system or other nerve pathologies which affect sight and unrelated to diabetes; subjects who systematically took steroid treatments and/or anti-VEGF therapies and/or medicines treating the central nervous system or with neuroprotective effects in the three months preceding the intervention; patients afflicted by poorly controlled systemic pathologies. In addition, the study has excluded patients whose studied eye presented: intraocular pressure greater or equal to 22 mmHg or with a history of glaucoma; other ocular pathologies of vascular, degenerative or inflammatory nature not ascribable to diabetes; topical therapies with ocular antihypertensive drugs, cortisone drugs, FANS or anti-VEGF drugs in the 30 days before intervention; eye surgery history in the three months before intervention; subjects who, in ophtalmoscopy or OCT exams, presented significant vitreo-retinal tractions which could affect retinal thickness; excessive opacity of the dioptric media which prevented ophtalmoscopic

evaluation or the acquisition of retinal images with OCT which could prevent a certain evaluation of presence/absence of DR or other retinal pathologies. Lastly, subjects who had participated in other clinical studies in the 90 days before intervention have been also excluded.

After collecting a clinical and pharmacological anamnesis, ocular and general, every patient has underwent a complete eye exam in accordance with the routine clinical preoperative procedure, which consisted in the evaluation of visual acuity and refraction, slit-lamp biomicroscopy, tonometry, and the ophtalmoscopic exam of the eye fundus with eventual grading of DR according to internationally established criteria. [28] Each subject has moreover undergone an OCT exam of the studied eye.

3.3. OCT Exam

Every subject's studied eye has undergone the spectral domain optical coherence tomography (SD-OCT) with Spectralis HRA+OCT(Heidelberg Engineering, Heidelberg, Germany). Two scan patterns have been used:

- "7 Line Raster Scan": 6mm linear scans centered at the fovea at 0°, 30°, 60°, 90°, 120°, 150° in ART mode (100 images averaged).
- "Dense Volume Scan": macular area of 6x6 mm centered at the fovea (512x456 pixel) in ART-mode (60 images averaged).

For each SD-OCT linear scan, an automatic algorithm has individuated different retinal layers based on the different shades of gray corresponding to the reflectivity indexes of each layer.

The available algorithm (version 5.5.0.5) was, however, able to elaborate this stratification in a reliable way only on the linear scans of the fovea, not on those of the macular mapping. For this reason, various linear scans have been extrapolated from the "7 Line Raster Scan" acquisition, and have undergone the automatic stratification with a consequent correction by an operator. Since the

error of the instrument consisted in the inexact identification of the border lines between retinal layers, the manual correction consisted in the repositioning into proper place of the incorrectly placed points, in order to redefine the retinal profile.

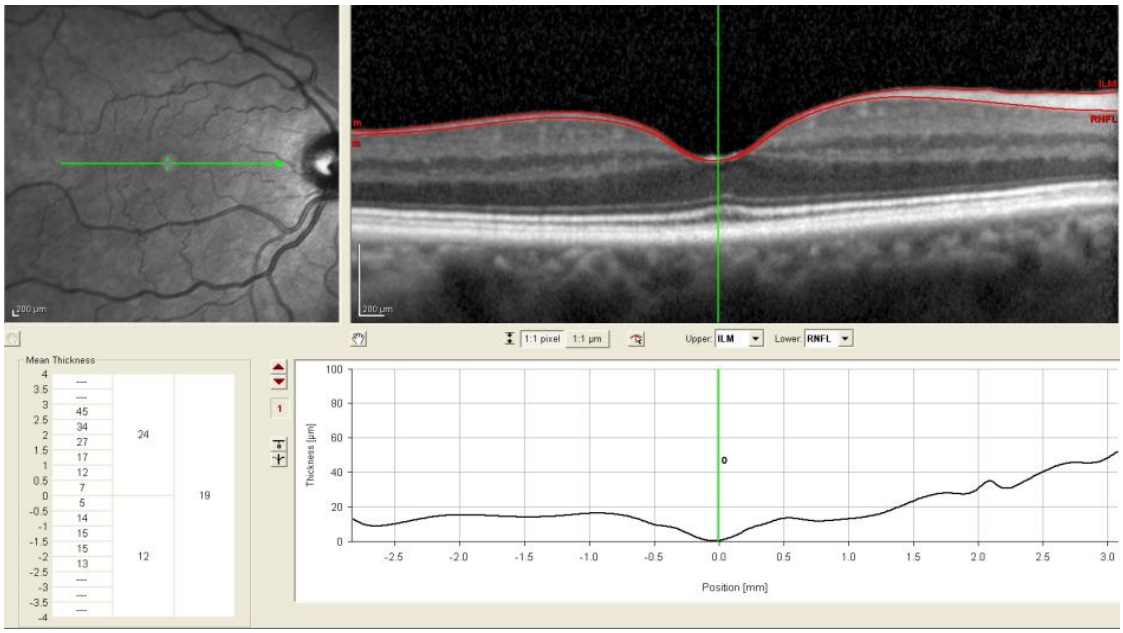


Figure 21. *Visualization screen at the SD-OCT exam with Spectralis HRA+OCT. On top left there is an infrared image of the eye fundus with the localization and the direction of the scanning; on top right, the scan image with the identification of RNFL (between the two dividing lines); on bottom left, the middle value of the considered layer in different distance intervals from the fovea; on bottom right, the graph representing the thicknesses of the considered layer in different intervals.*

For each of the 6 scans, 6 retinal layers of interest have been segmented, which, from inside to out, were:

- Retinal nerve fiber layer (RNFL): from the inner limiting membrane (ILM) to the border with the ganglion cell layer;
- Ganglion cell layer (GCL): from the border with the nerve fiber layer to the border with the inner plexiform layer;
- Inner plexiform layer (IPL): from the border with the ganglion cell layer to the border with the inner nuclear layer;

- Inner nuclear layer (INL): from the border with the inner plexiform layer to the border with the outer plexiform layer;
- Outer plexiform layer (OPL): from the border with the inner nuclear layer to the border with the outer nuclear layer;
- Outer nuclear layer (ONL): from the border with the outer plexiform layer to the border with the external limiting membrane (ELM).

For each layer of each linear scan (at 0°, 30°, 60°, 90°, 120°, 150°), the algorithm has automatically calculated the average thickness of 0.5mm side intervals starting from the fovea, the so-called ‘point 0’, up to 2.5 mm away from it (for the total length of 5mm). In this way, 5 intervals have been defined on each side (10 in total) starting from the point 0, which were: -2.5/-2mm, -2/-1.5mm, -1.5/-1mm, -1/-0.5mm, -0.5/0mm, 0/+0.5mm, +0.5/+1mm, +1/+1.5mm, +1.5/+2mm, +2/+2.5mm (the negative and positive values have been attributed based on the orientation with respect to the fovea). Each interval of each scan has been identified by the arbitrarily assigned progressive numbering (from 1 to 60), resulting in a diagram which has helped create a map of analyzed points (figure 22).

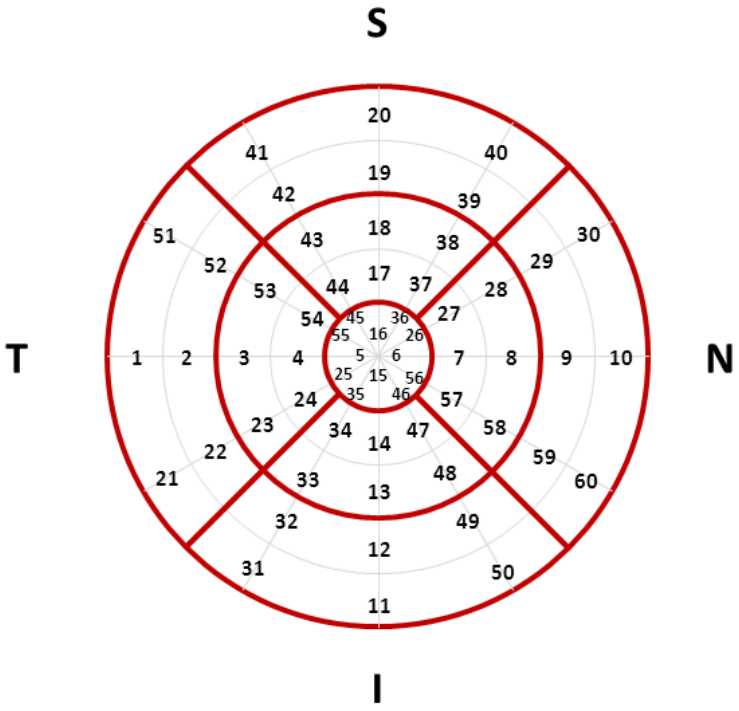


Figure 22. *Representation of the intervals considered (60 in total) in the analyzed scans. Spatial positioning with respect to the fovea: S: superior; N: nasal; I: inferior; T: temporal.*

The intervals have been compared in various patients considering the right eye as reference, while the relative points of the left eye have been analyzed according to the way in which the comparison of the areas in ETDRS chart functions.

3.4. Collection, conservation and analysis of aqueous humour

All patients have undergone the normal preoperative preparations for the phacoemulsification surgery and the intraocular lens insertion: disinfection of periocular skin with povidone-iodine 5%, instillation of sterile lidocaine, irrigation of the conjunctival sac with povidone-iodine 5% and abundant washing out of the eye with BSS (balanced salt solution).

A quantity of aqueous humor (between 150 and 200 µl) has been aspirated from the anterior chamber of the eye, carrying out a parecentesis under microscope

with a 30 gauge needle and an insulin syringe (1 ml), and avoiding all contact with the intraocular tissues, since they are possible sources of sample contamination.

Immediately after the aspiration of AH, the first operator has carried out the subsequent surgical maneuvers according to normal surgery procedure, while a second operator has handled the division and the conservation of material.

The collected sample has been divided into two 50µl aliquots which were placed into conical test tubes of 1.7ml each, previously numbered in a progressive way and conserved at temperature of +4°C.

Each of these contained 50µl of RIPA buffer (RadioImmunoPrecipitation Assay buffer) which was modified (25mM Tris-buffer, 150mM NaCl, 0.1% Tween20, 1mM EDTA -EthyleneDiamineTetraacetic Acid-, 10% glycerol, 0.1% SDS -Sodium Dodecyl Sulfate-, 10mM NaF e 1mM PMSF -Phenylmethanesulfonyl Fluoride-; ph=7.5) and to which 50µL T-PER (Protein Extraction Reagent) supplemented with a cocktail of protease inhibitors (code #0078510; Pierce Biotechnology, Rockford, IL) was added. The excess material was placed into a third empty test tube.

After closing and delicately shaking the test tubes in order to favor the complete mixing of the sample with the conservation medium, on each test tube only and exclusively the reference “PT: ID code” (Patient: identification code of the study subject) was added.

Within an hour from the collection, the samples were transported in portable thermal containers from the OR to the refrigeration cell where they were frozen at -20°C and conserved.

After having collected all the samples, they were shipped to the IRCCS (Institute for Recovery and Care of Scientific Characteristics), G.B. Bietti Foundation for Study and Research in Ophthalmology (Rome), in accordance with the international regulations regarding the transport of biological material.

In the laboratory unit, the samples were conserved at the temperature of -70°C. After the quality/quantity analyses of total protein, and before the subsequent protein analyses (ELISA and protein array), the AH has been treated sonically (VibraCell, Sonics, Newton, CT) in order to effectively extract cell protein (to share DNA/RNA), and then clarified by centrifugation.

3.4.1. Quantitative determination of total protein

According to the study design, the sampling of AH was carried out dividing the total collected amount in two different buffers (T-PER and RIPA) which allow specific analysis.

For RIPA sampling, the quantification of total protein was carried out according to the Bradford method. In brief, 2µl of AH were diluted with 18µl of ddw (water DirectQ5; millipore.com) and mixed with 200µl of a prediluted solution (code 500-0006; Biorad, bio-rad.com). After a brief incubation of 5 minutes at room temperature, 3µl of each sample and of a reference standard curve (0-1000µg/ml BSA; sigma-aldrich.com) were acquired at the digital spectrophotometer with the appropriate Bradford option (Nanodrop ND1000 UV-Vis Spettrofotometer; nanodrop.com). The protein concentration was calculated based on the linearized standard curve (BSA) as provided by the program.

For T-PER sampling, 3µL samples were directly evaluated by digital spectrophotometer, using 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 appropriate buffer. The data was used to evaluate possible variations in total protein content between groups and to normalize the samples before loading in the specific assay.

This analysis allowed the evaluation of the potential variations in total protein concentrations between and within different groups, and the normalization of the samples before proteomic analyses.

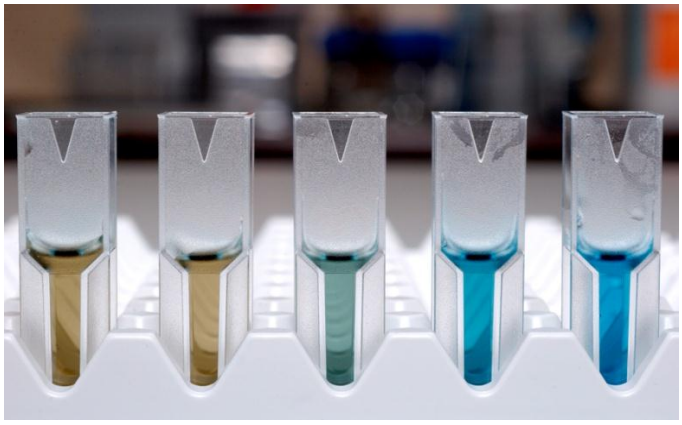


Figure 23. *Protein quantification with Bradford method: from left to right we notice the increasing protein concentrations.*

3.4.2. GFAP, AQP1 and AQP4 Analyses with ELISA test

The ELISA tests used to quantify GFAP, AQP4 and AQP1 were the following: GFAP-NS830 (millipore.com), AQP1-MBS700396 (mybiosource.com), AQP4-MBS705290 (mybiosource.com).

This double-sandwich ELISA is a solid phase assay which uses 96well-plastic pre-coated plates, which permits to contemporarily assay a high number of samples (up to 40 samples in duplicate) from different patients in order to analyze the same molecule. As in the protocol provided by the manufacturer, for every specific ELISA, both prediluted samples and standard curve, prepared in TBS buffer (20mM Tris-Cl and 150mM NaCl, containing 3% of BSA, 5mM EDTA and 1x protease inhibitor cocktail) were applied to the pre-coated plates appropriately pre-absorbed. Standard curves (range) and limit detections were as follows: GFAP, 1.5-100ng/mL and 1.5ng/mL; AQP4, 15.6-1000pg/mL and less than

6.6pg/mL; AQP1, 15.6-1000pg/mL and less than 3.9pg/mL. After an incubation period of 18hrs and the appropriate washes, the appropriate biotin-conjugated-antibodies and the streptavidin-peroxidase complex were added, as provided by the kit. The specific binding was visualized by adding the TMB (tetramethylbenzidine) substrate (ebiosource.com). In case of the presence of the antibody-biotin-streptavidin-peroxidase complex, the sublayer turns to yellow with the intensity proportional to the antigen concentration in the sample. The enzyme reaction was blocked through acidification (adding the 2N HCl stop solution) and the colorimetric signal was read by the Sunrise plate reader spectrophotometer of (tecan.com) at 490nm wavelength. Finally, the GFAP, AQP1 and AQP4 concentrations in the samples were estimated according to the 3rd grade polynomial standard curve provided by the manufacturer.

Figure 24. *ELISA kit used for AQP1 and AQP4 quantification*

3.4.3. Inflammatory profile analysis with protein-array

The protein array was conducted using the RayBiotech technology established by the manufacturer (raybiotech.com). This method offers the advantage of contemporary evaluation, for every single sample, of a significant number of proteins, depending on the precostumized array. Both membrane (array series with 4 pre-spotted membranes) and glasschips (G series comprising 1 slide with 4 or 8 identical subarrays) were used in this study. Normalizations, Positive/Negative and Internal controls were carried out according to the procedure.

Membrane-based arrays: All the samples were analyzed using the inflammatory array kit (#AAH-INF-3), providing all reagents suitable for analysis. As established by the manufacturer, the membranes were equilibrated in buffer solution and incubated with 300ng/ml of protein extract appropriately diluted in lysis buffer containing the protease-inhibiting cocktail. After an 18-hour incubation and the appropriate washes, the antigen-antibody binding was recognized by adding a cocktail of specific secondary biotin-conjugated antibodies, followed by the incubation with streptavidin conjugated to peroxidase. After further washes and the addition of the luminal substrate (ECL; SuperSignal West Pico Trial; pierce.com), the specific signals were acquired by Kodak Image working station equipped with the 1D Kodak Image Analysis software (Kodak 550, Eastman Kodak Company, Sci. Imaging Systems, Rochster, NY). The chemiluminescent signals (spots) were quantified using the single spot densitometry mode provided by the NIH 1D ImageJ software (Image J v1.43; http://rsb.info.nih.gov/ij/). The data was normalized according to the suggestion provided by the manufacturer and have subsequently been statistically confronted.

C

Inflammation profile

(protein array on membrane)

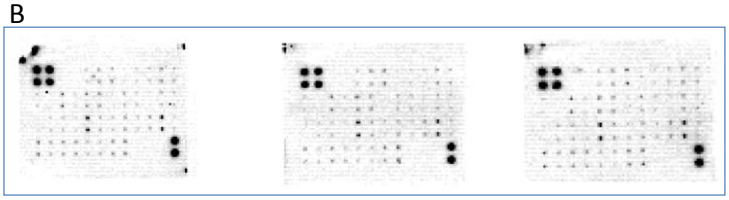
A

RayBio® Human Inflammation Antibody Array 3 Map

	A	B	C	D	E	F	G	H	I	J	K	L
1	POS	POS	NEG	NEG	EOTAXIN	EOTAXIN-2	GCSF	GM-CSF	ICAM-1	IFN-γ	I-309	IL-1α
2	POS	POS	NEG	NEG	EOTAXIN	EOTAXIN-2	GCSF	GM-CSF	ICAM-1	IFN-γ	I-309	IL-1α
3	IL-1β	IL-2	IL-3	IL-4	IL-6	IL-6sR	IL-7	IL-8	IL-10	IL-11	IL-12 p40	IL-12 p70
4	IL-1β	IL-2	IL-3	IL-4	IL-6	IL-6sR	IL-7	IL-8	IL-10	IL-11	IL-12 p40	IL-12 p70
5	IL-13	IL-15	IL-16	IL-17	IP-10	MCP-1	MCP-2	M-CSF	MIG	MIP-1α	MIP-1β	MIP-1δ
6	IL-13	IL-15	IL-16	IL-17	IP-10	MCP-1	MCP-2	M-CSF	MIG	MIP-1α	MIP-1β	MIP-1δ
7	RANTES	TGF-β1	TNF-α	TNF-β	s TNF RI	s TNF RII	PDGF-BB	TIMP-2	BLANK	BLANK	NEG	POS
8	RANTES	TGF-β1	TNF-α	TNF-β	s TNF RI	s TNF RII	PDGF-BB	TIMP-2	BLANK	BLANK	NEG	POS

Note: IL-12 p40 detects only IL-12 p40 and IL-12 p70 detects only IL-12 p70
Note: TGF-β1 detects only active form

B



Control Diabetes Diabetes with RD

Figure 25. Membrane based protein array related to the inflammatory pathway. ***A.*** *Schematic representation of the array.* ***B.*** *Representative chip-arrays specific for (from left to right: control, Diabetic w/o RP and Diabetic with RP). A dot represents a specific protein, as schematized in the map. The black intensity (ECL staining) represents the positive staining, as detected and quantified by Kodak. White color represent an over expression.*

Glasschip-based arrays. For the single staining, array-chips (#AAH-INF-G-3) were incubated with samples and the specific binging was labeled with a biotin-conjugated cocktail of Abs, followed by a cy3-conjugated streptAvidin complex, and directly acquired by the Genepix 4100 microarray scanner (Molecular Devices LLC, Sunnyvale, CA) equipped with the GenePix Pro 3.0 software (Axon Instruments, Foster City, CA), according to the manufacturer's procedure. The fluorescence signals were provided as median values, according to internal parameters.

An extension of the chip-technology was developed to evaluate simultaneously differences in samples from 11 patients with diabetic retinopathy (with or without edema) as single staining (cy3 labeling), compared to a mix of samples from 11

diabetic patients without diabetic retinopathy or 12 controls. In this double staining procedure, pathological and control groups were labeled with Cy3-dye and labeled proteins were purified in spin columns (GE Healthcare, gelifesciences.com). In parallel, a common reference protein was made from pooled samples from a selected group of individuals: total proteins were labeled with cy5 as previously reported. Each cy3-pathological/control sample was combined with an equal amount of pooled cy5-labeled common reference. A 1:1 mix pathological: control mix (70µL/well/chip) was hybridized for 18hrs at 4°C. After washes in stringency conditions, the glass-slides were removed from plastic tray-support and washed once in ddw to remove salts and quickly spin to dry the chips. The double-fluorescence signals were acquired with the Genepix 4100 microarray scanner. The software provides the cy3-signal for each spot to be compared with those of cy5-signal, represented by the common reference hybridized to the same chip/spot and expressed as ratio.

Figure 26. *Kit for cytokine quantification with protein array.*

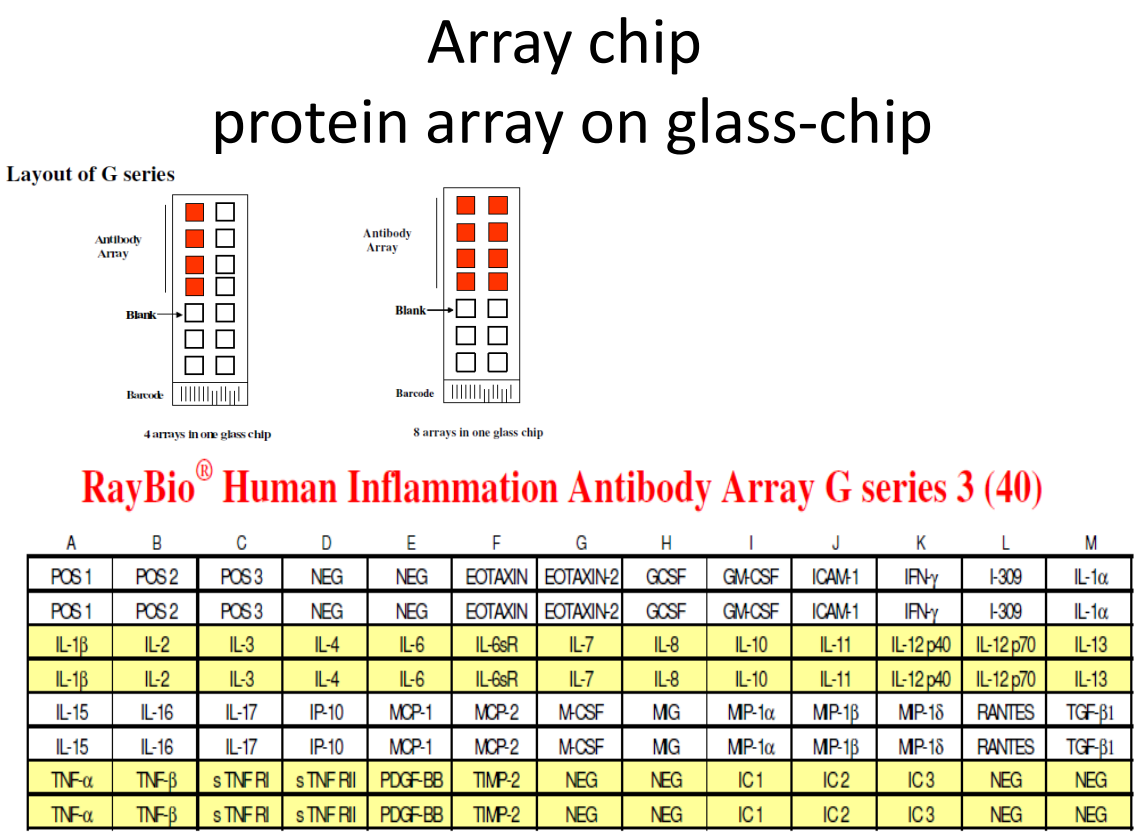
Array data analysis. Both chemiluminescence and fluorescence signals were analyzed and compared by NIH and StatView softwares. Normalization was carried out according to the instructions provided by the manufacturer. In the array approach, all comet tails were ignored and only median signal values were used for the identification of any biomarker variation. Particularly, the Internal Control (spiking-in proteins with no cross-reactivity with the samples) allows to normalize and compare signal intensities from array membranes and chips in different experiments/times. Only median signal values obtained after the acquisition with the same PMT settings, were used for the comparative analysis. Inter- and intra-assay CV≤10% was considered in the study and any ≥1.5-fold increase or ≤0.65 fold decrease in signal intensity, was considered to guarantee specific signals above background. A significance level of .05 was selected to limit the number of false-negative results.

To acquire the spot signals, hand-made circle was drawn around the spot of the membrane-array image (35-bit tiff image), permitting the software to quantify the chemioluminescent signal and express it as optical density (OD). Data is provided as pathologic OD/control OD ratio. In the chip-array, the genepix software directly circles and evaluates the fluorescence signal inside each spot and provides a ratio value (MF) as pathological/ referring signal),which served for the normalization calculations. This approach would imply that the chip-based array is more precise than the membrane-based array. In order to minimize the intra- and the inter-assay variability, only the chip-based array data was used for the final statistical evaluation. The single tester handled all the material and followed all the phases of the experiment.

The clusters of antibodies used for both arrays are as follows: eotaxin, eotaxin-2, G-CSF (granulocyte colony-stimulating factor), GM-CSF (granulocyte-macrophage colony-stimulating factor), ICAM-1 (intercellular adhesion molecule-

1), IFN-γ (interferon-γ), I-309 (chemokine (C-C motif) ligand 1), IL-1α (interleukin-1α), IL-1β (interleukin-1β), IL-2 (interleukin-2), IL-3 (interleukin-3), IL-4 (interleukin-4), IL-6 (interleukin-6), IL-6sr (interleukin-6 soluble receptor), IL-7 (interleukin-7), IL-8 (interleukin-8), IL-10 (interleukin-10), IL-11 (interleukin-11), IL-12p40 (interleukin-12p40), IL-12p70 (interleukin-12p70), IL-13 (interleukin-13), IL-15 (interleukin-15), IL-16 (interleukin-16), IL-17 (interleukin-17), IP-10 (interferon gamma-induced protein 10), MCP-1 (monocyte chemotactic protein-1), MCP-2 (monocyte chemotactic protein-1), M-CSF (macrophage colony-stimulating factor), MIG (monokine induced by gamma interferon), MIP-1α (macrophage inflammatory protein-1α), MIP-1β (macrophage inflammatory protein-1β), MIP-1δ (macrophage inflammatory protein-1δ), RANTES (regulated and normal T cell expressed and secreted), TGF-β1 (transforming growth factor-β1), TNF-α (tumor necrosis factor-α), TNF-β (tumor necrosis factor-β), sTNF-R1 (soluble tumor necrosis factor-receptor1), sTNF-R2 (soluble tumor necrosis factor-receptor2), PDGF-BB (platelet-derived growth factor) and TIMP-2 (tissue inhibitor of metalloproteinases-2).

Although membrane-array and chip-array techniques are both the semiquantitative methods of proteomic evaluation, the membrane-array technique consists in manual tester evaluation of the chemioluminescent signal versus control signal, whereas in the chip-array the genepix instrument directly evaluates the fluorescence signal of the spot versus control signal (which served for the normalization calculations). Therefore, in the chip-based array, the optical density values are displayed by the instrument. In order to minimize intra- and inter-assay variability, only the chip-based array data was used for the final statistical evaluation. The single tester handled all the material and followed all the phases of the experiment.



	A	B	C	D	E	F	G	H	I	J	K	L	M
1	POS 1	POS 2	POS 3	NEG	NEG	EOTAXIN	EOTAXIN-2	GCSF	GM-CSF	ICAM-1	IFN-γ	I-309	IL-1α
2	POS 1	POS 2	POS 3	NEG	NEG	EOTAXIN	EOTAXIN-2	GCSF	GM-CSF	ICAM-1	IFN-γ	I-309	IL-1α
3	IL-1β	IL-2	IL-3	IL-4	IL-6	IL-6sR	IL-7	IL-8	IL-10	IL-11	IL-12 p40	IL-12 p70	IL-13
4	IL-1β	IL-2	IL-3	IL-4	IL-6	IL-6sR	IL-7	IL-8	IL-10	IL-11	IL-12 p40	IL-12 p70	IL-13
5	IL-15	IL-16	IL-17	IP-10	MCP-1	MCP-2	M-CSF	MIG	MIP-1α	MIP-1β	MIP-1δ	RANTES	TGF-β1
6	IL-15	IL-16	IL-17	IP-10	MCP-1	MCP-2	M-CSF	MIG	MIP-1α	MIP-1β	MIP-1δ	RANTES	TGF-β1
7	TNF-α	TNF-β	s TNF RI	s TNF RII	PDGF-BB	TIMP-2	NEG	NEG	IC 1	IC 2	IC 3	NEG	NEG
8	TNF-α	TNF-β	s TNF RI	s TNF RII	PDGF-BB	TIMP-2	NEG	NEG	IC 1	IC 2	IC 3	NEG	NEG

Figure 27. *Protein array on glass-chip: the left one contains 4 chips, the right one 8 chips. According to the chip-based glass array, 40 inflammatory proteins were analyzed and quantified by GenePix platform. The schematic representation of the array is reported below. Each protein is assayed in duplicate. The chip also contains positive and negative controls, IC1, IC2 and IC3 which serve for identification of the normalization factor at the end of the analysis: thereafter, the computer calculates data based on the normalization factor.*

3.5. Statistical Analysis

In this study four groups of patients were confronted: controls, diabetics without DR, diabetics with diabetic retinopathy (DR), and diabetics with DR and macular edema.

The concentration in the AH of total protein, GFAP, AQP1 and AQP4 was quantitatively expressed in reference units (mg/ml, or pg/µg) and, for descriptive purposes, the results were reported in terms of mean ± standard deviation. The difference in mean protein concentration between the groups was confronted with unpaired T-test analysis. The difference in GFAP, AQP1 and AQP4 concentration between the groups was confronted using the ANOVA test which, in the case of a

statistically significant result ($p<0.05$), was followed by the Tukey-Kramer post-hoc analysis.

A similar analysis was used to evaluate the cytokine concentration, expressed in terms of intensity of the fluorescence signal.

The thickness of retinal layers was expressed in reference units (μm) and described in terms of the mean ± standard deviation.

The mean values of each group were confronted using the repeated measures analysis of variances (ANOVA-RM). There were 3 types of analyses conducted:

- comparison of mean total thickness: each patient has contributed with measurements of 60 intervals of the target reference;
- comparison of the thicknesses of concentric rings (as defined by the ETDRS): each patient has contributed with 12 measurements of the central ring of 1 mm diameter, 24 measurements of the inner ring of 3 mm diameter and 24 measurements of the outer ring of 5 mm diameter;
- comparison of thicknesses of four sectors (superior (S), nasal (N), inferior (I) and temporal (T): each patient has contributed with 15 measurements for each sector.

In cases of significant results ($p<0.05$), the ANOVA-RM was followed by the Bonferroni multiple comparisons post-hoc test.

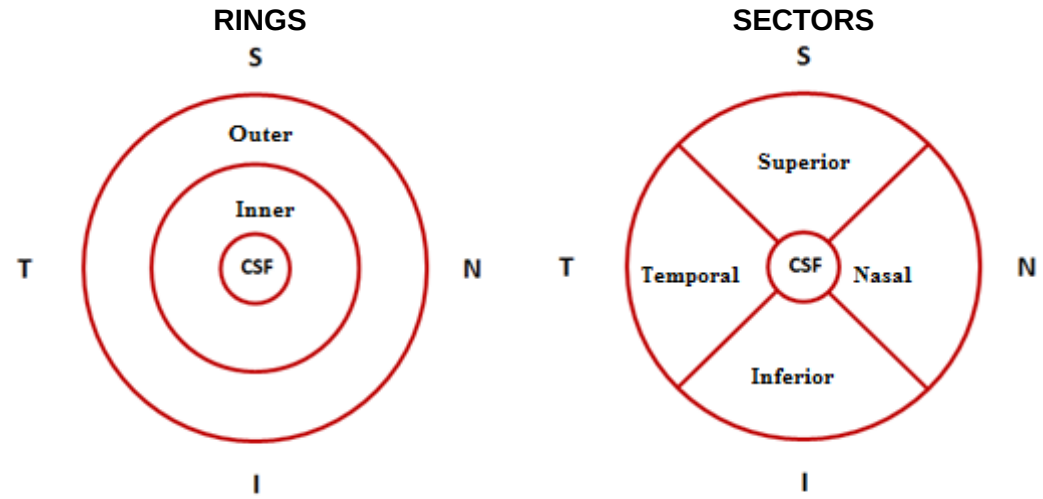


Figure 24. *Charts of the division of the macular area in rings, sector of the macular area. Position in space with respect to the fovea: S, superior; N, nasal; I, inferior; T, temporal, CSF, central subfield.*

The ANOVA model has also enabled the evaluation of the average profile trend of the thicknesses in different patient groups in different retinal areas considered. The statistical analysis has been carried out using the SAS® v.9.2 software on a personal computer.

RESULTS

4.1. Population

The data collected refers to 34 eyes of 34 patients hence divided: 12 normal subjects (controls), 11 diabetic patients without DR and 11 diabetic patients with DR (further divided in 5 patients without DME and 6 with DME). All the patients with DR presented non proliferative diabetic retinopathy (NPDR).

The control group was composed of 5 males and 7 females, between 66 and 87 years of age (mean age: 75.4±6.4 years), the group of diabetics without DR was formed by 3 males and 8 females, between 56 and 86 years of age (mean age 73.9±9.0 years), while the group of diabetics with DR consisted in 6 males and 5 females, between 49 and 83 years of age (mean age 69.9±11.4 years), ultimately divided in diabetics with DR and without macular edema and diabetics with DR and macular edema (tables III and IV).

Among the diabetic patients without DR, the duration of diabetes mellitus (DM) was between 2 and 15 years (mean 7.7±5.1 years), and in all case it was type 2 DM; among the diabetic patients with DR, the duration was between 10 and 40 years (mean 20.1±9.8 years); mean duration of DM was 13.4±9.6 years in DR without ME and 21.8±11.1 years in DR with ME; and there was 1 case of type 1 DM, while in the remaining 10 cases it was type 2 DM. In the first group (diabetics without DR), 1 patient was in therapy with oral hypoglycemic agents and insulin, 7 with only the oral hypoglycemic agents, 2 with only the dietary control, while 1 patient did not assume any preventive action; in the second group (diabetics with DR and without ME), 1 patient was in therapy with oral hypoglycemic agents and insulin, 1 only with oral hypoglycemic agents, 2 only with insulin, while 1 patient limited himself to dietary control; in the third group (diabetics with DR and with ME), 1 patient was in therapy with oral hypoglycemic agents and insulin, 1 only with oral hypoglycemic agents, 4 only with insulin.

In the control group, there were 8 right eyes and 4 left eyes analyzed, with an intraocular pressure (IOP) between 12 and 20 mmHg (mean 16.3±2.3 mmHg), and a best corrected visual acuity (BCVA) between +1.0 and +0.15 logMar (mean 0.41±0.22 logMar) in the studied eye; in the group of diabetics without DR, 4 right eyes and 7 left eyed were studied, with an IOP comprised between 14 and 22 mmHg (mean 17.5±2.4 mmHg) and BCVA between +1.0 and +0.1 logMar (mean 0.58±0.52 logMar) in the studied eye; in the group of diabetics with DR (both without and with ME), 6 right eyes and 5 left eyes were studied, with IOP comprised between 11 and 18 mmHg (mean 15.7±2.6 mmHg) and BCVA between +1.52 and +0.15 (mean 0.63±0.51 logMar) in the studied eye. In the last group, 2 patients presented mild NPDR, 8 moderate NPDR and 1 severe NPDR; Table IV shows separate data for DR without ME and DR with ME.

In all 34 cases, the collection of the AH was successful, without contamination by blood or by tissues from the anterior chamber of the eye. In average, the quantity of the AH collected was superior to 150 µl (150-200 µl). No intra-operative complication was registered, and the post-operative management was regular in all cases. For every sample, the test tube collection, the freezing, the conservation and the dispatch to the laboratory for analysis took place without problems.

Table III. *Characteristics of subjects pertaining to four groups. Diabetics w/o RD: diabetics without DR; Diabetics w/RD: diabetics with DR; Diabetics w/RD and edema: diabetics with DR and macular edema.*

PARAMETERS	Controls (N=12)	Diabetics w/oDR (N=11)	Diabetics w/DR ME- (N=5)	Diabetics w/DR ME+ (N=6)
Mean age ± SD (yrs)	75.4 ± 6.4	73.9 ± 9.0	75.8 ± 9.8	62.0 ± 8.6
Sex (M:F)	5:7	3:8	4:1	2:4
Mean duration ± SD DM (yrs)	n.a.	7.7 ± 5.1	13.4 ± 9.6	21.8 ± 11.1
Type DM (DM1:DM2)	n.a.	0:11	0:5	1:5
DM Treatment				
Oral Hypoglycemics + insulin	n.a.	1	1	1
Oral Hypoglycemics	n.a.	7	1	1
Insulin	n.a.	0	2	4
Diet	n.a.	2	1	0
No treatment	n.a.	1	0	0

Table IV. *Characteristics of studied eyes in the four groups. Diabetics w/o RD: diabetics without DR; Diabetics w/RD: diabetics with DR; Diabetics w/RD and edema: diabetics with DR and macular edema.*

PARAMETERS	Controls (N=12)	Diabetics w/o DR (N=11)	Diabetics w/DR ME- (N=5)	Diabetics w/DR ME+ (N=6)
Studied eye (RE:LE)	8:4	4:7	2:3	4:2
Mean intraocular pression±SD (mmHg)	16.3±2.3	17.5±2.4	15.5±2.7	16.2±2.4
Visual acuity±SD (logMar)	0.41±0.22	0.58±0.52	0.60±0.38	0.63±0.49
N° pt with DR				
Mild NPDR	-	-	1	1
Moderate NPDR	-	-	4	4
Severe NPDR	-	-	0	1
PDR	-	-	0	0

4.2. Protein Analysis

This study evaluated the mean concentrations in the AH of total protein and of GFAP, AQP1 and AQP4. The comparison between the four patient groups (controls, diabetics without DR, diabetics with DR and no macular edema and diabetics with DR and with macular edema) has helped form the following observations:

- Total proteins: no significant difference in mean protein concentration has been detected among the controls, diabetics without DR and diabetics with DR;
- Total proteins: a significant difference in mean protein concentration has been detected between the diabetics with DR (and no macular edema) and diabetics with macular edema

Table V. *Comparison of the mean concentrations and their standard deviations SD (in mg/ml) of total proteins present in the aqueous humour in the four groups. Diabetics w/o DR: diabetics without DR; Diabetics w/DR ME-: diabetics with DR and no macular edema; Diabetics w/DR ME+: diabetics with DR and macular edema*

MEAN CONCENTRATION OF TOTAL PROTEINS ± SD (mg/ml)			
Controls (N=12)	**Diabetics w/o DR (N=11)**	**Diabetics w/DR ME- (N=5)**	**Diabetics w/DR ME+ (N=6)**
0.44±0.07	0.45±0.10	0.50±0.08	0.37±0.03

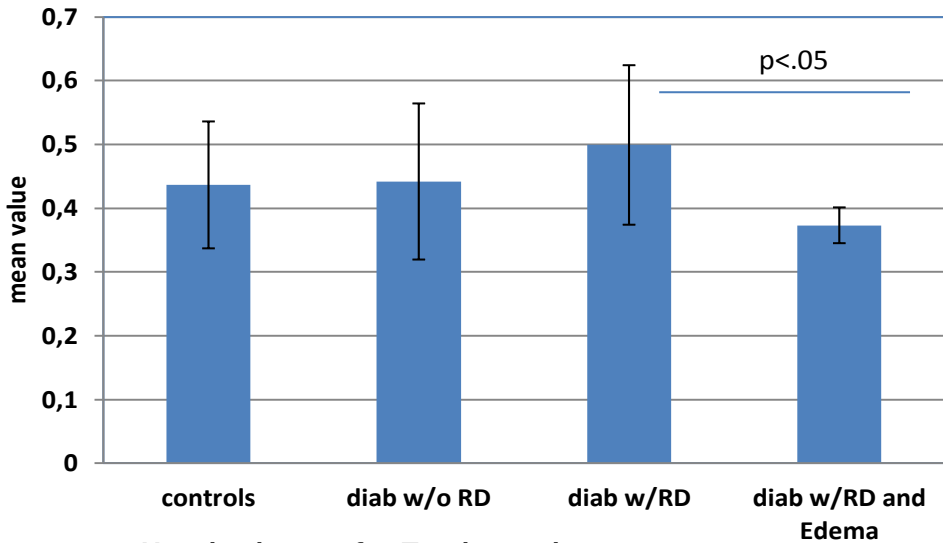


Figure 25. *Mean concentrations of total proteins (in mg/ml) present in the aqueous humour of the four groups as detected by Bradford microarray and Nanodrop specific evaluation (2uL/595nm; BSA as referring protein). Diab w/o RD: diabetics without DR; Diab w/RD: diabetics with DR; Diab w/RD and edema: diabetics with DR and macular edema.*

- GFAP: the mean concentration is significantly increased in diabetic patients with DR compared both to the controls (324.44 pg/µg vs 182.34 pg/µg; Tukey-Kramer *post-hoc* test $p<0.05$) and diabetics without DR (324.44 pg/µg vs 165.61 pg/µg; Tukey-Kramer *post-hoc* test $p<0.05$);
- AQP1: the mean concentration is significantly increased in diabetic patients with DR compared both to the controls (105.72 pg/µg vs 50.92 pg/µg; Tukey-Kramer *post-hoc* test $p<0.05$) and diabetics without DR (105.72 pg/µg vs 28.84 pg/µg; Tukey-Kramer *post-hoc* test $p<0.05$); it is significantly increased in diabetic subjects with retinopathy and without macular edema compared to those with macular edema (105.72pg/µg vs 37.09pg/µg).
- AQP4: the mean concentration is significantly increased in diabetic patients without DR compared to controls (206.87pg/µg vs 33,58 pg/µg, Tukey-Kramer *post-hoc* test $p<0.05$); it is significantly increased in

diabetic patients with DR, and in diabetic patients with DR and DME compared to controls (respectively 852.03 pg/µg vs 33.58 pg/µg, Tukey-Kramer *post-hoc* test $p<0.05$; and 403.08 pg/µg vs 33,58 pg/µg, Tukey-Kramer *post-hoc* test $p<0.05$); it is significantly increased in diabetic patients with DR compared to those without DR; (403.08 pg/µg vs 206.87 pg/µg, Tukey-Kramer *post-hoc* test $p=0.0002$); it is significantly increased in diabetic patients with DR and no DME compared to diabetic patients with DME; (respectively 852.03 pg/µg vs 403.08 pg/µg, Tukey-Kramer *post-hoc* test $p=0.02$).

- AQP4/AQP1 concentrations: The Kendall Rank analysis showed weak and non significant correlation ($Tau=0.21$, $p=0.3$) between these biomarkers, despite the trend in increase.

Table VI. *Comparison of the mean concentrations and their standard deviations (in pg/µg) of proteins GFAP, AQP1 and AQP4 present in aqueous humor of the four groups. Diabetics w/o DR: diabetics without DR; Diabetics w/DR ME-: diabetics with DR and no macular edema; Diabetics w/DR ME+: diabetics with DR and macular edema.*

	MEAN CONCENTRATION ± SD (pg/µg)			
	Controls (N=12)	**Diabetics w/o DR (N=11)**	**Diabetics w/DR ME- (N=5)**	**Diabetics w/DR ME+(N=6)**
GFAP	182.34±114.44	165.61±47.19	324.44±262.54	175.84±76.09
AQP1	50.92±20.36	28.84±29.96	105.72±15.69	37.09±15.5
AQP4	33.58±21.20	206.87±491.70	852.03±103.24	403.08±291.61

GFAP

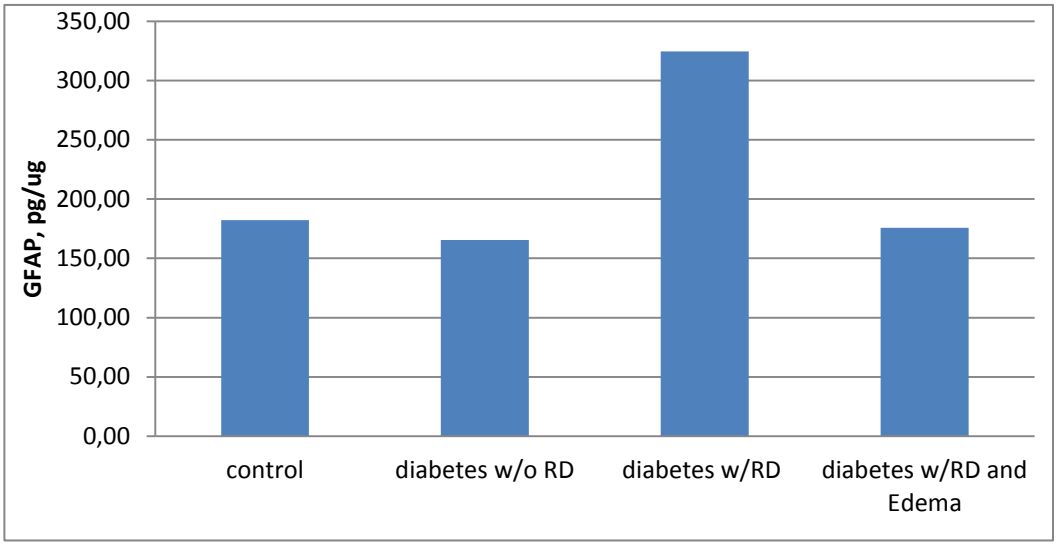


Figure 26. *Mean GFAP concentrations (in pg/µg) in the aqueous humour of the four groups. The mean concentration is significantly greater in diabetic patients with DR compared both to the controls and diabetics without DR, ($p<0.05$). Diabetes w/o RD: diabetics without DR; Diabetes w/RD: diabetics with DR; Diabetes w/RD and Edema: diabetics with DR and macular edema.*

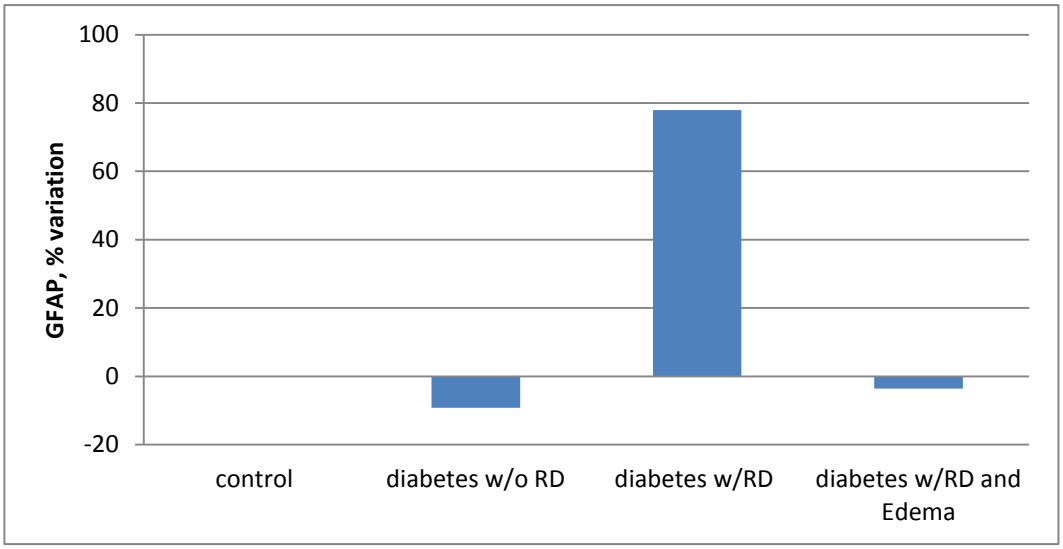


Figure 27*. Percentage (%) variation of GFAP expression in all groups, compared to controls. GFAP ELISA assay [millipore.com]: detection range 10 to 0.16 ng/mL (sensitivity less than 10pg/mL). Diabetes w/o RD: diabetics without DR; Diabetes w/RD: diabetics with DR; Diabetes w/RD and Edema: diabetics with DR and macular edema.*

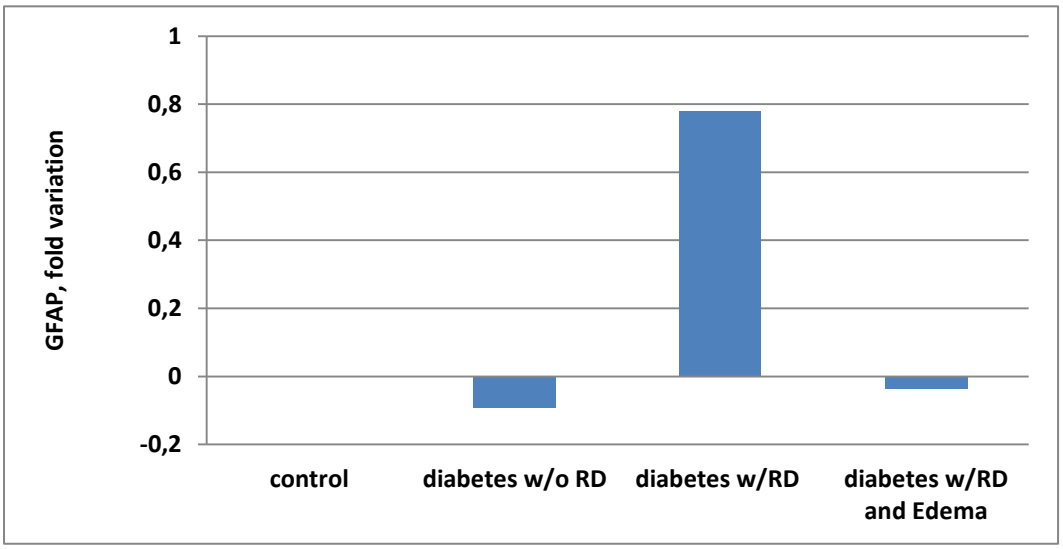


Figure 28. *Fold changes of GFAP expression in all groups compared to controls. Positive fold changes means increase in concentration; negative fold changes means decrease in concentration. Diabetes w/o RD: diabetics without DR; Diabetes w/RD: diabetics with DR; Diabetes w/RD and Edema: diabetics with DR and macular edema.*

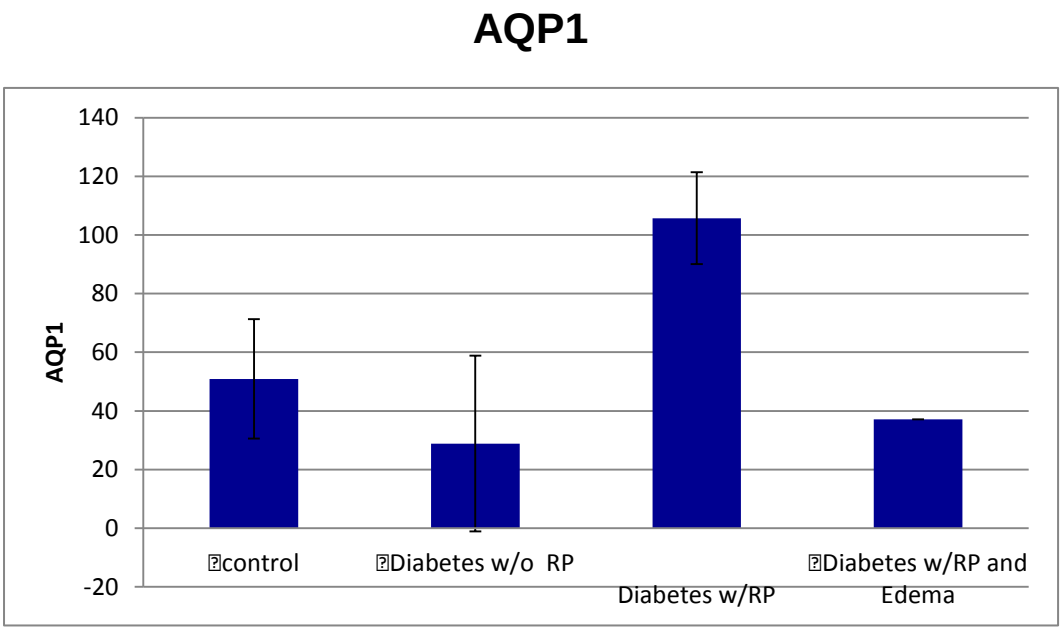


Figure 29. *Mean AQP1 concentrations (in pg/µg) in the aqueous humour of the four groups. The mean concentration is significantly greater in diabetic patients with DR compared both to the controls and diabetics without DR, ($p<0.05$); it is significantly increased in diabetic subjects with retinopathy and without macular edema compared to those with macular edema. Diabetes w/o RD: diabetics without DR; Diabetes w/RD: diabetics with DR; Diabetes w/RD and Edema: diabetics with DR and macular edema.*

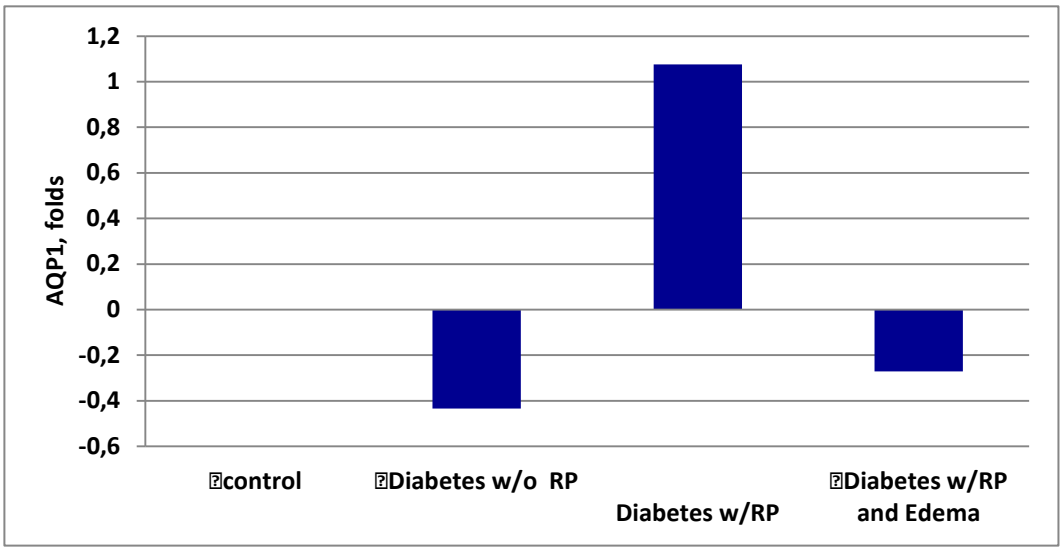


Figure 30. *Fold changes of AQP1 expression in all groups compared to controls. Positive fold changes means increase in concentration; negative fold changes means decrease in concentration. Diabetes w/o RD: diabetics without DR; Diabetes w/RD: diabetics with DR; Diabetes w/RD and Edema: diabetics with DR and macular edema.*

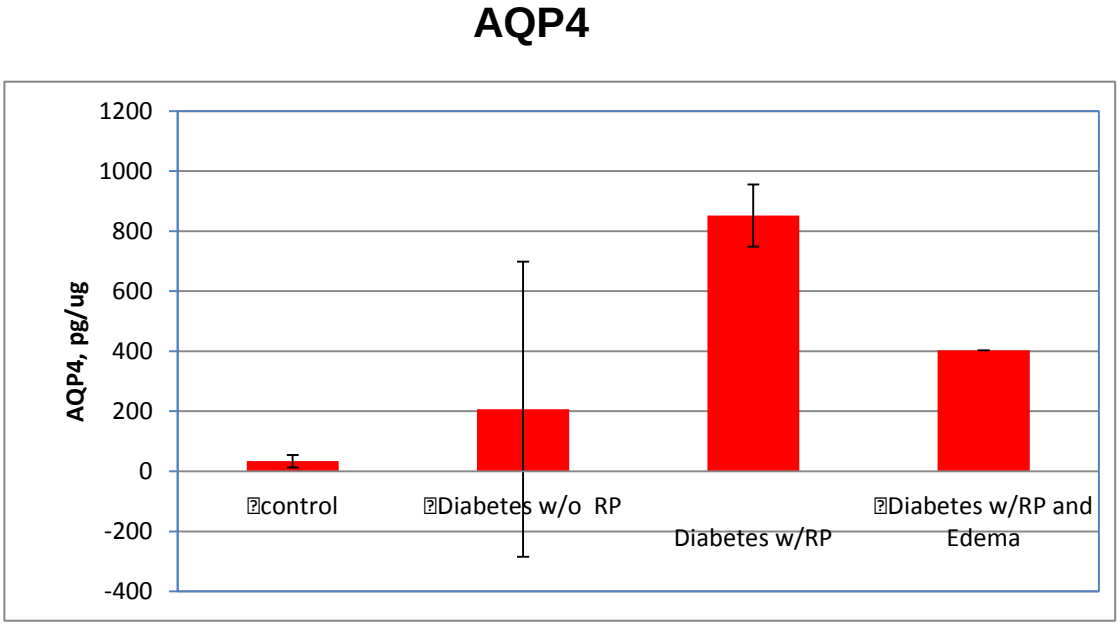


Figure 31. *Mean AQP4 concentrations (in pg/µg) in the aqueous humour of the four groups. AQP4 concentration was normalized based on the total protein concentration. The mean concentration is significantly increased: in diabetic patients without DR compared to controls, in diabetics with DR, and in diabetics with DR and DME compared to controls; in diabetics with DR compared to those without DR; in diabetics with DR and no DME compared to diabetics with DME. Diabetes w/o RD: diabetics without DR; Diabetes w/RD: diabetics with DR; Diabetes w/RD and Edema: diabetics with DR and macular edema.*

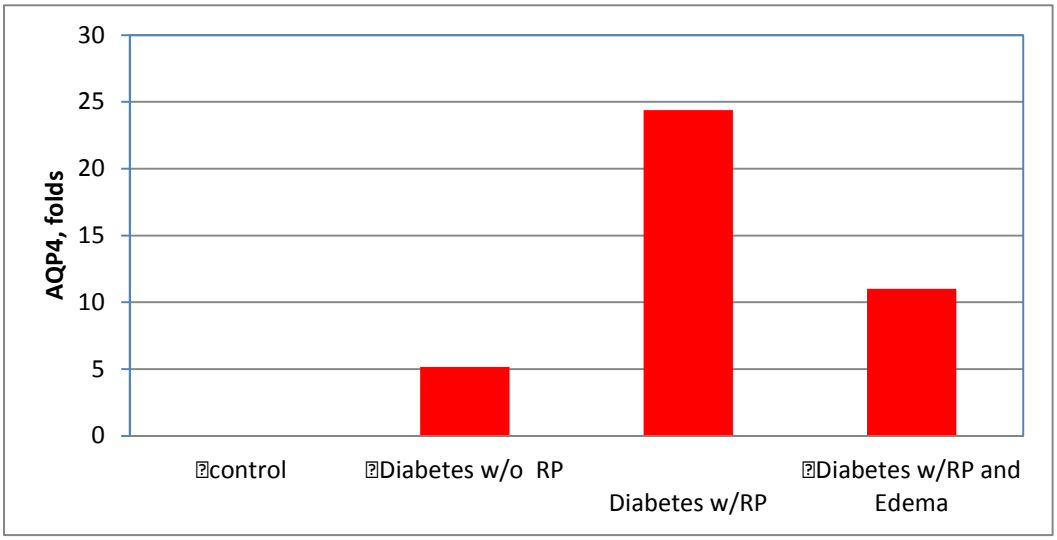


Figure 32. *Fold changes of AQP4 expression in all groups compared to controls. Positive fold changes means increase in concentration; there are no negative fold changes. Diabetes w/o RD: diabetics without DR; Diabetes w/RD: diabetics with DR; Diabetes w/RD and Edema: diabetics with DR and macular edema.*

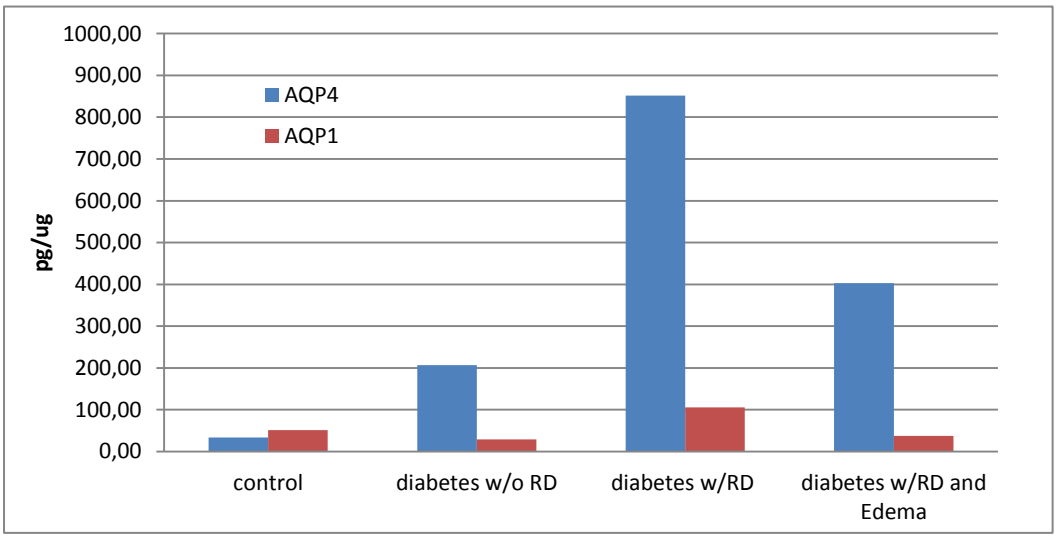


Figure 33. *Mean AQP4 and AQP1 concentrations (in pg/µg) in the aqueous humour of the four groups. The Kendall Rank analysis showed weak and non significant correlation (Tau=0.21, p=0.3) between these biomarkers, despite the trend in increase. Diabetes w/o RD: diabetics without DR; Diabetes w/RD: diabetics with DR; Diabetes w/RD and Edema: diabetics with DR and macular edema.*

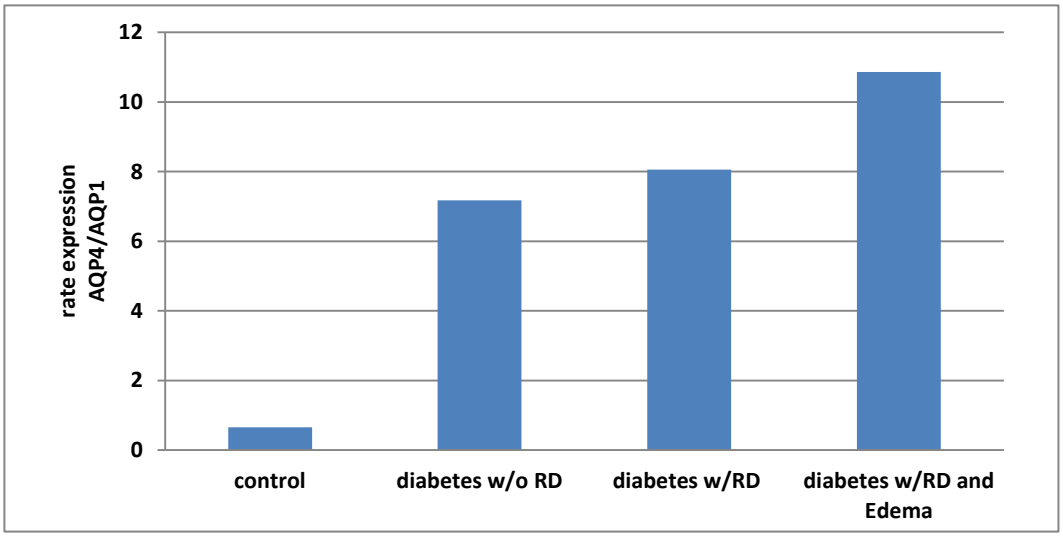


Figure 34. *AQP4/AQP1 ratio in all groups. Diabetes w/o RD: diabetics without DR; Diabetes w/RD: diabetics with DR; Diabetes w/RD and Edema: diabetics with DR and macular edema.*

For the different cytokines analyzed, the intensity of the fluorescent signal was confronted and the values are represented in table VII.

Table VII. *Mean intensity and statistical significance of the fluorescent signal (optical density values F532-B532) of the cytokines present in the aqueous humour of the four groups in all patients. Significant values are in bold (*p, 0.05, **p, 0.001, ***p,0.0001 (ANOVA followed by Tukey-Kramer post-hoc). Diabetics w/oDR: diabetics without DR; Diabetics w/DR ME-: diabetics with DR and no macular edema; Diabetics w/DR ME+: diabetics with DR and with macular edema; monocytochemotactic protein-1 (MCP-1); granulocyte colony-stimulating factor (G-CSF), granulocyte/macrophage colony-stimulating factor (GM-CSF),interferon (IFN)-, interferon-inducible 10-kDa protein(IP-10), macrophage inflammatory protein-1 alpha (MIP-1a), MIP-1b, platelet-derived growth factor (PDGF)-BB, regulated upon activation, normal T cell expressed and secreted, tumor necrosis factor alpha (TNF-a), intercellular adhesion molecule-1 (ICAM-1).*

	1 Controls		2 Diabetics w/o DR		3 Diabetics w/DR ME-		4 Diabetics w/DR ME+	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Eotaxin	**145.0**	299.0	422.0	453.9	**925.7 ***	944.0	**544.3 ***	581.2
Eotaxin2	***248.3***	142.8	298.8	557.7	575.2	768.8	***972.0***	1122
GCSF	609.2	623.3	381.7	643.0	682.8	682.7	589.5	641.9
GMCSF	**128.3**	287.3	210.8	448.7	685.5	802.2	**1213.8 ***	1485
ICAM1	109.2	310.4	290.8	382	432.5	503.2	176.5	297.0
IFNy	**154.8**	379.9	**495.3 ***	228.5	434.7	299.0	**842.8 *****	199.8
I309	21.5	288.4	30.8	260.4	240.3	381.8	201.0	326.9
IL1a	***310.0***	354.8	***855.2***	867.5	326.5	441.7	508.5	351.6
IL1b	***199.8***	452.3	***909.0***	1049	445.7	714.5	807.2	909.1
IL2	398.3	405.7	1161.5	1121	954.5	1032	1131.0	1196
IL3	**880.8**	211.8	**1307.3 ****	423.3	955.7	240.5	1050.5	389.1
IL4	**89.5**	158.0	**638.7 ***	710.1	**546.5 ***	540.3	**433.2 ***	515.3
IL6	894.2	733.2	1063.0	748.0	970.2	677.7	633.0	319.9
IL6R	323.2	467.5	607.0	500.1	488.3	650.2	458.2	606.5
IL7	**1805.8**	1030	**805.8 ***	755.8	1284.5	1041	**797.2 ***	472.4
IL8	764.8	661.1	995.2	633.2	859.5	403.9	902.2	328.1
IL10	***135.0***	321.0	376.3	353.6	476.7	710.2	***666.5***	580.3
IL11	***30.8***	483.7	95.7	452.9	216.2	519.9	***654.2***	868.1
IL12p40	338.0	501.3	186..3	502.8	155.7	604.9	240.8	630.0
IL12p70	226.2	413.8	68.7	297.9	132.3	408.5	104.5	167.0
IL13	434.8	506.5	441.5	662.0	418.7	954.2	701.3	697.1
IL15	863.3	401.7	920.2	585.7	880.8	662.0	1114.0	544.8
IL16	292.0	370.0	543.5	552.0	522.8	561.9	347.7	402.8
IL17	**90.0**	571.2	**1085.7 ***	1236	383.5	722.1	636.8	901.1
IP10	**676.5**	620.9	1431.5	943.4	1014.8	1228	**2260.7 ****	1087
MCP1	***6464.3***	4124	5755.5	3792	6446.0	4682	***10799.3***	7884
MCP2	**30.5**	396.4	**449.8 ***	299.7	351.0	465.4	64.5	448.0
MCSF	***7059.0***	11989	***420.7***	588.7	***274.2***	433.6	***327.0***	343.7
MIG	474.7	477.0	729.3	701.6	616.3	631.9	664.5	587.2
MIP1a	**100.5**	324.8	179.7	426.9	-19.8	119.0	**965.8 ***	1600
MIP1b	644.5	273.2	***360.8***	444.4	**830.3 ****	240.3	**828.0 ****	75.5
MIP1d	361.8	514.8	264.0	513.3	645.7	535.9	514.0	694.2
RANTES	**76.2**	159.1	59.2	328.5	***657.0***	981.8	461.3	652.3
TGFb1	514.7	613.0	209.2	334.1	255.7	338.6	374.2	395.1
TNFa	827.5	559.9	1193.5	817.3	1423.2	780.8	1225.5	428.5
TNFb	**790.8**	253.8	**1423.7 *****	190.2	**1579.2 *****	339.3	**1849.0 *****	281.8
sTNFRI	283.5	259.1	253.7	447.3	557.3	485.9	548.2	674.1
sTNFRII	**160.3**	435.4	235.8	245.1	**610.2**	548.2	843.3	990.6
PDGF-BB	356.2	387.4	91.8	170.1	225.2	440.9	440.2	414.1
TIMP2	11440	5074	8585.3	2665	11860.8	5802	13825.2	7013

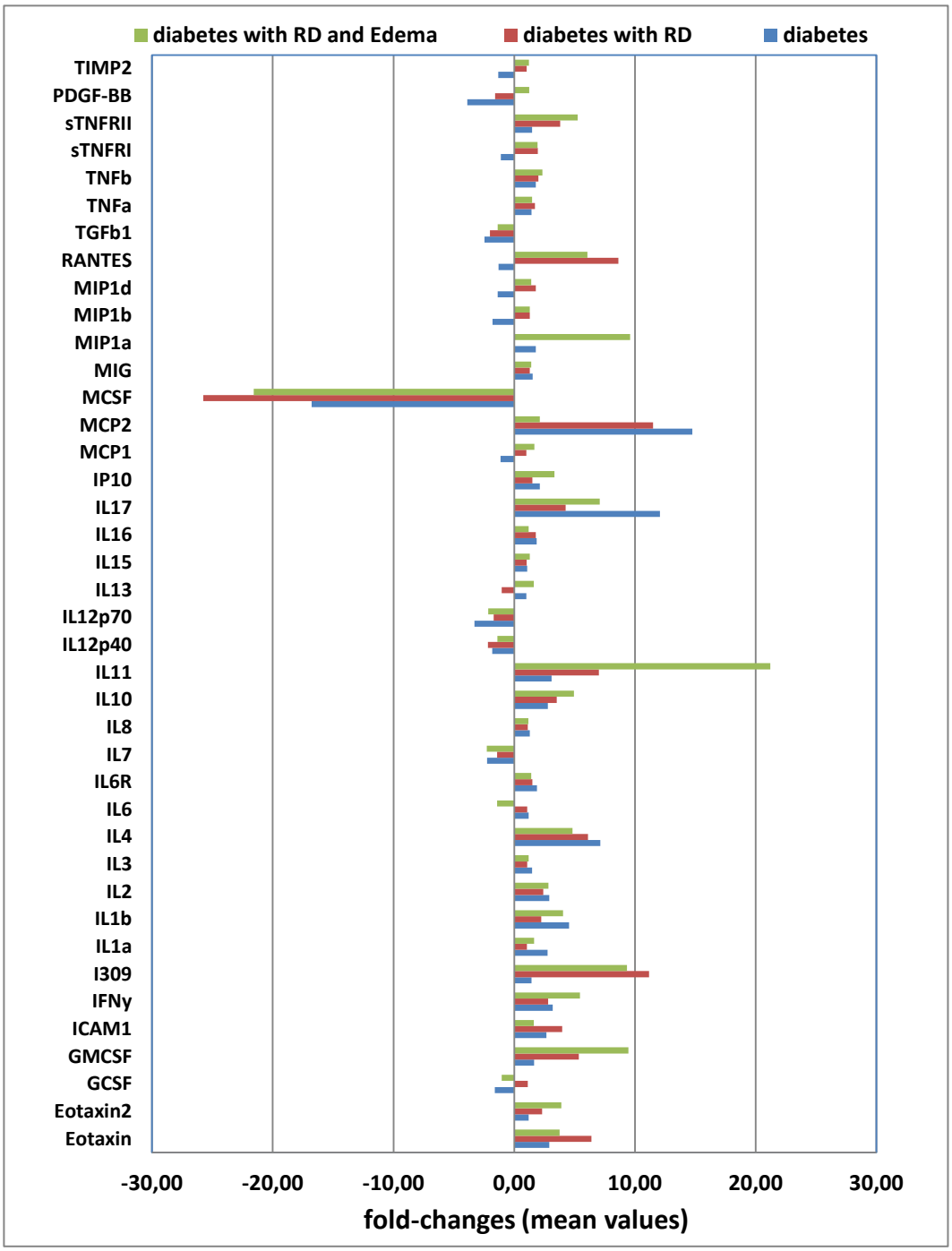


Figure 39. *Bar graph of folds changes of protein expression as inferred from the fluorescence detected by antibody microarray techniques in all patients. The instrument (genePix) detects the mean values of the column F532-B532 which represents the median optical density values of each patient. Statistical analysis was performed on single (F532-B532) values. Green: Diabetics with DR and macular edema; Red: Diabetics with DR and without macular edema; Blu: Diabetics without DR. Fold changes are expressed versus the control group.*

The intensity of the signal was significantly greater in diabetic subject without DR compared to controls for the following cytokines: IFNy (495.3 vs 154.8, p=0.01), IL-3 (1307.3 vs 880.8, p=0.0049), IL-4 (638.7 vs 89.5, p=0.03), IL-17 (1085.7 vs

90, p<0.05), TNF-ß (1423.7 vs 790.8, p<0.0001), MCP2 (449.8 vs 30.5, p<0.05), The intensity of the IL-7 signal was significantly decreased in diabetic subject without DR compared to controls (805.8 vs 1805.8, p=0.01). There was a borderline statistical significance in: IL-1a (855.2 vs 310), IL1b (909 vs 199.8), MIP1b (360.8 vs 644.5), and MCSF (420.7 vs 7059).

The intensity of the signal was significantly greater in diabetic subject with DR and no macular edema compared to controls for the following cytokines: Eotaxin (925.7 vs 145, p=0.03), TNF-ß (1579.2 vs 790.8). There was a borderline statistical significance in: IL-4 (546.5 vs 89.5, p=0.04), MCP2 (351 vs 30.5, p<0.05), RANTES (657.0 vs 76.2, p=0.04), sTNFRII (610.2 vs 160.3, p<0.05), IFNy (434.7 vs 154.8), TNFα (1423.2 vs 827.5) and MCSF (274.2 vs 7059).

The intensity of the signal was significantly greater in diabetic subject with DR and macular edema compared to controls for the following cytokines: GM-CSF (1213.8 vs 128.3, p=0.01), IFNy (842.8 vs 154.8, p<0.0001), IP-10 (2260.7 vs 676.515.6, p=0.0024), TNFb (1849 vs 790.8, p<0.0001), MIP1a (965.8 vs 100.5, p<0.05), sTNFRII (843.3 vs 160.3, p<0.05). The intensity of the IL-7 signal was significantly decreased in diabetic subject with DR and macular edema compared to controls (797.2 vs 1805.8, p=0.04). There was a borderline statistical significance in: Eotaxin 2 (972 vs 248.3), IL-10 (666.5 vs 135), IL-11 (654.2 vs 30.8), MCP1 (10799.3 vs 6464.3) and MCSF (327 vs 7059).

The intensity of the signal was significantly greater in diabetic subject with DR and no macular edema compared to diabetic subject without DR for MIP1b (830.3 vs 360.8, p<0.05).

The intensity of the signal was significantly greater in diabetic subject with DR and macular edema compared to diabetic subject without DR for the following cytokines: TNFb (1849 vs 1423.7, p=0.0031), MIP1b (830.3 vs 444.4, p=0.0096). (Table VII)

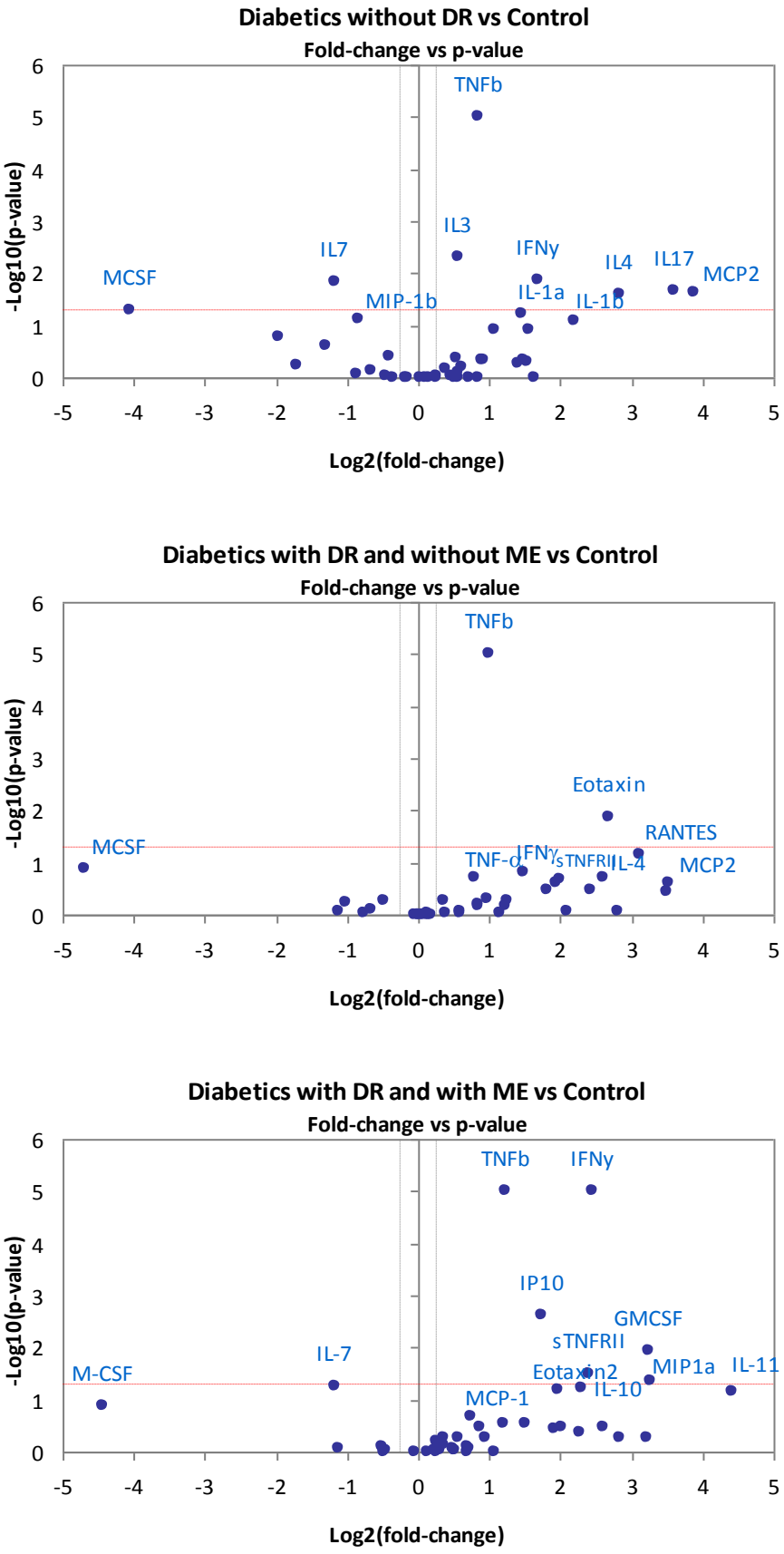


Figure 40. *Volcano plots of logarithm of fold-changes versus negative logarithm of statistical significance (p values) of cytokines' expression in aqueous humor of diabetics without diabetic retinopathy versus controls; diabetics with diabetic retinopathy and without macular edema versus controls, and diabetics with macular edema versus controls.*

Following are some representative chip arrays procedures showing the difference in fluorescence signal *or* fold changes detected by antibody microarray techniques in the aqueous humor between controls and different diabetic patients groups (diabetics without retinopathy, diabetics with retinopathy and no macular edema, and diabetics with retinopathy and macular edema.

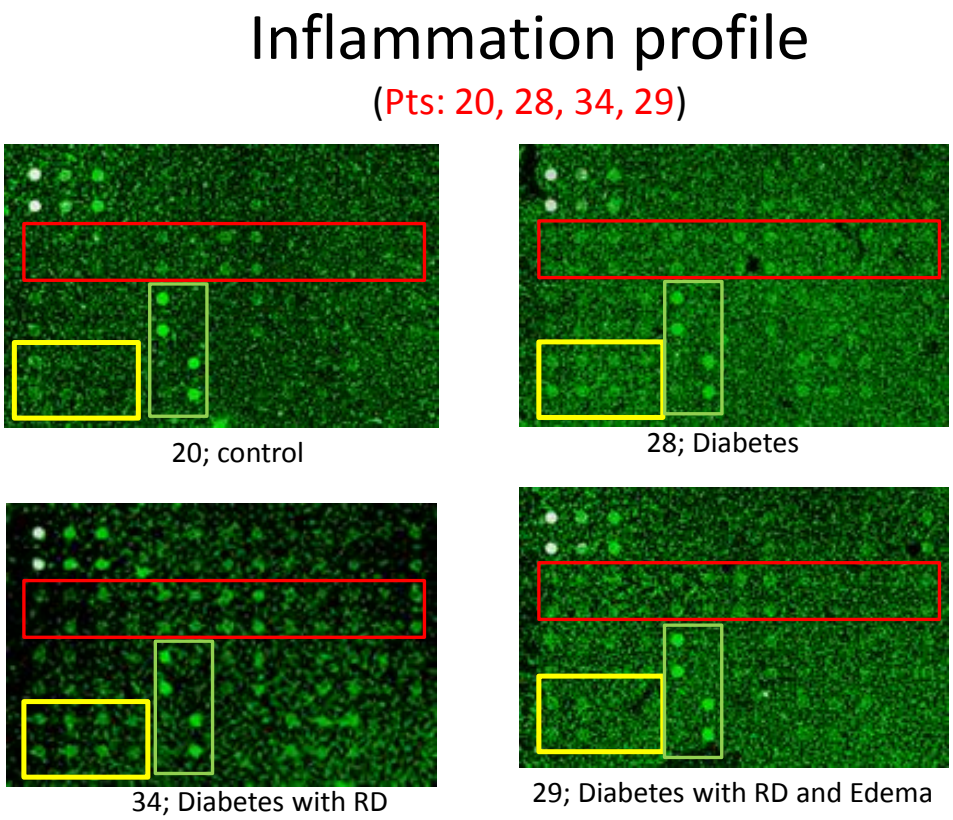


Figure 41. *Representative experimental chip-array procedure (Patients: 20, 28, 34, 29). All groups were tested in parallel (n=1 patient/group/slide). Green/cy3 signals (specific binding) were acquired by GenePix and expressed as F532-B532 median values. Positive and negative controls are different and are used by the instrument in order to regulate the fluorescent signal. Diabetes: diabetics without retinopathy; Diabetes with RD: Diabetics with retinopathy and no Edema, and Diabetes with RD and edema: Diabetics with retinopathy and Edema.*

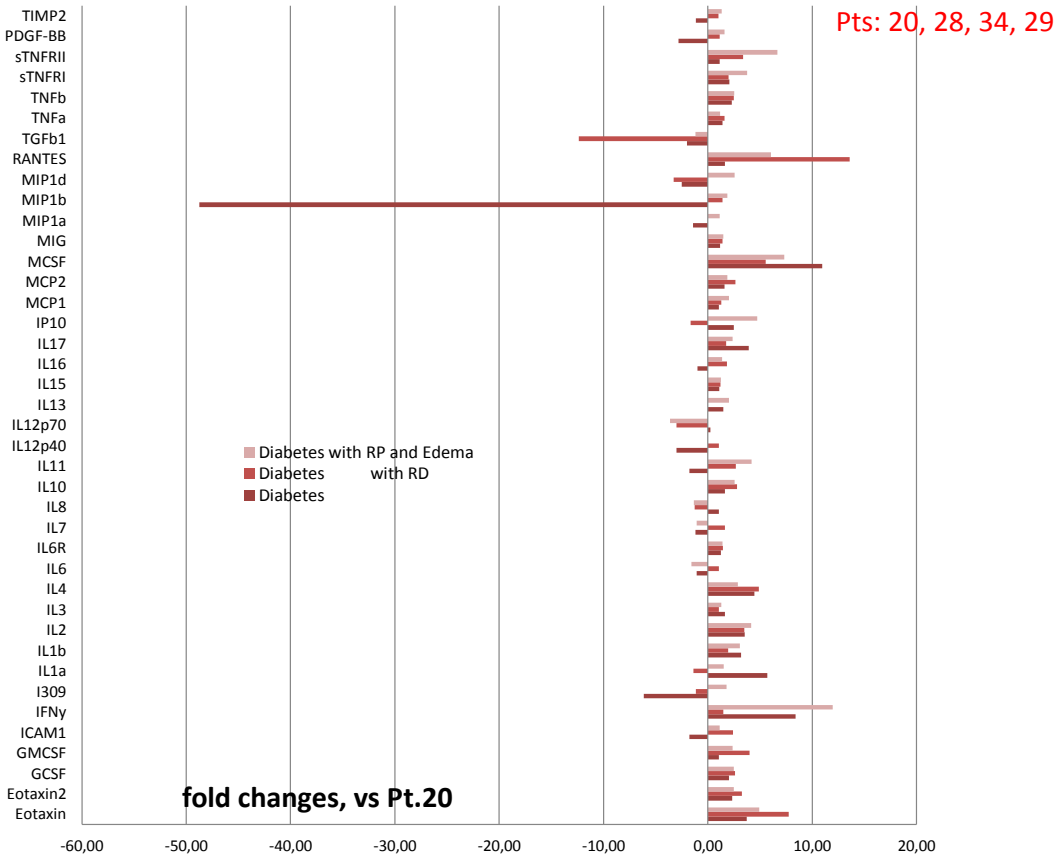


Figure 42. *Bar graph of Single Experiment: According to the chip-based array, 41 proteins were analysed. Intensity of protein expression as inferred from the fluorescence detected by antibody microarray techniques in the aqueous humor of controls, Diabetics without retinopathy, Diabetics with retinopathy and no Edema, and Diabetics with retinopathy and Edema. Fold changes (increase/decrease) are reported as the ratio between the expression intensity of each protein between experimental groups and related controls. In the case of results <1, the formula "-1/fold" was applied. Statistical analysis was performed according to: ANOVA/two-tailed t-test and Tukey-Kramer post hoc. A 1.2 fold cut-off was considered significant. Diabetes: diabetics without retinopathy; Diabetes with RD: Diabetics with retinopathy and no Edema, and Diabetes with RD and edema: Diabetics with retinopathy and Edema.*

Table VIII. *Fold changes and statistical significance of the fluorescent signal (optical density values F532-B532) of the cytokines present in the aqueous humour of the four groups in: patients 28, 34, 29 versus patient 20. Mean of a 1.2 fold cut-off was considered significant;. Diabetics w/oDR: diabetics without DR; Diabetics w/DR ME-: diabetics with DR and no macular edema; Diabetics w/DR ME+: diabetics with DR and with macular edema*

Chip	Folds					
	vs. control			vs. Diabetics w/o DR		vs. Diabetic w/DR ME-
Biomarkers	Diabetic w/o DR	Diabetic w/DR ME-	Diabetic w/DR ME+	Diabetic w/DR ME-	Diabetic w/DR ME+	Diabetic w/DR ME+
Eotaxin	3,73	7,74	4,91	2,07	1,32	-1,57
Eotaxin2	2,33	3,26	2,48	1,40	1,06	-1,31
GCSF	2,01	2,60	2,48	1,30	1,24	-1,05
GMCSF	1,04	3,99	2,36	3,83	2,26	-1,70
ICAM1	-1,77	2,39	1,13	4,23	1,99	-2,13
IFNy	8,40	1,48	11,95	-5,68	1,42	8,08
I309	-6,13	-1,16	1,81	5,28	11,06	2,10
IL1a	5,69	-1,38	1,50	-7,86	-3,79	2,07
IL1b	3,17	1,93	3,07	-1,65	-1,03	1,59
IL2	3,52	3,49	4,13	-1,01	1,18	1,18
IL3	1,64	1,06	1,27	-1,55	-1,29	1,20
IL4	4,47	4,90	2,86	1,10	-1,56	-1,71
IL6	-1,07	1,05	-1,58	1,12	-1,48	-1,66
IL6R	1,26	1,46	1,42	1,16	1,13	-1,03
IL7	-1,17	1,65	-1,09	1,93	1,08	-1,80
IL8	1,05	-1,25	-1,36	-1,32	-1,43	-1,09
IL10	1,65	2,79	2,55	1,69	1,55	-1,09
IL11	-1,76	2,68	4,19	4,72	7,37	1,56
IL12p40	-3,00	1,07	nd	3,22	nd	nd
IL12p70	0,25	-3,00	-3,61	1,35	1,12	-1,20
IL13	1,47	nd	2,02	nd	1,38	-16,62
IL15	1,08	1,23	1,25	1,13	1,15	1,02
IL16	-1,01	1,82	1,37	1,84	1,38	-1,33
IL17	3,91	1,75	2,36	-2,24	-1,66	1,35
IP10	2,49	-1,65	4,72	-4,11	1,89	7,79
MCP1	1,04	1,27	2,03	1,22	1,94	1,59
MCP2	1,61	2,63	1,88	1,63	1,17	-1,40
MCSF	10,95	5,55	7,33	-1,97	-1,49	1,32
MIG	1,17	1,40	1,48	1,20	1,26	1,05
MIP1a	-1,41	nd	1,15	nd	1,61	-7,55
MIP1b	-48,75	1,40	1,86	68,15	90,45	1,33
MIP1d	-2,49	-3,29	2,57	-1,32	6,39	8,47
RANTES	1,65	13,58	6,05	8,25	3,68	-2,24
TGFb1	-2,00	-12,39	-1,20	-6,20	1,66	10,30
TNFa	1,39	1,60	1,18	1,15	-1,17	-1,35
TNFb	2,29	2,50	2,53	1,09	1,11	1,01
sTNFRI	2,08	2,00	3,76	-1,04	1,81	1,88
sTNFRII	1,15	3,36	6,68	2,92	5,80	1,99
PDGF-BB	-2,82	1,15	1,61	3,24	4,54	1,40
TIMP2	-1,15	1,03	1,31	1,19	1,51	1,27

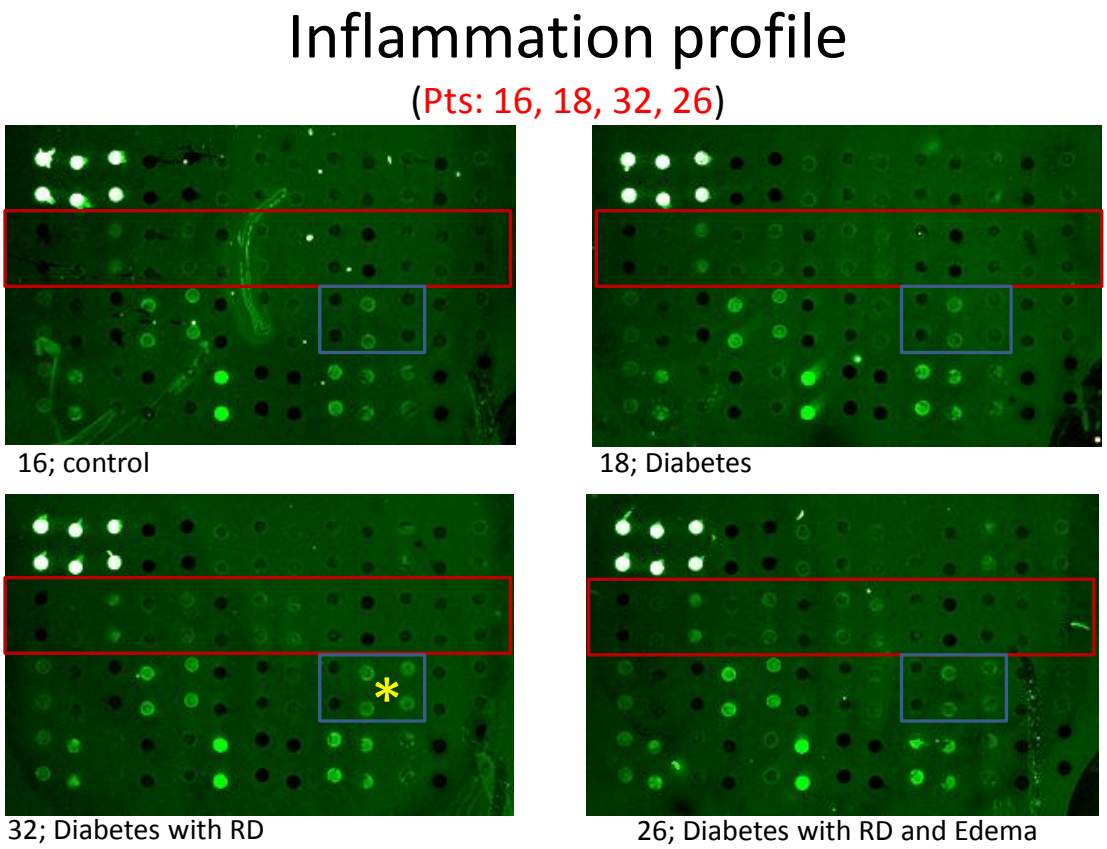


Figure 43. *Representative experimental chip-array procedure (Patients: 16, 18, 32, 26). All groups were tested in parallel (n=1 patient/group/slide). Green/cy3 signals (specific binding) were acquired by GenePix and expressed as F532-B532 median values. The asterisk presents the difference of 4 spots among 4 patients. The artifacts are due to the production or transportation damage. Diabetes: diabetics without retinopathy; Diabetes with RD: Diabetics with retinopathy and no Edema, and Diabetes with RD and edema: Diabetics with retinopathy and Edema.*

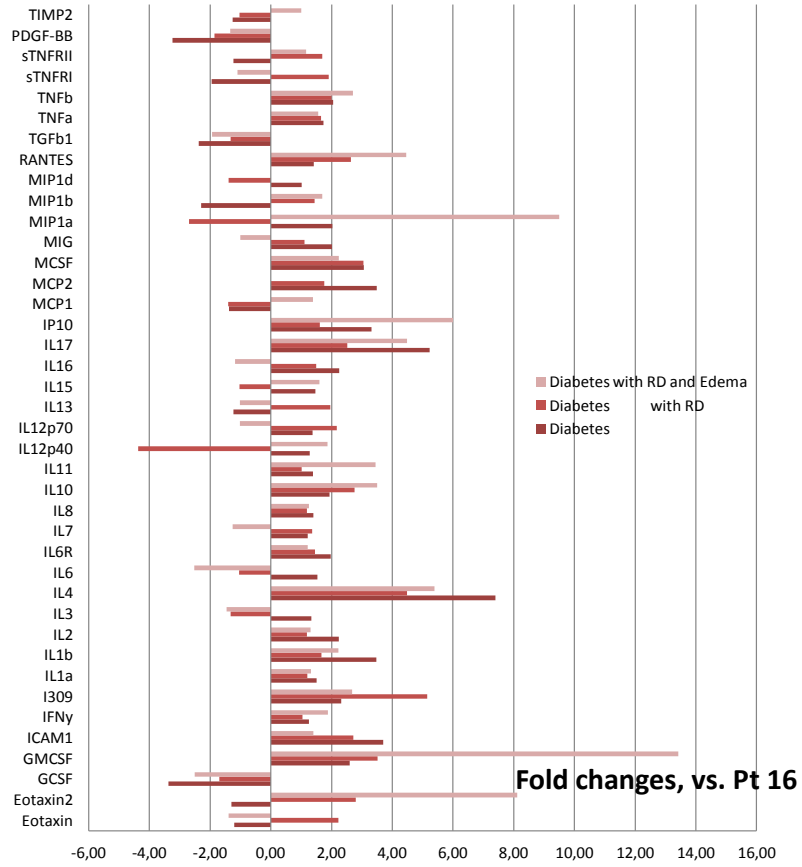
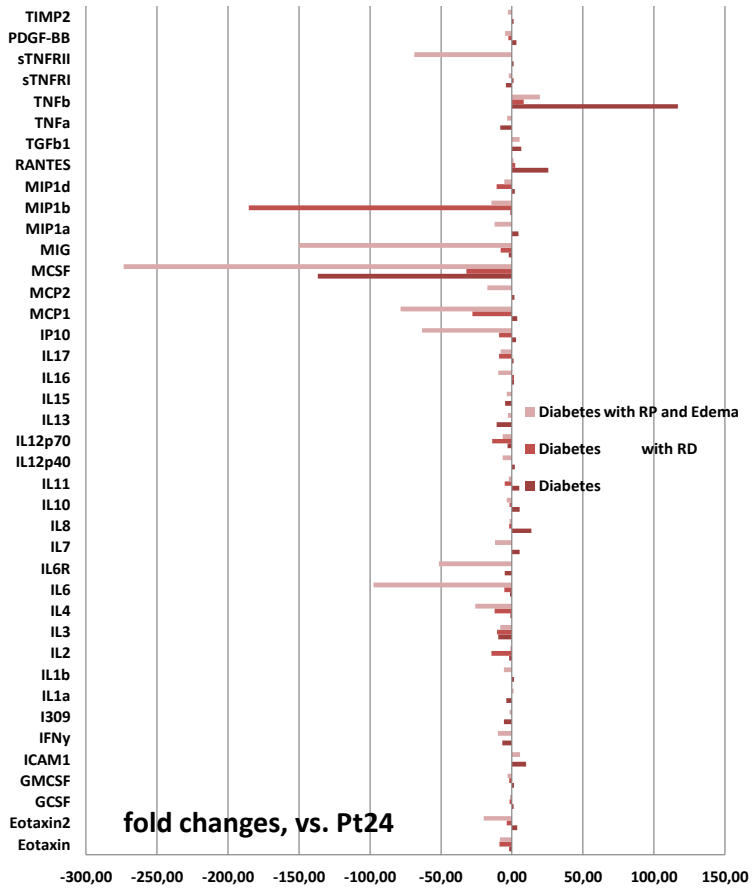


Figure 44. *Bar graph of Single Experiment: Intensity of protein expression as inferred from the fluorescence detected by antibody microarray techniques in the 1st detection. Fold changes (increase/decrease) are reported as the ratio between the expression intensity of each protein between experimental groups and related controls. In the case of results <1, the formula "-1/fold" was applied. Statistical analysis was performed according to: ANOVA/two-tailed t-test and Tukey-Kramer post-hoc. A 1.2 fold cut-off was considered significant. Diabetes: diabetics without retinopathy; Diabetes with RD: Diabetics with retinopathy and no Edema, and Diabetes with RD and edema: Diabetics with retinopathy and Edema.*

Table IX. *Fold changes and statistical significance of the fluorescent signal (optical density values F532-B532) of the cytokines present in the aqueous humour of the four groups in: patients 18, 32, 26 versus patient 16. Mean of a 1.2 fold cut-off was considered significant;. Diabetics w/oDR: diabetics without DR; Diabetics w/DR ME-: diabetics with DR and no macular edema; Diabetics w/DR ME+: diabetics with DR and with macular edema*

Chip	Folds					
	vs. control			vs. Diabetic w/o DR		vs. Diabetic w/DR ME-
Biomarkers	Diabetic w/o DR	Diabetic w/DR ME-	Diabetic w/DR ME+	Diabetic w/DR ME-	Diabetic w/DR ME+	Diabetic w/DR ME+
Eotaxin	-1,21	2,23	-1,39	2,69	-1,15	-3,69
Eotaxin2	-1,30	2,80	8,11	3,63	10,53	-21,36
GCSF	-3,37	-1,70	-2,51	1,98	1,34	1,07
GMCSF	2,59	3,52	13,42	1,36	5,17	-105,43
ICAM1	3,70	2,72	1,40	-1,36	-2,64	-6,12
IFNy	1,25	1,05	1,87	-1,20	1,50	-3,96
I309	2,32	5,15	2,68	2,22	1,16	-4,39
IL1a	1,50	1,21	1,32	-1,25	-1,14	-21,36
IL1b	3,48	1,67	2,23	-2,08	-1,56	-3,84
IL2	2,24	1,19	1,30	-1,88	-1,71	-3,80
IL3	1,34	-1,32	-1,46	-1,77	-1,95	-2,29
IL4	7,40	4,49	5,39	-1,65	-1,37	-4,74
IL6	1,53	-1,04	-2,51	-1,59	-3,86	-6,28
IL6R	1,97	1,45	1,22	-1,36	-1,61	-10,01
IL7	1,22	1,36	-1,25	1,12	-1,53	-5,75
IL8	1,40	1,19	1,25	-1,18	-1,12	-3,94
IL10	1,93	2,75	3,51	1,43	1,82	-3,60
IL11	1,39	1,02	3,45	-1,37	2,48	1,00
IL12p40	1,28	-4,37	1,87	-5,58	1,46	1,51
IL12p70	1,37	2,18	-1,02	1,58	-1,40	-6,36
IL13	-1,24	1,95	-1,01	2,41	1,22	-32,06
IL15	1,47	-1,03	1,60	-1,51	1,08	-2,76
IL16	2,25	1,50	-1,17	-1,51	-2,65	-18,59
IL17	5,23	2,52	4,49	-2,08	-1,16	-5,40
IP10	3,32	1,61	6,00	-2,06	1,81	-2,32
MCP1	-1,38	-1,40	1,39	-1,02	1,92	-2,77
MCP2	3,50	1,76	nd	-1,98	nd	-1,25
MCSF	3,07	3,05	2,24	-1,01	-1,37	-5,57
MIG	2,01	1,11	-1,01	-1,81	-2,02	-4,15
MIP1a	2,03	-2,69	9,50	-5,46	4,68	1,17
MIP1b	-2,29	1,44	1,70	3,31	3,90	-2,65
MIP1d	1,02	-1,39	nd	-1,42	nd	-1,69
RANTES	1,41	2,64	4,46	1,87	3,17	-11,12
TGFb1	-2,37	-1,33	-1,94	1,79	1,22	-7,56
TNFa	1,74	1,66	1,56	-1,05	-1,11	-3,22
TNFb	2,06	2,00	2,71	-1,03	1,32	-23,34
sTNFRI	-1,95	1,90	-1,10	3,71	1,76	-20,93
sTNFRII	-1,24	1,70	1,16	2,10	1,44	-1,02
PDGF-BB	-3,24	-1,86	-1,34	1,75	2,42	1,98
TIMP2	-1,26	-1,03	1,00	1,22	1,27	-43,74



Pts: 24, 31, 35, 33

Figure 45. *Bar graph of a Single Experiment: Intensity of protein expression as inferred from the fluorescence detected by antibody microarray techniques in the 2nd detection. Fold changes (increase/decrease) are reported as the ratio between the expression intensity of each protein between experimental groups and related controls. In the case of results <1, the formula "-1/fold" was applied to calculate the decrease. Statistical analysis was performed according to: ANOVA/two-tailed t-test and Tukey-Kramer post-hoc. A 1.2 fold cut-off was considered significant. Diabetes: diabetics without retinopathy; Diabetes with RD: Diabetics with retinopathy and no Edema, and Diabetes with RD and edema: Diabetics with retinopathy and Edema.*

Table X. *Fold changes and statistical significance of the fluorescent signal (optical density values F532-B532) of the cytokines present in the aqueous humour of the four groups in: patients 31, 35, 33 versus patient 24 (control). Mean of a 1.2 fold cut-off was considered significant;. Diabetics w/oDR: diabetics without DR (patient 31); Diabetics w/DR ME-: diabetics with DR and no macular edema (patient 35); Diabetics w/DR ME+: diabetics with DR and with macular edema (patient 33)*

Chip	Folds					
	vs. control			vs. Diabetic w/o DR		vs. Diabetic w/DR ME-
Biomarkers	Diabetic w/o DR	Diabetic w/DR ME-	Diabetic w/DR ME+	Diabetic w/DR ME-	Diabetic w/DR ME+	Diabetic w/DR ME+
Eotaxin	-1,88	-8,77	-8,45	-2,45	-2,11	1,16
Eotaxin2	3,67	-3,72	-19,75	nd	nd	-1,88
GCSF	1,05	-1,83	-1,41	nd	nd	-36,14
GMCSF	1,54	-2,09	-3,09	nd	nd	18,67
ICAM1	9,86	nd	5,71	nd	nd	2,13
IFNy	-6,88	nd	-9,83	-5,57	-4,52	1,23
I309	-5,60	nd	-1,60	nd	nd	1,50
IL1a	-3,86	nd	1,22	-3,22	-8,18	-2,54
IL1b	1,55		-5,73	nd	nd	2,93
IL2	-2,07	-14,61	-1,24	1,12	-4,74	-5,29
IL3	-9,75	-10,56	-8,16	-408,71	-30,85	13,25
IL4	-1,15	-12,16	-25,80	nd	nd	1,74
IL6	-1,33	-5,52	-97,50	-6,12	-2,68	2,28
IL6R	-4,97	nd	-51,33	nd	nd	6,07
IL7	5,54	nd	-12,00	14,95	-3,45	-51,60
IL8	13,52	-2,03	-1,64	-22,77	-11,91	1,91
IL10	5,51	-1,67	-3,55	-30,05	4,14	124,50
IL11	5,22	-5,19	-2,39	nd	nd	2,14
IL12p40	1,97	nd	-6,44	nd	nd	15,13
IL12p70	-3,00	-13,97	-6,58	nd	nd	-2,18
IL13	-10,67	nd	-2,81	nd	nd	1,87
IL15	-4,69	nd	-3,57	-1,86	-6,84	-3,68
IL16	1,38	1,52	-9,75	nd	nd	3,46
IL17	1,23	-8,99	-7,88	nd	nd	-1,83
IP10	2,89	-8,98	-63,50	-12,17	-55,47	-4,56
MCP1	3,63	-27,75	-78,50	-13,79	-6,88	2,00
MCP2	1,65	nd	-17,25	nd	nd	-9,17
MCSF	-136,75	-32,23	-273,50	nd	nd	-242,63
MIG	-2,17	-7,86	-150,00	-9,66	1,12	10,80
MIP1a	4,52	nd	-12,20	nd	nd	1,35
MIP1b	-1,22	-185,26	-14,56	-266,40	-3,24	82,25
MIP1d	2,08	-10,88	-5,26	-2,87	-4,29	-1,49
RANTES	25,65	2,33	1,06	nd	nd	3,92
TGFb1	6,53	nd	5,47	nd	nd	2,65
TNFa	-8,25	nd	-3,49	-15,11	-103,24	-6,83
TNFb	116,86	8,28	19,71	-3056,12	-4,84	632,00
sTNFRI	-4,22	1,14	-2,24	nd	nd	-1,46
sTNFRII	1,27	nd	-68,75	nd	nd	-31,20
PDGF-BB	3,01	-2,50	-4,72	33,94	10,68	-3,18
TIMP2	1,19	nd	-2,96	-64,76	-2,37	27,30

4.3. Thickness analysis of the retinal layers

This study has also evaluated the thickness of various retinal layers via the SD-OCT, considering 60 acquired intervals. The comparison between the 4 different groups has been carried out: healthy controls, diabetics without DR, diabetics with DR and without DME, and diabetics with DR and DME.

The mean thickness of various layers (60 intervals per layer) has shown significant differences between the 4 groups in the following cases (table VIII):

- In the patient group with DR and DME, the average thicknesses of RNFL, INL and ONL have resulted significantly greater compared to those of the other 3 groups ($p<0.05$);
- In the patient group with DR and without DME, the RNFL thickness has resulted significantly diminished, and the ONL thickness significantly increased compared to the controls ($p<0.05$); in addition, though not reaching the level of statistical significance, also in other groups there was a tendency to RNFL reduction and ONL increase in diabetic patients without DR compared to the controls.

Table XI. *Comparison of the mean thickness and its standard deviation (in μm) of different retinal layers in the 4 groups of patients. Diabetics w/o DR: diabetics without retinopathy; diabetics w/DR ME-: diabetics with retinopathy and without macular edema; diabetics w/DR ME+: diabetics with retinopathy and DME; RNFL: nerve fiber layer; GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer.*

LAYER	MEAN THICKNESS OF RETINAL LAYERS ± SD (μm)			
	Controls (N=12)	Diabetics w/o DR (N=11)	Diabetics w/DR ME- (N=5)	Diabetics w/DR ME+ (N=6)
RNFL	26.8±3.4	26.5±2.8	24.3±2.6	37.3±9.9
GCL	39.4±3.3	40.2±4.6	38.5±2.3	44.1±5.6
IPL	30.3±1.8	29.7±3.8	29.9±2.9	33.8±2.9
INL	35.6±4.0	35.1±2.2	36.4±4.3	46.4±1.6
OPL	23.5±4.5	22.7±3.9	23.9±3.2	30.8±10.1
ONL	70.3±9.0	72.4±5.6	77.3±7.4	91.8±20.6

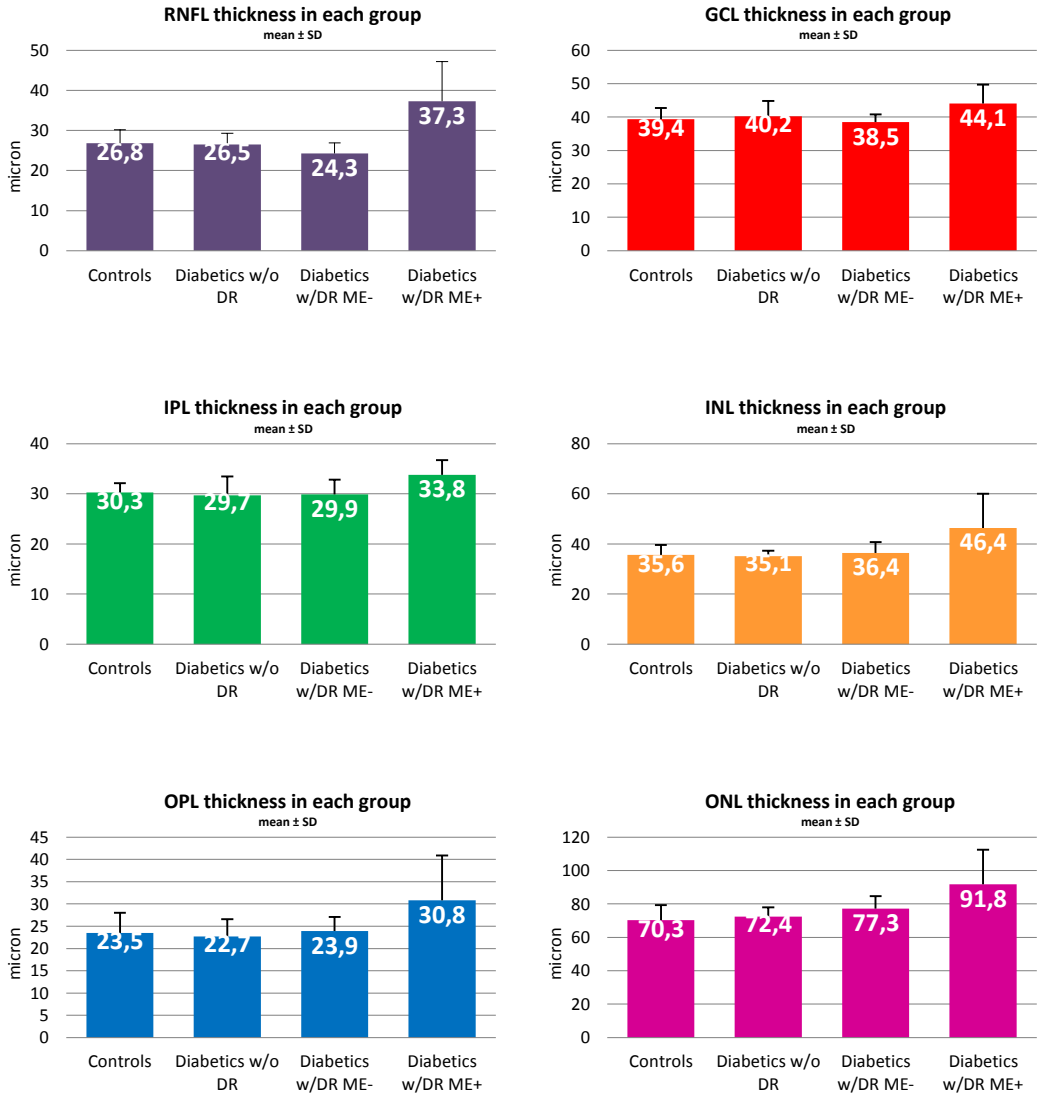


Figure 46. *The mean thickness of different retinal layers (in μm). There is significantly higher thicknesses of RNFL, INL and ONL in diabetics with DR and DME compared to the other groups ($p<0.05$); the RNFL thickness is significantly reduced in diabetics with DR and without DME compared to controls ($p<0.05$); significantly greater ONL thickness in diabetics with DR and without DME compared to controls ($p<0.05$). Diabetics w/o DR: diabetics without retinopathy; diabetics w/DR ME-: diabetics with retinopathy and without macular edema; diabetics w/DR ME+: diabetics with retinopathy and DME; RNFL: nerve fiber layer; GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer.*

For each layer, the mean thicknesses of the central ring (12 intervals per ring), of the inner ring (24 intervals per ring) and of the outer ring (24 intervals per ring)

have evidenced the significant differences between the 4 groups in the following cases (table IX):

- in RNFL, the thicknesses of the inner and the outer rings are significantly greater in diabetics with DR and DME compared to the other groups ($p<0.05$);
- in INL, the thicknesses of the central and the inner rings are significantly greater in diabetics with DR and DME compared to diabetics without DR and the controls ($p<0.05$);
- in OPL, the thickness of the central ring is significantly greater in diabetics with DR and DME compared to the one in the other groups ($p<0.05$);
- in ONL, the thicknesses of the central ring and the external one are significantly greater in diabetics with DR and DME compared to diabetics without DR and the controls ($p<0.05$).

It has been moreover detected that the course of the RNFL profile is significantly different in diabetics with DR and DME compared to the other groups ($p<0.05$), because of the accentuated increase of the thickness moving from the inside to the outside. No significant differences have been found in the course of the profiles of the other layers.

Table XII. *Comparison of the mean thickness and its standard deviation (in µm) per ring in different retinal layers of the 4 patient groups. Diabetics nDR: diabetics without retinopathy; diabetics DR ME-: diabetics with retinopathy and without macular edema; diabetics DR ME+: diabetics with retinopathy and DME; RNFL: nerve fiber layer; GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer.*

RING	MEAN THICKNESS PER RING ± SD (µm)				
	LAYER	Controls (N=12)	Diabetics w/o DR (N=11)	Diabetics w/DR ME- (N=5)	Diabetics w/DR ME+ (N=6)
CSF	RNFL	9.3±3.5	9.0±2.6	8.0±1.8	10.3±2.3
	GCL	13.3±4.5	13.8±5.5	14.6±3.0	16.5±5.1
	IPL	12.4±3.6	12.1±4.3	12.8±5.2	16.2±4.1
	INL	19.3±5.3	18.4±5.1	22.3±3.2	33.2±14.3
	OPL	15.3±5.7	14.6±5.8	13.5±3.7	26.7±11.9
	ONL	97.4±12.9	101.0±9.9	106.9±9.3	129.0±31.1
INNER	RNFL	24.3±3.4	25.0±3.8	22.0±2.1	33.4±8.1
	GCL	53.5±6.2	53.5±6.4	50.3±4.1	57.5±9.2
	IPL	36.2±2.6	35.3±5.4	36.8±4.9	41.2±3.4
	INL	43.4±6.0	42.4±4.0	43.7±4.9	56.2±16.9
	OPL	27.3±6.8	26.2±5.1	27.0±4.7	34.0±9.8
	ONL	70.8±10.2	71.5±6.4	78.2±9.1	88.8±23.5
OUTER	RNFL	38.1±5.0	36.7±3.0	34.6±4.5	54.7±15.9
	GCL	38.4±4.4	40.2±5.0	38.6±3.3	44.4±4.8
	IPL	33.4±3.6	32.9±3.9	31.5±1.2	35.2±4.8
	INL	36.0±3.9	36.3±2.4	36.1±4.7	43.1±11.0
	OPL	23.8±2.7	23.2±2.7	26.1±3.2	29.7±10.0
	ONL	56.1±8.6	59.1±6.6	61.6±6.5	76.2±22.0

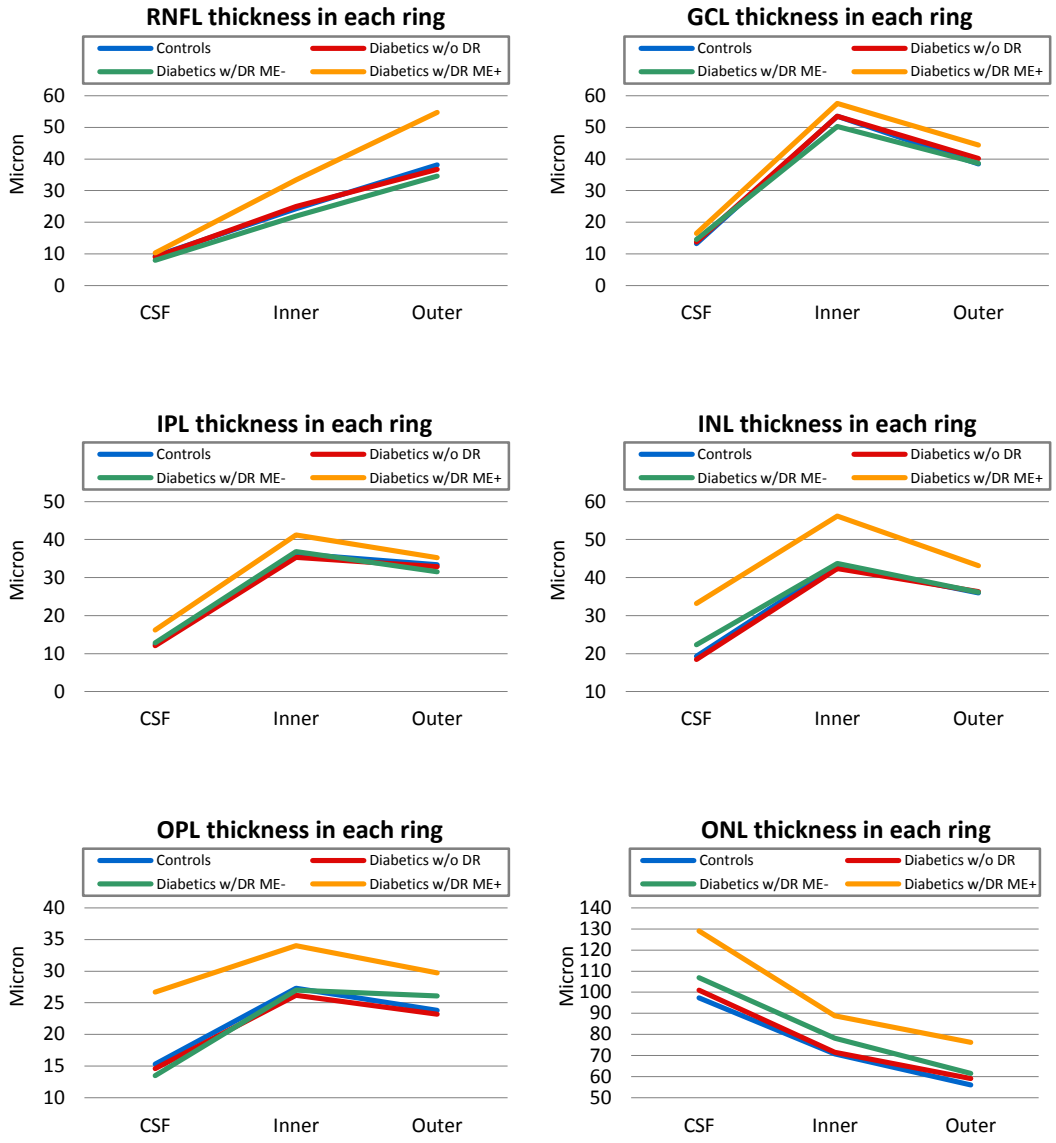


Figure 47. *Mean thickness per each ring in the different retinal layers (in μm). Diabetics w/o DR: diabetics without retinopathy; diabetics w/DR ME-: diabetics with retinopathy and without macular edema; diabetics w/DR ME+: diabetics with retinopathy and DME; RNFL: nerve fiber layer; GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer.*

For each layer, the mean thicknesses of the sectors superior, nasal, inferior and temporal (15 intervals per sector), have evidenced significant differences between the groups in the following cases (table X):

- in RNFL, the thicknesses of the sectors superior, inferior and temporal have resulted significantly greater in diabetics with DR and DME compared to the other groups ($p<0.05$);
- in GCL, the thickness of the superior sector has resulted significantly greater in diabetics with DR and DME compared to diabetics without DR and the controls ($p<0.05$);
- in INL, the thicknesses of the sectors superior, nasal, inferior and temporal have resulted significantly greater in diabetics with DR and DME compared to diabetics without DR and the controls ($p<0.05$);
- in ONL, the thicknesses of the superior and the inferior sectors have resulted significantly greater in diabetics with DR and DME compared to diabetics without DR and the controls ($p<0.05$), while the thickness of the temporal sector resulted significantly greater only compared to the controls. ($p<0.05$);

No significant differences have been detected in the course of the profiles in the different sectors.

Table XIII. *Comparison of the mean thickness and its standard deviation (in µm) per sector in different retinal layers of the 4 patient groups. Diabetics w/o DR: diabetics without retinopathy; diabetics w/DR ME-: diabetics with retinopathy and without macular edema; diabetics w/DR ME+: diabetics with retinopathy and DME; RNFL: nerve fiber layer; GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer.*

SECTOR	MEAN THICKNESS PER SECTOR ± SD (µm)				
	LAYER	Controls (N=12)	Diabetics w/oDR (N=11)	Diabetics w/DR ME- (N=5)	Diabetics w/DR ME+ (N=6)
Superior	RNFL	29.5±4.1	29.2±3.7	26.1±3.6	40.3±11.6
	GCL	39.5±3.5	40.4±3.6	39.9±2.6	45.9±3.8
	IPL	30.1±3.2	29.6±4.7	29.9±1.5	31.4±2.9
	INL	35.5±4.1	36.0±2.7	36.3±4.5	43.1±5.7
	OPL	23.4±3.2	23.8±6.1	28.2±5.8	32.0±10.0
	ONL	70.4±7.7	69.4±6.6	74.4±8.2	85.6±11.2
Nasal	RNFL	29.8±5.7	28.6±3.3	27.6±3.3	39.3±17.2
	GCL	41.0±2.9	41.9±6.3	41.5±4.1	46.0±8.3
	IPL	30.1±2.3	29.6±4.2	29.3±4.4	34.6±3.9
	INL	37.2±4.8	35.7±2.4	37.7±3.9	46.8±13.4
	OPL	24.8±9.6	23.8±6.6	23.7±4.9	28.4±4.3
	ONL	71.9±12.5	75.4±6.4	80.8±7.1	84.4±15.0
Inferior	RNFL	30.1±3.6	29.1±3.6	27.2±3.2	44.3±12.8
	GCL	38.2±4.5	39.6±4.9	37.3±2.9	42.0±7.6
	IPL	30.2±2.7	29.1±4.0	30.0±2.7	33.2±1.7
	INL	35.4±4.2	34.5±2.1	36.4±3.6	48.7±21.7
	OPL	23.7±4.5	22.6±4.0	24.1±2.8	37.6±27.7
	ONL	66.5±9.1	69.6±6.8	73.7±9.0	98.4±40.3
Temporal	RNFL	18.0±2.8	18.9±2.9	16.8±1.5	26.3±1.7
	GCL	39.1±4.7	39.4±5.0	35.6±2.4	42.8±4.0
	IPL	30.8±2.4	30.5±4.8	30.5±6.2	35.8±3.4
	INL	34.4±4.5	34.3±3.3	35.2±6.3	46.7±13.5
	OPL	22.0±4.6	20.6±3.5	20.3±2.6	25.6±1.6
	ONL	72.2±10.7	75.3±8.1	79.9±7.9	98.0±35.0

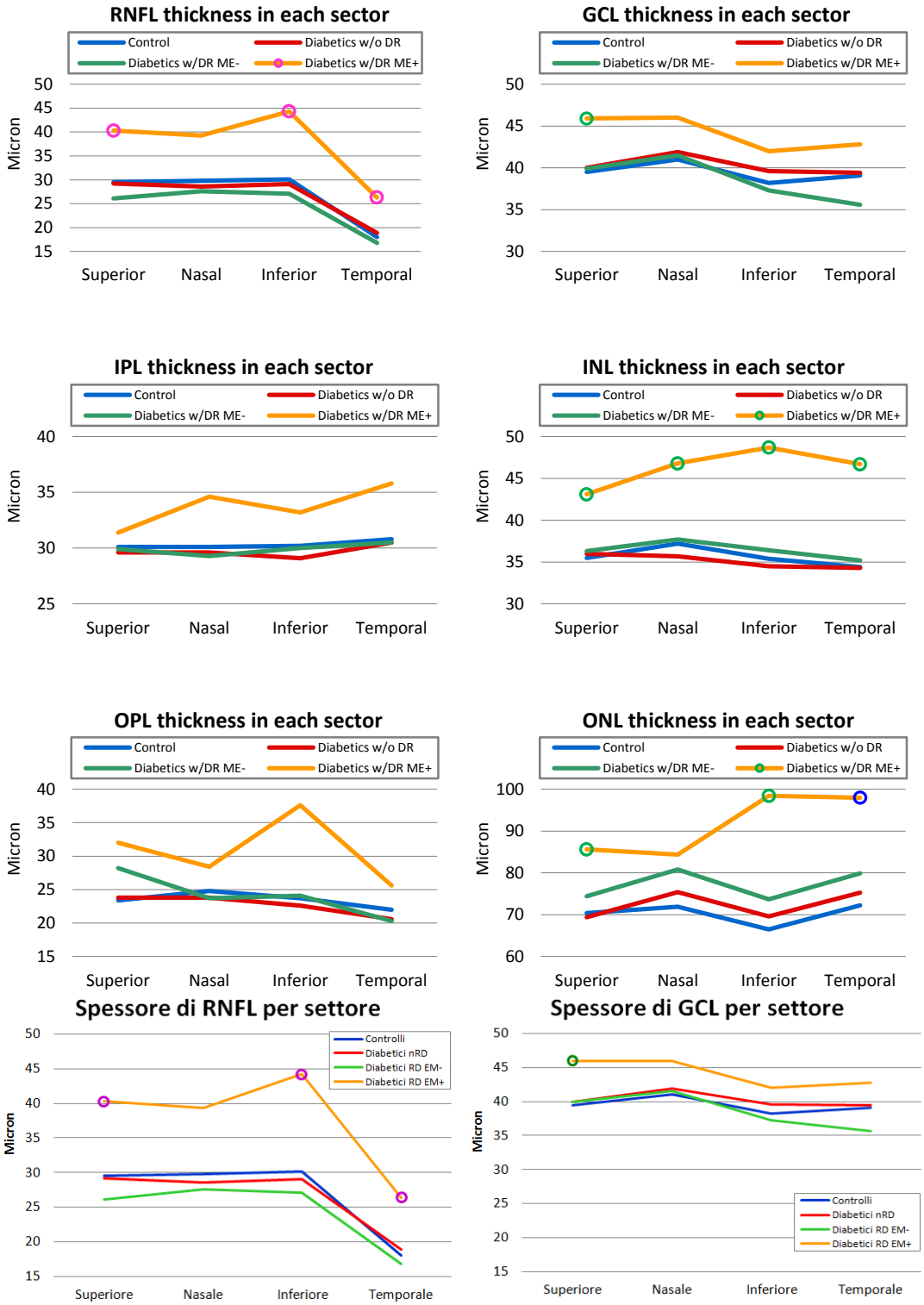


Figure 48. *Mean thickness per each sector of the different retinal layers (in µm). Pink circles indicate the significant difference between the thickness of the considered layer between diabetics with DR and DME compared to other groups (p<0.05); green circles indicate the significant difference between diabetics with DR and DME compared to diabetics with DR and the controls (p<0.05); blue circles indicate the significant difference between diabetics with DR and DME and the controls (p<0.05). Diabetics w/o DR: diabetics without retinopathy; diabetics w/DR ME-: diabetics with retinopathy and without macular edema; diabetics w/DR ME+: diabetics with retinopathy and DME; RNFL: nerve fiber layer; GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer*

DISCUSSION

Our study has analyzed for the first time in literature the concentrations in the aqueous humour of the proteins of glial origin GFAP, AQP1 and AQP4 in subjects afflicted and not afflicted by DM, detecting a significant increase in the first group compared to the second one, both in presence of non proliferative DR and in its absence. Ours is, moreover, the first study to demonstrate a significant increase of different inflammatory cytokines in the AH of diabetic subjects, whether they presented a clinically detectable retinal involvement, or not.

The AH, the vitreous and the lachrymal fluid constantly exchange substances with the blood and with ocular tissues: their protein content may therefore be considered an extremely useful instrument for the evaluation of the integrity and the functionality of the structure of the ocular bulb.[27] Since there have been various studies which had demonstrated that the microvascular complications caused by DM could alter quantitatively and qualitatively the protein content of ocular fluids, it is reasonable to consider possible to identify certain proteins as markers which could anticipate the onset of DR. [14, 27, 210-219] It is well known that microvascular lesions typical of DR are preceded first by the activation and the dysfunction of glial cells and then by the retinal neurodegeneration: for this reason, our research was concentrated on the identification of the early biomarkers of glial origin found in AH.

Even though this fluid is not in direct contact with the retina, the proteins released by the retina may overflow in it thanks to the diffusion through the vitreous and the intraocular structures and to the crossing of the blood-retinal and the blood-aqueous barriers.[14] In addition, it is known that the collection of the AH is much easier and much less invasive than the collection of the vitreous, and that it does not imply any additional risk with respect to a normal cataract intervention: for these reasons, we believed that this fluid constituted the most adequate choice for our research. [220]

Despite the ongoing debate on the existence of a connection between the protein composition of the AH and the vitreous, the Funatsu and the Ecker groups have demonstrated that there is good relationship between the two for many of the contained proteins. [215, 217, 220] Our interest has, moreover, been to individuate the biomarkers of glial activation which present themselves in different concentrations in healthy subjects compared to diabetic subjects with or without signs of retinopathy, independently of their vitreous concentration.

One of the greatest limitations of the use of AH consists in the fact that only small volumes, usually around 100-200µl, can be obtained in the collection process. These quantities are sufficient only for the evaluation of a limited number of proteins via the ELISA test, especially considering that protein concentrations in this liquid are extremely low. For this reason, we have decided to use, along with the ELISA tests for AQP1, AQP4 and GFAP, also the protein array technique, which is able to analyze contemporarily many molecules, which in our case were constituted by 40 different cytokines. It is a recently introduced technique in continuous evolution, whose validity in recognizing the presence of studied molecules was demonstrated by the Sharma group, and confirmed by various studies on different cytokines in ocular fluids.[156, 221-223]

In our study, the mean GFAP concentration results significantly increased in diabetic patients with DR compared both to the controls (324.44 pg/µg vs 182.34 pg/µg) and diabetics without DR (324.44 pg/µg vs 165.61 pg/µg). This protein is usually expressed by the glial cells of the CNS, and, in particular, on the retinal level in physiological conditions it is expressed only by the astrocytes, while it is absent in Müller cells. GFAP, together with other proteins, participates in the formation of intermediate filaments of the cellular cytoskeleton, and has an important role in the mitotic process, in the maintenance of the blood-encephalic and the blood-retinal barriers, as well as in neuronal communication and repair. [224-227] During the course of DM, in spite the fact that its expression decreases

in astrocytes, this protein is expressed on the retinal level thanks to its increased production by the Müller cells. [83, 97-99, 101] The fact that there is a hyper-expression of GFAP during the course of different neurodegenerative pathologies involving not only the retina, but also, more generally, the CNS, explains the reason why it is considered a valid marker of glial activation. [149] In particular, on the ocular level, the importance of GFAP is evidenced especially after retinal insults, since it has been observed that it is very important for the integrity of the Müller cells and consequently for the integrity of the internal and the external limiting membranes. [149]

The available studies in literature have analyzed the GFAP expression exclusively on the histological level, both in animal and in human tissue; in this study, however, we have evaluated the concentration *in vivo* in AH both in normal conditions and in the course of diabetic disease. At the very beginning (in diabetics without DR) we found a moderate decrease in GFAP when compared to the normal subjects. This may be due to the early atrocyte loss, already demonstrated as one of the first signs of neuronal dysfunction in DM. [83] Later on, there is a significant increase of GFAP in presence of very initial stages of clinically detectable signs of DR, confirming the early activation of the glial component in the course of DR (Muller cells).

Different studies have, in fact, confirmed such hypothesis, suggesting that the glial activation could be responsible for the instauration of functional and metabolic imbalances of the retina which are the reasons behind both the neuronal and the microvascular damage. [89, 90, 93, 94] The increase of GFAP in AH would therefore be the consequence both of the normal exchange process of the Müller cells, which become hypertrophic and more numerous, and the increased apoptotic rate of the astrocytes, and to a lesser degree, of the very Müller cells, according to many studies done on animal and human histological samples during the course of DM.[93, 94, 150, 228-230]

There were no studies in literature which analyzed the aquaporin concentrations in ocular fluids, with the exception of two studies by the Ohashi and the Hirai groups on AQP5 in lachrymal fluid, respectively during the course of the dry eye syndrome and the dacryoadenitis. [231, 232] In order to quantify this protein, these studies have made use, as in our case, of the ELISA test, and both studies have detected a reduction of AQP5. [231, 232]

In our study, the AQP4 concentration resulted significantly greater in diabetic patients with retinopathy compared both to diabetics without retinopathy and the healthy subjects (respectively 852.03pg/µg *vs* 206.87pg/µg *vs* 33.58 pg/µg); such increase resulted significant also in diabetics without DR compared to healthy subjects (206.87pg/µg *vs* 33.58 pg/µg). AQP4 is a channel protein which permits the flow of free water through the cell membrane, and which results present in the CNS, in the eye, in the kidney and in the respiratory apparatus. [233] In the eye in particular, AQP4 is normally expressed especially by the Müller cells and the astrocytes, as well as by the cilliary body epithelium and the lachrymal gland. [118, 126, 234] Thanks to its ability to transport free water and its functional pairing with potassium channels, AQP4 maintains the osmotic balance between the intra- and the extra-cellular matrixes, thus guaranteeing optimal cell functioning. [235] The Zhang and the Cui groups have recently evidenced, in the experiments on rat retina, that during the course of DM there is an increase in AQP4 on the retinal level: it is plausible to suppose that this constitutes a compensation attempt with respect to the altered osmotic equilibrium in the retina during diabetes.[146, 147] While there are different histological studies done so far regarding the variation in AQP4 expression in the retina during the course of DM, there are no researches which evaluate its concentration in ocular fluids either in healthy subjects or in subjects afflicted with pathologies regarding the eye. [97, 121, 146-148] Our study has investigated AQP4 concentrations in healthy subjects and in diabetics, noticing a significant increase of this aquaporin

in the AH, not only in presence of microvascular alterations typical of DR, but also in its subclinical stage. We consider this increase caused, on one part, by the natural process of retinal cell metabolism, and, on the other, by the increased cell apoptosis which strikes the astrocytes and only marginally the Müller cells: both factors would entail the release of this protein in the AH, making it therefore detectable at this location. Since AQP4 is expressed especially in the retinal glial cells, it is possible to consider this molecule an early and very effective marker of retinal damage during the course of DM.

In our research, we have also detected a significant increase in AQP1 in diabetic subjects with retinopathy compared both to diabetics without DR and to healthy subjects (respectively 105.72pg/µg *vs* 28.84pg/µg *vs* 50.92pg/µg); and in diabetic subjects with retinopathy and without macular edema compared to those with macular edema (105.72pg/µg vs 37.09pg/µg). This aquaporin, present in the CNS, the eye, the kidney and the respiratory apparatus, is usually expressed in amacrine cells and the photoreceptors, as well as in the corneal endothelium and the iridocorneal trabeculae, in the cilliary body epithelium, in the lens and iris and in RPE. [119, 132, 136, 233, 236] Like AQP4, its function is also the regulation of the transcellular flow of free water, contributing to the maintenance of osmotic balance between the intra- and the extracellular matrixes, hence exercising an essential role in the functional maintenance of the cells.[235] There are various histological studies both of animal and human tissue, which have analyzed the changes in AQP1 expression during the course of DM: they have evidenced its presence also in the inner retinal layers, in particular in Müller cells, and the substitution of AQP4 with AQP1 in retinal surface vessels, entailing an increase in its production. [116, 121, 144, 145] Such hyper-expression would constitute a compensation attempt regarding the altered osmotic balance inside the retina during the course of DM.[116, 126] Even though many studies were directed at the different histological expressions of AQP1 during this pathology, nobody has

analyzed the concentrations of this protein in ocular fluids. With the present study, however, we have examined the AQP1 concentrations in the AH both in the healthy subjects and in the diabetic ones, evidencing the increase of this protein in subjects afflicted by DR. In this case, too, the increase is connected to the normal process of retinal cell metabolism and to the increased apoptosis which affects the astrocytes in particular, and only marginally the Müller cells, during the course of DM, with the consequent release of molecules inside ocular fluid, especially inside the AH. Since we have not noticed an increase in AQP1 in diabetic patients without DR compared to controls, it is possible to consider that such increase can be detected exclusively when the retinal damage is clinically evident and that it therefore constitutes a modification of glial cells subsequent to those previously mentioned.

AQP1 could therefore constitute a useful marker of glial activation, capable of indicating the aggravation of retinal damage during the course of DM.

A curious finding was the observation of decrease in GFAP, AQP1 and AQP4 in diabetics with macular edema compared to diabetics with DR and without ME. This might be explained by the anatomic alterations in Muller cells induced by cystic spaces caused by edema. The cells are stretched, and reduced in number, and therefore this may be the reason for the observed decrease in glial markers concentration in ME.

The data concerning the concentration of different inflammatory cytokines, presented in the form of fluorescent signal, has enabled the detection, both in diabetic subjects without retinopathy and in those with retinopathy, of a general increase in such molecules, in compliance with the hypothesis according to which the DR would also be inflammatory in nature. [31] Our study has evidenced how the levels of some inflammatory cytokines result higher in diabetic subjects without retinopathy compared to normal subjects: IFNy, IL-1a, IL-1b, IL-3, IL-4, IL-17, TNF-ß, MCP2; whereas, the IL-7 was significantly decreased in diabetic

subjects without DR; an increase has been observed also between the diabetics with DR and normal subjects for: Eotaxin, TNF-ß, IL-4, MCP2, RANTES, sTNFRII, IFNy, TNF-α ; an increase has been observed also between the diabetics with DME and normal subjects for: Eotaxin2, GM-CSF, IFNy, IP-10, TNFb, MIP1a, sTNFRII, Il-10, IL-11, MCP1; a significant difference has been observed also between the diabetics with DR and those without for: MIP1b, TNFb. Therefore, this may be explained by the fact that these cytokines are affected by the diabetes-caused retinal damage only in a later stage.

In our study, therefore, we have noticed especially the significant increase in the cytokines produced by the glial cells and in those which attract the circulating macrophages to the site of the lesion. This observation confirms the key role that these cells hold, not only in presence of microvascular lesions caused by DR, but also in the stage before the lesions become clinically detectable. For this reason, glial activation would entail the triggering of the inflammatory process responsible for the retinal damage, both vascular and nervous.

In contrast to what has been said for GFAP, AQP1 and AQP4, there are numerous studies which have investigated the presence of the inflammatory cytokines in AH and the vitreous of diabetic subjects. [155, 219, 222, 237, 238] Nevertheless, most of these studies have considered only the subjects who presented advanced stages of DR, in many cases already in its proliferative form or associated with the macular edema. [215, 217, 219, 222, 223, 237-240] The peculiarity of this study consists in the search for the signs of glial activation before the onset of microvascular lesions, confirming that both the glial activation and the inflammatory response in general can have a crucial role in the formation of retinal lesions from the earliest stages of diabetes mellitus. Many studies have evidenced an increase in VEGF, a proangiogenic cytokine, and an increase in PEDF, with antiangiogenic effect, in diabetic patients afflicted by retinopathy compared to non diabetic subjects.[239, 241]

We have not, however, analyzed these molecules, since our main interest was to concentrate specifically on the glial activation caused by diabetes, and not on the retinal angiogenic process.

With respect to the AH, there are many works which have evidenced an increase in inflammatory cytokines in diabetic subjects with retinopathy compared to healthy subjects. The cytokines which resulted significantly increased are: MIP-1β, sICAM-1, IL-6, IL-8, IL-12, IP-10, MCP-1, TGF-β.[155, 156, 215, 219, 222, 223, 237, 238] Moreover, our study, evidences that IFNy, IL-1a, IL-1b, IL-3, IL-4, IL-17, TNF-ß, and MCP2 do not increase only when there are visible signs of microvascular lesions, but are increased already at the subclinical stage.

The works which have analyzed the cytokine concentration in the vitreous or AH in presence of proliferative diabetic retinopathy or of macular edema, have reported a significant increase in IL-1, IL-3, IL-6, IL-8, IL-10, IL-13, IP-10, MCP-1, MIP-1β, PDGF, TNF-α, VEGF, sICAM-1, sVCAM-1, MMP-1, MMP-9, TGF-b, PIGF and RANTES compared to non diabetic subjects. [63, 66, 67, 219, 239, 242, 242-250] In compliance with this data (although we have not included patients with PDR and we have evaluated samples of AH which contain a significantly lower concentration of cytokines), our study has demonstrated that, GM-CSF, IFNy, IP-10, TNFb, MIP1a, sTNFRII, Eotaxin 2, IL-10, IL-11, MCP1 are increased in the AH of patients with DME, whereas, IFNy, IL-3, IL-4, IL-17, TNF-ß, MCP2, IL-1a, IL-1b are increased during the course of DM even when there are no signs of retinopathy. MCP-2 is a monocyte chemotactic factor and increased levels of this chemokine suggest an early inflammatory response. [223] On the other hand, the late inflammatory response in DR (before the onset of new-vessels) was demonstrated by Kyung Oh et al. with higher concentrations of MCP1, IP-10, IL-8 in eyes with severe NPDR and PDR, than in eyes with less severe DR and normal subjects. [223] This may partially explain differences in our results: we have evaluated patients in earlier stages of DR, and therefore

these cytokines were increased just in DME patients.

One of the major limits of this study is the relatively low number of involved patients, especially as we divided patients with DR into two groups which were separately analyzed. Therefore, statistical significance was not reached (although absolute numbers were increased) for several cytokines that have already been demonstrated increased in DR. These include: IL-2, IL-13, ICAM1 (see table VII) On the other side, a decrease in TGF-b was found, a potent neuroprotective factor, even in diabetics without DR.[98]

We can therefore affirm that, considering the results obtained in our study, there are various cytokines which can be considered as early biomarkers of glial activation, confirming again the central role of these cells in the pathogenic process of DR.

The obtained results evidence for the first time that all of the glial cell categories are activated since the very beginnings of DM, and that their modifications constitute a crucial element in the pathogenetic process of DR. The only previous work which focused on these cells, conducted by the Nishoikiori group, has evidenced in the vitreous of diabetic patients an increase in GDNF (glial cell-derived neurotrophic factor): this was probably a compensation attempt caused by the damage in the blood-retinal barrier during the course of diabetes.[251] Nevertheless, our study detects signs of glial activation in the AH already at the subclinical stages of DR, when it is still not possible to make any diagnosis based on microvascular lesions.

The identification of retinopathy biomarkers is important for early diagnosis, therapy and retinal lesions prognosis during the course of DM. We believe that the evidence of the pathogenetic role of glial cells in this microvascular complication might lead to new and more efficient therapeutic solutions able to improve the outcome of the disease. So far, there is only one study in the literature which has investigated the risk markers for the onset of retinopathy

during the course of DM; none of the 11 proteins it has individuated is connected to glial activation, but only to mechanisms of molecular transport, angiogenesis, cellular oxidation, tissue remodeling and cytoprotection. [14]

The glial activation detected during the course of diabetes mellitus is also confirmed by the OCT analysis of the thicknesses of retinal layers.

In our study, the mean thickness of RNFL in diabetic patients with retinopathy and macular edema results significantly increased compared both to controls and diabetic subjects without or with retinopathy not associated to macular edema. Such increase is statistically significant in the inner and the outer rings and in the sectors superior, inferior and temporal of the macular area. We have, moreover, observed that in diabetics with retinopathy and macular edema, the behavior of RNFL is contrary to the one in the other groups, where there its thickness tends to decrease. The increase in RNFL thickness in diabetic subjects with retinopathy and edema can be explained, more than as the instauration of the interstitial edema (which involves only minimally the nerve fiber layer), with the fact that the instrument we used does not allow the distinction of ILM from RNFL, entailing its inclusion in the RNFL measurements. Regarding this matter, histopathologic studies have demonstrated that diabetic subjects present an ILM thickening caused mainly by the Müller cell alteration, which become hypertrophic and are afflicted by proliferation. [252-254] We therefore believe that the RNFL thickening we observed is caused prevalently by the increase in number of the Müller cells and the swellings of their end-feet on neuronal degeneration, which is thus masked by this phenomenon. The tendency of progressive reduction of RNFL thickness in the other groups, which reaches statistical significance in diabetic patients with retinopathy without macular edema compared to controls, is explained by the equally progressive neuronal loss induced by diabetic disease. It is well known that the reason behind such phenomenon is the glial cell dysfunction, which are no longer able to maintain an adequate osmotic

equilibrium between the intra- and the extra-cellular matrixes and which, by expressing different proinflammatory cytokines, induce the apoptosis of neuronal cells, especially of the ganglion cells, with their consequent axonal degeneration. .[73, 83, 98, 99, 108, 109] In compliance with a similar hypothesis, two recent studied presented at the ARVO (Association for Research in Vision and Ophthalmology) have evidenced certain morphological alterations in vivo of the retina of diabetic subjects: the first one has observed a significant increase in thickness of ILM, IPL, INL and a decrease in those of GCL and RNFL in patients with non proliferative retinopathy, while the second one noticed the thickening of the INL+IPL complex and the presence of microaggregates in ILM and RNFL corresponding to the microglia, confirming the early activation of the glial cells in the retina during the course of diabetes mellitus. [207, 208] Finally, many works have observed the thinning of RNFL and, in some cases, of the GCL+IPL complex as well, demonstrating that retinal neurodegeneration constitutes an early event in the course of diabetes mellitus, present from the subclinical stages of the retinopathy. [201, 204, 255, 256]

We can therefore affirm that both the protein analysis and the measurement of the thicknesses of retinal layers carried out with SD-OCT may provide useful information on the activation of the retinal glia during the course of diabetes mellitus.

CONCLUSIONS

In conclusion, we have demonstrated that, during the course of diabetes mellitus, there is a significant increase in the aqueous humour of the protein biomarkers of glial activation, not only in presence of advanced stages of diabetic retinopathy, but also in its subclinical and early clinical stages. This has confirmed the physiopathological role of these cells in the development of this microvascular complication, and is a valid reason for considering the proteins derived in this way as potential predictors of the development of retinopathy. The proteins for which an increase was registered in the aqueous humour of diabetic patients (with or without DR) compared to healthy subjects are: GFAP, AQP1, AQP4, IFNy, IL-1a, IL-1b, IL-3, IL-4, IL-10, IL-11, IL-17, TNF- α, TNF-ß, MCP1, MCP2, Eotaxin, Eotaxin 2, RANTES, sTNFRII, GM-CSF, IP-10, MIP1a, MIP1b; GFAP, AQP1 and AQP4 were reduced in diabetic patients with macular edema compared to diabetic patients with diabetic retinopathy and no macular edema, probably due to anatomic changes of Muller cells. The 23 proteins we have individuated may therefore be used in future as possible therapeutic targets for the prevention and care of diabetic retinopathy, helping to avoid its onset and improving the visual prognosis.

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